Transdermal Fentanyl for Pain Management in Cancer Patients

Sudeep Raj Bista
BPharm MSPharm MMedRes

School of Pharmacy
Griffith Health
Griffith University

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Doctor of Philosophy

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'The woods are lovely, dark and deep.
But I have promises to keep, and miles to go before I sleep'

Robert Frost (1874-1963)

ॐ भूभुः स्व तत्सवितुर्वरणं। भर्गो देवस्य धीमहि धीयो यो न प्रचोदयात्।।

Gayatri Mantra; Rig Veda (10:16:3)

(O thou existence absolute, creator of the three dimensions, we contemplate upon thy divine light. May he stimulate our intellect and bestow upon us true knowledge)
Abstract

Moderate to severe pain is common among cancer patients and affects 70–80 % of patients with advanced cancer. We have the means and the knowledge to relieve pain in many patients, but evidence from surveys and observational studies shows that many patients have troublesome or severe pain and do not get adequate relief. Although opioids remain the only class of drug with the ability to ameliorate severe pain, even in developed countries with access to a range of opioids, opioid formulations and adjuvant therapies, pain management is still a major problem in cancer care. As there is a narrow therapeutic window between pain control and toxicity, there is also substantial potential for side-effects, and, therefore, current practice when starting patients on fentanyl (an opioid class of drug) is to begin with a low dose and titrate the dose up slowly according to pain response and adverse events. As a consequence, it is often several days before a patient’s pain is controlled. Little is known about how factors such as patient demographics, organ function, effect of enzyme inhibitor/inducer, or the drug delivery system itself influence the pharmacokinetics (PK) of fentanyl in cancer patients. Better methods are required to monitor, individualise and improve opioid dosing.

Patients with advanced malignant disease are by definition frail and have poor performance status. There is considerable reluctance on the part of health professionals to subject these individuals to non-essential tests and investigations, including the repeated venepuncture that has been necessary in PK studies to date. The use of saliva rather than plasma has been shown to be an attractive alternative for therapeutic drug monitoring (TDM) because the collection is painless, simple and cheaper than venesection. Relatively little is known about the PK profile of fentanyl in cancer patients. If the PK profile of fentanyl could be studied in a heterogeneous group of cancer patients, this could help in optimising fentanyl dosing through population PK analysis. It would further enhance the safety and efficacy of fentanyl in clinical practice. This study has examined various factors and variables that influence the PK of fentanyl, thus potentially improving the effective management of pain in cancer patients. Additionally, this study has measured drug concentrations in saliva to investigate its potential as a substitute for plasma analysis, for use in future monitoring of therapeutic drug concentrations and in PK studies.
This study was conducted using both analytical and observational methods. Paired saliva and blood samples were taken from in-patients and out-patients with malignant disease at an oncology/palliative care service at the Mater Adults Hospital, Brisbane, Australia. A visual and descriptive scale of 0–4 (0: perfectly adhered to; 4: completely peeled off the skin) developed by the Food and Drug Administration (FDA) for pharmaceutical manufacturing purposes, was used to grade the degree of fentanyl patch adhesion at the time of sampling. A study was conducted to validate this scoring tool for use in clinical practice. At the time of sampling, participants were also asked to identify their pain score on a numerical rating scale of 0–10 (0: no pain; 10: worst pain). Wherever possible, samples were taken at the same time as routine pathology testing.

A sensitive, accurate and precise method of quantifying fentanyl and nor-fentanyl in plasma and saliva samples was developed and validated using high performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS). Total and free fentanyl and nor-fentanyl concentrations were quantified using the method developed. A protein binding study was also performed, which demonstrated that fentanyl was bound extensively to albumin (ALB) rather than α-1 acid glycoprotein (AAG). The protein binding study determined the free fraction of fentanyl available for the pharmacodynamic (PD) effect. Almost 96% of fentanyl was bound to plasma protein. This study found saliva drug concentrations to far exceed plasma concentrations, suggesting the possibility of a mechanism of active transport into saliva for fentanyl. No correlation between plasma and saliva concentration was observed, and no correlation was found between the concentration of fentanyl and its metabolite, nor-fentanyl, in either of the matrices. However both plasma and saliva mean concentrations of fentanyl were well correlated with dose, with considerable inter-patient variation at each dose. Pain score data revealed that the majority of patients had adequate pain control. A preliminary study to examine several polymorphisms in the ARRB2, BDNF and KCNJ6 genes to determine any association with fentanyl dosing showed no association with any of the genotypes investigated in our population. Population PK analysis was performed using non-linear mixed effects modelling (NONMEM) software. Various cofactors such as pain score, effect of enzyme inhibitor/inducer, liver function, renal clearance and patch adhesion were included in the modelling. Besides a priori included weight, no other patient characteristic could be identified that significantly influenced fentanyl pharmacokinetics in a predictive manner. Patch adhesion, while not identified as a significant covariate is likely to influence fentanyl exposure and should be monitored in
clinical practice. The overall degree of patch adhesion within the study cohort was high (>90% patients scored 0) and potentially the reason why incomplete patch adherence did not significantly impact on overall bioavailability in PK studies.

This study investigated many aspects of the use of transdermal fentanyl in cancer patients. Though no significant factors were found that could change the current dosing practices of fentanyl for pain management in cancer patients, various crucial findings were demonstrated. Data on protein binding, novel extraction methods and adsorption minimizing techniques in analysis should assist, and have an impact on, future clinical research and trials. A useful tool for scoring patch adhesion has been validated and deemed reliable to use in clinical practice. Detection of higher fentanyl concentrations in saliva than plasma, with a good correlation to dose, may allow saliva to be used as an alternative to plasma in PK/PD studies of fentanyl in cancer patients.
Statement of Originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

_____________________________
Sudeep Raj Bista
Acknowledgements

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<td>AAG</td>
<td>α-1 acid glycoprotein</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP binding cascade</td>
</tr>
<tr>
<td>ABCB1</td>
<td>ATP-binding cassette B1 gene</td>
</tr>
<tr>
<td>ADME</td>
<td>absorption, distribution, metabolism, excretion</td>
</tr>
<tr>
<td>ALB</td>
<td>albumin</td>
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<tr>
<td>ALT</td>
<td>alanine aminotransferase</td>
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<td>AST</td>
<td>aspartate aminotransferase</td>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<td>APCI</td>
<td>atmospheric pressure chemical ionisation</td>
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<tr>
<td>ARRB2</td>
<td>arrestin, beta 2 gene</td>
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<tr>
<td>BBB</td>
<td>blood-brain barrier</td>
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<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
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<td>body mass index</td>
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<td>BOV</td>
<td>between occasion variability</td>
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<td>BPI</td>
<td>brief pain inventory</td>
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<td>BSA</td>
<td>body surface area</td>
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<td>BSV</td>
<td>between subject variability</td>
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<tr>
<td>CDB</td>
<td>cotton dental bud</td>
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<td>CE</td>
<td>collision energy</td>
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<td>CI</td>
<td>confidence interval</td>
</tr>
<tr>
<td>CL</td>
<td>clearance</td>
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<td>creatinine clearance</td>
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<td>CRF</td>
<td>case report form</td>
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<tr>
<td>CTCAE</td>
<td>common terminology criteria for adverse events</td>
</tr>
<tr>
<td>CV</td>
<td>coefficient of variation</td>
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<tr>
<td>CXP</td>
<td>collision cell exit potential</td>
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<tr>
<td>DP</td>
<td>declustering potential</td>
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<tr>
<td>DRD2</td>
<td>dopamine receptor D2 gene</td>
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<tr>
<td>DT</td>
<td>dialysis tubing</td>
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<tr>
<td>EDTA</td>
<td>ethylene diamine tetra acetic acid</td>
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<td>Term</td>
<td>Definition</td>
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<tr>
<td>EMA</td>
<td>European Medicines Agency</td>
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<tr>
<td>EP</td>
<td>entrance potential</td>
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<td>ESI</td>
<td>electrospray ionisation</td>
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<td>FDA</td>
<td>Food and Drug Administration</td>
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<tr>
<td>FOCE</td>
<td>first order conditional estimation</td>
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<tr>
<td>GC-MS</td>
<td>gas chromatography mass spectrometry</td>
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<tr>
<td>GIRK</td>
<td>G protein-activated inward rectifier potassium channel</td>
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<tr>
<td>HC</td>
<td>high control</td>
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<td>HPLC-MS/MS</td>
<td>high performance liquid chromatography tandem mass spectrometry</td>
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<td>HREC</td>
<td>human research ethics committee</td>
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<tr>
<td>IS</td>
<td>internal standard</td>
</tr>
<tr>
<td>ka</td>
<td>absorption rate</td>
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<tr>
<td>KCNJ6</td>
<td>G protein-activated inward rectifier potassium channel 2 gene</td>
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<tr>
<td>LC</td>
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<tr>
<td>LLOQ</td>
<td>lower limit of quantification</td>
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<tr>
<td>LOQ</td>
<td>limit of quantification</td>
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<td>MAF</td>
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<td>mass balance approach</td>
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<td>MF</td>
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<td>megaohm</td>
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<td>MOR</td>
<td>µ opioid receptor</td>
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<td>mobile phase b</td>
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<td>MR</td>
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<td>multiple reaction monitoring</td>
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<tr>
<td>MWCO</td>
<td>molecular weight cut off</td>
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<tr>
<td>m/z</td>
<td>mass/charge</td>
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<tr>
<td>NCBI</td>
<td>National Centre for Biotechnology Information</td>
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<tr>
<td>NONMEM</td>
<td>non-linear mixed effects modelling</td>
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<tr>
<td>OFV</td>
<td>objective function value</td>
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<tr>
<td>OPRM1</td>
<td>opioid receptor, mu 1 gene</td>
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<tr>
<td>PBS</td>
<td>phosphate buffer solution</td>
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<tr>
<td>PCI</td>
<td>post column infusion</td>
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</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>p-glycoprotein</td>
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<td>residual ‘unexplained’ variability</td>
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<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>TDDS</td>
<td>transdermal drug delivery system</td>
</tr>
<tr>
<td>TDM</td>
<td>therapeutic drug monitoring</td>
</tr>
<tr>
<td>UF</td>
<td>ultrafiltration</td>
</tr>
<tr>
<td>ULOQ</td>
<td>upper limit of quantification</td>
</tr>
<tr>
<td>Vd</td>
<td>volume of distribution</td>
</tr>
<tr>
<td>VPC</td>
<td>visual predictive check</td>
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<td>WT</td>
<td>weight</td>
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\[ Cn = \frac{(Cu \times Ws)}{Wu} \quad \text{Eq- 2.1} \]

\[ \% \text{Loss} = \frac{Cs - Cds}{Cs} \times 100 \quad \text{Eq- 3.2} \]

\[ \% \text{Recovery} = 100 - \% \text{Loss} \quad \text{Eq- 3.3} \]

\[ F_b = F_t - F_f \quad \text{Eq- 3.4} \]

\[ \% \text{bound} = \frac{F_b}{F_t} \times 100 \quad \text{Eq- 3.5} \]

\[ \% \text{PPB} = \frac{(C_2 - C_3) \times V_2 + C_3 \times V_3}{C_1 \times V_1} \times 100 \quad \text{Eq- 3.6} \]

\[ \% \text{Recovery} = C_2 \times V_2 + C_3 \times V_3 \quad \text{Eq- 3.7} \]

\[ \text{Unbound fraction (}F_u) = \frac{F_f}{F_t} \quad \text{Eq- 3.8} \]

\[ \text{Bound fraction} = 1 - F_u \quad \text{Eq- 3.9} \]

\[ \frac{S}{P} = 1 + 10(pKa - pHs) \times fp/1 + 10(pKa - pHp) \times fs \quad \text{Eq- 5.10} \]

\[ c(t) = \frac{\text{Dose}}{V} \times \exp\left\{\frac{CL}{V}\right\} \times t \quad \text{Eq- 7.1} \]

\[ C_{ij} = C_{\text{pred,ij}} + \varepsilon_{ij} \quad \text{Eq- 7.2} \]

\[ C_{ij} = C_{\text{pred,ij}} \times \exp\varepsilon_{ij} \quad \text{Eq- 7.3} \]

\[ C_{ij} = C_{\text{pred,ij}} \times \exp\varepsilon_{ij} + \varepsilon_{ij} \quad \text{Eq- 7.4} \]

\[ \Theta_i = \Theta_{\text{pop}} \times e^{\eta_i} \quad \text{Eq- 7.5} \]

\[ \Theta_{ik} = \Theta_{\text{pop}} \times e^{(\eta_i + \kappa_{ik})} \quad \text{Eq- 7.6} \]
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Publications


Conferences


Queensland Translating Research into Practice (TRIP) symposium, 25 July, Brisbane, Australia.


**Grants**


Chapter 1. Fentanyl for use in pain management

1.1 Opioids in pain management
Traditionally, the term ‘opioid’ refers to all compounds, both natural and synthetic, that act in a similar way to morphine, whereas ‘opiate’ refers only to compounds that are derived from *Papaver somniferum*, such as morphine and codeine. Opioids are subdivided into those based on the 4 and 5-epoxymorphinan ring structure (such as morphine, codeine, diamorphine and naloxone), piperidine and phenylpiperidines (such as meperidine, loperamide, and fentanyl), and the diphenylheptlamines (such as methadone and dextropropoxyphene). Although these drugs have quite different chemical structures and mechanisms, as well as differing rates of drug distribution and elimination, their one common feature is interaction with the mu opioid receptor as the primary target.

Approximately 80 % of cancer patients in advanced stages of illness have moderate to severe pain, and more than 10 million cancer patients worldwide are prescribed opioids. Other pain settings in which opioids are used are non-malignant chronic pain and postsurgical acute pain [1]. The means and the knowledge exist to relieve pain in the majority of patients, but evidence from surveys and observational studies shows that many patients have troublesome or severe pain and do not get adequate relief. The skilled use of opioid analgesics is crucial to the relief of cancer pain, but there is a ‘shocking lack of evidence to support clinical practice’ [2].

Opioids remain the only class of drug with the ability to ameliorate severe pain. Even in developed countries with access to a range of opioids, opioid formulations and adjuvant therapies, pain management is still a major problem in cancer care. As there is a narrow therapeutic window between pain control and toxicity, there is also substantial potential for side-effects. For this reason, when patients are first given opioids, current practice is to start at a low dose and titrate the dose slowly according to pain response and adverse events. As a consequence, it is often several days before a patient’s pain is controlled. Little is known about how factors such as age, sex and organ function influence the pharmacokinetics (PK) of commonly used opioids in cancer patients. Most studies to date have been undertaken in patients with end-stage organ failure (e.g. renal or liver failure) and do not reflect the population in which these drugs are commonly used. Pain control and total plasma opioid concentrations have been shown to correlate poorly [3].
This is perhaps related to factors at the site of action, although PK and pharmacogenetic (PG) variations have yet to be fully investigated. The PG factors contributing to the efficacy and adverse events of opioids are complex and multiple. Moreover, it is unclear how significant these factors are in clinical practice [4]. Better methods are required to monitor, individualise and improve opioid dosing.

1.2 Fentanyl
Fentanyl \([\text{N-(1-phenethyl-4-piperidyl) propionanilide}]\) (Figure 1.1) is a synthetic, highly selective opioid agonist that acts primarily at the mu opioid receptor, with minor activity at the delta and kappa receptors [5]. Along with morphine and oxycodone, it is the opioid used most commonly in cancer patients. Compared to morphine, it is approximately 100 times more potent, 1000 times more lipophilic and has a lower molecular weight. It exhibits a more rapid onset of action and a shorter duration of effect [6]. In opioid-naive postoperative patients, fentanyl produces rapid and effective analgesia at minimum plasma concentrations between 0.6 and 1.5 \(\mu\text{g/L}\) [7] with an increase in drug related adverse events at concentration > 2 \(\mu\text{g/L}\) [8]. Moreover, the development of opioid tolerance varies between individuals. Both the minimum effective fentanyl concentration for analgesia and the threshold concentration for increased adverse events increase with the development of tolerance [8].

The oral (enteral) bioavailability of fentanyl is poor [6]. Hence the routes of administration used include intravenous, subcutaneous, transdermal, transmucosal, and spinal [9-11]. The transdermal patch is the most common means of delivery of fentanyl to patients with cancer. There is no ‘standard’ dose of fentanyl, and marked variation occurs in the dose necessary to obtain adequate pain relief in different patients. The recommendation is to replace each patch every 72 hours; however, the analgesic benefit for some patients is lost after 48 hours [12]. Relatively little is known about the drug’s PK profile in cancer patients. One study has shown that fentanyl PK changes with age, with serum drug concentrations increasing more slowly in the elderly than in younger patients [13]. It is not known how much body mass (especially in cachectic patients) and patch adherence contribute to inter- and intra-individual variation in PK profile, efficacy or toxicity. In cancer patients the effectiveness of the patch may be impaired by muscle wasting and changes in subcutaneous fat content (cachexia), as well as by poor adherence of the patch to skin due to increased ambient temperature and humidity in certain climates [14-16]. In practice these problems are often overcome by utilising a
higher dose patch to increase plasma fentanyl concentration, or by placing an adhesive
dressing over the fentanyl patch to increase patch adherence to skin. Both these
approaches have the potential to result in toxicity, and there is little evidence for their
effectiveness and/or safety. Only one clinical study of fentanyl patches in a cancer
population exhibiting cachexia has been reported. In this study, intravenous blood
sampling demonstrated lower total fentanyl concentrations at 48 and 72 hours after
applying the patch in cachectic patients than in normal-weight patients [17]. Fentanyl
concentrations were used as a surrogate for the effectiveness of the treatment only, and
a more detailed PK analysis was not performed.

1.3 PK of transdermal fentanyl
Fentanyl has a molecular weight of 336.47 g/mol. It is a highly lipophilic drug with high
lipid solubility, having an octanol: water partition coefficient of greater than 700 [18]
and pKa of 7.89–8.6 (at 15–47.5 °C) [19]. The structure of fentanyl and nor-fentanyl is
shown in Figure 1.1.

![Structure of fentanyl (left) and nor-fentanyl (right)](image)

**Figure 1.1 Structure of fentanyl (left) and nor-fentanyl (right)**

1.3.1 Absorption
After application of the first patch, there is a mean lag period of 24 hours (range 1–30
hours) before therapeutic serum concentrations are reached [20, 21]. These results
suggest that the plateau of plasma fentanyl concentration is achieved during the second
12-hour period of the first patch [22]. Hence steady state is usually achieved with the
second patch system [23]. However, steady-state plasma fentanyl concentrations can
vary from 4- to 5-fold between individual patients [24], though intraindividual
variability is relatively low [7]. A slight, but not clinically relevant, further increase in
trough concentrations may be observed until the second or third patch is removed [25]. The absolute extent of bioavailability is close to 100 %, considering the dose derived from the claimed absorption rate [7]. Resulting serum fentanyl concentrations are thus similar to those observed with intravenous fentanyl infusions and are stable over time [26-28]. Daily peak concentrations are often observed in the early morning hours; these may result from increased body surface temperature during bed stay as a result of occlusive thermal effects [14]. There is substantial evidence demonstrating that increasing skin temperature enhances the permeability of various drugs such as nitroglycerin [29, 30], nicotine [31], methyl salicylate [32], clonidine [33], and fentanyl [34]. External heating induces changes in skin permeability, hemodynamics, blood flow distribution, and vasodilation [35-37]. Local heat exposure to transdermal delivery systems has been shown to increase total drug absorption and plasma drug concentrations more than 3-fold [21].

PK studies indicate that a depot of drug is present in the upper layers of the skin after the first application. From this depot, drug is released to the circulation. Once steady-state blood levels have been reached, these concentrations can be maintained as long as the fentanyl patches are replaced every 48 to 72 hours [25, 38]. Half-life values after the removal of the patch vary between 13 and 25 hours [20, 24, 26, 39, 40]. This range is 2 to 3 times higher than values obtained after intravenous administration (6–8 hours) [20, 22] and is probably related to the slow diffusion of the drug from the skin depot into the systemic circulation [22]. Indeed, when the transdermal patch is removed, a depot of fentanyl (about 10 % of the dose) remains in the stratum corneum, and absorption continues maintaining blood fentanyl concentrations for almost 24 hrs. Therefore, patients who have experienced clinically relevant adverse events or overdosing require continued monitoring for up to 24 hours post patch removal [41]. After the initial transdermal fentanyl patch application, there is a significant delay before steady-state blood levels are achieved, resulting in prolonged time before there is adequate pain relief. This lag period can vary from one hour to over 30 hours (mean value, 13 hours). Therefore, the most common approach to managing baseline pain is to prescribe two medications, a long-acting medication such as transdermal fentanyl and a short-acting medication to treat the breakthrough pain [42, 43].
1.3.2 Distribution
After an intravenous bolus, fentanyl is rapidly distributed from plasma into highly vascularised compartments before redistribution to muscle and fat tissue. Fentanyl includes a large volume of distribution (3.5–8 L/kg, average 6 L/kg) and a high total body clearance (30–72 L/h) [44]. After an initial equilibration phase, fentanyl is released back into plasma from these storage sites. This accounts for its long elimination half-life of 3–8 hours [7].

Fentanyl has also been reported to be a substrate for the p-glycoprotein (P-gp) efflux transporter. In addition to passive diffusion, active transport systems have also been described for fentanyl uptake via the brain endothelium [45]. Metabolites of fentanyl have been shown to appear in plasma [46] stool and urine [47]. The high lipophilicity of fentanyl is also thought to be responsible for its rapid penetration into the central nervous system (CNS) [48, 49]. As it has been demonstrated that fentanyl and its metabolite transport into various body fluids, it is assumed that its passage into saliva is also functional. If fentanyl concentrations in saliva could be quantified, saliva concentrations could be substituted for plasma drug concentrations in PKPD studies and/or for supporting dose titration in clinical practice. If this relationship could be established, it could be a significant contribution to fragile cancer patients who are already in pain and reluctant to provide regular plasma samples for clinical observations.

1.3.3 Metabolism and elimination
Fentanyl is primarily (>75%) metabolised [50, 51] by CYP3A4 [52] and the inactive [53] nor-fentanyl by CYP3A5. N-dealkylation appears to be the primary pathway of fentanyl metabolism in humans [46]. The main metabolite is nor-fentanyl; minor metabolites are hydroxypropionyl-fentanyl and hydroxypropionyl-norfentanyl that do not have any relevant pharmacological activity [50]. Approximately 75% of fentanyl is excreted via the kidney, less than 10% of it as unchanged fentanyl. About 9% of metabolites are recovered in the faeces [50]. After removal of the transdermal patch, serum fentanyl concentrations decline gradually. Approximately 30% of the total delivered dose from the patch remains in the skin. This depot explains the mean apparent terminal elimination half-life of 22–25 hours, 2–3 times longer than that of intravenous fentanyl [26].
Free drug is the active component, in equilibrium with the concentration at the site of action. It is also available for metabolism in the liver; thus protein binding of fentanyl is likely to be of significance to both its efficacy and its PK. Protein binding of fentanyl is controversial, and some reports suggest that it may bind primarily to ALB [54, 55], though binding to AAG has also been reported [56, 57]. Protein binding in human plasma has been reported as being 80–85 % bound [54-58].

1.3.4 Adverse effects
Fentanyl displays all opioid-related adverse effects, such as nausea and vomiting (20–60 %), pruritus (0–30 %) and urinary retention (40 %) [44]. As with all other opioids, dose-dependent respiratory depression is the most serious and potentially life-threatening adverse effect. However, the most common formulation-specific adverse effects are related to the adhesive, and include erythema (10 %), itching and occasional pustules (<1 %) [7].

1.3.5 Drug interactions and PK effects of fentanyl
As it does for intravenous fentanyl, age influences the PK of transdermal fentanyl, and doses should be reduced and regular dose assessments made, especially in geriatric, debilitated and cachectic patients [59]. The PK of transdermal fentanyl in children appears to be similar to, and perhaps less variable than, that in adults [60]. Caution should be observed in patients with renal or hepatic insufficiency or unstable conditions with rapidly varying metabolism. Fentanyl is metabolised mainly via the human cytochrome P450 3A4 isoenzyme system (CYP3A4); therefore, potential interactions may occur when fentanyl is given concurrently with agents that affect CYP3A4 activity. Co-administration with agents that induce CYP3A4 activity may reduce the efficacy of fentanyl. The concomitant use of transdermal fentanyl with all CYP3A4 inhibitors (such as ritonavir, ketoconazole, itraconazole, troleandomycin, clarithromycin, nelfinavir, nefazadone, amiodarone, amprenavir, aprepitant, diltiazem, erythromycin, fluconazole, fosamprenavir, verapamil, or grapefruit juice) may result in an increase in fentanyl plasma concentrations, which could increase or prolong adverse drug effects and may cause potentially fatal respiratory depression. It is necessary to carefully monitor patients receiving transdermal fentanyl and any CYP3A4 inhibitor for signs of respiratory depression for an extended period of time and adjust the dosage if warranted [61]. Concomitant administration of fentanyl with ethanol may result in additive or
synergistic CNS depression and may cause respiratory depression, hypotension, profound sedation, or coma [61].

1.4 Significance and outcome
Pain is under-assessed and poorly treated the world over. One of the reasons for this is a lack of knowledge of the effects of various factors on the PKPD of analgesics. The reasons for the considerable intra- and inter-patient variability with regard to opioid effectiveness are poorly understood. Many of the drugs used in routine clinical practice have a very narrow therapeutic window, i.e. the dose resulting in analgesia may be very similar to that causing toxicity. This study aims to address some of the knowledge gaps that currently prevent dose optimisation of these drugs. The study will also analyse the necessary PK models to investigate if any patient-related factors have an influence on pain management. Findings from this study may be able to contribute to the understanding of how fentanyl should be prescribed to cancer patients. The clinical implementation of the relevant findings from this study can be the subject of further clinical research into individualised dosing strategies and TDM of opioids for pain management in cancer patients.

1.5 Aims and hypothesis
1.5.1 Aims
The primary aim of this study was to determine the best means of measuring drug concentrations (plasma, saliva, free or total concentrations) for PK studies and/or PKPD models and apply this information to improve clinical practice with respect to fentanyl dosing in individual patients with cancer pain. Overall this study addressed the following aims:

i. to develop and validate a HPLC-MS/MS method for the determination of fentanyl and nor-fentanyl in human plasma (total and free concentrations) and saliva

ii. to investigate the protein binding behaviour of fentanyl, and its metabolite nor-fentanyl, in human plasma, ALB and AAG

iii. to develop and validate a transdermal patch adhesion scoring tool to be used in clinical practice at the time of sampling

iv. to determine if saliva is a surrogate for plasma drug concentration in PKPD studies of fentanyl in cancer patients
v. to conduct a preliminary study to examine several polymorphisms in the *ARRB2*, *BDNF* and *KCNJ6* genes to determine any association with fentanyl dosing

vi. to perform population PK modelling for fentanyl to investigate the relationship between various cofactors influencing pain control

1.5.2 Hypothesis

i. The measurement of the more lipophilic opioid fentanyl in saliva provides a non-invasive means of studying PK in patients with cancer to monitor efficacy and reduce toxicity.

ii. There are many cofactors that affect the PK of fentanyl and influence effectiveness and toxicity, such as patch adhesion, effect of enzyme inducer/inhibitor, liver function and renal clearance.

1.6 Outline of the thesis

Each of the six aims of this research study is detailed as a separate chapter in the thesis. Due to the interdisciplinary nature of the research study, each chapter will include background, materials and methods, results and discussion, and conclusion. The first chapter of the thesis provides an introduction to the research area and the final chapter provides a conclusion with future directions.
Chapter 2. Development and validation of an HPLC-MS/MS method for the determination of fentanyl and nor-fentanyl in human plasma and saliva

2.1 Introduction

Despite the advantages of saliva sampling, there are innumerable problems with quantifying the drugs of choice in saliva. The major problems reported are low volume obtained after centrifugation [62], adsorption of drugs in collecting devices and variation in recovery depending upon the specific product used [63]. Hence, a method that allows the extraction of the drug of choice in saliva from the collecting device with acceptable recovery should be of benefit in PK studies. A typical saliva collection device (Salivette®) is shown in Figure 2.1. The Salivette® comprises a cap, cotton dental bud (CDB), CDB holder, and a collecting tube. The CDB is a cotton roll/ bud designed to be placed in the mouth for saliva collection.

Figure 2.1 A typical Salivette® showing the various components

A simple HPLC-MS/MS method is required to be developed and validated to quantify fentanyl and nor-fentanyl in plasma and saliva using identical conditions in terms of instrumentation and analysis. Koch et al. [64] have developed a method to quantify fentanyl and its metabolite in rat plasma; however, the instrumentation conditions are different for both the parent compound and its metabolite, requiring time-consuming re-injection. Verplaetse and Tytgat [65] have also developed a method to quantify fentanyl and nor-fentanyl in plasma and urine for forensic studies. However, no saliva matrix was included in their method. A detailed review of previous methods for quantifying fentanyl and nor-fentanyl in plasma is described later in the chapter (Section 2.1.4) including a comparison of various methods and instrumentation, and their limitations.
2.1.1 High Performance Liquid Chromatography (HPLC)

In chromatography, a fluid (mobile phase) moves over a non-moving (stationary) phase. When there is a strong interaction between a given compound and the stationary phase, the migration of the component will slow down. When the interaction is minimal, the compound will migrate with the same velocity as the mobile phase. This results in the separation of the various components of a mixture [66]. Chromatography yields two basic pieces of information on the separated components: the degree of retention (characteristic of molecular structure) and the signal intensity (related to the amount of the component). HPLC is a highly advanced form of column chromatography. This technique allows substantially improved chromatographic efficiency, resulting in better separation of the components in a mixture. It allows the solvent to flow through the column, which is packed with very small particle-size column material under high pressure. This provides a large surface area for interaction between the stationary phase and the eluting solvents, increasing the number of theoretical plates and providing a measure of efficiency. Flow rate is also optimised to achieve optimal separation. A typical HPLC instrumentation consists of a reservoir of mobile phase, a pump, an injector, a separation column and a detector. Compounds are separated by injecting the sample mixture onto the column. The various components in the mixture pass through the column at different rates due to differences in their partitioning behaviour between the mobile liquid phase and the stationary phase. A schematic representation of a typical HPLC system is shown in Figure 2.2 [67].
2.1.2 Mass Spectrometry

Mass spectrometry is an analytical tool used to measure the mass/charge (m/z) ratio of ions produced in an ion source. Mass spectrometers have three basic components, namely the ion source, the analyser and the detector. Once the sample is introduced into the ion source, the analyte molecules entering the analyser are converted into ions. They are then selected, based on their m/z ratio [68, 69]. A general mechanism of typical tandem mass spectrometry is shown in Figure 2.3. MS/MS (usually called tandem mass spectrometry) typically has more than one m/z analyser separated by a collision cell. The first m/z analyser selects the ions arising from the particular analyte in the positive ion mode [M+H]+ or in the negative ion mode [M-H]−. When these ions pass through the collision cell they are fragmented in a characteristic manner, based on their bond energies, by collision with low pressure inert gas (nitrogen, argon or xenon) to form the product ions [69]. In the initial stages of quantitative assay development using mass spectrometry, the precursor ion is identified (usually by molecular mass) representing the addition of H+ during ionisation. The ionisation method used depends on the analyte under investigation. The modes of ionisation available include Electrospray Ionisation (ESI) and Atmospheric Pressure Chemical Ionisation (APCI).
ESI, represented in Figures 2.4 and 2.5 [70], is the most widely used mode of ionisation and is applicable to a wide range of samples that are polar and ionic in nature [71]. ESI is a sensitive and efficient method of ionising compounds in solution, such as eluent from an HPLC containing non-volatile, thermally labile polar analytes (i.e. analytes likely to become charged under the ion source conditions). Compounds with molecular weights <600 atomic mass unit (amu) can be analysed using this technique. Mixed aqueous and organic mobile phase are the optimum for ESI, making it readily amenable to reverse phase liquid chromatography. The sample is sprayed (in solution) into the source at the high potential of 3–5 kV. This process is charge-dependent, and since the analyte used will be of low concentration, electrolytes must also be in the liquid at a minimum concentration of 10–5 mol/L to assist in promoting ionisation. The recommended flow rate is from nL/min to 0.3 mL/min. In this high-flow interface, heat is applied to aid the evaporation of mostly aqueous droplets. ESI mode may be influenced by the composition of the mobile phase, and, most importantly, this technique is more sensitive to ion-suppression effects, which is a major challenge in the handling of biological matrices [69].

Figure 2.3 Schematic representation of the general mechanism of a typical tandem mass spectrometry (copyright permission obtained; ©2002-2014 University of Bristol)
To carry out the process of APCI represented in Figures 2.6 and 2.7 [70], the instrument used for ESI is reconfigured to introduce a corona discharge needle at the point where the stream of solvent that contains the analyte/s enters. A potential of 3-6 kV is then applied to the corona discharge needle [66]. This technique is mass sensitive, as compared to ESI which is concentration sensitive, however analytes should be thermally stable to be assayed using APCI. This ionisation mode is suitable for non-polar, thermally stable compounds of molecular weight < 1300 amu. The heated nebuliser has a high flow, with a flow rate of 0.2-2.0 mL/min. The probe is heated to facilitate vaporisation. Because of the nature of the ionisation process, APCI is more
robust when there are mobile phase additives. A typical comparison between ESI and APCI is listed in Table 2.1.

Figure 2.6 Schematic representation of APCI interface (copyright permission obtained; ©2002-2014 University of Bristol)

Figure 2.7 Schematic representation of mechanism of APCI (copyright permission obtained; ©2002-2014 University of Bristol)
Table 2.1 Comparison of ESI and APCI

<table>
<thead>
<tr>
<th>Feature</th>
<th>ESI</th>
<th>APCI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular ions formed</td>
<td>singly and multiply charged [M±H]; [M±nH]</td>
<td>singly charged [M±H]</td>
</tr>
<tr>
<td>Analyte polarity</td>
<td>polar – highly polar</td>
<td>non polar – polar</td>
</tr>
<tr>
<td>Mass range</td>
<td>150-200,000 u</td>
<td>150-1000 u</td>
</tr>
<tr>
<td>Flow rate</td>
<td>nL/min to 0.3 mL/min</td>
<td>0.2 to 2 mL/min</td>
</tr>
<tr>
<td>Analyte thermal stability</td>
<td>low</td>
<td>high</td>
</tr>
<tr>
<td>Tolerance to impurities</td>
<td>low</td>
<td>high</td>
</tr>
</tbody>
</table>

2.1.3 HPLC coupled with tandem mass spectrometry

HPLC coupled with tandem mass spectrometry has been considered one of the most widely accepted techniques [69], showing better sensitivity, specificity and high throughput [72] in various analytical methods. This method can produce adequate precision and accuracy at a very low concentration, short retention times and high flow rates. Either ESI or APCI can be used in HPLC-MS/MS.

2.1.4 Literature review of methods for quantifying fentanyl and nor-fentanyl

A review of previous methods for quantifying fentanyl and nor-fentanyl in plasma and saliva was performed. The review included sample preparation techniques, methods of analysis and cost effectiveness. Electronic searching was performed using various databases, such as MEDLINE, EMBASE and PUBMED. There was no restriction placed on the date of publication. Some of the methods for quantifying fentanyl and nor-fentanyl that were reported in the literature are described in Tables 2.2 and 2.3.
Table 2.2 Methods for quantifying fentanyl and nor-fentanyl other than using HPLC-MS/MS

<table>
<thead>
<tr>
<th>Reference</th>
<th>Sample</th>
<th>Sample preparation</th>
<th>Instrumentation</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wang et al. [73]</td>
<td>Breath</td>
<td>Solid-phase micro extraction</td>
<td>GC-MS</td>
<td>No plasma or saliva</td>
</tr>
<tr>
<td>Egan et al. [74]</td>
<td>Plasma</td>
<td>Radio immunoassay</td>
<td>Radio immunoassay</td>
<td>No saliva</td>
</tr>
<tr>
<td>Silverstein et al. [75]</td>
<td>Urine and saliva</td>
<td>Solvent extraction</td>
<td>GC-MS</td>
<td>No plasma, no deuterated IS</td>
</tr>
<tr>
<td>Malkawi et al. [76]</td>
<td>Plasma (rabbit)</td>
<td>Solvent extraction</td>
<td>GC-MS</td>
<td>No saliva, no deuterated IS, no ion-suppression</td>
</tr>
</tbody>
</table>

*IS=internal standard

Table 2.3 Methods for quantifying fentanyl and nor-fentanyl using HPLC-MS/MS

<table>
<thead>
<tr>
<th>Reference</th>
<th>Sample</th>
<th>Sample preparation</th>
<th>Instrumentation</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Verplaetse and Tytgat [65]</td>
<td>Urine and whole blood</td>
<td>Solid phase extraction</td>
<td>LC-MS/MS</td>
<td>No saliva or plasma</td>
</tr>
<tr>
<td>Solassol et al. [77]</td>
<td>Plasma</td>
<td>Solid phase extraction</td>
<td>LC-MS</td>
<td>No saliva, no ion-suppression</td>
</tr>
<tr>
<td>Koch et al. [64]</td>
<td>Plasma (primate)</td>
<td>Multi-step liquid extraction</td>
<td>LC-MS</td>
<td>No saliva, no ion-suppression</td>
</tr>
<tr>
<td>Saito et al. [78]</td>
<td>Plasma</td>
<td>Solid phase extraction</td>
<td>LC-MS/MS</td>
<td>No saliva, no deuterated IS, no ion-suppression</td>
</tr>
<tr>
<td>Heltsley et al. [79]</td>
<td>Saliva and Urine</td>
<td>Liquid liquid extraction</td>
<td>LC-MS/MS</td>
<td>No plasma, no ion-suppression</td>
</tr>
</tbody>
</table>

*IS=internal standard

2.1.5 Limitations of the methods published to date

HPLC coupled with tandem mass spectrometry is a widely accepted method in bioanalytical assays. The method generates better sensitivity, specificity and reproducibility [72] when operated with suitable specifications. Moreover, method selection also depends upon the resources available in the research facility. As our research facility was equipped with HPLC-MS/MS, we were more interested in methods that were performed precisely with HPLC-MS/MS. However, other methods were also considered to make the comparisons in general. Further, the studies reported in the literature, as in Table 2.2, were performed using different methods and
instrumentation to HPLC-MS/MS. Hence such methods were not included in our criteria when a method was developed.

Furthermore, although the methods displayed in Table 2.3 used HPLC-MS/MS, there were many limitations, such as the lack of the use of a deuterated internal standard (IS) and a rigorous sample preparation method. They were less cost effective, and matrix effects (ion suppression/enhancement) were not assessed. Most of the methods did not include saliva as a matrix, with only two study by Silverstein et al. [75] and Heltsley et al. [79], quantifying fentanyl and nor-fentanyl in saliva samples. The method used by Silverstein et al., however, was Gas Chromatography (GC-MS), and additionally, no deuterated IS was used. Likewise the method used by Heltsley et al., use large amount of saliva sample which is not always possible especially with cancer patients with dry mouth. Further they did not include plasma sample and also did not performed ion-suppression test. This necessitated the development of a new HPLC-MS/MS method for the quantitation of fentanyl in human plasma and saliva.

2.2 Material and Methods
2.2.1 Reference materials and reagents
Fentanyl, nor-fentanyl, deuterated (d₅) fentanyl and deuterated (d₅) nor-fentanyl were obtained from Cerilliant®, Round rock, Texas, USA. Acetonitrile, dichloromethane, methanol, NaCl and formic acid were obtained from Merck, New Jersey, USA. All the chemicals and reagents obtained were of highest purity (≥ 99 %, HPLC-graded). Salivettes® were obtained from Sarstedt, Nümbrecht, Germany. 18 megaohm (MΩ) water was obtained from a Labmate water purification system (Aquacure, Brisbane, Australia). A centrifuge machine operating at 500 g (IEC CL2, Thermofischer Scientific) and 2000 g (IEC Micromax, Thermofischer Scientific) was obtained from Scoresby, Victoria, Australia.

2.2.2 HPLC-MS/MS instrumentation and conditions
Samples were analysed using a Shimadzu HPLC system (DGU-20A3 Degasser; LC-20 AD Liquid Chromatography; CBM-20A Communication Module; SIL-20AC Auto Sampler) (Nakagyo-Ku, Koyoto, Japan) coupled with an API 3200 tandem mass spectrometer (MS/MS) (Applied Biosystems, Mount Waverly, Victoria, Australia). Analyst software version 1.4.2 from Applied Biosystems was used for data acquisition. The ESI was operated in positive ion mode, employing multiple reaction monitoring
(MRM) with Declustering Potential (DP), Entrance Potential (EP), Collision Energy (CE) and Collision Cell Exit Potential (CXP) set up, as mentioned in Table 2.4.

Table 2.4 MRM parameters set up for quantifying fentanyl, nor-fentanyl, fentanyl-d₅ and nor-fentanyl-d₅ in plasma and saliva samples

<table>
<thead>
<tr>
<th></th>
<th>Q1 Mass</th>
<th>Q3 Mass</th>
<th>Time (mSec)</th>
<th>DP (V)</th>
<th>EP (V)</th>
<th>CE (V)</th>
<th>CXP (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fentanyl</td>
<td>337</td>
<td>188</td>
<td>70</td>
<td>40</td>
<td>12</td>
<td>30</td>
<td>4</td>
</tr>
<tr>
<td>fentanyl-d₅</td>
<td>342</td>
<td>188</td>
<td>70</td>
<td>40</td>
<td>12</td>
<td>30</td>
<td>4</td>
</tr>
<tr>
<td>Nor-fentanyl</td>
<td>233</td>
<td>84</td>
<td>70</td>
<td>46</td>
<td>11</td>
<td>25</td>
<td>6</td>
</tr>
<tr>
<td>Nor-fentanyl-d₅</td>
<td>238</td>
<td>84</td>
<td>70</td>
<td>46</td>
<td>11</td>
<td>25</td>
<td>6</td>
</tr>
</tbody>
</table>

Nitrogen was used as the source gas. Curtain gas pressure was maintained at 10 psi and collision gas at 10 psi; ion source gas 1 and ion source gas 2 were maintained at 25 and 20 psi respectively. Likewise, ion spray was maintained at 5500 V, and drying gas temperature at 450°C. The assay was performed using an Alltima C18 3 µm 50×2.1 mm column fitted with an Alltech C18 5 µm 7.5×2.1 mm guard column (Deerfield, Illinois, USA). Mobile phase A (MP-A) comprised 0.1 % formic acid in 18 MΩ water. Similarly, mobile phase B (MP-B) comprised 15 % methanol in acetonitrile with 0.1 % formic acid. Gradient elution was performed starting at 90 % MP-A/10 % MP-B, progressing to 100 % MP-B over 4.5 mins; it was held at 100 % MP-B for 1.5 mins, and then reduced to 90 % MP-A/10 % MP-B for the remainder of the run to re-equilibrate. Column temperature was maintained at ambient (22 ± 2°C) and the auto sampler operated at 10°C. Total flow rate of the mobile phase was 0.3 ml/min. Run time was set to 8 mins, with a retention time of 3.80 mins for fentanyl and 3.20 for nor-fentanyl.

2.2.3 Preparation of calibration standards, internal standards and spiked controls

2.2.3.1 Fentanyl stock solution (0.662 mg/mL fentanyl as free base)

Approximately 10 mg of fentanyl citrate was weighed and recorded and made up to 10 mL in a volumetric flask with methanol (equivalent to 6.62 mg of fentanyl). The solution was stored in 12 mL screw-capped glass vials (appropriately labelled) at −70°C until required.
2.2.3.2 Fentanyl storage solution (10 mg/L)
A 10 mg/L storage solution was produced by transferring 151 µL of stock solution to a 10 mL volumetric flask and filling the flask to the mark with methanol. The storage solution was then transferred to appropriately labelled 12 mL screw-capped culture tubes and stored at –70 °C.

2.2.3.3 Nor-fentanyl storage solution (10 mg/L)
A 10 mg/L storage solution was produced by transferring 100 µL of 1 mg/mL nor-fentanyl stock solution to a 10 mL volumetric flask and filling the flask to the mark with methanol. The storage solution was then transferred to appropriately labelled 12 mL screw-capped culture tubes and stored at –70 °C.

2.2.3.4 Fentanyl/Nor-fentanyl working solutions (100 µg/L)
One hundred µL of the fentanyl storage solution (10 mg/L) and 100 µL of the nor-fentanyl storage solution (10 mg/L) were placed in a 10 mL volumetric flask. The methanol was evaporated. Ten mL of plasma/saliva was added. This produced 100 µg/L of fentanyl/nor-fentanyl working solution. The solution was transferred to appropriately labelled 12 mL screw-capped culture tubes and stored at –20 °C.

2.2.3.5 Fentanyl/Nor-fentanyl internal standard storage solution (150 µg/L)
One hundred µL of fentanyl-d5 stock solution (15 µg/mL) was transferred to a 10 mL volumetric flask. One hundred and fifty µL of nor-fentanyl-d5 stock solution (10 µg/mL) was transferred to the same volumetric flask, which was then filled to the mark with 18 MΩ of water to produce 150 µg/L of fentanyl/nor-fentanyl internal standard storage solution. This solution was transferred to appropriately labelled 12 mL screw-capped culture tubes and stored at –70 °C.

2.2.3.6 Fentanyl/Nor-fentanyl internal standard working solution (1.5 µg/L)
One hundred µL of internal standard storage solution (150 µg/L) was transferred to a 10 mL volumetric flask, which was then filled to the mark with acetonitrile. This solution was transfer to an appropriately labelled 12 mL screw-capped culture tube for dispensation.
2.2.3.7  **Fentanyl and nor-fentanyl standard dilution solutions for calibration curve**

Fentanyl and nor-fentanyl standard dilution solutions for the calibration curve were prepared from the working solution in a concentration range of 0.02 µg/L, 0.1 µg/L, 0.5 µg/L, 2 µg/L and 10 µg/L (Table 2.5). Standard dilution solutions were prepared in both blank plasma and saliva.

<table>
<thead>
<tr>
<th></th>
<th>10 µg/L (A)</th>
<th>2 µg/L (B)</th>
<th>0.5 µg/L (C)</th>
<th>0.1 µg/L (D)</th>
<th>0.02 µg/L (E)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>*1 mL</td>
<td>*200 µL</td>
<td>*50 µL</td>
<td>100 µL</td>
<td>100 µL</td>
</tr>
<tr>
<td></td>
<td>(From A)</td>
<td>(From B)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Sufficient plasma/saliva to make up to 10 mL

*From fentanyl/nor-fentanyl working solution

A blank sample was prepared by spiking IS in a drug-free plasma and a saliva matrix. A second blank sample was prepared with just blank plasma and a saliva matrix. The working solution and the standard dilution solutions were all stored in –20 °C until required. The quality control (QC) samples with a concentration of 9 µg/L as high control (HC) and 0.045 µg/L as low control (LC) were prepared in both the blank plasma and the saliva matrix respectively. QC samples were quantified in order to evaluate the precision and accuracy of the assay.

2.2.4  **Sample preparation**

2.2.4.1  **Extraction of fentanyl and nor-fentanyl from plasma samples**

Sample preparation involved protein precipitation. 200 µL of sample (standard, control or unknown) and 600 µL of internal standard working solution (1.5 µg/L) were added to an appropriately labelled Eppendorf tube. Samples were vortex-mixed for 30 seconds and centrifuged at 2000 g for 10 mins. The supernatant was transferred to a 12 mL glass culture tube and evaporated to dryness at 40 °C under a stream of nitrogen gas. Samples were reconstituted in 200 µL of MP-A, vortex-mixed for 30 seconds, and then centrifuged at 2000 g for 10 mins. The supernatant 150 µL was then transferred into auto sampler vials and 50 µl injected.
2.2.4.2 Extraction of fentanyl and nor-fentanyl from saliva samples
Where adsorption and low saliva volume wasn’t the limiting factor, extraction of fentanyl and nor-fentanyl from saliva samples was performed similarly to the process used with the plasma sample mentioned in Section 2.2.4.1.

2.2.4.3 Extraction of fentanyl and nor-fentanyl in saliva from CDB
When adsorption of fentanyl and nor-fentanyl occurred in the cotton roll/ bud of the collecting device (CDB), the preparation method was different. The method involved measuring the initial weight of five individual Salivettes® before adding saliva. Once the initial weight had been measured, 300 µL of fentanyl and nor-fentanyl in saliva was pipetted into the CDB at five different concentration ranges, each prepared as a standard dilution solution. Then the final weight was measured. The five concentration ranges were plotted for the calibration curve. Similarly, 300 µL of HC and LC in saliva was pipetted into the CDB, and the initial and final weights were measured. The difference between the initial and final weights should give the weight of the saliva present in the CDB. Likewise, approximately two different unknown volumes of standard dilution solution, each from three concentrations at 2 µg/L, 0.1 µg/L and 0.02 µg/L, were pipetted into the CDB. The initial and final weights were measured. The same procedure was followed for a blank sample and a double blank sample prepared as mentioned in Section 2.2.3. Further, 500 µL of IS working solution was added in each Salivette® containing the CDB. All Salivettes® were allowed to stand for approximately 10 mins. The CDB was removed from the Salivettes® by forceps and placed in a 200 mL glass beaker. Approximately 4 mL of acetonitrile was poured on the CDB. The beaker was shaken and the contents allowed soaking uniformly in the acetonitrile. The acetonitrile was poured into a 12 mL glass culture tube.

The extraction with acetonitrile was repeated three consecutive times. All the acetonitrile was combined in a 12 mL glass culture tube. The extraction procedure was followed for every CDB. Then the acetonitrile was evaporated under nitrogen gas. Once dried, 200 µL of MP-A was added and vortex-mixed for 30 seconds. All the solution was pipetted out into a 1.5 mL Eppendorf tube and centrifuged at 2000 g for 10 mins. 150 µL of supernatant was then transferred into the autosampler vials ready for assay. Two different unknown volumes of saliva from three different concentrations were analysed to normalise the concentration with the standard volume using the weight
difference method. The validation of this extraction procedure was performed according to European Medicines Agency (EMA) guidelines, with some modifications [80].

2.2.4.4 Recovery of fentanyl and nor-fentanyl from CDB

To confirm whether the extraction procedure provides acceptable recovery after normalisation with the standard volume, 150 µL (adjusted volume) each from three standard concentrations (0.02 µg/L, 0.5 µg/L and 2 µg/L) were pipetted into the CDB. Initial and final weights were measured. The extraction procedure was similar to that described in Section 2.2.4.3. Adjusted volumes of saliva samples (150 µL), each from three different concentrations, were analysed to normalise the concentration with the standard volume (200 µL).

The weight difference method was used to calculate the amount of drug in saliva from unknown samples, based on the weight normalised with the standard known volume. The equation below was used to calculate the normalised concentration of analyte from unknown or adjusted volumes of saliva samples:

\[
C_n = \frac{(C_u \times W_s)}{W_u}
\]

where

- \(C_n\) = normalised concentration of analyte in unknown volume of saliva present in CDB
- \(C_u\) = concentration of analyte in unknown volume of saliva present in CDB
- \(W_s\) = weight of standard sample (difference of initial and final weight of Salivette®)
- \(W_u\) = weight of unknown sample (difference of initial and final weight of Salivette®)
- \(C_k\) = calculated concentration of analyte in known volume of saliva present in CDB

If the normalised concentration (\(C_n\)) of the unknown volume of analyte in saliva equals the calculated concentration of standard sample present in the known volume of saliva samples (\(C_k\)), the method should be deemed valid. A recovery rate of 80–120% is considered an acceptable value.

2.2.5 Bioanalytical method validation for plasma

For the purpose of validation, the most recent EMA guidelines were followed, with some modifications [80]. These guidelines are applicable to the validation of analytical methods for PK studies. This study followed all the specified criteria for quantifying fentanyl and nor-fentanyl in human plasma matrix. The validation demonstrated the
selectivity, linearity, accuracy, precision, matrix effect, extraction efficacy and stability of the analyte.

2.2.5.1 Specificity
Specificity was demonstrated by preparing chromatograms taking six samples of blank plasma and saliva each spiked with fentanyl, nor-fentanyl and IS. For acceptable specificity, the chromatogram is expected to have any peak greater than one third of the lower limit of quantitation at the retention time of the analyte.

2.2.5.2 Calibration and Linearity
For the purpose of determining linearity, a calibration curve was generated at five concentration ranges (0.02 µg/L, 0.1 µg/L, 0.5 µg/L, 2 µg/L and 10 µg/L) each for plasma and saliva. A least-square linear regression weighted 1/x², 1/x and none was plotted for each plasma and saliva sample to correlate the best possible % CV (coefficient of variation) for each weighted plot. To be accepted, the linearity should have a correlation coefficient (R²) of 0.99 or better. The back calculated concentration of calibration samples should fall within ± 20 % of the original value. For each experimental session, a new calibration curve was constructed.

2.2.5.3 Inaccuracy and Imprecision
Inaccuracy and imprecision were determined by intra- and inter-day assay. Intra-day assay was done by running five batches of standard samples along with five high control and low control samples in different time frames. Similarly, inter-day assay was performed by running all the specified standards and controls for three different days. The inaccuracy was assessed as a percent deviation of the measured concentration from the original value. Any value within a range of 80–120 % as a mean percent deviation of measured concentration was deemed acceptable for the purpose of determining inaccuracy. The imprecision was evaluated by calculating the % CV. Similarly, any % CV value within 20 % was deemed acceptable for the purpose of determining imprecision. Inaccuracy and imprecision data were then analysed by analysis of variance (ANOVA).

2.2.5.4 Matrix effect (ion suppression)
The assay was tested for matrix effects, including ion suppression, in plasma using the post column infusion (PCI) technique and as mentioned in EMA guidelines (IS-
normalised matrix factor (MF)). Ion suppression using PCI was also performed. 9 µg/L fentanyl and nor-fentanyl in 18 MΩ of water was prepared. Blank plasma samples, each n=6, were prepared from different individuals with IS (deuterated fentanyl and nor-fentanyl). A constant infusion of 9 µg/L fentanyl and nor-fentanyl in 18 MΩ of water was passed through syringe (post column) directly into the ion source. Further blank plasma with IS was passed through the HPLC system using the C18 column simultaneously.

Furthermore, ion-suppression as EMA was performed by taking plasma samples from each of six subjects and spiked with either 0.045 or 9.00 µg/L of fentanyl and nor-fentanyl respectively. For each analyte and IS, the MF was calculated as the ratio of the peak area in the presence of matrix spiked after extraction, to the peak area of the analyte (or IS) prepared in mobile phase A. The IS normalised MF was calculated by dividing the MF of the analyte by the MF of the IS.

2.2.5.5 Stability
A stability study for the analytes in plasma was done using high and low controls. The entire stability test was done by preparing solutions from the same stock, except for (e) and (f), as follows:

a) Auto sampler stability at 4 °C: performed by extracting the samples prepared from the stock solutions, keeping them in the auto sampler for 72 hr and then re-injecting them.

b) Bench-top stability: performed by extracting the controls prepared from the same stock solutions and allowing the controls to stand for 6 hr at laboratory ambient temperature (22 ± 2 °C) before being analysed.

c) Long term stability: performed by storing the controls prepared from the same stock solutions for 60 days at –70 °C. After 60 days the controls were extracted and analysed.

d) Freeze-thaw stability: performed by freezing and thawing the controls prepared in plasma and saliva samples from the same stock solutions for three cycles and analysing them.

e) Eight-months stability: performed by spiking the plasma samples with fentanyl at two different known concentrations (0.05 and 0.5 µg/L) and storing them for 8 months at –70 °C. After 8 months they were analysed using the freshly prepared
standards. The per cent recovery was calculated against the original spiked concentrations.

f) Two--years stability: performed by storing the patient samples for 2 years at –70 °C. The samples were analysed for both fentanyl and nor-fentanyl as soon as they were received. After 2 years they were analysed again using the freshly prepared standards. The per cent recovery was then calculated. Similarly, plasma samples spiked with three known concentrations (0.5, 2 and 10 µg/L) of fentanyl and nor-fentanyl were also stored for 2 years at –70 °C. After 2 years all the samples were analysed using freshly prepared standards. The per cent recovery was calculated against the original spiked concentrations.

2.2.5.6 Limit of quantification
The limit of quantification (LOQ) was determined for each analyte and matrix for which linearity, accuracy and imprecision had been demonstrated to be adequate.

2.2.6 Bioanalytical method validation for saliva
The bioanalytical method validation for saliva was performed in the same way as for plasma, as described in Section 2.2.5, in general following the most recent EMA guidelines. The validation was performed for both the extraction procedures described in Sections 2.2.4.2 and 2.2.4.3.

2.3 Results and discussion
Preliminary sampling demonstrated that patients were often unable to provide adequate saliva volumes to allow collection from the CDB by centrifugation. Further, significant adsorption was demonstrated to the CDB when tested using spiked samples. Fentanyl and nor-fentanyl at two different concentrations (0.045 and 9 µg/L) were prepared in duplicate in saliva and passed through the CDB. After centrifuging, the samples were assayed and compared with the solution without passing through the CDB. The result showed that fentanyl and nor-fentanyl was adsorbed up to almost 50 % and 70 % in the CDB respectively (Table 2.6).
Table 2.6 Adsorption of fentanyl and nor-fentanyl in CDB of Salivette®

<table>
<thead>
<tr>
<th>Spiked conc. (µg/L)</th>
<th>Fentanyl (n=3)</th>
<th>Nor-fentanyl (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean assayed conc. (µg/L)</td>
<td>Mean recovered conc. (µg/L)</td>
</tr>
<tr>
<td>9 µg/L</td>
<td>8.57</td>
<td>3.69</td>
</tr>
<tr>
<td>0.045 µg/L</td>
<td>0.062</td>
<td>0.037</td>
</tr>
</tbody>
</table>

Hence this study also demonstrated a practical approach to using a Salivette® to collect saliva by overcoming two major drawbacks: firstly, when drugs adsorb to the CDB, and secondly, when low saliva volumes are obtained. The amount of drug in saliva was calculated based upon the weight normalised with standard known volume (control). All the values obtained were within the acceptable criteria. This demonstrated that the extraction method was valid and should be applicable for PKPD studies.

Moreover, there are also various devices commercially available which could collect saliva as low as 0.03 mL [62], but such low volumes are not sufficient for some HPLC assay methods, for which samples as high as 200-300 µL are required. Hence this method should be able to calculate the amount of drug in low saliva volume by normalising with the standard known volume. Furthermore, ion suppression can have a significant deleterious impact on HPLC-MS/MS assays [72], and previously reported assay methods have not considered the issue of ion suppression and/or other matrix effects on plasma and saliva [64, 65]. Current data demonstrate that this has limited, if any, impact on the method developed. Salivette® was used in this study; however, this method should be useful for any commercially available oral fluid collecting device similar to Salivette®.

In the initial method development, various organic solvents were used in extracting fentanyl and nor-fentanyl from the CDB, and several methods of achieving an acceptable recovery were tried. Preliminary extraction using acetonitrile and dichloromethane generated better recovery than when other solvents were used. However, acetonitrile produced a higher recovery rate than dichloromethane in repeated extraction. Hence acetonitrile was used in the extraction protocol. Based on the recovery from preliminary extraction by acetonitrile from the CDB, a pre-validation, single-day study was also performed with just one set of standards and controls (Table 2.7). The
pre-validation study generated a good recovery and linearity. Hence a full validation study was performed for the extraction of fentanyl and nor-fentanyl from the CDB.

Table 2.7 Pre-validation data using acetonitrile to extract fentanyl and nor-fentanyl from CDB

<table>
<thead>
<tr>
<th>Spiked conc. (µg/L)</th>
<th>#Sample volume (µL)</th>
<th>Fentanyl</th>
<th>Nor-fentanyl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Calculated conc. (µg/L)</td>
<td>Normalised conc. (µg/L)</td>
</tr>
<tr>
<td>0.02</td>
<td>299</td>
<td>0.021</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>296</td>
<td>0.561</td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>295</td>
<td>2.07</td>
<td></td>
</tr>
<tr>
<td>0.045†</td>
<td>298</td>
<td>9.48</td>
<td></td>
</tr>
<tr>
<td>9.0†</td>
<td>297</td>
<td>0.050</td>
<td></td>
</tr>
<tr>
<td>0.02*</td>
<td>198</td>
<td>0.014</td>
<td>0.022</td>
</tr>
<tr>
<td>0.5*</td>
<td>200</td>
<td>0.378</td>
<td>0.566</td>
</tr>
<tr>
<td>2.0*</td>
<td>200</td>
<td>1.44</td>
<td>2.15</td>
</tr>
</tbody>
</table>

# Difference of initial and final weight of Salivette® after pouring 300 and 200 µl of saliva as standard and test concentrations respectively

*test concentration
†quality controls

2.3.1 Bioanalytical method validation

2.3.1.1 Specificity

Blank and spiked chromatograms for both analytes and both internal standards in plasma and saliva typical of the six obtained demonstrated the absence of any chromatographic and/or mass spectrometric interference. Specificity for both the plasma and saliva samples was within the accepted criteria. The mean interfering peak was observed well below one third of the lower limit of quantification at the retention time of analyte, fulfilling the criteria for both plasma (Figure 2.8) and (Figure 2.9). No interfering peaks were observed with either of the matrix at the retention time. Internal standards for both plasma (Figure 2.10) and saliva (Figure 2.11) also did not produce any interference peak at the retention time.
Figure 2.8 Specificity: Plasma (fentanyl and nor-fentanyl). Chromatogram showing extract of blank human plasma and analyte at the lower limits of quantification (LLOQ) from bottom to top: (a) blank plasma at nor-fentanyl transition (b) blank plasma at fentanyl transition (c) nor-fentanyl at LLOQ (d) fentanyl at LLOQ

Figure 2.9 Specificity: Plasma (fentanyl IS and nor-fentanyl IS). Chromatogram showing extract of blank human plasma and IS from bottom to top: (a) blank plasma at nor-fentanyl-d₅ transition (b) blank plasma at fentanyl-d₅ transition (c) nor-fentanyl-d₅ (d) fentanyl-d₅
Figure 2.10 Specificity: Saliva (fentanyl and nor-fentanyl). Chromatogram showing extract of blank human saliva and analyte at LLOQ from bottom to top: (a) blank saliva at nor-fentanyl transition (b) blank saliva at fentanyl transition (c) nor-fentanyl at LLOQ (d) fentanyl at LLOQ

Figure 2.11 Specificity: Saliva (fentanyl IS and nor-fentanyl IS). Chromatogram showing extract of blank human saliva and IS from bottom to top: (a) blank saliva at nor-fentanyl-d$_5$ transition (b) blank saliva at fentanyl-d$_5$ transition (c) nor-fentanyl-d$_5$ (d) fentanyl-d$_5$
2.3.1.2 Limit of quantification and sensitivity

Based on acceptable imprecision, accuracy and linearity for both analytes at 0.030 µg/L for plasma and 0.045 µg/L for saliva, these values have been set as the respective lower limits of quantification (LLOQs). Similarly, based on demonstrated acceptable imprecision, accuracy and linearity at a concentration of 9.0 µg/L for both plasma and saliva, this has been set as the upper limit of quantification (ULOQ).

2.3.1.3 Calibration curve and linearity

Calibration curves were plotted for fentanyl and nor-fentanyl prepared from both human plasma (Figure 2.12 and Figure 2.13) and saliva (Figure 2.14 and Figure 2.15) respectively in a range of 0.02, 0.1, 0.5, 2 and 10 µg/L, using a 1/x^2 weighting factor. This weighting factor was used because it was found to produce optimal accuracy and imprecision data. The linearity in plasma was demonstrated by an average (n=4) $R^2$ (± SEM) of 0.9989 ± 0.0002 for fentanyl and 0.9988 ± 0.0003 for nor-fentanyl. Similarly, linearity in saliva was demonstrated by an average (n=4) $R^2$ (± SEM) value of 0.9993 ± 0.0002 for fentanyl and 0.9994 ± 0.0002 for nor-fentanyl. Likewise the calibration curve for saliva extracted directly from the CDBs was plotted for fentanyl and nor-fentanyl respectively. Linearity was demonstrated by an average (n=4) $R^2$ (± SEM) value of 0.9991 ± 0.0003 for fentanyl and 0.9971 ± 0.0007 for nor-fentanyl.

![Figure 2.12 Standard calibration curve for fentanyl spiked in blank human plasma at different concentrations (0.02, 0.1, 0.5, 2 and 10 µg/L).](image)
Figure 2.13  Standard calibration curve for nor-fentanyl spiked in blank human plasma at different concentrations (0.02, 0.1, 0.5, 2 and 10 µg/L)

\[ y = 0.4803x + 0.026 \]

\[ R^2 = 0.9998 \]

Figure 2.14  Standard calibration curve for fentanyl spiked in blank human saliva at different concentrations (0.02, 0.1, 0.5, 2 and 10 µg/L)

\[ y = 0.4642x + 0.0033 \]

\[ R^2 = 0.9999 \]
2.3.1.4 Inaccuracy and Imprecision

Intra- and inter-day imprecision and inaccuracy data are presented in Table 2.8, Table 2.9 and Table 2.10. The inaccuracy for fentanyl and nor-fentanyl in both plasma and saliva was within 96-108 %, and imprecision expressed as % CV was below 9 % in all cases except for nor-fentanyl in plasma, where it was 15.5 %.

Table 2.8 Intra- and inter-day inaccuracy and imprecision of fentanyl and nor-fentanyl in human plasma

<table>
<thead>
<tr>
<th></th>
<th>Inaccuracy (%)</th>
<th>Intra-day imprecision (% CV)</th>
<th>Inter-day imprecision (% CV)</th>
<th>Total imprecision (% CV)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Spiked conc. (µg/L)</strong></td>
<td><strong>Mean assayed conc. (µg/L)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fentanyl</td>
<td>100.8</td>
<td>4.39</td>
<td>7.21</td>
<td>8.44</td>
</tr>
<tr>
<td>Nor-fentanyl</td>
<td>96.2</td>
<td>9.64</td>
<td>12.4</td>
<td>15.5</td>
</tr>
<tr>
<td><strong>0.030</strong></td>
<td><strong>0.030</strong></td>
<td><strong>8.84</strong></td>
<td><strong>3.10</strong></td>
<td><strong>4.02</strong></td>
</tr>
<tr>
<td><strong>9.0</strong></td>
<td><strong>8.80</strong></td>
<td><strong>97.8</strong></td>
<td><strong>6.87</strong></td>
<td><strong>7.69</strong></td>
</tr>
</tbody>
</table>
### Table 2.9 Inaccuracy and imprecision data for fentanyl and nor-fentanyl in human saliva

<table>
<thead>
<tr>
<th></th>
<th>Spiked conc. (µg/L)</th>
<th>Mean assayed conc. (µg/L)</th>
<th>Inaccuracy (%)</th>
<th>Intra-day imprecision (% CV)</th>
<th>Inter-day imprecision (% CV)</th>
<th>Total imprecision (% CV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fentanyl</td>
<td>0.045</td>
<td>0.044</td>
<td>98.6</td>
<td>2.22</td>
<td>2.71</td>
<td>3.50</td>
</tr>
<tr>
<td></td>
<td>9.0</td>
<td>8.97</td>
<td>99.7</td>
<td>2.15</td>
<td>0.94</td>
<td>3.08</td>
</tr>
<tr>
<td>Nor-fentanyl</td>
<td>0.045</td>
<td>0.046</td>
<td>102.8</td>
<td>3.65</td>
<td>2.77</td>
<td>4.58</td>
</tr>
<tr>
<td></td>
<td>9.0</td>
<td>9.14</td>
<td>101.6</td>
<td>2.59</td>
<td>1.14</td>
<td>3.73</td>
</tr>
</tbody>
</table>

Furthermore, fentanyl and nor-fentanyl concentration in some of the saliva samples from patients exceeded the linearity range, i.e. 10 µg/L. Since the saliva extraction method from the CDB as it developed is a one-off process that utilises all the saliva samples, no leftover saliva samples were available for further repetition to dilute the sample and quantify the analyte within the linear range. Hence a new standard calibration curve was generated with concentrations ranging from 0.02 µg/L to 50 µg/L.

### Table 2.10 Intra- and inter-day inaccuracy and imprecision of fentanyl and nor-fentanyl in human saliva, performed by extracting analyte from CDB (Salivette®) for calibration curve ranging from 0.02 to 10 µg/L

<table>
<thead>
<tr>
<th></th>
<th>Spiked conc. (µg/L)</th>
<th>Mean assayed conc. (µg/L)</th>
<th>Inaccuracy (%)</th>
<th>Intra-day imprecision (% CV)</th>
<th>Inter-day imprecision (% CV)</th>
<th>Total imprecision (% CV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fentanyl</td>
<td>0.045</td>
<td>0.045</td>
<td>100.7</td>
<td>2.85</td>
<td>2.89</td>
<td>4.20</td>
</tr>
<tr>
<td></td>
<td>9.0</td>
<td>9.42</td>
<td>104.7</td>
<td>3.79</td>
<td>1.79</td>
<td>5.58</td>
</tr>
<tr>
<td>Nor-fentanyl</td>
<td>0.045</td>
<td>0.047</td>
<td>104.1</td>
<td>4.83</td>
<td>4.25</td>
<td>6.44</td>
</tr>
<tr>
<td></td>
<td>9.0</td>
<td>9.71</td>
<td>107.9</td>
<td>5.65</td>
<td>6.76</td>
<td>7.38</td>
</tr>
</tbody>
</table>

### Table 2.11

Furthermore, fentanyl and nor-fentanyl concentration in some of the saliva samples from patients exceeded the linearity range, i.e. 10 µg/L. Since the saliva extraction method from the CDB as it developed is a one-off process that utilises all the saliva samples, no leftover saliva samples were available for further repetition to dilute the sample and quantify the analyte within the linear range. Hence a new standard calibration curve was generated with concentrations ranging from 0.02 µg/L to 50 µg/L. Three quality controls were used: 0.045, 25 and 45 µg/L. Inaccuracy and imprecision were within the acceptable range (Table 2.11).
Table 2.11 Inaccuracy and imprecision data for fentanyl and nor-fentanyl in human saliva, obtained by extracting analyte from CDB (Salivette®) for calibration curve ranging from 0.02 to 50 µg/L

<table>
<thead>
<tr>
<th></th>
<th>Spiked conc. (µg/L)</th>
<th>Mean assayed conc. (µg/L)</th>
<th>Inaccuracy (%)</th>
<th>Intra-day imprecision (% CV)</th>
<th>Inter-day imprecision (% CV)</th>
<th>Total imprecision (% CV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fentanyl</td>
<td>0.045</td>
<td>0.047</td>
<td>105.30</td>
<td>1.85</td>
<td>2.34</td>
<td>2.09</td>
</tr>
<tr>
<td></td>
<td>25.0</td>
<td>25.59</td>
<td>102.40</td>
<td>1.92</td>
<td>3.23</td>
<td>2.57</td>
</tr>
<tr>
<td></td>
<td>45.0</td>
<td>45.61</td>
<td>101.35</td>
<td>1.03</td>
<td>4.71</td>
<td>2.88</td>
</tr>
<tr>
<td>Nor-fentanyl</td>
<td>0.045</td>
<td>0.045</td>
<td>101.07</td>
<td>2.36</td>
<td>2.33</td>
<td>2.35</td>
</tr>
<tr>
<td></td>
<td>25.0</td>
<td>23.17</td>
<td>92.63</td>
<td>7.74</td>
<td>11.36</td>
<td>9.36</td>
</tr>
<tr>
<td></td>
<td>45.0</td>
<td>43.62</td>
<td>96.92</td>
<td>3.28</td>
<td>7.55</td>
<td>5.35</td>
</tr>
</tbody>
</table>

2.3.1.5 Matrix effect (ion suppression)

Matrix factors and IS-normalised MFs are summarised in Table 2.12. While ion suppression occurs for fentanyl and nor-fentanyl, the reduced % CV of the IS-normalised MFs for both analytes in both matrices suggests that, provided the matching blank matrix is used to prepare calibrators (as opposed to pure solution), the IS will normalise adequately for the matrix effects occurring.

Table 2.12 Matrix factor for fentanyl and nor-fentanyl in plasma and saliva

<table>
<thead>
<tr>
<th></th>
<th>Plasma</th>
<th>Saliva</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MF</td>
<td>MF</td>
<td>MF</td>
<td>MF</td>
<td>MF</td>
<td>MF</td>
<td>MF</td>
<td>MF</td>
<td>MF</td>
<td>MF</td>
<td>MF</td>
<td>MF</td>
</tr>
<tr>
<td></td>
<td>(Analyte)</td>
<td>(IS)</td>
<td>(IS normalised)</td>
<td>(Analyte)</td>
<td>(IS)</td>
<td>(IS normalised)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fentanyl</td>
<td>HC</td>
<td>1.11</td>
<td>1.04</td>
<td>1.07</td>
<td>1.03</td>
<td>0.95</td>
<td>1.09</td>
<td>(15.3)</td>
<td>(15.3)</td>
<td>(1.9)</td>
<td>(20.8)</td>
<td>(21.5)</td>
</tr>
<tr>
<td></td>
<td>LC</td>
<td>1.86</td>
<td>1.85</td>
<td>1.02</td>
<td>1.05</td>
<td>1.05</td>
<td>0.991</td>
<td>(9.8)</td>
<td>(10.0)</td>
<td>(2.1)</td>
<td>(23.7)</td>
<td>(23.8)</td>
</tr>
<tr>
<td>Nor-fentanyl</td>
<td>HC</td>
<td>1.24</td>
<td>1.13</td>
<td>1.09</td>
<td>0.993</td>
<td>0.965</td>
<td>1.03</td>
<td>(11.4)</td>
<td>(10.9)</td>
<td>(2.30)</td>
<td>(22.9)</td>
<td>(24.4)</td>
</tr>
<tr>
<td></td>
<td>LC</td>
<td>1.19</td>
<td>1.20</td>
<td>0.99</td>
<td>1.08</td>
<td>1.05</td>
<td>1.03</td>
<td>(10.6)</td>
<td>(10.9)</td>
<td>(1.60)</td>
<td>(26.5)</td>
<td>(26.0)</td>
</tr>
</tbody>
</table>

Post column infusion (PCI) was also performed to test ion suppression for the extracted blank plasma (Figure 2.16) and saliva (Figure 2.17) samples. However, the chromatogram obtained didn’t have a stable base line; rather it increased with the run and dropped down at the end of the run.
Figure 2.16 PCI of fentanyl and nor-fentanyl in extracted human plasma using gradient flow (progressing organic phase from 10 % to 100 % over 4.5 mins and held at 100 % organic phase for 1.5 mins, then reduced to 10 % organic phase for the remainder of the run)

Figure 2.17 PCI of fentanyl and nor-fentanyl in extracted human saliva using gradient flow (progressing organic phase from 10 % to 100 % over 4.5 mins and held at 100 % organic phase for 1.5 mins then reduced to % organic phase for the remainder of the run)

A PCI was performed with mobile phase to see if the chromatogram behaved similarly to the saliva and plasma matrices using our usual gradient flow method. However, the mobile phase itself behaved similarly to the plasma and saliva matrices. This could have been due to a change in the organic phase concentration (gradient flow) as the run progressed. Hence more experiments were performed to find out whether gradient flow had any effect on the run.
Initially a PCI was performed using 10% organic phase for the entire run (isocratic flow). The chromatogram generated had a stable lower base line (Figure 2.18). Further, the organic phase was changed to 100 % for the entire run. This also generated a stable base line, but the base line was observed at much higher counts per second (Figure 2.19). Likewise, a PCI was performed with gradient flow, as developed for our method. The gradient run showed an increase in the base line as the concentration of organic phase increased, reaching 100 % before finally dropping down once the gradient fell back to 10 % (Figure 2.20).

![Figure 2.18 PCI of fentanyl and nor-fentanyl in mobile phase using 10 % organic phase](image-url)
Figure 2.19 PCI of fentanyl and nor-fentanyl in mobile phase using 100 % organic phase

Figure 2.20 PCI of fentanyl and nor-fentanyl in mobile phase using gradient flow

Hence it was assumed that in gradient flow, the organic phase enhances ionisation in the ion source as it increases, thus increasing the base line. Therefore, the PCI of fentanyl and nor-fentanyl in plasma and saliva resulted in a similar behaviour with the background matrix effect due to the changing mobile phase composition over time. However, as deuterated IS was used for both fentanyl and nor-fentanyl, any possible ion
suppression/enhancement should have been compensated for, as observed for the IS-normalised MFs for both analytes in both matrices, as per the EMA method.

2.3.1.6 Stability
The stability study was performed under various laboratory conditions (Table 2.13 and Table 2.14). No significant loss and degradation of analyte and IS was observed. Stability within the auto sampler for 72 hr at 4 °C and at bench top for 6 hr at 22 ± 2 °C was well maintained. Similarly, repeated freezing and thawing did not significantly affect any analyte assayed. Likewise, no effect on analyte was observed with long-term storage at –70 °C. The result achieved suggested that none of the steps taken during sample preparation, sample analysis and storage affected the analysis.

Table 2.13 Stability study of fentanyl and nor-fentanyl in human plasma

<table>
<thead>
<tr>
<th>Stability condition</th>
<th>Spiked conc. (µg/L)</th>
<th>Mean assayed conc. (n=3) (µg/L)</th>
<th>Imprecision (% CV)</th>
<th>Inaccuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fentanyl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autosampler (4 °C) for 72 hr</td>
<td>0.045</td>
<td>0.043</td>
<td>1.80</td>
<td>95.6</td>
</tr>
<tr>
<td></td>
<td>9.0</td>
<td>8.73</td>
<td>1.08</td>
<td>97.0</td>
</tr>
<tr>
<td>Bench-top (24 °C) for 6 hr</td>
<td>0.045</td>
<td>0.045</td>
<td>3.62</td>
<td>99.1</td>
</tr>
<tr>
<td></td>
<td>9.0</td>
<td>8.77</td>
<td>0.46</td>
<td>97.4</td>
</tr>
<tr>
<td>Freezer (–80 °C) for 60 days</td>
<td>0.045</td>
<td>0.045</td>
<td>0.95</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>9.0</td>
<td>8.61</td>
<td>2.32</td>
<td>95.7</td>
</tr>
<tr>
<td>Freeze-thaw for three cycles</td>
<td>0.045</td>
<td>0.045</td>
<td>4.01</td>
<td>99.8</td>
</tr>
<tr>
<td></td>
<td>9.0</td>
<td>8.81</td>
<td>1.43</td>
<td>97.9</td>
</tr>
<tr>
<td>Nor-fentanyl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autosampler (4 °C) for 72 hr</td>
<td>0.045</td>
<td>0.042</td>
<td>1.08</td>
<td>92.9</td>
</tr>
<tr>
<td></td>
<td>9.0</td>
<td>8.55</td>
<td>4.20</td>
<td>95.0</td>
</tr>
<tr>
<td>Bench-top (24 °C) for 6 hr</td>
<td>0.045</td>
<td>0.041</td>
<td>4.98</td>
<td>91.6</td>
</tr>
<tr>
<td></td>
<td>9.0</td>
<td>8.45</td>
<td>1.60</td>
<td>93.9</td>
</tr>
<tr>
<td>Freezer (–80 °C) for 60 days</td>
<td>0.045</td>
<td>0.043</td>
<td>7.02</td>
<td>96.2</td>
</tr>
<tr>
<td></td>
<td>9.0</td>
<td>8.32</td>
<td>0.87</td>
<td>92.4</td>
</tr>
<tr>
<td>Freeze-thaw for three cycles</td>
<td>0.045</td>
<td>0.044</td>
<td>5.99</td>
<td>97.8</td>
</tr>
<tr>
<td></td>
<td>9.0</td>
<td>8.40</td>
<td>2.15</td>
<td>93.3</td>
</tr>
</tbody>
</table>
Table 2.14 Stability study of fentanyl and nor-fentanyl in human saliva

<table>
<thead>
<tr>
<th>Stability condition</th>
<th>Spiked conc.(µg/L)</th>
<th>Mean assayed conc. (n=3) (µg/L)</th>
<th>Imprecision (% CV)</th>
<th>Inaccuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fentanyl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autosampler (4 °C) for 72 hr</td>
<td>0.045</td>
<td>0.043</td>
<td>0.76</td>
<td>94.4</td>
</tr>
<tr>
<td></td>
<td>9.0</td>
<td>9.02</td>
<td>0.56</td>
<td>100.2</td>
</tr>
<tr>
<td>Bench-top (24 °C) for 6 hr</td>
<td>0.045</td>
<td>0.042</td>
<td>0.46</td>
<td>92.4</td>
</tr>
<tr>
<td></td>
<td>9.0</td>
<td>8.80</td>
<td>6.62</td>
<td>97.8</td>
</tr>
<tr>
<td>Freezer (–80 °C) for 60 days</td>
<td>0.045</td>
<td>0.044</td>
<td>3.36</td>
<td>96.9</td>
</tr>
<tr>
<td></td>
<td>9.0</td>
<td>9.24</td>
<td>0.24</td>
<td>102.7</td>
</tr>
<tr>
<td>Freeze-thaw for three cycles</td>
<td>0.045</td>
<td>0.044</td>
<td>0.81</td>
<td>97.3</td>
</tr>
<tr>
<td></td>
<td>9.0</td>
<td>8.88</td>
<td>2.17</td>
<td>98.7</td>
</tr>
<tr>
<td>Nor-fentanyl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autosampler (4 °C) for 72 hr</td>
<td>0.045</td>
<td>0.044</td>
<td>3.03</td>
<td>98.7</td>
</tr>
<tr>
<td></td>
<td>9.0</td>
<td>9.32</td>
<td>1.59</td>
<td>103.6</td>
</tr>
<tr>
<td>Bench-top (24 °C) for 6 hr</td>
<td>0.045</td>
<td>0.046</td>
<td>3.32</td>
<td>101.8</td>
</tr>
<tr>
<td></td>
<td>9.0</td>
<td>8.86</td>
<td>9.77</td>
<td>98.4</td>
</tr>
<tr>
<td>Freezer (–80 °C) for 60 days</td>
<td>0.045</td>
<td>0.045</td>
<td>1.98</td>
<td>100.7</td>
</tr>
<tr>
<td></td>
<td>9.0</td>
<td>9.26</td>
<td>0.87</td>
<td>102.9</td>
</tr>
<tr>
<td>Freeze-thaw for three cycles</td>
<td>0.045</td>
<td>0.045</td>
<td>2.38</td>
<td>100.4</td>
</tr>
<tr>
<td></td>
<td>9.0</td>
<td>9.42</td>
<td>0.17</td>
<td>104.7</td>
</tr>
</tbody>
</table>

Similarly, 8 months storage stability did not show significant deterioration of fentanyl, and the mean per cent recovery for both of the spiked concentrations was 94.25% (Table 2.15). However, a 2-year stability study with plasma samples (both patient samples and spiked plasma) showed a mean per cent recovery of 84.5% for fentanyl, suggesting long-term storage could cause the compound to deteriorate. However, there was no significant deterioration of nor-fentanyl, with a mean per cent recovery of 98.7% (Table 2.16). This could possibly be due to a change in the pH of the plasma matrix over the time of the study, resulting in the ionisation of fentanyl and/or its conversion to nor-fentanyl.
Table 2.15 Stability study (8 months) of fentanyl in human plasma

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Conc. (µg/L) (Before 8 months)</th>
<th>Conc. (µg/L) (After 8 Months)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05–8M</td>
<td>0.05</td>
<td>0.048</td>
<td>95.4</td>
</tr>
<tr>
<td>0.5–8M</td>
<td>0.5</td>
<td>0.466</td>
<td>93.1</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td>94.25</td>
</tr>
</tbody>
</table>

Table 2.16 Stability study (2 years) of fentanyl and nor-fentanyl in human plasma

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Fentanyl Conc. (µg/L) (under 2 yrs)</th>
<th>Fentanyl Conc. (µg/L) (after 2 yrs)</th>
<th>Fentanyl Recovery (%)</th>
<th>Nor-fentanyl Conc. (µg/L) (under 2 yrs)</th>
<th>Nor-fentanyl Conc. (µg/L) (after 2 yrs)</th>
<th>Nor-fentanyl Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5–2 yrs</td>
<td>0.500</td>
<td>0.43</td>
<td>86.0</td>
<td>0.50</td>
<td>0.473</td>
<td>94.6</td>
</tr>
<tr>
<td>2–2 yrs</td>
<td>2.0</td>
<td>1.73</td>
<td>86.3</td>
<td>2.0</td>
<td>1.94</td>
<td>97.0</td>
</tr>
<tr>
<td>10–2 yrs</td>
<td>10.0</td>
<td>8.51</td>
<td>85.1</td>
<td>10.0</td>
<td>9.93</td>
<td>99.3</td>
</tr>
<tr>
<td>S00174</td>
<td>1.110</td>
<td>0.938</td>
<td>84.5</td>
<td>0.024</td>
<td>0.022</td>
<td>91.5</td>
</tr>
<tr>
<td>S00176</td>
<td>0.561</td>
<td>0.460</td>
<td>82.0</td>
<td>0.235</td>
<td>0.253</td>
<td>107.4</td>
</tr>
<tr>
<td>S00186</td>
<td>0.920</td>
<td>0.784</td>
<td>85.2</td>
<td>0.487</td>
<td>0.500</td>
<td>102.6</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td>84.8</td>
<td></td>
<td></td>
<td>98.7</td>
</tr>
</tbody>
</table>

2.3.1.7 Recovery of fentanyl and nor-fentanyl from CDB

The normalised value and per cent recovery for adjusted volumes of analyte in saliva for three concentrations (0.02 µg/L, 0.5 µg/L and 2 µg/L) was within the acceptable value (Table 2.17).

Table 2.17 Normalised concentration and percent recovery of fentanyl and nor-fentanyl in saliva from CDB extraction using three different spiked concentrations (0.02, 0.5 and 2 µg/L)

<table>
<thead>
<tr>
<th>Fentanyl (n=3)</th>
<th>Nor-fentanyl (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean assayed conc. (µg/L) (Ck)</td>
<td>Mean assayed conc. (µg/L) (Ck)</td>
</tr>
<tr>
<td>Mean normalised conc. (µg/L) (Cn)</td>
<td>Mean normalised conc. (µg/L) (Cn)</td>
</tr>
<tr>
<td>Mean recovery (%)</td>
<td>Mean recovery (%)</td>
</tr>
<tr>
<td>0.020 ± 0.0</td>
<td>0.019 ± 0.0</td>
</tr>
<tr>
<td>0.487 ± 0.0</td>
<td>0.489 ± 0.0</td>
</tr>
<tr>
<td>2.011± 0.0</td>
<td>2.10 ± 0.0</td>
</tr>
</tbody>
</table>

Ck = calculated concentration of analyte in known volume of saliva present in CDB
Cn = normalised concentration of analyte in unknown volume of saliva present in CDB
2.4 Conclusion

A simultaneous assay was developed and validated according to EMA guidelines for both fentanyl and nor-fentanyl in both human plasma and saliva. This method was successful in quantifying fentanyl and nor-fentanyl at concentrations as low as 0.045 and 0.03 µg/L respectively for saliva and plasma. In contrast to previous reports, the current method uses a very simple one-step sample preparation. Such simple and cost-effective methods are very useful when analysing multiple samples in routine laboratory tests. This extraction method should be able to be utilised without difficulty for those drugs that do not adsorb to saliva collection devices. However, in situations where saliva samples need to be collected using a collecting device such as a Salivette®, a different extraction method was developed that should be useful for any lipophilic drug, such as fentanyl, that displays adsorption issues in the collecting device.

Hence, the method presented here is sensitive, accurate and precise for quantifying plasma and saliva samples for both fentanyl and nor-fentanyl. It is also free of any impact from ion suppression. Further, this study also presents validation of the direct extraction of the analytes from a CDB as used in a Salivette®.

The results from this study have been published [81], and presented at conferences and other professional forums.
Chapter 3. Protein binding of fentanyl and its metabolite nor-fentanyl in human plasma, albumin and α-1 acid glycoprotein

3.1 Introduction

The plasma protein binding (PPB) of drugs has been shown to have significant effects on many aspects of clinical PK and PD. The variability in protein plasma levels and the subsequent impact on the extent of drug binding can cause modifications in the mode of drug action, distribution, disposition and elimination. Generally, it is believed that only the unbound drug is available for PD interactions with receptors. Protein binding also has a large influence on drug distribution and elimination [82]. In the early stages of drug development, PPB experiments advance understanding of absorption, distribution, metabolism, excretion (ADME) properties, assisting in candidate selection [83]. Highly plasma protein bound drugs such as warfarin, valproic acid and valproate sodium, as well as the penicillins, typically has a relatively low volume of distribution, since their strong association with plasma proteins confines them to vascular spaces. On the other hand, drugs that remain largely unbound in plasma are generally available for distribution out of the vascular system. However, the volume of distribution is also affected by the magnitude of drug binding by tissue proteins. Several drugs, such as amiodarone, digoxin, and tricyclic antidepressants, although highly bound to plasma proteins, are bound with greater affinity to tissue proteins, resulting in large volumes of distribution [84]. Unbound drug concentration is also important in the consideration of drug clearance. Only the unbound drug is available for glomerular filtration and, in some cases, hepatic clearance. However, for high extraction ratio drugs, clearance is independent of protein binding [85].

Serum ALB, with a molecular weight of 62 kD, is the most abundant plasma protein produced by the mature liver and functions as a transporter of various substances [86]. ALB is exclusively synthesised in the liver, at approximately 200 mg/kg per day, with a concentration of 35–50 g/L in human plasma [87]. ALB possesses a net cationic charge and thus attracts weakly acidic drugs by means of ionic binding. ALB also binds lipophilic compounds via hydrophobic binding forces [88]. While ALB is the most abundant plasma protein, many drugs exhibit significant binding to other plasma proteins as well.
AAG, an acute phase protein, is another plasma protein reported to bind drugs. Acute-phase proteins are proteins whose plasma concentrations increase in response to inflammation. One of the most important acute phase proteins is AAG; it is the principal binding protein for basic drugs, including lidocaine, verapamil, imipramine, propranolol amongst others. However, it can also bind acidic drugs such as Phenobarbital \[89\] and endogenous steroids (cortisol) \[90\]. AAG has a molecular weight of 42 kD, with a single chain of 183 amino acids \[58\]. The normal plasma concentration range of AAG is 0.4 to 1.0 g/L \[91\]. AAG has an anionic charge, and thus many basic drugs bind with high affinity to it \[92\].

Similarly, many other blood components, such as erythrocytes, lipoproteins, and \(\alpha\), \(\beta\) and \(\gamma\) globulins, are also capable of drug-protein interactions \[82, 93\]. Lipoproteins have been described as binding some basic drugs such as amitriptyline and nortriptyline \[94\]. Complement C3 has been reported to be related to imipramine binding \[95\] and the plasma protein binding of the non-steroidal anti-inflammatory agent, timegadine, was increased by enrichment of plasma with C-reactive protein, \(\alpha\)-antitrypsin and AAG \[96\]. Hence drugs may bind to more than one plasma protein.

Many methods have been reported to determine free and bound drug in plasma samples. Methodologies including microdialysis, equilibrium dialysis, ultrafiltration (UF), dynamic dialysis, ultracentrifugation, gel filtration, electrophoresis, spectrophotometry and enzyme kinetic methods have been reported \[97-100\]. However, many of these techniques are seldom used. Micro dialysis, equilibrium dialysis and UF have been used more widely in recent years \[82\]. Moreover, the UF method has been more popular due to its simplicity, which has enabled many researchers to use it, \[100\] with various modifications \[72, 101\]. It can also be applied to different, types of biological matrices, including tissue homogenates \[82\]. The major drawback of the UF device is the adsorption of drug to the membrane. However, there are a number of UF devices being manufactured, based upon the physiochemical property of the drug. The selection of a UF device that is specific to the compound of interest is usually beneficial. A typical UF device is simple to operate. The drug containing plasma is placed in an UF unit (these are commercially available and consist of two reservoirs separated by a filter); the filter allows plasma water and lower molecular weight compounds to pass through, while larger molecules such as plasma proteins are retained. The plasma water is forced through the filter by either negative pressure (centrifugation) or positive pressure (\(\text{N}_2\)).
gas or a syringe). As the plasma sample is filtered, the ultrafiltrate that emerges in the bottom reservoir is considered to contain a free or unbound concentration.

Fentanyl has been reported to bind to plasma proteins ranging from 80 to 85% \([54-58]\); however, there is controversy surrounding its binding specificity. Some studies have indicated that it binds to ALB \([54, 55]\), whereas others have reported binding primarily to AAG \([56, 57]\), which would be more consistent with its basic chemical nature. Moreover, there are no studies reporting the binding of its metabolite nor-fentanyl in human plasma. Hence an extensive evaluation was undertaken to perform PPB of fentanyl and its metabolite nor-fentanyl in phosphate buffer solution (PBS) using human serum ALB and AAG. PPB was also performed in human plasma to determine total plasma protein binding of fentanyl and its metabolite. Furthermore, fentanyl, being a lipophilic drug, has been reported to adsorb to UF devices \([72, 101]\). Hence adsorption to UF devices and plasticware commonly used during PPB experiments was also assessed.

### 3.2 Materials and Methods

#### 3.2.1 Reference material and reagents

Acetonitrile, dichloromethane, methanol, sodium chloride, formic acid, AAG and ALB were obtained from Merck, New Jersey, USA. Sodium dihydrogen orthophosphate \((\text{NaH}_2\text{PO}_4)\) and di-sodium hydrogen orthophosphate \((\text{NaH}_2\text{PO}_4)\) were obtained from ChemSupply, Gillman, South Australia. All the chemicals and reagents obtained were of highest purity \((\geq 99\%, \text{HPLC-graded})\). Salivettes® and Eppendorf tubes were obtained from Sarstedt, Nümbrecht, Germany. 10 mL and 5 mL plastic tubes were obtained from Technoplas, St. Mary’s, South Australia. Centifree® YM-30 (Cat #4104) and Amicon® Ultra UF devices were obtained from Merck Millipore, Tullagreen, Ireland. Dialysis tubing (Spectra/Por® 14 and 25 molecular weight cut off (MWCO)) was obtained from Spectrum Laboratories Inc., Rancho Dominguez, CA, USA. 18 MΩ water was obtained from a Labmate water purification system (Aquacure, Brisbane, Australia). A centrifuge operating at 500 g (IEC CL2, Thermofisher Scientific) and 2000 g (IEC Micromax, Thermofisher Scientific) was obtained from Scoresby, Victoria, Australia.
3.2.2 LC-MS/MS method

Fentanyl and nor-fentanyl in test samples was quantified using the method described in Chapter 2, with minor modifications to sample preparation.

3.2.3 Preparation of working solutions and reagents

3.2.3.1 Preparation of 0.1M phosphate buffer solution (PBS) of pH 7.4 in 0.3 % NaCl

0.45 g NaCl was weighed and dissolved in 150 mL of water. This produced 0.3 % NaCl solution. In addition, 1.1998 g of NaH₂PO₄ and 1.4196 g of Na₂HPO₄ was weighed and made up to volume in a 10 mL volumetric flask, using 0.3 % NaCl. This produced a 1M PBS. A 1/10 dilution was made with 0.3 % NaCl as needed, to produce a 0.1M solution. The pH was adjusted to 7.4 with 10M NaOH and stirred well.

3.2.3.2 Preparation of fentanyl and nor-fentanyl standard and working solutions

Fentanyl and nor-fentanyl standard and working solutions were prepared as explained in Chapter 2 (Section 2.3). However, the plasma/saliva matrix was replaced with buffer solutions wherever required.

3.2.3.3 Preparation of AAG stock solution (5 g/L)

50 mg AAG was weighed accurately and transferred to a 10 mL volumetric flask. PBS was added up to the mark to produce 5 g/L AAG solution.

3.2.3.4 Preparation of AAG working solution (1 g/L AAG in 2 µg/L and 0.1 µg/L fentanyl)

1 mL of AAG stock solution (5 g/L) was transferred to a 5 mL volumetric flask. Further, 100 µL of fentanyl/nor-fentanyl working solution (100 µg/L) was transferred to the same 5 mL volumetric flask. The volume was made up to the mark with PBS. This produced 1 g/L of AAG in 2 µg/L of fentanyl/nor-fentanyl in PBS.

Similarly 1 mL of AAG stock solution (5 g/L) was transferred to a 5 mL volumetric flask. Further, 50 µL of fentanyl/nor-fentanyl standard dilution solution (10 µg/L) was transferred to the same 5 mL volumetric flask. The volume was made up to the mark with PBS. This produced 1 g/L of AAG in 0.1 µg/L of fentanyl/nor-fentanyl in PBS.
3.2.3.5 *Preparation of ALB stock solution (200 g/L)*

2 g of ALB was weighed accurately and transferred to a 10 mL volumetric flask. PBS was added up to the mark to produce 200 g/L of ALB solution.

3.2.3.6 *Preparation of 40 g/L ALB in 2 µg/L and 0.1 µg/L fentanyl solution*

1 mL of ALB stock solution (200 g/L) was transferred to a 5 mL volumetric flask. Additionally, 100 µL of fentanyl/nor-fentanyl working solution (100 µg/L) was transferred to the same 5 mL volumetric flask. The volume was made up to the mark with PBS. This produced 40 g/L of ALB in 2 µg/L of fentanyl/nor-fentanyl in PBS solution.

Similarly, 1 mL of ALB stock solution (200 g/L) was transferred to a 5 mL volumetric flask. Fifty µL of fentanyl/nor-fentanyl standard dilution solution (10 µg/L) was transferred to the same 5 mL volumetric flask. The volume was made up to the mark with PBS. This produced 40 g/L of ALB in 0.1 µg/L of fentanyl/nor-fentanyl in PBS solution.

Fentanyl and nor-fentanyl calibrators were prepared identically in PBS and human plasma at 0.02, 0.1, 0.5, 2 and 10 µg/L, as mentioned in Chapter 2. IS (fentanyl D-5 and nor-fentanyl D-5) 1.5 µg/L was prepared in acetonitrile. Similarly, 0.1 and 2 µg/L solutions of fentanyl and nor-fentanyl respectively were prepared in human plasma, as mentioned in Chapter 2, Section 2.3.

3.2.4 *Adsorption studies*

3.2.4.1 *Adsorption of fentanyl and nor-fentanyl in PBS by UF device (traditional approach)*

Calibrators containing both fentanyl and nor-fentanyl at concentrations of 0.02, 0.1, 0.5, 2 and 10 µg/L were prepared in PBS. 500 µL of each standard was loaded into individual UF devices and centrifuged at 500 g for 10 mins. 125 µL of each calibrator pre- and post UF was transferred directly to glass autosampler vial inserts. 25 µL of IS (1.5 µg/L of each of deuterated fentanyl and deuterated nor-fentanyl in acetonitrile) was added to each insert and mixed well. All samples were then assayed against the pre-ultrafiltration standards as calibrators. The per cent loss (adsorption) and recovery were calculated using the equations below:
\[
\% \text{ Loss} = \frac{C_{cs} - C_{ds}}{C_{cs}} \times 100 \quad \text{ Eq- 3.2}
\]

\[
\% \text{ Recovery} = 100 - \% \text{ Loss} \quad \text{ Eq- 3.3}
\]

where

\[C_{cs} = \text{concentration of fentanyl/nor-fentanyl spiked into standard before UF}\]
\[C_{ds} = \text{assayed concentration of fentanyl/nor-fentanyl after UF}\]

Experiments for protein binding were performed using IEC CL2, Thermofischer Scientific centrifuge operating at 500 g and IEC Micromax, Thermofischer Scientific centrifuge operating at 2000 g at ambient temperature of 23 °C.

3.2.4.2 Adsorption of fentanyl and nor-fentanyl to plasticware

Adsorption of fentanyl to plasticware was tested in four matrices: PBS, AAG (1 g/L) and ALB (40 g/L) spiked in PBS, as well as in human plasma, at two concentrations (0.1 and 2 µg/L) (n=8 solutions). Five plastic (polypropylene) containers were tested: 10 ml tubes, 5 ml tubes, sample collection components of Salivette®, Eppendorf tubes and sample collection components of the Centrifree© UF device. One mL of each test solution prepared in PBS, AAG in PBS, ALB in PBS and plasma were stored capped at 4 °C for 72 hours. The samples that were prepared in plasma were assayed by the addition of 200 µL of sample and 600 µL of IS to labelled 12 mL glass culture tubes. Samples were then vortex-mixed for 30 seconds and centrifuged at 500 g for 30 mins. The supernatant was transferred to 12 mL glass culture tubes and evaporated to dryness under a nitrogen stream at 40 °C. Samples were reconstituted in 200 µL of mobile phase A (0.1% formic acid in water), then transferred to 12 mL glass culture tubes and centrifuged for a further 30 mins at 500 g to remove any final particulates. The supernatants were then transferred to glass autosampler vials and assayed against standards prepared in identical fashion, except that the standards were prepared in glass and did not have 72 hours storage at 4 °C. Fentanyl and nor-fentanyl at concentrations of 0.02, 0.1, 0.5, 2 and 10 µg/L were also prepared in plasma as calibrators. For samples prepared in PBS, AAG in PBS and ALB in PBS, 125 µL was transferred directly to glass autosampler vial inserts. 25 µL of IS was added to each insert and mixed well. All samples were then assayed against the standard calibrators containing both fentanyl and nor-fentanyl at concentrations of 0.02, 0.1, 0.5, 2 and 10 µg/L prepared in PBS in
identical fashion, except that all the standards were prepared in glass. The recovery of samples stored in plastic tubes was then calculated.

3.2.5 PPB studies using Centrifree® UF device

3.2.5.1 Protein binding of fentanyl and nor-fentanyl in PBS containing ALB and AAG

From a working solution containing 1 g/L AAG, both 0.1 and 2.0 µg/L fentanyl and nor-fentanyl were prepared (n=6 solutions), and 125 µL of each was transferred to individual glass autosampler vial inserts. Before analysis, 25 µL of IS (1.5 µg/L) was added to each and mixed well. These samples were then assayed to determine the total initial concentrations. 500 µL of the respective working solutions containing 0.1 and 2 µg/L fentanyl and nor-fentanyl in 1 g/L AAG were added to respective UF devices (Centrifree®) and centrifuged at 500 g for 10 mins. 125 µL of the ultrafiltrate obtained was transferred to HPLC glass inserts. Before analysis, 25 µL of IS (1.5 µg/L) was added and mixed well. This was used to calculate the free concentration. Similarly, samples containing 0.1 and 2 µg/L fentanyl and nor-fentanyl in 40 g/L ALB solutions were processed as above. Fentanyl and nor-fentanyl standard solutions for the calibration curve were prepared similarly in PBS at a concentration range of 0.02, 0.1, 0.5, 2 and 10 µg/L to quantify fentanyl and nor-fentanyl in respective samples. Bound concentrations of fentanyl and nor-fentanyl were calculated using the equations below:

\[ F_b = F_t - F_f \quad \text{Eq-3.4} \]

\[ \% \text{ bound} = \left( \frac{F_b}{F_t} \right) \times 100 \quad \text{Eq-3.5} \]

where

- \( F_b \) = Bound fentanyl or nor-fentanyl concentration
- \( F_t \) = Total fentanyl or nor-fentanyl concentration
- \( F_f \) = Free fentanyl or nor-fentanyl concentration

3.2.5.2 Protein binding of fentanyl and nor-fentanyl in human plasma

From the working solutions, four spiked human plasma samples were prepared containing 0.1 and 2 µg/L fentanyl or nor-fentanyl respectively. 1 mL of each was loaded into respective UF devices and centrifuged at 500 g for 30 mins. 200 µL of standard or filtrate and 600 µL of IS were added to individual, appropriately labelled Eppendorf tubes. Samples were vortex-mixed for 30 seconds and centrifuged at 2,000 g
for 10 mins. The supernatant was transferred to 12 mL glass culture tubes and evaporated to dryness under a nitrogen stream at 40 °C. Residues were reconstituted in 200 µL of mobile phase A (0.1 % formic acid in water), vortex-mixed for 30 seconds, then centrifuged at 2,000 g for 10 mins. The supernatants (150 µL) were then transferred to individual autosampler vials ready for assay. The percent of PPB was calculated using the equations above. Fentanyl and nor-fentanyl calibrators were prepared in blank human plasma at concentrations of 0.02, 0.1, 0.5, 2 and 10 µg/L.

### 3.2.5.3 Plasma protein binding study using mass balance approach (MBA)

The MBA employed to determine protein binding using an UF device was also used [101] to compare the results obtained by our usual method. The MBA has been shown [101] to minimise adsorption in protein binding studies using UF devices, thereby achieving a more accurate PPB result. Hence, an experiment to determine the percent of PPB and the percent of recovery was also performed as a completely separate experiment as described by Wang et al. [101]. The experiments were performed by first spiking 1 mL of human plasma at two different concentrations (0.1 and 2 µg/L) with fentanyl and nor-fentanyl, removing an aliquot of 200 µL, then placing the compounds in a Centrifree® device. The aliquot was immediately processed to measure the initial compound concentration (C₁). The top and bottom chambers of the Centrifree® device were weighed before the addition of the compounds and again after spinning to obtain the approximate volume of the top plasma (V₂) and bottom ultrafiltrate (V₃) samples, assuming a density of one for both samples. The Centrifree® device was spun at 500 g for 30 mins. A 200 µL aliquot of the top plasma (C₂) and bottom ultrafiltrate (C₃) were then removed for detection. The results were calculated using the following equations:

\[
\% \text{ PPB} = \frac{(C₂ - C₃) \times V₂}{(C₂ \times V₂) + (C₃ \times V₃)} \times 100 \quad \text{Eq- 3.6}
\]

\[
\% \text{ Recovery} = \frac{(C₂ \times V₂) + (C₃ \times V₃)}{(C₁ \times V₁)} \times 100 \quad \text{Eq- 3.7}
\]

where

C₁ is initial drug concentration in plasma, analysed directly from the pool of spiked plasma before centrifugation; V₁ is the plasma volume loaded into the Centrifree® device, estimated as the weight difference between the UF device before and after loading the plasma samples. C₂ and V₂ are the plasma drug concentration and volume in the reservoir of the Centrifree® device after centrifugation, and C₃ and V₃ are the ultrafiltrate drug concentration and volume in the collection chamber of the Centrifree®
device. V₂ and V₃ are calculated in the same way as V₁, using the weight difference before and after. The results obtained by the MBA were compared to the traditional UF method, calculated using equations (3.2) and (3.4).

### 3.2.5.4 Protein binding of fentanyl and nor-fentanyl in cancer patients

Protein binding of fentanyl and nor-fentanyl was also assessed clinically in in-patients and out-patients (n=56) of an oncology/palliative care service within a metropolitan hospital who were receiving fentanyl at various doses, each via a transdermal patch (Durogesic©). The samples were collected randomly, irrespective of any specified time period. Blood samples (3-4 mL) were collected in standard 5 mL ethylene diamine tetra acetic acid (EDTA) tubes without a serum separator plug. The samples were centrifuged within one hour of collection and the plasma stored at –70 °C until analysis. Protein binding studies and the analysis of plasma samples were performed as specified above. Bound concentrations for both fentanyl and nor-fentanyl were calculated from the total plasma concentration, as well as the free (unbound) concentration, using equation 3.3 above. The unbound and bound fraction of fentanyl and nor-fentanyl was also calculated using the equations below:

\[
\text{Unbound fraction (F}_u\text{) } = \frac{F_f}{F_t} \quad \text{Eq- 3.8}
\]

\[
\text{Bound fraction } = 1 - F_u \quad \text{Eq- 3.9}
\]

where

- \(F_t\) = Total fentanyl or nor-fentanyl concentration
- \(F_f\) = Free fentanyl or nor-fentanyl concentration
- \(F_u\) = Unbound fraction

### 3.3 Results and Discussion

The PPB study was performed using UF devices. Initially, however, an attempt was made to use dialysis tubing (DT) to calculate the free fentanyl concentration. As our lab was not equipped with dialysis equilibrium instrumentation, a method as described previously [102] was adopted. In brief, the experiment was performed by inserting approximately 20 cm of DT in a 12 mL glass culture tube with both open ends of the DT facing outside. The DT was coiled or tied with a rubber band at the neck of glass culture tube. Before inserting the DT inside the glass culture tube, it was soaked in distilled water for 30 mins, rinsed and washed. 500 µL of fentanyl in PBS was placed
inside the DT. The glass culture tube holding DT was then centrifuged to obtain the free concentration. Initially we used 14,000 molecular weight cut off (MWCO) DT and centrifuged for 30 mins at 500 g. This yielded a very low volume, from which was not possible to quantify the analytes. Hence the time was increased to 60 mins and then to 90 mins. However, after 90 mins of spin it was observed that there was loss of volume in the collection tube, and the concentration obtained was higher than the initial spiked concentration. This might be due to loss of volume due to evaporation by the heat generated by the centrifuge machine after 90 mins of spin. The DT MWCO was increased from 14,000 to 25,000 on the assumption that enough volume could be obtained with just a 30 minute run. However, a similar problem was observed with 25,000 MWCO DT at 30 mins, with low volume yielded; and at the higher time spin there was more concentrated analyte than in the initial standard spiked solution (Table 3.1). Hence the UF device was preferred as the appropriate method for PPB.

Table 3.1  Percent recovery of fentanyl in PBS using DT with two different MWCO at 90 mins of centrifugation

<table>
<thead>
<tr>
<th>25 MWCO DT (n=2)</th>
<th>14 MWCO DT (n=2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Std. calculated conc. (µg/L)</td>
<td>At 90 mins (µg/L)</td>
</tr>
<tr>
<td>0.513</td>
<td>1.52</td>
</tr>
<tr>
<td>0.05</td>
<td>0.161</td>
</tr>
<tr>
<td>0.010</td>
<td>0.092</td>
</tr>
</tbody>
</table>

*cps=counts per second

The choice of UF device is an important consideration when doing PPB studies due to the adsorption of the drug at varying degrees. An adsorption study was performed with Centrifree® and Amicon® Ultra using PBS. Centrifree® demonstrated a higher recovery rate than Amicon® Ultra (Table 3.2) therefore this device was used in all subsequent PPB studies. Plasma protein binding in PBS was performed at two different concentrations (0.1 and 2 µg/L) for both fentanyl and its metabolite using a Centrifree® UF device.
Table 3.2 Percent recovery of fentanyl and nor-fentanyl in PBS with two different UF devices at various concentrations

<table>
<thead>
<tr>
<th>Conc. (µg/L)</th>
<th>Amicon® Ultra (% Recovery)</th>
<th>Centrifree® (% Recovery)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fentanyl</td>
<td>Nor-fentanyl</td>
</tr>
<tr>
<td>0.02</td>
<td>0.0</td>
<td>76.2</td>
</tr>
<tr>
<td>0.1</td>
<td>0.0</td>
<td>83.6</td>
</tr>
<tr>
<td>0.5</td>
<td>0.1</td>
<td>66.7</td>
</tr>
<tr>
<td>2</td>
<td>0.6</td>
<td>63.0</td>
</tr>
<tr>
<td>10</td>
<td>0.4</td>
<td>77.4</td>
</tr>
</tbody>
</table>

Adsorption of drugs to materials used in devices for the determination of protein binding has been widely considered to be a significant issue in PPB studies. To avoid such adsorption researchers have used many techniques \([72, 101]\). Adsorption has largely been assessed using solutions devoid of plasma proteins, often in PBS solution. In contrast, Wang et al. \([101]\) have recently reported the impact of adsorption on PPB experiments (which invariably contain plasma proteins) to be minimal. A traditional approach to assessing adsorption, compared with the mass balance approach of Wang et al. \([101]\), is therefore reported in this present study. As ultrafiltrates, however, do not contain proteins, adsorption from this matrix may more closely parallel that of PBS. Hence adsorption from collection devices for ultrafiltrates has also been assessed, as has adsorption from whole plasma during the storage of samples.

Samples prepared in PBS, AAG in PBS, ALB in PBS and human plasma were used to demonstrate the adsorption behaviour of fentanyl in different plastic devices (polypropylene). The results are shown in Table 3.3. The results clearly demonstrate that the adsorption of fentanyl to plasticware was significantly higher for PBS than for plasma proteins and a human plasma matrix. One reason given in the literature for this phenomenon is that plasma proteins could occupy and adhere to the binding sites and form a layer \([103, 104]\), thereby preventing drugs from accessing binding sites. Surprisingly, fentanyl did not adsorb to Eppendorf tubes as to other plasticware. Likewise, the adsorption of fentanyl (prepared in PBS) in the sample-collecting component of a UF device was also negligible. Results suggested that not all plasticware has similar adsorption behaviour. However, these data demonstrate that fentanyl does adsorb to plastic devices, though at varying degrees. Hence glassware is recommended for storing and preparing fentanyl solutions. Moreover, when spiked plasma protein solutions or plasma are used, it is unlikely to be a matter of concern.
Table 3.3  Adsorption of fentanyl to plasticware, as prepared in different solutions

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Test device</th>
<th>% Recovery*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>10 ml plastic tube</td>
<td>88.1 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>5 ml plastic tube</td>
<td>88.7 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>Salivette® (collection tube)</td>
<td>91.3 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>Eppendorf tube</td>
<td>95.5 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>UF device (collection tube)</td>
<td>109.9 ± 0.00</td>
</tr>
<tr>
<td>AAG in PBS</td>
<td>UF device (collection tube)</td>
<td>102.0 ± 0.00</td>
</tr>
<tr>
<td>ALB in PBS</td>
<td>UF device (collection tube)</td>
<td>103.9 ± 0.00</td>
</tr>
<tr>
<td>Plasma</td>
<td>Stored in Eppendorf</td>
<td>101.9 ± 0.00</td>
</tr>
</tbody>
</table>

*Reported as mean % recovery ± SEM (n=3)

Plasma protein binding in PBS was performed at two different concentrations (0.1 and 2 µg/L) for both fentanyl and its metabolite using a Centrifree® UF device. ALB and AAG were prepared at the usual medium physiological concentrations for humans, namely 40 g/L for ALB and 1 g/L for AAG [87, 91]. PPB of fentanyl in PBS solutions of ALB and AAG at concentrations of 0.1 and 2 µg/L is shown in Table 3.4. Additionally, the total percentage of PPB obtained using human plasma is shown in Table 3.5. Hence the binding behaviour of fentanyl in PBS and human plasma showed a similar pattern. Nor-fentanyl binding in PBS solutions of ALB and AAG did not demonstrate any considerable binding; neither was nor-fentanyl significantly bound to plasma proteins when human plasma was used (Table 3.4 and Table 3.5). As a highly polar inactive metabolite, nor-fentanyl does not have any direct clinical significance [50]. However, the potential exists for metabolites to compete with the parent drug for binding sites, having an indirect impact on the protein binding and hence the free concentration and/or clearance of the parent drug. These results demonstrate that the bound fentanyl ratio was similar across the therapeutic range of concentrations. The results demonstrated that fentanyl, although a basic drug with a pKa value of 8.43, does not bind predominantly to AAG, even in the absence of ALB. This is quite surprising, given the number of basic drugs reported to bind to AAG.

Furthermore, some researchers have suggested that fentanyl may bind to lipoproteins in the blood [55, 56]. The present data, however, demonstrate that fentanyl extensively
binds to ALB, and to a much lesser extent to AAG, with a total PPB of about 86-89 per cent. Based on the extent of binding to ALB that has been shown here, it is unlikely that binding to lipoproteins is of any significance.

Table 3.4  Protein binding of fentanyl and nor-fentanyl in phosphate buffer solution containing ALB and AAG

<table>
<thead>
<tr>
<th>% Binding of fentanyl</th>
<th>% Binding of nor-fentanyl</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALB</td>
<td>AAG</td>
</tr>
<tr>
<td>*0.1 µg/L</td>
<td>77.9 ± 1.1</td>
</tr>
<tr>
<td>*2 µg/L</td>
<td>77.5 ± 1.0</td>
</tr>
</tbody>
</table>

* % binding of 0.1 µg/L and 2 µg/L of fentanyl and nor-fentanyl prepared in PBS containing 40 g/L and 1 g/L of human serum ALB and AAG respectively. Reported as mean ± SEM (n=3)

Table 3.5  Protein binding of fentanyl and nor-fentanyl in human plasma: comparing MBA with direct UF method

<table>
<thead>
<tr>
<th>Total % binding using direct UF method</th>
<th>Total % binding using MBA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fentanyl</td>
<td>Nor-fentanyl</td>
</tr>
<tr>
<td>*0.1 µg/L</td>
<td>89.3 ± 1.2</td>
</tr>
<tr>
<td>*2 µg/L</td>
<td>89.7 ± 0.4</td>
</tr>
</tbody>
</table>

*Reported as mean ± SEM (n=3)

As a second approach to testing for the impact of adsorption on the protein binding experiments presented here, PPB studies were performed using the MBA as described by Wang et al. [101]. Adsorption, calculated as per cent recovery, was also compared for both the methods used. The results generated by the MBA and its comparisons are shown in Table 3.5 and Table 3.6. The comparisons suggest that the percent of PPB is less when the MBA method is used. However, the per cent recovery obtained using this approach did not exhibit any significant improvement. To sum up, the mass balance approach could be widely used to calculate PPB and avoid errors due to adsorption when UF devices are used, as advocated by the author [101]. Perhaps this approach could have a wider application, with the selection of the method depending on the properties of the drug/s tested and the physicochemical characteristics of UF devices. However, in the case of fentanyl the comparisons did not demonstrate any benefit of the MBA.
### Table 3.6 Adsorption of fentanyl and nor-fentanyl from Centrifree® ultrafiltration device

<table>
<thead>
<tr>
<th></th>
<th>% Recovery using direct UF method</th>
<th>% Recovery using MBA</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fentanyl</td>
<td>Nor-fentanyl</td>
<td>Fentanyl</td>
</tr>
<tr>
<td>*0.1 µg/L</td>
<td>85.0 ± 5.6</td>
<td>106.0 ± 5.7</td>
<td>81.5 ± 2.2</td>
</tr>
<tr>
<td>*2 µg/L</td>
<td>80.8 ± 2.5</td>
<td>103.0 ± 4.1</td>
<td>79.3 ± 0.8</td>
</tr>
</tbody>
</table>

*Reported as mean % recovery ± SEM (n=3)*

Cancer patients demonstrated a wider range of protein binding results, as shown in Table 3.7. A total of 56 patient samples were collected. However, due to the lower signal detection for nor-fentanyl, only 50 patient samples could be quantified.

### Table 3.7 Protein binding of fentanyl and nor-fentanyl in cancer patients receiving transdermal fentanyl at varying doses

<table>
<thead>
<tr>
<th>Patient demographics</th>
<th>Median (range)</th>
<th>Fentanyl (n=56)</th>
<th>Median (range)</th>
<th>Nor-fentanyl (n=50)</th>
<th>Median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>69.5 (39-90)</td>
<td>0.623 (0.043-9.72)</td>
<td>0.225 (0.009-14.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex: (Male: Female)</td>
<td>34:22</td>
<td>0.024 (0.002-0.466)</td>
<td>0.194 (0.011-4.45)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body surface area (BSA) (m²)</td>
<td>1.80 (0.06-2.26)</td>
<td>24.80 (15-41.9)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>35.0 (4.2-135)</td>
<td>0.046</td>
<td>0.511</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Protein binding</td>
<td>95.1 ± 3.52</td>
<td>32.4 ± 21.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average ± SD (range)</td>
<td>(76.7-99.8)</td>
<td>(0.6-99.3)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Such variability in protein binding could be due to a myriad of factors, including concurrent drug intake, variation in expression of binding proteins, or other chronic illnesses, including cancer itself. Fentanyl has been used in palliative care for pain control as an alternative to morphine due to various adverse effects associated with morphine, as well as it being highly potent and lipophilic [6, 100]. However, the PK behaviour of fentanyl is highly variable, with large intra- and inter-patient variability, thereby necessitating individual dose titration [77]. Studies have suggested that the free fraction of the drug is responsible for the receptor site effect [82]. In the clinical setting,
pharmacological and PK variability have been described among patients receiving fentanyl. These differences could be explained by the fact that fentanyl is bound extensively to plasma proteins [50]. Hence protein binding of fentanyl would play a pivotal role in PKPD studies to individualise the dose in cancer pain patients to manage cancer pain effectively. In this study we have only tried to assess the protein-binding behaviour of fentanyl in chronic cancer patients. However, investigating various factors associated with the broad range of binding affinity in chronic conditions would warrant dose optimisation studies in clinical settings.

3.4 Conclusion
Surprisingly for a basic drug, results presented here suggest that fentanyl binds primarily to ALB. However, AAG may contribute to a minor proportion of the binding seen in plasma. Nor-fentanyl does not have any significant binding (less than 20 %) to plasma proteins. Both the methods (MBA and UF method) used in this study showed total plasma protein binding of fentanyl to be close to that reported in the literature (80-85 %). However, protein binding to plasma samples from cancer patients receiving transdermal fentanyl was higher (average 95.1%) with a wider range (76.7–99.8%). Further, adsorption to plastic devices was higher in PBS alone than in solutions containing protein. This demonstrates that adsorption of fentanyl from plasma should not be a concern in PPB studies when ultrafiltration devices and/or plasticware are used.

The results from this study have been published [105], and presented at conferences and other professional forums.
Chapter 4. Validation of a fentanyl patch adhesion scoring tool for clinical application

4.1 Introduction
Transdermal drug delivery systems (TDDS), also known as “patches”, are dosage forms designed to deliver a therapeutically effective amount of drug across a patient’s skin. The adhesive of the TDDS is critical to the safety, efficacy and quality of the product. The therapeutic effect of the drug is linked to the adhesive performance of the TDDS. Reduction in the surface area of contact as a result of patch lift, or even the patch falling off, diminishes the delivery of drug from the patch. In other words, poor adhesion results in improper dosing of patients [16].

The degree of patch adhesion is important not only for therapeutic effectiveness, but also in dose monitoring and PK studies, since the therapeutic efficacy and/or blood concentration is directly related to the percentage of the patch that is adhered. If the extent of adhesion is not known, the dose of fentanyl could be incorrectly adjusted and PK studies could be misinterpreted. A validated scoring system is therefore needed to assess the degree of adhesion at the time of sampling.

4.1.1 TDDS design
Two different types of transdermal fentanyl delivery systems are currently available commercially, namely; reservoir or matrix patches. In a reservoir formulation, fentanyl is sandwiched between an occlusive backing layer and an adhesive controlled-released membrane and delivery is determined by the special rate-controlling membrane (Figure 4.1). More recently, matrix delivery systems have been introduced. In a matrix formulation, fentanyl is completely dissolved in an adhesive polymer matrix, from which the drug is continuously released into the skin. The dose of drug delivered depends on the amount of drug held in the matrix and the area of the patch applied to the skin. The active ingredient is distributed evenly throughout the patch, so one-half of a patch will have half the original surface area and deliver half the original dose per hour. Good adhesion of the transdermal patch to the skin is essential for maximum efficacy; therefore patients must be instructed on the proper technique for patch application. The matrix and reservoir transdermal delivery systems of fentanyl have been equally well tolerated. The PK profiles of reservoir and matrix patches are similar,
and the two delivery systems were considered bioequivalent since they resulted in similar rates and extents of exposure of fentanyl [23, 106, 107]. However, the matrix formulation has a number of advantages over the reservoir formulation. Compared to the old system, there is a lower risk of accidental overdose with membrane damage for the matrix type. Furthermore, the new system is expected to be more convenient and more comfortable to wear than the old system due to its smaller size and better adhesive properties. Therefore, the reservoir patch is currently being phased out in nearly all markets and replaced with the new design, e.g. current matrix based patch, Durogesic©. Matrix patches were introduced in Australia in 2006 and were expected to replace supplies of reservoir patches by August 2006, when the reservoir patches were discontinued and withdrawn from the market. Two batches of 50 µg/hr patches were recalled in Australia in October 2005 because of reports of leaking [108]. In addition, the new matrix patch design is effective and safe in treating patients with moderate to severe cancer pain, and can significantly improve quality of life [109].

![Figure 4.1 Matrix (a) and reservoir (b) TDDS design](image)

**Figure 4.1 Matrix (a) and reservoir (b) TDDS design**

4.1.2 Factors affecting transdermal drug delivery of fentanyl

The absorption of fentanyl from a transdermal therapeutic system is proportionate to the surface area of the patch. The anatomical site of application (chest, abdomen and thigh) of the patch does not have a clinically significant effect on fentanyl absorption [39]. However, absorption of fentanyl from the patch is governed by skin permeability and by local blood flow. Because permeation of fentanyl through the skin is a much slower process than its removal by the cutaneous blood flow, only extreme variations in blood supply should influence fentanyl absorption [110, 111]. Additionally heat has also been shown to increase the rate of fentanyl absorption by up to 30% [21]. Constant TDDS contact with the skin over the whole application period is required for consistent delivery and absorption of the drug [112]. In other words, the quality of contact between patch and skin is directly reflected in the consistency of drug delivery. Absorption of
drug through the skin is affected by a number of factors such as skin site, skin thickness, skin temperature, body temperature, blood flow, lipid concentration, number of hair follicles, skin cleansing, hydration status, sweat gland function, ethnic group, pH of skin surface and the state and integrity of the stratum corneum [14, 20, 21, 113-116]. Additionally average skin thickness varies as a function of age, gender and race [14]. Aged skin has lower moisture content and is less elastic, while younger skin is more hydrated and consequently more elastic [15].

The transdermal permeation of a drug substance requires a low molecular weight (<1000g/mol) and adequate oil and water solubility (lipophilicity). Hence the high lipid solubility (reflected by a high octanol/water coefficient of 717 as opposed to 0.77 for morphine) and low molecular weight (336.47 g/mol) of fentanyl make it suitable for transdermal administration [18, 110]. Such administration has shown to provide effective, continuous pain relief in chronic cancer-related pain [117]. Despite its feasibility for use in transdermal application, adhesion or the lack of adhesion of such transdermal systems to the skin is a critical factor directly related to drug delivery and therapeutic effectiveness. Since the drug absorption process is related to the drug partition between the TDDS and the skin and the drug permeation process, complete skin contact over the entire delivery surface for the entire label application period is essential. If the TDDS lifts or partially detaches, the effective area of TDDS/skin contact, and thus the drug absorption, changes in an unpredictable manner. Therapeutic failure can then occur [16]. Hence, fentanyl patches are manufactured such that the amount of drug released is proportionate to the surface area of the patch. A related problem concerns the adherence of TDDS in patients with excessive sweating, in whom there is a possible reduction in effective surface area for absorption [118].

Hence, as mentioned above, the absorption of fentanyl from a transdermal therapeutic system is proportionate to the surface area of the patch; the adhesion percentage of surface area would reflect the absorbed fentanyl from the reservoir and correspond to its therapeutic effectiveness. Consequently several factors have an influence on the adhesion property. For example cachexia would have an effect on skin permeability, thereby reducing fentanyl absorption which has a significant effect on the PKPD of transdermal fentanyl patches. One study has analyzed the PK characteristics of transdermal fentanyl in cachectic and normal weight cancer patients with pain [17]. The clinical implication of the study suggests that transdermal fentanyl is not the opioid of
choice for cachectic cancer patients with pain. Poor transdermal absorption of fentanyl in these patients may result in inadequate analgesia despite an apparently large dose of opioid [17]. However, before any conclusion may be drawn, the adhesion properties must also be taken into consideration, since the poor availability of the drug in the bloodstream may be related to impaired adhesion of patches on the skin. Since the delivery of drug from transdermal patches is closely related to its release pattern and skin adhesiveness, the effectiveness of drug and its PKPD also depends upon these patch properties. Therefore future research in this area is focused on developing efficient adhesive systems for transdermal patches to allow for complete adhesion over the skin surface area and delivery of the targeted drug dose [119].

4.1.3 Assessment methods for the adhesion properties of TDDS
The adhesion of TDDS is one of the most critical parameters for product safety, efficiency and quality. Several in vitro techniques have been proposed to monitor adhesive performance, such as peel adhesion, or tack and shear strength. However, in vivo human skin testing is still the most reliable method for the evaluation of the TDDS [16]. Nevertheless, these measurements are focused mainly on initial adhesion for industrial application, rather than the long-term adhesion to human skin [120]. Despite the fact that the adhesion of TDDS is one of the most critical parameters for product safety, efficiency and quality; clinical TDDS studies are mainly focused on medical effectiveness and less on adhesive properties. Though, in some clinical studies the adherent area is estimated visually by a specialized person or by the volunteers themselves [121-123]. However, no such validated visual scoring tools have been developed for clinical practice.

The FDA has provided a scoring system based on the percentage adherent area of the patch (Table 4.1), but guidance for further data analysis and evaluation is not given [124]. This recommended scoring system is used by the pharmaceutical industry when designing new generic transdermal fentanyl patches or for product redevelopment in cases where new adhesives or other patch components are under investigation. Given that this scoring system is useful for defining the adhesion area of fentanyl patches, the aim of this study was to validate this scale for use in clinical practice.
Table 4.1  FDA scoring system for patch adhesion

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>≥ 90 % adhered</td>
<td>Essentially no lift off the skin</td>
</tr>
<tr>
<td>1</td>
<td>≥ 75 % to &lt; 90 % adhered</td>
<td>Some edges only lifting off the skin</td>
</tr>
<tr>
<td>2</td>
<td>≥ 50 % to &lt; 75 % adhered</td>
<td>Less than half of the patch lifting off the skin</td>
</tr>
<tr>
<td>3</td>
<td>&gt; 0 % to &lt; 50 % adhered but not detached</td>
<td>More than half of the patch lifting off the skin without falling off</td>
</tr>
<tr>
<td>4</td>
<td>0 % adhered - patch detached</td>
<td>Patch completely off the skin</td>
</tr>
</tbody>
</table>

4.2 Materials and Methods

This was a two part study. Part 1 involved the development of the photo database (survey tool). In Part 2, the survey tool was tested on a number of volunteers to assess its validity and reliability. This study was granted ethical approval by Mater Health Services Human Research Ethics Committee (HREC) and Griffith University HREC (Appendix 1 and 2).

4.2.1 Part 1: Survey Tool development

4.2.1.1 Recruitment of patients and volunteers

Patients recruited to this part of the study were in-patients and out-patients of an oncology/palliative care service within a metropolitan hospital who were receiving fentanyl via a transdermal patch (Duragesic©) at any dose, were > 18 yrs, were willing to have a photograph taken of their fentanyl patch, were able to read and understand the participant information and consent form (PICF) (Appendix 3), and provide written consent. Research staff reviewed the drug prescriptions and liaised with participating clinicians at the site to identify eligible patients.

Volunteers consisted of the members of the palliative care research team and/or hospital staff willing to wear a placebo patch on their chest or arm for a number of days, were willing to have a photograph taken of their placebo patch, were able to read and understand the PICF (Appendix 4), and provide written consent.

This was a convenience sample of patients and staff selected to obtain an adequate numbers of photographs demonstrating patches at varying levels of adhesion. Attempts were made to recruit patients and volunteers demonstrating a range of skin textures, skin colours, skin fold thickness, propensity to sweat, and hair cover.
4.2.1.2 Data collection

Demographic information was collected to ensure a representative sample of both patients and volunteers. No changes were made to the patches worn by patients. A close-up photograph of consenting patients’ patch, or staff members’ placebo patch, was taken by research staff and numbered and coded appropriately. Images used in the survey were cropped so that the participant number was not visible. Similarly, it was not possible to identify the patient or volunteer from the photographs taken. Photographs were taken until 10 samples were obtained at each FDA adhesion level across a range of participants in order to build a photo library of 50 photographs reflecting varying degrees of patch adhesion.

4.2.1.3 Calculation of patch photo area

Photo editing software (Adobe Photoshop C6 Extended, Version 13.0 X64) was used to calculate the patch adhesion area and assign scores to the photographs as a control. The data obtained from the software measuring tool was exported to Microsoft Excel (Professional Plus 2010, Version 14.0.6129.5000, 32bit). The software allowed the area of the adhered portion of the patch to be calculated, which was then converted into a percentage of the total patch area, and subsequently assigned a score of 0-4 according to the FDA tool. This was used as the standard comparator against which visual assessment was judged.

4.2.1.4 Preparation of survey tool

The library of 50 photographs was used to create two surveys (survey A and survey B). Ten randomly selected photographs were included in both surveys to determine intrarater variability. Of the remaining 40 photographs, 20 were included in survey A and the other 20 in survey B, with a spread of difficulty across each adhesion level (Figure 4.2).

The FDA scoring tool chart and patch photographs surveys were laminated. A prevalidation pilot study was performed on research staff to ensure that the photographs were of a good quality, easy to view and that the survey could be completed in a time efficient manner.
4.2.2 Part 2: Testing the tool

4.2.2.1 Recruitment of survey participants
Participants in this part of the study were health care professionals (doctors, pharmacists and nurses) recruited from within the oncology/palliative care service. Inclusion criteria included only the willingness to complete the survey and to provide demographic information such as age and years of experience.

4.2.2.2 Survey
Each of the 30 consenting staff members were given either survey A or B in random order by research staff. Approximately one week later, they were given the other survey in order to assess intra-observer along with inter-observer reliability. Participants were asked to score each of the photographs for patch adhesion according to the FDA scoring tool on the scoring sheet.

4.2.3 Data analysis
An index of rater reliability and validity was used to examine the extent to which participants concurred in their scoring and also the extent to which scores concurred with control scores judged through photo editing software.

Spearman’s Rank Correlation was used for validity analysis whereby the participant scores were compared to the control scores obtained from the photo editing software.
Cohen’s kappa statistic ($k$) was used to test intra- and inter-rater reliability. Inter-rater reliability was calculated amongst the 30 participants of survey A, survey B and the combination of surveys A and B. Intra-rater reliability was calculated using the scores of the ten patches common to both surveys. Stata/SE, ver12.1 was used for the calculations. A pre-defined correlation of 0.7-0.8 was considered sufficient and $>0.8$ a strong correlation for the validity test [125, 126]. Similarly, $k>0.75$ was considered to be excellent, 0.40 to 0.75 fair to good, and below 0.40, poor [127]. Given that the sample size should be at least five times larger than the total of score categories in validity and reliability studies, our sample size of 30 participants was considered adequate [128].

4.2.4 Data management

Information collected from the participants (patient and volunteers) were recorded onto a case report form (CRF) (Appendix 5, 6 and 7). The participants were assigned a unique study number (coded) which was used to identify them on all CRF's. It may be necessary to confirm details and check data in the CRF against the original medical record and therefore it had to be possible to identify the correct records. Paper records, which included identifiers, were stored in secure, locked conditions at the Mater with access limited to the principal investigators. An electronic record was password protected. It is anticipated that the information will be retained for at least 5 years. This period is consistent with National Health and Medical Research Council guidelines regarding retention of clinical research data. At the end of this period all paper records will be shredded and electronic records wiped.

4.2.5 Dissemination of results

The results from this study have been published [129], and presented at several conferences and other professional forums. However in any publication or presentation the information was provided in such a way that the participants were not identified. Results of the study were intended to be provided to the participants, if they so required.

4.3 Results and discussion

Validity was strong for both series (Spearman’s rank 0.954 and 0.978 for survey A and B respectively; $p <0.001$) (Table 4.2).
Table 4.2  Validity test (Spearman’s Rank Correlation) for survey series A and B including p values

<table>
<thead>
<tr>
<th>Survey series</th>
<th>Spearman’s Rank Correlation</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Series A</td>
<td>0.954</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Series B</td>
<td>0.978</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Inter-rater reliability ranged from 0.392 to 0.858 (combined $k =0.547$) and 0.327 to 0.910 in series A and B respectively, with lower $k$’s being associated with FDA scores 1 and 2 (patch adhesion between 75 and 90%). The combined agreement across both series was 0.585 (Table 4.3). The intra-rater reliability of the tool was also satisfactory with the overall kappa being 0.605 ($p <0.001$) for two measurements of the ten common patches scored by each of 30 participants (Table 4.4).

Table 4.3  Inter-rater reliability for survey A, B and overall (A and B) expressed as $k$ for different FDA scores with mean $k$ values

<table>
<thead>
<tr>
<th>FDA Score</th>
<th>Inter-rater reliability*</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Overall (k)</td>
<td>Survey A (k)</td>
<td>Survey B (k)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.478</td>
<td>0.417</td>
<td>0.554</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.475</td>
<td>0.358</td>
<td>0.592</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.392</td>
<td>0.327</td>
<td>0.433</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.683</td>
<td>0.794</td>
<td>0.575</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.885</td>
<td>0.858</td>
<td>0.910</td>
<td></td>
</tr>
<tr>
<td>Combined k</td>
<td>0.585</td>
<td>0.547</td>
<td>0.620</td>
<td></td>
</tr>
</tbody>
</table>

*All $p < 0.001$

Table 4.4  Intra-rater reliability for survey A and B for two measurements of ten common patches scored by each of 30 raters

<table>
<thead>
<tr>
<th>Agreement</th>
<th>Expected agreement</th>
<th>$k^*$</th>
<th>Std error</th>
</tr>
</thead>
<tbody>
<tr>
<td>68.7%</td>
<td>20.6%</td>
<td>0.605</td>
<td>0.029</td>
</tr>
</tbody>
</table>

*p < 0.001

There was no correlation between age and years of experience when scoring the level of patch adhesion (Table 4.5). Further, no difference was found between male and female participants.
Table 4.5 Correlation between survey results and control results by years of experience and gender of rater

<table>
<thead>
<tr>
<th>Survey</th>
<th>Experience (in years)*</th>
<th>Gender*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt; 10 (n=13)</td>
<td>≥ 10 (n=17)</td>
</tr>
<tr>
<td>Survey A</td>
<td>0.961</td>
<td>0.942</td>
</tr>
<tr>
<td>Survey B</td>
<td>0.966</td>
<td>0.981</td>
</tr>
</tbody>
</table>

*All \( p < 0.001 \)

Overall, the patch adhesion score determined by the participants visually in this study corresponded well to those generated by the photo editing software thus rendering the scoring system highly valid. The reliability of the scoring system was not as strong. This can be explained by the nature of the scoring tool. The inter-rater reliability for score “4” (patch completely detached) was excellent \((k>0.75)\), whereas the reliability for scores “1” and “2” (partial patch adhesion between 75 and 90%) were low \((k<0.4)\), especially for Survey A (Table 4.3). FDA score “4” (patch detached) is easier to score correctly, than score “2” \((≥ 50\% \text{ to } < 75\% \text{ adhered})\), especially where a score is close to the ‘borderline’, for example 48% or 51%. In order to make the study more robust, the photograph library was constructed to include a number of photographs that were very close to the borderline between two scores (Figure 4.3).

![Figure 4.3 Examples of closely scored photographs: (a) score 1 (75.8%) and (b) score 2 (73.49%)](image)

This may have resulted in a lower than expected score. There were also limitations in the two dimensional nature of the survey tool. This was highlighted during the pre-validation of the survey tool where it was noted by health care staff that the assessment of a laminated photograph was significantly more challenging than assessment of the degree of adhesion of a patch in clinical practice.
A recent study assessing genetic, pathological and physiological determinants of transdermal fentanyl PK in over 620 cancer patients found marked variation in serum fentanyl concentrations. The genotypes and clinical factors considered accounted for only a small proportion of variability in the study [130]. The degree of patch adhesion was not measured in this study and may be an important factor that has been overlooked. A standardised scoring tool such as this is essential for future PK studies of fentanyl patches.

The amount of drug released in a fentanyl patch is proportionate to the surface area of the patch. Constant contact between the skin and the patch over the whole application period is essential for consistent delivery and absorption of the drug. Therefore, the amount of adhesion of the patch to the skin is a critical factor, directly related to drug delivery and therapeutic effectiveness.

Nine randomised controlled trials were identified in a recent Cochrane review on the analgesic efficacy of transdermal fentanyl for relief of cancer pain [131]. Of the 9 RCTs that met the inclusion criteria for the Cochrane Review, none of these studies recorded the level of adhesion of the fentanyl patches. The studies that were included were small, used different study designs, and compared fentanyl with many different drugs. While the quality of evidence in these studies was limited, the authors concluded that if patients were able to tolerate the medication and survived to the end of the study, pain appeared to be improved and the majority of patients would have “no worse than mild pain”. Randomised trial literature for the effectiveness of transdermal fentanyl is limited. The authors of the Cochrane review suggested that given the difficulty of conducting randomised controlled trials in the palliative care setting, observational studies that meet criteria for quality, validity and size could make a significant contribution to studies of cancer pain treatment. The recording of the degree of patch adhesion would be critical in these future studies.

4.4 Conclusion

The adhesion of a transdermal drug delivery system to a patients’ skin is directly related to the therapeutic efficacy of the medicine. A validated scoring system is therefore needed to assess the degree of adhesion during clinical practice and at the time of sampling for PKPD studies. The FDA has a recommended scoring system for the adhesion of transdermal patches that is used by pharmaceutical industry when designing
new transdermal patches; however this tool has not been validated in a clinical setting. This study has validated the FDA scoring system for adhesion of fentanyl patches used for the management of cancer pain in adult patients. The FDA scoring tool has been shown to be reliable and valid for use in routine clinical practice for assessing patch adhesion and should be used as a standard for any future PK studies.
Chapter 5. Saliva as a surrogate for plasma drug concentration in PKPD studies

5.1 Introduction

5.1.1 The measurement of drugs in saliva

Patients with advanced malignant disease are by definition frail and have poor performance status. There is considerable reluctance on the part of health professionals to subject these individuals (and even more so, terminally ill children) to non-essential tests and investigations, including repeated venepuncture that has been necessary in PK studies to date. The measurement of drug concentrations in saliva can be a convenient substitute for plasma analysis, both in monitoring therapeutic drug concentrations and in PK and biopharmaceutic studies in adults, children, and animals. Examples include; nicotinamide [132], fluconazole [133], sulfamethazine in sheep [134], irinotecan [135, 136] diethylcarbamazine [137], ethanol [138] and a number of drugs used in paediatrics [139]. The use of saliva rather than blood has been shown to be an attractive alternative for TDM in children because its collection is painless, simpler and cheaper than venesection [140]. In a previous study [141] the majority of patients voiced a preference for saliva sampling over venesection (apart from those patients with indwelling central lines).

As saliva is a nearly protein-free ultrafiltrate of plasma, only free drug in the plasma should pass into the saliva. Thus, a drug’s saliva concentration could represent the portion of the drug that reaches the intended target tissue [142]. This approach has been exploited for phenytoin, where saliva concentrations correlate better with cerebrospinal fluid concentration than with serum concentrations [143]. A review of the relationship of saliva/plasma drug concentrations suggests that saliva concentrations may relate better to efficacy and/or toxicity than plasma levels [144]. As drugs must pass through cellular membranes to enter saliva, they also must pass through membranes to reach their site of action in the central nervous system. It is possible that the PKPD relationship of saliva concentrations will be of greater utility in dose adjustment than plasma concentrations in the event that the PK shows sufficient variability to justify dose individualisation of these drugs.
However, there are four major physiochemical determinants of drug penetration into saliva: (i) molecular mass, (ii) lipid solubility, (iii) ionisation in plasma (pKa), and (iv) saliva pH (which increases with flow rate) [142]. Salivary pH has been shown to be a vital factor in determining the concentration of drug in saliva. As the pH increases, basic drug gets ionised. Similarly, as the pH decreases, acidic drug becomes ionised, and hence the concentration varies. A study [145] reviewing therapeutic drug monitoring in saliva concluded that plasma concentrations could be predicted from saliva concentrations without pH correction for those drugs with pKa between 5.5 and 8.5. Fentanyl has a pKa of 8.4, i.e. within this range. The authors also concluded that for some drugs the saliva concentration related better to efficacy than did plasma concentrations. Hence the utility of saliva concentrations is not necessarily dependent on a consistent correlation between saliva and plasma concentrations.

Usually saliva sampling is done by one of two methods, either by stimulation or without stimulation. Unstimulated sampling is done by direct pouring of saliva in to a suitable device, for example, a wide-mouthed beaker. Stimulation is done by the patient’s chewing a cotton bud or suitable material. Saliva sampling by stimulation has several advantages, as a large volume can be obtained. Further stimulation also increases saliva flow rate. As saliva flow rate increases, the concentration of bicarbonate in the saliva increases, as does the saliva pH. The pH of stimulated saliva is reported to fall within a narrow range around 7.4, corresponding to normal plasma pH [146]. Hence the use of stimulated saliva (using a non-citrated cotton bud) rather than resting saliva is more promising because of the benefits of larger sample volume, smaller pH gradient between plasma and saliva, and smaller variability in saliva/plasma concentration ratios. In some instance, a citrated bud is used to reduce variation in saliva pH [145]. However, citrated sampling would decrease the saliva pH and increase the pH gradient between plasma and saliva, consequently increasing the saliva/plasma variability [144].

In the event that there is a huge variability in saliva and plasma pH, the correlation between saliva/plasma concentrations for a particular drug is estimated using the mathematical model shown below, which is based on the Henderson-Hasselbach equation [147]. This equation normalises the saliva concentration at a particular pH corresponding to its plasma pH, which is usually fixed at 7.4.
\[
S/P = \frac{1+10^{(pK_a-pH_s)}*f_p}{1+10^{(pK_a-pH_p)}*f_s}
\]

Eq-5.10

where

\( S/P \) = saliva/plasma ratio, \( pH_s = \) saliva \( pH \), \( pH_p = \) plasma \( pH \), \( f_p = \) unbound drug concentration in plasma, and \( f_s = \) unbound drug concentration in saliva.

However, in the current study this equation was disregarded because a very low saliva volume was available to measure the \( pH \). Further, a non-citrated dental bud was used to collect the saliva, so that the sample would be in a range close to the plasma \( pH \) of 7.4, and hence saliva concentration would better predict plasma concentration.

5.1.2 The measurement of opioids in saliva

Strong correlations between plasma and saliva concentrations have been described for the analgesics paracetamol [148] and hydromorphone [149]. Other opioids, including codeine [150], diamorphine [151], methadone [152], morphine [153], and dihydrocodeine [154], have also been analysed to investigate saliva/plasma / (S/P) ratios of drug concentration. Mixed results have been reported, with marked variability in S/P ratios between studies.

In a recent study undertaken by Hardy et al. [141], 139 paired S/P samples were collected from 43 cancer patients who had been taking sustained release (SR) oxycodone for more than 5 days at doses ranging from 10–600 mg/day (median 40 mg/day). Plasma and saliva oxycodone and metabolite concentrations were measured using HPLC-MS/MS. Saliva concentrations of oxycodone were much higher than plasma concentrations. There was a poor correlation between concentrations of both oxycodone and noroxycodone in plasma and saliva over a range of times following dosing. No correlation was shown between salivary \( pH \) and oxycodone or noroxycodone concentrations. The study concluded that saliva does not provide a valid substitute for plasma when monitoring oxycodone levels for PK studies or therapeutic monitoring.

In another study, plasma and saliva samples were taken before the daily dose of methadone in 60 patients undergoing methadone maintenance treatment. Saliva \( pH \) was measured immediately after sampling, and concentrations of (LD)-, (L)-, and (D)-
methadone in saliva and plasma were measured. The study concluded that the measurement of methadone in saliva, even after allowing for variable saliva pH, does not provide an accurate estimate of corresponding total or unbound plasma concentrations of methadone or its enantiomers [144]. In the only study detected to date, Silverstein et al. [75] attempted to quantify fentanyl and nor-fentanyl in saliva and urine to detect and monitor substance abuse in seven female patients receiving intravenous fentanyl. They reported that neither fentanyl nor nor-fentanyl was detected in saliva at any time of sampling. Based upon the result they concluded that saliva testing does not appear to be a viable alternative to urine testing. However, the research of Silverstein et al. was performed only with a small sample size. This study has therefore been performed with a bigger sample size in order to estimate any possible relationship between saliva concentrations with either free, or total, fentanyl plasma concentrations. If fentanyl concentrations in saliva could be quantified, saliva could be used instead of plasma for PKPD studies. If this relationship could be established, it could provide a significant advantage to frail cancer patients who are reluctant to give regular plasma samples for clinical observations.

A pilot study of 19 paired plasma and saliva samples from subjects receiving fentanyl for cancer pain control via a transdermal patch was initially conducted to observe any possible relationship. Pain scores were also obtained from the subjects at the time of sampling. No significant correlation was found at this sample size. However, the pilot study demonstrated the validity of an assay method using HPLC-MS/MS and the ability to collect and process qualitative data. Moreover, the pilot study also gave insight into the adsorption of fentanyl in a Salivette®, which led to the development and validation of a novel method of quantifying fentanyl and nor-fentanyl in saliva samples from a Salivette® (Chapter 2).

5.2 Materials and Methods
5.2.1 Research Plan
5.2.1.1 Study Locations
The locations were: Mater Adults Hospital, Australian Centre for Paediatric Pharmacokinetics (Mater pathology Service) and Department of Palliative and Supportive Care, Mater Health Services, Brisbane, Australia. This study was granted ethics approval by Mater Health Services HREC and Griffith University HREC (Appendix 8 and 9).
5.2.1.2 Sample size

Population modelling for the most part, does not lend itself to ‘traditional’ statistical analysis such as is often used in a randomised clinical trial, hence traditional methods of determining sample size are not applicable with this approach. The purpose is to estimate the parameters of a model (e.g. clearance, volume of distribution of a drug) and a number of covariates (e.g. patients’ characteristics) which help explain variability about the parameters of the model, not to compare differing treatments per se (as might be done with standard hypothesis testing). Previous population modelling studies [141, 155] have indicated that with the planned sampling design, 50 patients contributing 4 to 6 samples over several occasions, is the minimum number necessary to give satisfactory estimates of the structural parameters (e.g. clearance, volume of distribution) and the variance parameters (e.g. inter-patient and inter-occasion variability) [141]. Therefore, in this study fifty-six patients were approached to collect 4 to 6 samples over several occasions, if possible.

5.2.1.3 Participants

Patients were in-patients and out-patients of an oncology/palliative care service at Mater Health Services. Inclusion and exclusion criteria were as listed below.

Inclusion criteria:

- patients who
  - had a diagnosis of malignant disease
  - were receiving fentanyl via a transdermal patch (Durogesic© any dose)
  - were willing and able to provide up to 6 saliva and blood sample(s)
  - were able to read and understand PICF (Appendix 10) and provide written consent.

Exclusion criteria:

- patients who
  - had oral mucositis, infection and/or xerostomia (such that it is painful/not possible to collect a saliva sample)
  - were using fentanyl for breakthrough medication.

5.2.1.4 Recruitment

Research staff reviewed drug prescriptions and liaised with participating clinicians at each site to identify potentially eligible patients. Identified patients were approached and asked if they were willing to participate in the study and informed consent obtained.
5.2.1.5 Participant withdrawal
All patients were eligible to withdraw from the study at any time. If a participant withdrew consent during the study, the study doctor and relevant study staff would not collect additional blood or saliva or personal information from the participants. However, results from the blood and saliva tests will be retained, along with the personal information already collected, to ensure that the results of the study could be measured adequately. Data collected up to the time the participant withdrew from the study will form part of the study results.

5.2.1.6 Safety considerations / Patient safety
For participants, the risks of being involved in this project were largely the same as those involved in their normal treatment for cancer. For blood collection there was the usual small risk of haematoma, infection and/or slight pain. All blood collection was undertaken by a member of staff trained in phlebotomy. There was also a risk of slight discomfort to the participant when providing the saliva sample, as they had to chew the dental cotton bud to provide saliva.

5.2.2 Assessments
5.2.2.1 Baseline Documentation
Patient age, height, and weight, recent blood tests (within 1 week of sampling, to assess renal function and liver function), fentanyl dose, duration of treatment, site of placement, timing of samples in relation to timing of fentanyl patch change, and concomitant medications were documented. BMI and BSA were determined at baseline and at the time of each sample collection.

5.2.2.2 Pain scores
At the time of sampling, adult participants were asked to complete a Brief Pain Inventory (BPI) which scores pain at its best, worst and average over the preceding 24 hours and ‘right now’. Research nurses in the cancer/palliative care unit were experienced in the use of these measures.

5.2.2.3 Patch Adhesion
A visual and descriptive scoring system recommended by the FDA for adhesion of transdermal patches was used to grade the degree of patch adhesion at the time of
sampling. This patch adhesion scale was validated for use in clinical practice, as described in Chapter 4.

5.2.2.4 Toxicity
Adverse events commonly associated with opioids (e.g. nausea, somnolence, hallucinations, itch) were assessed at the time of sampling using the common terminology criteria for adverse events (CTCAE) [156].

5.2.3 Methods and analysis
5.2.3.1 Sample collection and storage
Blood samples (3–4 ml) were collected in standard 5 ml EDTA tubes without a serum separator plug. The samples were centrifuged within one hour of collection and the plasma stored at –70 °C until analysis for both free and total fentanyl and nor-fentanyl concentrations. Saliva samples were obtained by having the participant chew a CDB (non-citrated) that was supplied to them in a Salivette®. The Salivette® was pre-weighed and the weight recorded before it was supplied to the patient. Thorough rinsing of the mouth was required at least 5 mins prior to specimen collection, as remnants of orally administered medicines may contaminate saliva specimens. Saliva samples thus obtained were post-weighed. The weight was recorded and the samples stored at –70 °C until analysis. Blood and saliva samples were collected at times convenient for the patient. Wherever possible, blood samples were salvaged from leftover plasma from routine pathology testing. A minimum of 4 pairs of samples were collected from each consenting patient at approximately 12 and 24 hours after patch application and daily thereafter until the patch was changed. Fixed time points were not necessary with this study, and this facilitated the use of routine samples. Steady-state conditions were not required; samples were taken from patients who had just been started on a fentanyl patch. There was no interruption to, or adjustment of, the prescribed medication in any patient in order to facilitate PK sampling. Fentanyl and nor-fentanyl was quantified, as mentioned in Chapter 2, for both plasma and saliva matrices. Patients were also asked whether they had a preference for the sampling type, i.e. saliva over blood.

5.2.3.2 High Performance Liquid Chromatography – Tandem Mass Spectrometry (HPLC-MS/MS)
Plasma samples were assayed for total fentanyl and nor-fentanyl concentrations. Plasma samples were also assayed for free fentanyl and nor-fentanyl concentrations. The
concentration of fentanyl and nor-fentanyl in saliva was also assayed. The assay was performed according to the validated method Chapter 2. Free concentrations of fentanyl and nor-fentanyl were determined after UF using Centrifree© Ultrafiltration units, as described in Chapter 3.

5.2.3.3 Analysis of saliva/plasma relationship
The concentration of fentanyl in saliva was plotted against the concentration of fentanyl (total, bound / unbound) in plasma. All possible relationships were evaluated with respect to time and dose. This plot was also performed for the metabolite concentrations. Since samples were taken before the next patch change, the validity of any correlation between saliva and plasma concentrations was assessed after most of the drug was absorbed and distributed (i.e. when clearance is the main PK process operating).

5.2.3.4 Pain scores
The pain scores were plotted against total and free fentanyl plasma concentration. The saliva fentanyl concentration was also plotted against the pain score. The same relationship was also plotted for the metabolites. Likewise, the pain score was plotted against the dose. The strength of these relationships was further evaluated using either linear or non-linear models, as deemed appropriate from the data.

5.2.3.5 Patch adhesion
Patch adhesion scores were plotted against total and free fentanyl plasma concentration. The saliva fentanyl concentration was also plotted against the pain score. The same relationship was also plotted for the metabolites. Likewise, patch adhesion was plotted against dose and pain score.

5.2.3.6 Protein binding of fentanyl
Protein binding of fentanyl was performed, and free fentanyl concentration was calculated using the method described in Chapter 3 for all plasma samples.

5.2.3.7 Data management
Information collected from the participants was recorded in a CRF (Appendix 11). All the data and the documents were managed as mentioned in Chapter 4, Section 4.2.4.
5.2.3.8 Dissemination of results and publication

The results of the study described in this chapter have been published [157] and presented at conferences and other professional forums. No information about the identification of participants was provided in any publication or presentation. It was intended that the results of the study be provided to the participants if they wished to have them.

5.3 Results and discussion

5.3.1 Participants

One hundred and sixty three paired plasma/saliva samples were collected from 56 participants over the 24-month study period. The median sample per patient was 2 (range: 1–10). The majority of participants had cervical, prostate, ovarian or breast cancer and were attending the day oncology unit on a regular basis for chemotherapy or bisphosphonate infusions. The median age of participants was 69.5 years (range: 39–90). All participants were at steady state, i.e. they had been taking fentanyl for more than 5 days. Fentanyl doses ranged from 12.5 to 200 µg, (median 50 µg). The median BSA and BMI of patients was 1.80 m\(^2\) (range 0.06-2.26 m\(^2\)) and 24.80 kg/m\(^2\) (range 15–41 kg/m\(^2\)) respectively. Sampling times after each patch change ranged from 0 to 77 hours (median 27 hr). Patient demographics are shown in Table 5.1.

Table 5.1 Patient demographics

<table>
<thead>
<tr>
<th>Details</th>
<th>Median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paired sample</td>
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</tr>
<tr>
<td>Age (yrs)</td>
<td>69.5</td>
<td>39–90</td>
</tr>
<tr>
<td>Dose (µg)</td>
<td>50</td>
<td>12.5–200</td>
</tr>
<tr>
<td>Diagnosis</td>
<td></td>
<td>Prostate, Ovary, Cervical, Breast</td>
</tr>
<tr>
<td>BSA (m(^2))</td>
<td>1.80</td>
<td>0.06–2.26</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>24.80</td>
<td>15–41</td>
</tr>
<tr>
<td>Pain score</td>
<td>2</td>
<td>0–10</td>
</tr>
<tr>
<td>Patch adhesion</td>
<td>0</td>
<td>0–3</td>
</tr>
<tr>
<td>Fentanyl PPB (%)</td>
<td>95.1</td>
<td>76.7–99.8</td>
</tr>
<tr>
<td>Sampling time (hrs)</td>
<td>27</td>
<td>0–77</td>
</tr>
</tbody>
</table>
5.3.2 Sample size
Fifty-six patients were approached, and 163 paired samples were collected. However, not all collected plasma and saliva samples were quantifiable due to low sample volume and LLOQ. Some patients passed away or were transferred to another health care setting before being able to provide 4 paired samples. Saliva samples from some patients were very low or nil due to dry mouth. Hence, only 162 samples for plasma and 157 samples for saliva were quantifiable for fentanyl concentration. For the fentanyl plasma/saliva correlation study only 156 paired samples could be used. Similarly, in the case of the nor-fentanyl concentration, 159 plasma and 151 saliva samples were quantifiable, and 147 paired samples were used for the correlation study.

5.3.3 Fentanyl and nor-fentanyl analysis in plasma and saliva samples
Plasma concentrations of fentanyl and nor-fentanyl ranged from 0.043 to 9.72 (mean 0.877) and 0.09 to 14.6 (mean 0.465) μg/L respectively. Saliva concentrations of fentanyl and nor-fentanyl ranged from 0.012 to 38.41 (mean 4.84) and 0.004 to 4.21 (mean 0.336) μg/L respectively. Saliva fentanyl concentrations were much higher than plasma fentanyl concentrations, raising the possibility of active transport into saliva. However, saliva nor-fentanyl concentrations were less than plasma nor-fentanyl concentrations, though to a lesser extent. There was no significant correlation between fentanyl and nor-fentanyl concentrations in plasma ($r^2 = 0.3599$, Figure 5.1).

![Figure 5.1 Correlation showing fentanyl and nor-fentanyl measured in plasma samples](image-url)
Similarly, no correlation was observed with saliva fentanyl and nor-fentanyl concentration ($r^2 = 0.1041$, Figure 5.2).

![Figure 5.2 Correlation showing fentanyl and nor-fentanyl measured in saliva samples](image)

There was also a poor correlation between concentrations of both fentanyl and nor-fentanyl in plasma and saliva when samples were taken concurrently over a range of times following dosing ($r^2 = 0.3733$ and $0.0014$ respectively; Figures 5.3 and 5.4). Table 5.2 shows various parameters calculated from plasma and saliva samples obtained from 56 cancer patients.

<table>
<thead>
<tr>
<th>Description</th>
<th>Median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total plasma fentanyl conc. (µg/L)</td>
<td>0.623</td>
<td>0.043–9.72</td>
</tr>
<tr>
<td>Free plasma fentanyl conc. (µg/L)</td>
<td>0.024</td>
<td>0.002–0.467</td>
</tr>
<tr>
<td>Total plasma nor-fentanyl conc. (µg/L)</td>
<td>0.225</td>
<td>0.009–14.5</td>
</tr>
<tr>
<td>Saliva fentanyl conc. (µg/L)</td>
<td>2.51</td>
<td>0.012–38.4</td>
</tr>
<tr>
<td>Saliva nor-fentanyl conc. (µg/L)</td>
<td>0.125</td>
<td>0.004–4.2</td>
</tr>
<tr>
<td>Plasma MR (fentanyl/nor-fentanyl)</td>
<td>0.374</td>
<td>0.078–4.55</td>
</tr>
<tr>
<td>Saliva MR (fentanyl/nor-fentanyl)</td>
<td>0.063</td>
<td>0.002–4.47</td>
</tr>
<tr>
<td>Plasma/saliva ratio (fentanyl)</td>
<td>0.193</td>
<td>0.033–1.48</td>
</tr>
<tr>
<td>Plasma/saliva ratio (nor-fentanyl)</td>
<td>1.35</td>
<td>0.027–7.07</td>
</tr>
</tbody>
</table>

MR = Metabolic Ratio
Figure 5.3 Correlation showing total fentanyl concentration measured in plasma and saliva samples

Figure 5.4 Correlation showing nor-fentanyl concentration measured in plasma and saliva samples
5.3.4 Pain score

An analogue scale of 0 to 10 was used to score the severity of pain in increasing order. The median pain score was recorded at 2 on a scale of 0 to 10. In addition, responses to the question of “pain scores now” were grouped into ≤3/10 for ‘good’ pain control and >3/10 for ‘bad’ pain control. One hundred and seventeen (72.3 %) of the sampling points showed “pain now” scores of ≤3/10, and 45 (27.7%) demonstrated scores of >3/10 (Table 5.3), showing that patients were adequately managed for their pain. This could be due to the breakthrough medication that each patient was receiving in addition to their regular fentanyl patch. Further, the results showed that “pain now” scores of ≤3/10 were not associated with higher fentanyl concentrations; instead, uneven distribution of fentanyl concentration was observed in both cut-off groups.

Table 5.3 Comparison between pain score ‘Now’ ≤3/10 and pain score ‘Now’ >3/10

<table>
<thead>
<tr>
<th>Pain score ‘Now’ Cut-off groups</th>
<th>Pain score ‘Now’ ≤3/10</th>
<th>Pain score ‘Now’ &gt;3/10</th>
<th>Z-score</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample number n (%)</td>
<td>117 (72.3)</td>
<td>45 (27.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calculated concentration (µg/L):Median (range)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma fentanyl (total)</td>
<td>0.781 (0.043–3.53)</td>
<td>0.949 (0.150–3.14)</td>
<td>-1.47</td>
<td>0.142</td>
</tr>
<tr>
<td>Plasma fentanyl (free)</td>
<td>0.045 (0.002–0.466)</td>
<td>0.043 (0.003–0.218)</td>
<td>0.343</td>
<td>0.728</td>
</tr>
<tr>
<td>Plasma nor-fentanyl (total)</td>
<td>0.341 (0.009–1.81)</td>
<td>0.404 (0.024–1.68)</td>
<td>-0.953</td>
<td>0.342</td>
</tr>
<tr>
<td>Saliva fentanyl</td>
<td>4.84 (0.012–38.4)</td>
<td>5.03 (0.114–22.6)</td>
<td>0.068</td>
<td>0.944</td>
</tr>
<tr>
<td>Saliva nor-fentanyl</td>
<td>2.92 (0.004–3.07)</td>
<td>0.368 (0.010–3.80)</td>
<td>-0.549</td>
<td>0.582</td>
</tr>
</tbody>
</table>

*Mann-Whitney U test for significance performed as P ≤0.05.

Pain scores measured against the total and free fentanyl plasma concentrations did not show any significant relationship (Figures 5.5 and 5.6).
The saliva fentanyl concentration was also plotted against pain scores (Figure 5.7) and the pain scores against the dose (Figure 5.8). No correlation was observed. The result showed no relationship between the pain score and any of the plasma or saliva concentrations, thus concurring with previous reports for opioids such as morphine [3, 158]. However, one report was found on the association between opioid concentration and pain intensity [159]. Likewise no relationship was observed between pain score and time since last patch application.
5.3.5 Patch adhesion

It was necessary to ensure that patches were adequately sticking to the application site so that the drug concentration in the plasma or saliva did not misrepresent the correlation study. To this end a validated tool developed by the FDA was used to score the level of patch adhesion at each sampling point (Chapter 4). An analogue scale of 0 to 4 was used to score the level of patch adhesion at the site of application. Patch adhesion scores ranged from 0 to 3 with a median score of 0. The scoring tool showed
that the majority of patients’ patches were adequately sticking to the site of application (Table 5.4). The table shows that more than 90% of the patches at different sampling points \((n = 130)\) had good adhesion scores of ‘0’ (≥90%) or ‘1’ (≥75% to <90%). Only a small number \((n = 13)\) showed very low patch adhesion (9%).

Table 5.4 Patch adhesion score observed in cancer patients with respect to dose and concentration

<table>
<thead>
<tr>
<th>Patch adhesion score</th>
<th>Score ‘0’</th>
<th>Score ‘1’</th>
<th>Score ‘2’</th>
<th>Score ‘3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample no. (n) (%)</td>
<td>79 (55.2)</td>
<td>51 (35.7)</td>
<td>9 (6.30)</td>
<td>4 (2.80)</td>
</tr>
<tr>
<td>Median dose (µg) (range)</td>
<td>49.3 (12–150)</td>
<td>45.4 (12–100)</td>
<td>79 (37–200)</td>
<td>46.5 (37–75)</td>
</tr>
<tr>
<td>Calculated concentration (µg/L): Median (range)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma fentanyl (total)</td>
<td>0.918 (0.043–3.53)</td>
<td>0.689 (0.130–2.63)</td>
<td>1.19 (0.249–3.14)</td>
<td>0.749 (0.451–1.31)</td>
</tr>
<tr>
<td>Saliva fentanyl</td>
<td>5.91 (0.012–38.4)</td>
<td>3.96 (0.114–31.58)</td>
<td>6.03 (0.640–15.9)</td>
<td>4.52 (0.537–9.99)</td>
</tr>
</tbody>
</table>

Patch adhesion scores were plotted against saliva and total and free fentanyl plasma concentrations. A trend was observed, in that the fentanyl concentration decreased with an increase in the patch adhesion score (Figures 5.9, 5.10 and 5.11). However, to strongly correlate its effect in clinical application, population PK modelling using patch adhesion as a cofactor will be discussed in detail in Chapter 7.

Figure 5.9 Correlation showing total fentanyl concentration measured in plasma and patch adhesion
Figure 5.10  Correlation showing free fentanyl concentration measured in plasma and patch adhesion

\[ y = 0.003x + 0.0462 \]
\[ R^2 = 0.0015 \]

Figure 5.11  Correlation showing fentanyl concentration measured in saliva and patch adhesion

\[ y = 0.1462x + 5.0578 \]
\[ R^2 = 0.0003 \]

In the same way, the patch adhesion score was plotted against the dose and the pain score (Figures 5.12 and 5.13). However, no correlation was observed.
Figure 5.12  Correlation showing fentanyl dose administered through transdermal patch and patch adhesion

\[ y = 2.3901x + 48.297 \]
\[ R^2 = 0.0032 \]

Figure 5.13  Correlation showing pain score and patch adhesion in cancer patients receiving transdermal fentanyl at various doses

\[ y = 0.2417x + 2.1638 \]
\[ R^2 = 0.0065 \]
5.3.6  Protein binding of fentanyl

The PPB of fentanyl was calculated as described in Chapter 3 of this thesis. Protein binding to plasma samples from cancer patients receiving transdermal fentanyl was higher (average 95.1 %) with a wider range (76.7–99.8 %) than reported in literature (80–85 %).

5.3.7  Patient preference

The majority of patients questioned chose saliva sampling over plasma sampling as the preferred method. The exception was in those with indwelling central lines from which blood could be taken easily with no further discomfort; in these patients, saliva sampling offered no advantage.

Dose versus plasma and saliva fentanyl concentration (averaged for each sampling point) were plotted. There was a linear relationship between the dose administered and the corresponding concentrations (Figure 5.14). This linearity was because the patients were at steady-state concentration over the time period. It also helps to affirm that the method developed to assay fentanyl and nor-fentanyl concentration in both plasma and saliva matrix was valid. Time (interval of patch change time at each sampling point) versus plasma and saliva concentration showed no significant relationship (Figures 5.15 and 5.16).

![Graph showing correlation between total plasma and saliva fentanyl concentration and dose administered to cancer patients]

Figure 5.14  Correlation showing total plasma and saliva fentanyl concentration and dose administered to cancer patients
Figure 5.15 Correlation showing total plasma fentanyl concentration and sampling time after patch change

Figure 5.16 Correlation showing saliva fentanyl concentration and sampling time after patch change
Our results demonstrated that both fentanyl and nor-fentanyl are readily detected in saliva samples. However, no meaningful relationship between saliva and plasma fentanyl concentrations could be established. We have demonstrated significant intra- and inter-individual variation in both plasma and saliva concentrations of fentanyl when delivered via the transdermal route. This is consistent with other studies and justifies the wide dose-equivalent opioid ranges published [77].

Another important factor impacting on fentanyl concentration in body fluid is the degree of patch adhesion, which is important not only for therapeutic effectiveness, but also in dose monitoring and PK studies, since the therapeutic efficacy and/or blood concentration is directly related to the percentage of the patch that is adhered. If the extent of adhesion is not known, the dose of fentanyl could be incorrectly adjusted and PK studies could be misinterpreted. The median patch adhesion score was recorded to be 0 in a range of 0 to 3. This showed that the patients’ patches were adequately sticking to the site of application, allowing targeted drug concentration to reach the body compartment. However no relationship was observed between patch adhesion score and time since last patch application.

The data also showed saliva fentanyl concentrations exceeding plasma concentrations, suggesting that the possibility of active transport into saliva is likely, as previously demonstrated for oxycodone [141]. While saliva does contain protein [160], it is generally accepted that with respect to protein binding of drugs, saliva behaves as a protein-free ultrafiltrate of plasma, whereby only free drug in the plasma should pass into the saliva by passive diffusion [142, 144, 146, 147]. It has been previously described that drugs enter saliva through passive diffusion [161] due to the concentration gradient between plasma and saliva. Furthermore, if the drug exhibits low plasma protein binding, the free (unbound) concentration can be diffused from plasma to saliva [141]. Free drug is the active component, in equilibrium with the concentration at the site of action. It is also available for metabolism in the liver; thus protein binding of fentanyl is likely to be of significance to both its efficacy and its PK. However, as fentanyl is almost 85–90 % protein-bound [58], according to the literature and to our result as demonstrated in Chapter 3, there is less free fentanyl available in plasma. Furthermore, the median PPB of fentanyl of patients in the current study is also 96 %, which is very high, allowing less free fentanyl to diffuse through the biological
membrane. Hence our results showed that saliva concentration exceeded plasma concentration, and that active transport into saliva may be likely.

5.4 Conclusion
No predictive correlation was observed between plasma and saliva fentanyl concentration. However the detection of higher fentanyl concentrations in saliva than plasma, with a good correlation to dose, may allow saliva to be used as an alternative to plasma in PK/PD studies of fentanyl in cancer patients. Fentanyl was readily detectable in saliva in higher concentrations than plasma raising the possibility of an active transport mechanism. The dose versus mean concentration relationship was also higher for saliva than for plasma.

Pain scores were also assessed, to see whether saliva concentration could better predict pain control. The study found that pain was adequately managed, though no correlation was shown between plasma or saliva fentanyl concentration. Patch adhesion was also recorded in order to estimate whether a given dose was delivered from the delivery system, so as to evaluate the relationship that could exist between patch adhesion, drug concentration and pain control. Patch adhesion was good in most of the patients and a trend was revealed linking patch adhesion to fentanyl concentration, which is further explored in Chapter 7.

Despite both plasma and saliva concentrations showing large inter-patient variation at each dose and that neither plasma nor saliva demonstrated a correlation between drug concentration and pain 'right now' even when corrected for dose, these data suggest that saliva may have advantages over plasma for the determination of fentanyl levels, supporting its use as an alternative to plasma in PK studies. The large variation in plasma and saliva fentanyl concentrations at each dose level suggests that there are a number of factors influencing fentanyl PK. The influence of patient demographics, organ function, pain score and patch adhesion will be the focus of the population PK study in Chapter 7.
Chapter 6. Pharmacogenetics and pain control

6.1 Introduction

6.1.1 Pharmacogenetics of opioids
Pharmacogenomics is a broader application of genomic technologies to drug discovery, PK, PD and therapeutic outcomes, while pharmacogenetics (PG) is the study of genetic variations affecting drug response, though the terms are sometimes used interchangeably. The genes and deoxyribonucleic acid (DNA) determine the structure and function of proteins. Genetic variations can understandably alter the structures and functions of proteins and play a major role in the PK and PD of drugs such as opioids [162].

As variability in patient responses to pharmacotherapies seems evident even among well-designed studies, a consideration of why this is a common occurrence in many types of therapies has the potential to enhance clinical outcomes. The rapidly progressing field of PG offers insight into the variation in responses observed clinically, and in particular for the variability observed among patients administered µ opioid analgesics [163]. Examples of the variability of responses by individuals to drug therapies can easily be found throughout peer-reviewed literature. A well-known placebo-controlled study on controlled-release oxycodone for treating post-herpetic neuralgia found that 42 % of the individuals did not respond to the treatment, and even among those who derived benefit, there were varying levels of response [164]. An indication of the variation in individuals’ responses to analgesics is that opioid dose requirements can vary in the clinical setting as much as 40-fold [163]. A wide variance of morphine dose requirements was observed in a study of 3045 postoperative patients [1, 165]. Moreover, 10–30 % of patients treated with oral morphine do not have a successful outcome, primarily owing to side effects, as well as inadequate analgesia in some cases [166]. Furthermore, it is questionable whether a person should really be classified as “opioid-non-responsive” or whether the right opioid has just not been identified. There are several reasons why some individuals are unresponsive to analgesics in the same setting in which other patients obtain benefit. Certainly, an important determinant that gives rise to different responses is the heterogeneity among an individuals’ underlying disease. Thus the study of PG has lent insight into the role of genetics on the interindividual variability in responses to drugs.
Genetic variation influences interindividual variability in drug absorption, distribution, metabolism, and excretion [167, 168]. Ultimately these processes have an important impact on the observed efficacy and toxicity of a drug. PG may very well play a critical role in personalising medicine in the future, not only for managing pain, but also for treating ailments such as cancer and hypertension [163]. PG research can offer insights into some commonly observed clinical phenomena, including why individuals have different responses, in both efficacy and side effects, to the same dose of a given drug [169]. Many recent studies have identified and characterised the important roles that genetic variability plays in clinical opioid therapy outcomes.

6.1.2 Single nucleotide polymorphisms
The most common genetic variation is the single nucleotide polymorphism (SNP), which occurs at an appreciable frequency of >1% [170]. Other variations are called mutations (incidence <1%), which may be duplications, deletions, insertions, translocation or inversion of DNA segments. The nomenclature of SNPs is based either on the location of the base pair and the nucleotide change caused by the SNP (for example, A118G or 118A>G, which is the SNP of the μ opioid receptor subunit gene (OPRM1) at base pair 118, which codes for change of adenine to guanine); or on the alteration of amino acid sequence induced by the SNP (for example, A118G of OPRM1 can be abbreviated as Asn40Asp, which indicates a change of amino acid at position 40 from asparagine to aspartate); or on changes at the allele level (for example, the second variant of the wild-type CYP450 enzyme CYP2C9*1 is CYP2C9*3) [171].

6.1.3 Commonly observed polymorphisms in opioids
In addition to pathology and environmental factors such as diet, smoking, drug-drug and food-drug interactions, it is increasingly recognised that genetic factors may play a role in shaping the PK and PD aspects of the individual response to opioids [1]. Thus, patient genotype could be used to further guide dose individualisation, helping to reduce the incidence of withdrawal and adverse effects, particularly during the induction phase of treatment, improving treatment retention.

PG factors contributing to the efficacy and adverse events of opioids are complex and multiple. Moreover, it is unclear how significant these factors are in clinical practice [4]. There are a large number of pain targets where genetic polymorphisms can influence the experience, sensation, and behavioural response of pain. These include receptors,
transport proteins, neurotransmitter synthesising and catabolic enzymes [172, 173]. Hence, identification of the factors that contribute to variability in response to opioids also needs to take into consideration the genetic contribution and variability in pain perception and behavioural response.

Several literature reviews suggest various frequently observed gene encoding polymorphisms for opioids, of which some polymorphisms are consistent with fentanyl. Such polymorphisms can occur at various levels, for example, OPRM1, ARRB2 (arrestin, beta 2 gene) and DRD2 (dopamine receptor D2 gene), which are the genes encoding µ opioid receptors (MOR) and the ligands for opioids which affect the responses of patients to opioid drugs [167]. Likewise, metabolic enzyme polymorphisms affect opioid metabolism and excretion (e.g. CYP 3A4/5) [130]. Similarly, polymorphisms in transport proteins (ABCB1) impact the absorption, distribution, and elimination of opioids [168]. Other genes associated with opioids response include KCNJ6 (G protein-activated inward rectifier potassium channel 2 gene), which is responsible for opioid signalling [174] and brain-derived neurotrophic factor (BDNF) gene, which is associated with opioid addiction [175].

6.1.4 OPRM1
The MOR is the primary binding site target for the opioid drugs. About 100 variants in the MOR gene OPRM1 have been identified [176] with more than 20 producing amino-acid changes and having polymorphic frequencies greater than 1 % [177].

SNPs in the gene OPRM1 encoding the MOR have been linked to the variability of the analgesic effects of morphine [178]. A significant association was identified between the substitution of A118 by G118 in OPRM1 and a reduced response to morphine. Other PG studies have shown that patients with the A118G polymorphism of OPRM1 show variability in pain relief from morphine, alfentanil, morphine-6-glucuronide and levomethadone [179]. Hence the most commonly identified SNP in the OPRM1 gene is A118G [1].

The MOR, encoded by the OPRM1 gene, has been the subject of several genetic studies in the context of obstetric analgesia because this receptor is the main site of action of many endogenous peptides, including β -endorphin and encephalin [180]. β-endorphin is an opioid peptide produced primarily in the anterior lobe of the pituitary gland [181].
Following release from its precursor protein, pro-opiomelanocortin (POMC), β-endorphin is circulated via the blood stream to interact with specific opioid receptors located throughout the body [182]. The peptide interacts primarily with the MOR, although it can also bind to, and activate, other opioid receptors, e.g. the δ receptor [183]. It produces analgesia by inhibiting the firing of peripheral somatosensory fibres. Stress-induced increases in the release of β-endorphin are positively correlated with the amelioration of pain, whereas administration of exogenous opioids, such as fentanyl, reduces plasma levels of the peptide [181]. In experimental animals, exogenous opioids such as morphine have been shown to down-regulate the expression of POMC and subsequently induce a decrease in the biosynthesis of β-endorphin [184]. It has been suggested that decreased β-endorphin concentrations may play a role in a variety of chronic pain disorders [181].

Hence there has been a great interest in polymorphisms of OPRM1, 118A/G, because the G118 allele has been shown to result in the substitution of the amino acid asparagine with aspartate at position 40, which may increase the binding affinity and potency of β-endorphin [185]. Because of the relatively high prevalence of the G118 variant, if it does alter the clinical response to opioids, this may impact the dose required to produce effective analgesia [186].

6.1.5 ARRB2

β-arrestin 2 encoded by the gene ARRB2, is a component of the G-protein-coupled receptor complex and is involved in MOR and dopamine receptor D2 signalling processes in signal transduction [187]. ARRB2 is an intracellular protein that can regulate the number of functional receptors expressed on the cell surface at a given time. The MOR and the dopamine receptor D2, both of which mediate rewarding properties of common drugs of abuse, are regulated by ARRB2 [188]. ARRB2 spans approximately 11 kb of genomic DNA and consists of 14 exons. It is expressed in multiple tissues and organs, with especially high expression levels in the brain. It regulates opioid signal transduction through promotion of receptor desensitisation and internalisation [189]. Apart from its classical function in receptor desensitisation, ARRB2 also functions as a signalling intermediate, through a kinase/phosphatase scaffold, in response to dopamine receptor activation. This implicates ARRB2 as a positive mediator of dopaminergic synaptic transmission [188].
6.1.6 **DRD2**

Illicit opioid use is a significant public health issue with approximately 74,000 Australians and 900,000 Americans dependent upon these drugs [190]. Worldwide it is estimated that approximately 10 million people abuse illicit opioids [191]. Opioid abuse is not only associated with high mortality rates and poor health among users but also imposes disproportionately large economic and social costs upon the community in general [192, 193]. There have been several studies and discussions on opioid abuse and dependence in association with genetic polymorphisms on dopamine receptors. Dopamine–opioid interactions have been widely studied to identify association between opioids and dopamine receptors. Anatomically, opioids and dopamine systems are closely related [194] and their interactions are functionally significant [195]. Neurochemically, opioids influence dopamine release [196]. The dopamine system also influences opioid behaviours. Lowered opioid analgesia after activation of dopamine systems has been reported, as well as enhanced analgesia with dopamine receptor antagonists [195].

The dopamine receptor 2 is a G protein-coupled receptor located on postsynaptic dopaminergic neurons that are centrally involved in reward-mediating pathways [197]. The dopamine receptor is encoded by the **DRD2** gene [198] with signalling through the receptor governing physiological functions related to locomotion, hormone production and drug abuse [197]. Further, ankyrin repeat and kinase domain containing 1 (**ANKK1**) is an enzyme that in humans is encoded by the **ANKK1** gene. **ANKK1** is a member of a protein kinase superfamily involved in signal transduction pathways. This gene contains a single nucleotide polymorphism that causes an amino acid substitution within the 11th of 12 ankyrin repeats of **ANKK1** (Glu713Lys of 765 residues) [197]. This polymorphism, which is commonly referred to as Taq1A, can influence **DRD2** receptor expression [199]. It is located in the coding region of the **ANKK1** gene that controls the synthesis of dopamine in the brain [197].

6.1.7 **CYP 3A4/5 metabolising enzyme**

Polymorphisms within metabolic enzymes that process and eliminate opioids and their metabolites also have an important effect on an individual’s response to opioid medications. Such polymorphisms could result in many adverse effects including those caused by poor metabolism as well as ultrarapid metabolism of drugs due to CYP 450 gene variants [200]. In clinical studies, CYP 450 enzymes have been directly linked to
variability in responses to codeine (CYP2D6), tramadol (CYP2D6), fentanyl (CYP3A4/5), and methadone (CYP2B6) [167, 201-203]. If these enzymes are inhibited (e.g., owing to drug-drug interactions), then the drugs’ opioid effects will also be enhanced [167]. However, opioids such as oxymorphone, morphine, and hydromorphone are not metabolised by CYP 450 enzymes [167].

When metabolism is to be investigated, there are several enzyme classes that are involved in opioid metabolism [163]. However, the CYP 450 enzymes mediate the metabolism of almost 40 % to 50 % of all medications [168], and therefore drug-drug interactions are more likely, with medications processed through this enzyme family. Furthermore, CYP3A subfamilies are the most abundant of all the CYP isoenzymes, and they mediate the metabolism of a broad range of drugs. Within the subfamily, CYP3A4 and CYP3A5 are the two most important enzymes that metabolise many drugs in the liver [204].

Fentanyl is metabolised by CYP3A4, and there is a marked variability between patients due to metabolism [52]. CYP3A5 is yet another important genetic contributor to interindividual differences in CYP3A-dependent drug clearance in humans [205]. The CYP3A4 enzyme mediates the dealkylation of fentanyl to norfentanyl in the liver as well as in the intestinal epithelium. An in vitro study showed that CYP3A5 is also a catalyst of fentanyl oxidation [206]. CYP3A4 and CYP3A5 are polymorphic. The most frequent and common CYP3A4 single nucleotide polymorphism is -392A>G (3A4*1B), and the polymorphism of CYP3A5 is 6986A>G (3A5*3), which result in variable fentanyl metabolism. Although fentanyl abuse results in toxicity, fentanyl toxicity may partially be due to CYP3A4 or CYP3A5 variant alleles, resulting in impaired fentanyl metabolism. Therefore, identifying and correlating an individual’s fentanyl toxicity with genotype (CYP3A4 and CYP3A5), may aid in fentanyl dosing adjustments [200].

A study investigating the genetic determinants of transdermal fentanyl PK in Japanese cancer patients has suggested that CYP3A5 nonexpressors (CYP3A5*3/*3) had significantly increased plasma fentanyl concentrations and central adverse events [207]. Another study conducted to assess genetic and non-genetic factors influencing interindividual variability in fentanyl metabolism in cancer pain patients using transdermal fentanyl patches, has shown that CYP3A4*22 SNP, in combination with the CYP3A5*3 SNP [130] influenced transdermal fentanyl PK [207].
In a study conducted in postmortem samples, *CYP3A4* and/or *3A5* variant alleles resulted in variable fentanyl metabolism, which contributed to fentanyl toxicity. The data provided evidence that *CYP3A5* was involved in fentanyl metabolism. Homozygous *CYP3A5*\(^*3\) results in impaired metabolism of fentanyl, and genotyping *CYP3A4*\(^*IB\) and *3A5*\(^*3\) variants, may help to certify fentanyl toxicity [19]. Another study investigated the impact of *CYP3A4*\(^*1G\) on the intraoperative plasma concentration of fentanyl and postoperative fentanyl consumption in the patient-controlled analgesia in Chinese Han patients undergoing lower abdominal surgery, and to evaluate whether the *CYP3A4*\(^*1G\) polymorphism could serve as a predictive biomarker of fentanyl serum levels and therapeutic efficacy in postoperative pain control. The study concluded that the *CYP3A4*\(^*1G\) polymorphism was related to the PK of fentanyl, and patients with the *CYP3A4*\(^*1G\) variant A allele appears to have a lower metabolic rate of fentanyl. Thus the *CYP3A4*\(^*1G\) polymorphism may predict the individual requirement of fentanyl [208].

Hence with regard to metabolism, polymorphisms of *CYP* loci have been a focus of attention, and a better understanding of such polymorphisms could produce better clinical implications in opioid toxicity and dose adjustments.

### 6.1.8 *ABCB1*

Drug transporters are important structural proteins that can influence the absorption, distribution, and elimination of opioids. In the gastrointestinal tract and hepatocytes, they have the ability to influence the bioavailability of orally administered opioids by restricting or facilitating intestinal absorption and facilitating presystemic biliary elimination [209, 210]. Their activity in the liver, kidneys, and small intestine can play an important role in hepatobiliary, renal, and intestinal clearances, as well as alter the distribution of opioids into different body compartments and tissues respectively; and the same applies to their metabolites [211, 212]. In particular, transporter expression at the blood-brain barrier has the potential to significantly influence the clinical efficacy and safety of opioids, whose major site of action lies within the CNS [1]. Therefore, polymorphisms in genes encoding proteins important for opioid transport have also been linked to variability in responsiveness to opioids.

The ATP binding cascade (ABC) super family of efflux transporters consists of nearly 50 known human members divided into several sub-families. The most characterised of
the ABC transporters is the P-gp which is characterised as an efflux transporter. The P-gp has a wide tissue distribution, but its most important role relating to opioid drugs is likely at the blood-brain barrier, where it can directly influence CNS exposure to substrates [213-215]. P-gp significantly influences the bioavailability of many opioids and is encoded by the ATP-binding cassette B1 (ABCB1)/multiple drug resistance 1 (MDR1) gene. However, ABCB1 gene encoding of P-gp is highly polymorphic, with over 100 SNPs identified, each possessing the potential to affect the expression and/or function of the transporter [1]. Polymorphisms of the P-gp transporter have also been linked to variations in morphine, fentanyl, and methadone efficacy [178, 216].

Originally identified as a multiple drug resistance gene in cancer cells, it has now been established that P-gp is involved in drug efflux in the small intestine and out of the brain via the blood-brain barrier (BBB). P-gp expression at the BBB is of particular importance to opioids, regulating access to their site of action and consequently affecting efficacy [216]. Hence the transport of opioid substrates by P-gp is an important consideration in the study of opioid PG because of the significant interindividual variability observed in P-gp expression and function.

6.1.9 KCNJ6

G protein-activated inward rectifier potassium channel (GIRK) is a protein that in humans is encoded by the KCNJ6 gene [217] and is associated with altered opioid antinociception [218]. To date, four subtypes (GIRK1-GIRK4) have been identified in mammals [219]. This GIRK channel activation is important for opioid receptor transmission, which is achieved by the activation of several G-protein-coupled receptors, including opioid receptors [220]. It is also involved in opioid effects on postsynaptic inhibition [174] and mediating a significant component of analgesia [219], [221]. GIRK channels are expressed in many tissues, including the heart [222], spinal cord [221], and various regions in the brain with different subunit compositions [223-225].

6.1.10 BDNF

BDNF is a secreted protein [226] that in humans is encoded by the BDNF gene located at chromosome 11 position 14 [227]. It is a member of the neurotrophin family, and is widely expressed in adult mammalian brain regions, especially in the cerebral cortex, hippocampus, and amygdala complex [228]. BDNF regulates neuronal survival,
promotes neurite outgrowth and maintains synaptic connectivity in the adult nervous system. Evidence indicates that *BDNF* is involved in the pathophysiology of various neurodegenerative and neuropsychiatric disorders [229-231] and it also plays an important role in reward-related processes and opioid-induced plasticity [232, 233].

Furthermore, linkage and association studies with markers in the *BDNF* genomic region have been associated with personality traits [234] and several neurological disorders including Parkinson’s disease, schizophrenia, bipolar disorder, obsessive-compulsive disorder and eating disorders [235]. Many polymorphism-related effects have been reported for *BDNF* with most studies focused on a single polymorphism in the pre-domain of *BDNF* (Val66Met) [38], showing strong evidence that the Met66 allele of this functional Val66Met polymorphism is associated with substance abuse [236-238] and specifically with opioid addiction [175]. Other research has also shown that chronic morphine exposure decreases *BDNF* expression in the ventral tegmental area in mice [239].

### 6.1.11 Pharmacogenetics of transdermal fentanyl in cancer patients

In summary, variability in response to opioids and dosage requirements can be influenced by a myriad of PG factors. Research in this area has progressed from the extensive investigation of genetic variability in drug-metabolising enzymes to now also include drug receptors and transporters [240, 241]. Therefore variability in opioid response may be due to differences in concentrations of either the parent drug or its active metabolites due to the metabolism of opioids. In addition, differences in absorption, distribution and elimination due to the transport of drugs into and out of tissues can also affect the rate and extent of drug absorption, distribution, and elimination [1]. Similarly, polymorphisms in the genes encoding various receptors and signalling pathways also impact an individual’s response to opioids. Hence, PG in addition to cofactors such as age, pathology, and sex, is now recognized as a determinant of interindividual variability in drug efficacy and toxicity.

A thorough PG study of all relevant SNPs potentially associated with interindividual variation in PK and PD of fentanyl in cancer patients is beyond the scope of this study. This study included a preliminary investigation into whether genetic polymorphisms in three genes, namely *ARRB2, BDNF* and *KCNJ6* contributed to variability in therapeutic response.
6.2 Materials and Methods

6.2.1 Patients and sample size
Patient demographics, exclusion and inclusion criteria, ethics, assessment, documentation and patch size (dose) are described in the previous chapters of this thesis. Fifty-six patients were recruited and blood samples (3–4 mL) collected in standard 5 ml EDTA tubes without a serum separator plug. The samples were centrifuged within one hour of collection. Supernatant (plasma) was pipetted in an appropriately labelled 5 mL plastic tube. Similarly, a single 3 mL EDTA sample was taken from the remaining matrix for PG study and stored at –70 °C until analysis.

6.2.2 Genotyping
DNA was extracted from whole blood collected in EDTA collection tubes (BD Australia) using Qiagen Midi DNA extraction kits (Qiagen Science, Germantown, MD, USA) as previously described [242].

The choice of candidate SNPs was based on those previously described in the literature with associations with pain and opioid pharmacology. To select the SNPs in the ARRB2 gene, the study performed by Oneda et al [187] was followed, where four SNPs (rs34230287 in the promoter, rs3786047 in intron 1, rs1045280 in exon 11 and rs2036657 in the 30 UTR) were selected on the basis of high heterozygosity (minor allele frequency > 0.14). Similarly SNPs in the BDNF genes were selected on the basis of a study conducted by Levran et al [243], where three SNPs in the BDNF gene (rs10835210, rs1491850, rs7934165) were shown to be associated with opioid (methadone) dose. KCNJ6 was selected based on a study by Lotsch et al [218], where the studied SNP (rs2070995 AA genotype) was shown to be associated with increased opioid dose requirements.

For KCNJ6 and BDNF, pyrosequencing assays were designed using the Pyromark Assay Design software. The primers used for pyrosequencing the three BDNF and one KCNJ6 SNPs are shown in Table 6.1.
Table 6.1 Primers for pyrosequencing (BDNF and KCNJ6)

<table>
<thead>
<tr>
<th>SNP</th>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDNF rs1491850</td>
<td>BDNF rs1491850 T_C L</td>
<td>CGCATATGAGACCTCAACATCTTCC</td>
</tr>
<tr>
<td></td>
<td>bio-BDNF rs1491850 T_C R</td>
<td>biotin-TTTCAGTTTCCCCGAACAT</td>
</tr>
<tr>
<td></td>
<td>BDNF rs1491850 T_C_seq</td>
<td>AATCATACAGATTTTACGTG</td>
</tr>
<tr>
<td>BDNF rs7934165</td>
<td>BDNF rs7934165 G_A R</td>
<td>GGGAGCATGCCAGGAATTG</td>
</tr>
<tr>
<td></td>
<td>bio-BDNF rs7934165 G_A L</td>
<td>biotin-GGAAGATGCCAAGTAGATATGC</td>
</tr>
<tr>
<td></td>
<td>BDNF rs7934165 G_A_seq</td>
<td>TTTGTGTCTTTGCACC</td>
</tr>
<tr>
<td>BDNF rs10835210</td>
<td>BDNF rs10835210 C_A L</td>
<td>TTGTCCTTCGGGTTATTTTGCAAT</td>
</tr>
<tr>
<td></td>
<td>bio-BDNF rs10835210 C_A R</td>
<td>biotin-TGCTTTACTCGTGCTGTTGAAAT</td>
</tr>
<tr>
<td></td>
<td>BDNF rs10835210 C_A_seq</td>
<td>TGTAACAGCAGGAAGT</td>
</tr>
<tr>
<td>KCNJ6 rs2070995</td>
<td>KCNJ6 rs2070995 G_A L</td>
<td>TTGACAAATGAGACCCCAACA</td>
</tr>
<tr>
<td></td>
<td>bio-KCNJ6 rs2070995 G_A R</td>
<td>biotin-TGGTTATGGCTACCGGCTCA</td>
</tr>
<tr>
<td></td>
<td>KCNJ6 rs2070995 G_A_seq</td>
<td>TTAAGAAGAAGATAATTCCC</td>
</tr>
</tbody>
</table>

Pyrosequencing is a method of DNA sequencing to determine the order of nucleotides in DNA based on the “sequencing by synthesis” principle. It involves taking a single strand of the DNA to be sequenced and then synthesizing its complementary strand enzymatically [244]. Pyrosequencing uses a mixture of three primers, including one biotinylated primer for attachment to the Streptavidin-magnetic beads for specific amplicon selection, and a sequencing primer. Pyrosequencing was performed on a QSeq platform (Biomolecular Systems, QIAGEN) using Pyromark Gold Q24 reagents (QIAGEN) and data analysed to identify genotypes using QSeq software version 2.1.3 (Biomolecular Systems, QIAGEN).

For ARRB2, the four SNPs were genotyped using Taqman assays specifically designed for amplification and genotype identification of each SNP. Briefly, 2.5 µL of 2x Taqman® Master Mix, 0.25 µL 20x Assay Working Stock (Taqman primer/probe mix), 1 µL of DNA (20 ng/ µL concentration) and 1.25 of nuclease free water was added in each 5 µL reaction sample with the samples assayed and analysed in triplicate. The cycling conditions are shown in Table 6.2.

Table 6.2 Cycling conditions for Taqman assay (ARRB2)

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Duration</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>AmpliTaq Gold, UP, Enzyme Activation</td>
<td>95°C</td>
<td>10 mins</td>
<td>Hold</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>15 seconds</td>
<td>40</td>
</tr>
<tr>
<td>Annealing/Extension</td>
<td>60°C</td>
<td>1 minute</td>
<td>40</td>
</tr>
</tbody>
</table>
One-way analysis of variance (ANOVA) was performed to determine if the mean levels of the fentanyl doses were significantly different among subjects in comparison to the genotypes identified for each SNP \( (p < 0.05) \). The minor allele frequency (MAF) was obtained from the dbSNP database compiled by the National Centre for Biotechnology Information (NCBI). MAF refers to the frequency at which the least common allele occurs in a given population. In other words it is the ratio of chromosomes in the population carrying the less common variant to those with the more common variant. The MAF from the NCBI was compared with the genotype frequency obtained from our study.

### 6.3 Results and Discussion

#### 6.3.1 BDNF

Table 6.3 shows the mean fentanyl dose for each of the genotypes for the three SNPs in the BDNF gene. The pain scores are also presented, where, similarly to Chapter 5, responses to the question of ‘pain scores now’ were grouped into \( \leq 3/10 \) for ‘good’ pain control and \( >3/10 \) for ‘bad’ pain control (an analogue scale of 0 to 10 was used to score the severity of pain in increasing order).

<table>
<thead>
<tr>
<th>Genotype (BDNF)</th>
<th>n</th>
<th>*Freq</th>
<th>†MAF</th>
<th>Pain score (n=35)</th>
<th>Dose</th>
<th>p &lt;0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mean ± SD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs1491850</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.998</td>
</tr>
<tr>
<td>CC</td>
<td>6</td>
<td>0.17</td>
<td></td>
<td>4 (66.7)</td>
<td>2 (33.3)</td>
<td>44.7 ± 27.8</td>
</tr>
<tr>
<td>TC</td>
<td>21</td>
<td>0.60</td>
<td>0.37</td>
<td>16 (76.2)</td>
<td>5 (23.8)</td>
<td>44.5 ± 26.9</td>
</tr>
<tr>
<td>TT</td>
<td>8</td>
<td>0.23</td>
<td></td>
<td>7 (87.5)</td>
<td>1 (12.5)</td>
<td>45.1 ± 21.0</td>
</tr>
<tr>
<td>rs7934165</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.869</td>
</tr>
<tr>
<td>CC</td>
<td>10</td>
<td>0.29</td>
<td></td>
<td>7 (70.0)</td>
<td>3 (30.0)</td>
<td>41.3 ± 21.7</td>
</tr>
<tr>
<td>TC</td>
<td>18</td>
<td>0.51</td>
<td>0.43</td>
<td>14 (77.8)</td>
<td>4 (22.2)</td>
<td>46.7 ± 28.0</td>
</tr>
<tr>
<td>TT</td>
<td>7</td>
<td>0.20</td>
<td></td>
<td>6 (85.7)</td>
<td>1 (14.3)</td>
<td>44.4 ± 24.7</td>
</tr>
<tr>
<td>rs10835210</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.714</td>
</tr>
<tr>
<td>AA</td>
<td>8</td>
<td>0.23</td>
<td></td>
<td>7 (87.5)</td>
<td>1 (12.5)</td>
<td>43.5 ± 23.0</td>
</tr>
<tr>
<td>AC</td>
<td>16</td>
<td>0.46</td>
<td>0.26</td>
<td>12 (75.0)</td>
<td>4 (25.0)</td>
<td>48.4 ± 28.7</td>
</tr>
<tr>
<td>CC</td>
<td>11</td>
<td>0.31</td>
<td></td>
<td>8 (72.7)</td>
<td>3 (27.3)</td>
<td>40.2 ± 22.1</td>
</tr>
</tbody>
</table>

*Frequency; † Minor allele frequency

In a previous study [243] it was shown that three SNPs in the BDNF gene were associated with the methadone dose required for effective methadone maintenance.
treatment for opioid addiction (n=227). The study showed that the CC genotype in rs1491850 was associated with lower methadone dose, and the TT genotype in rs7934165 and CC genotype in rs10835210 gene were associated with a higher methadone dose. Our study found no significant association for any of the genotypes in the three SNPs investigated with fentanyl dose. However, the MAF for the BDNF SNPs showed consistency with a previous report [243] where the TC genotype for rs1491850 and rs7934165 and the AC genotype for rs10835210 showed the highest frequency in the studied population.

### 6.3.2 KCNJ6

In a previous study on opioids (n=488), the KCNJ6 rs2070995 AA genotype was associated with increased opioid requirements [218]. Another study on genetic variants in KCNJ6 also showed increased opioid requirements in Japanese patients after abdominal surgery [245]. In our study, no significant association was observed between the rs2070995 genotypes and fentanyl dose (Table 6.4). Though non-significant, KCNJ6 showed a reduced $p$ value of 0.07 in comparison to other polymorphisms studied. This could be due to AA genotype been found in only 1 patient (n=1), therefore the significance level is skewed to the lower value.

#### Table 6.4 Mean fentanyl dose and pain scores by genotype groups for KCNJ6

<table>
<thead>
<tr>
<th>Genotype (KCNJ6)</th>
<th>n</th>
<th>*Freq</th>
<th>†MAF</th>
<th>Pain score (n=35)</th>
<th>Dose</th>
<th>p &lt;0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs2070995</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>27</td>
<td>0.77</td>
<td>0.76</td>
<td>20 (74.1)</td>
<td>45.3</td>
<td>0.073</td>
</tr>
<tr>
<td>GA</td>
<td>7</td>
<td>0.20</td>
<td>0.18</td>
<td>6 (85.7)</td>
<td>35.1</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>1</td>
<td>0.29</td>
<td>0.18</td>
<td>1 (100.0)</td>
<td>95.8</td>
<td></td>
</tr>
</tbody>
</table>

*Frequency; † Minor allele frequency

### 6.3.3 ARRB2

Oneda et al [187] reported that the SNPs rs3786047, rs1045280 and rs2036657 of ARRB2 were significantly associated with response to methadone maintenance treatment (n=238). Our results do not correlate with these results with no association was observed for any of the genotypes and fentanyl dose (Table 6.5). However, the MAF for ARRB2 SNPs in the studied population showed consistency with a previous study [187].
Table 6.5 Mean fentanyl dose and pain score by genotype groups for ARRB2

<table>
<thead>
<tr>
<th>Genotype (ARRB2)</th>
<th>n</th>
<th>*Freq</th>
<th>†MAF</th>
<th>Pain score (n=35)</th>
<th>Dose</th>
<th>p &lt;0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>n (≤3) (%)</td>
<td>n (&gt;3) (%)</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>rs2036657</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.415</td>
</tr>
<tr>
<td>AA</td>
<td>21</td>
<td>0.60</td>
<td></td>
<td>16 (76.2)</td>
<td>5 (23.8)</td>
<td>45.0 ± 24.6</td>
</tr>
<tr>
<td>GA</td>
<td>12</td>
<td>0.34</td>
<td>0.33</td>
<td>10 (83.3)</td>
<td>2 (16.7)</td>
<td>47.9 ± 23.7</td>
</tr>
<tr>
<td>GG</td>
<td>2</td>
<td>0.57</td>
<td></td>
<td>1 (50.0)</td>
<td>1 (50.0)</td>
<td>22.1 ± 14.3</td>
</tr>
<tr>
<td>rs37866047</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.745</td>
</tr>
<tr>
<td>AA</td>
<td>2</td>
<td>0.57</td>
<td></td>
<td>1 (50.0)</td>
<td>1 (50.0)</td>
<td>22.1 ± 14.3</td>
</tr>
<tr>
<td>GA</td>
<td>11</td>
<td>0.31</td>
<td>0.34</td>
<td>9 (81.8)</td>
<td>2 (18.2)</td>
<td>52.3 ± 24.8</td>
</tr>
<tr>
<td>GG</td>
<td>22</td>
<td>0.63</td>
<td></td>
<td>17 (77.3)</td>
<td>5 (22.7)</td>
<td>43.0 ± 25.2</td>
</tr>
<tr>
<td>rs34230287</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.304</td>
</tr>
<tr>
<td>CC</td>
<td>28</td>
<td>0.80</td>
<td></td>
<td>22 (78.6)</td>
<td>6 (21.4)</td>
<td>48.1 ± 26.4</td>
</tr>
<tr>
<td>TC</td>
<td>6</td>
<td>0.17</td>
<td>0.08</td>
<td>5 (83.3)</td>
<td>1 (16.7)</td>
<td>30.7 ± 13.8</td>
</tr>
<tr>
<td>TT</td>
<td>1</td>
<td>0.29</td>
<td></td>
<td>0 (0.00)</td>
<td>1 (100.0)</td>
<td>32.2 ± 0.0</td>
</tr>
<tr>
<td>rs1045280</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.529</td>
</tr>
<tr>
<td>CC</td>
<td>4</td>
<td>0.11</td>
<td></td>
<td>3 (75.0)</td>
<td>1 (25.0)</td>
<td>46.4 ± 35.7</td>
</tr>
<tr>
<td>TC</td>
<td>15</td>
<td>0.43</td>
<td>0.33</td>
<td>13 (86.7)</td>
<td>2 (13.3)</td>
<td>43.2 ± 20.8</td>
</tr>
<tr>
<td>TT</td>
<td>16</td>
<td>0.46</td>
<td></td>
<td>11 (68.8)</td>
<td>5 (31.3)</td>
<td>45.7 ± 27.7</td>
</tr>
</tbody>
</table>

*Frequency; † Minor allele frequency

The results in this study did not demonstrate any significant associations between fentanyl dose and the genotypes studied. However, our results did demonstrate genotypes were associated with good pain control. Unfortunately, only samples from 35 of the 56 patients were available for PG studies. Of the 125 sampling points, 91 (72.8%) of the sampling points showed “pain now” scores of ≤3/10, and 34 (27.2%) demonstrated scores of >3/10, showing that these patients were adequately managed for their pain. However, as mentioned earlier in chapter 5, this could be due to the breakthrough medication that each patient was receiving in addition to their regular fentanyl patch.

Given the small sample size in these preliminary PG studies it is difficult to conclude any association between fentanyl dosing and the variability resulting from genetic polymorphisms in the ARRB2, BDNF and KCNJ6 genes examined in this study. All SNPs included in this study were identified following research by Oneda et al [187], Lotsch et al [218] and Levrans et al [243], where the average sample sizes were 317 patients and/or volunteers.
In addition, although the genes examined have previously been identified to contribute to influence fentanyl dosing, several other genes not examined in this study have also been suggested to play a role.

Once such gene is \textit{OPRM1}, where the analgesic effect of fentanyl was related to genotypes in the \textit{OPRM1} gene in a study conducted on Japanese patients who underwent cosmetic surgery \cite{246}. Subjects with the G allele of the \textit{OPRM1} A118G SNP were less sensitive to fentanyl and tended to be more sensitive to cold pressor-induced pain. Additionally, subjects with the G allele of the IVS3+A8449G SNP of the \textit{OPRM1} gene required less fentanyl for postoperative analgesia \cite{246}. In contrast, in another study it was shown that the \textit{OPRM1} A118G variant significantly reduced intrathecal fentanyl ED50 for labour analgesia, suggesting that women with the G variant may be more responsive to opioids and require fewer analgesic drugs \cite{247}. These findings for fentanyl PG may have implications for cancer patients receiving transdermal fentanyl.

The polymorphisms within the metabolic enzymes (\textit{CYP 3A4/5}) have also not been included in this study. Recently, a study was conducted on 620 cancer patients receiving transdermal fentanyl \cite{130}. Serum fentanyl concentrations and metabolic ratio vary considerably between cancer pain patients on transdermal fentanyl patches. \textit{CYP3A4*22} and \textit{CYP3A5*3} genotypes, and multiple clinical factors, combine to influence transdermal fentanyl pharmacokinetics, but accounted for only a small proportion of variability (<2%) in this study \cite{130}. More recently, a study conducted to investigate the clinical implication of \textit{CYP3A5} and \textit{ABCB1} polymorphisms on transdermal fentanyl administration in 60 Japanese cancer patients \cite{207} found that \textit{CYP3A5*3} and \textit{ABCB1 C1236T} affected the PK and clinical response to fentanyl in cancer patients receiving fentanyl via transdermal delivery. The study suggested that these polymorphisms could predict the incidence of adverse effects and permit the individualisation of fentanyl dosages in cancer patients.

Molecular genetic studies investigating the TaqI A polymorphism of the \textit{DRD2} gene have produced mixed results regarding its involvement in various drug addictions \cite{248-256}. However, evidence has arisen that the TaqI A polymorphism of the \textit{DRD2} gene may act as a genetic indicator of poor treatment outcomes among methadone maintenance patients \cite{257}. Overall these studies have indicated that the TaqI A
polymorphism may be involved as a more general risk factor for substance abuse and dependence, rather than being specific to any one class of drug [258]. As yet, fentanyl dependence has not been reported elsewhere with respect to DRD2 gene polymorphisms, meaning, its effect in fentanyl dosing is unknown. A larger study to predict the association between the DRD2 gene polymorphism and fentanyl dosing is warranted.

Similarly ABCB1 is also an important structural protein with a key role in in drug transport that can influence the absorption, distribution, and elimination of opioids. Transport of opioid substrates by P-gp is an important consideration in the study of opioid PG because of the significant interindividual variability observed in P-gp expression and function.

Hence an expanded study with a larger sample size incorporating the genes not included in this study may provide a better understanding of the variability observed in cancer patients receiving transdermal fentanyl at varying doses.

6.4 Conclusion

A preliminary study was conducted to examine several polymorphisms in the ARRB2, BDNF and KCNJ6 genes to determine any association with fentanyl dosing. Our results demonstrate no association with any of the genotypes investigated in our population. However, good pain control was associated with almost all of the genotypes studied. Further studies will be required with a larger sample size to verify the nature of the effect and the association of these variants to fentanyl dose and to assess the potential clinical applications of these findings. Similarly, several studies are also needed to replicate these results, to determine the functional significance the genetic variants and to explore their significance in populations of different ethnicities. Such studies could provide valuable information to better modulate individual opioid analgesic doses required to achieve satisfactory pain control and open new avenues for personalised pain treatment.
Chapter 7. Population pharmacokinetics of fentanyl: identifying patient-related factors influencing the dose-exposure variability

7.1 Introduction
7.1.1 Population pharmacokinetics

Pharmacokinetics is the process describing drug exposure in the body. It characterises the concentration-time courses of a drug after dose administration and is commonly thought of as “what the body does to the drug” [259]. Pharmacokinetics describes the drug movement mechanisms through the rate of absorption, distribution and elimination [260]. There are several methods of characterising the PK of a drug. One method is a model-based compartmental approach, and this is what is implemented in this chapter. With this method the body is represented by one or several homogenous, interconnected compartments, to which the drug is administrated, between which it is transferred and from which it is eliminated. The model structure is defined by the data and route of administration, and the model chosen determines the number of parameters to be estimated. One-, two- or three-compartment models are most commonly used with this approach [261]. Population analysis is used to describe data that arose from more than one individual. The approach does not require each individual to provide sufficient data to completely characterise their own PK profile. Population analysis methods allow borrowing of information between individuals to fill in gaps in the PK and PD profiles [262] allowing for the use of sparse sampling study designs. The influence of patient characteristics, such as body weight, on variability in drug exposure can be quantified, as well as any remaining unexplained variability between patients. Population analysis methods provide information for dose individualisation in order to reach a target that will meet an individual patient’s needs [263]. The population modelling approach is also referred to as nonlinear mixed-effects modelling, in which the fixed and random effects are estimated simultaneously giving rise to the term ‘mixed-effects’. Fixed effects are the typical population PK parameters; unknown constants, e.g. clearance (CL), volume of distribution (Vd) and absorption rate (ka) and their relationship to covariates such as weight, age and creatinine clearance (CrCL); they hence quantify the “structural” aspects of the model. The random effects (Stochastic model) represent the variability among subjects (Between Subject Variability, BSV), e.g. due to differences in genetics, age, sex, race and weight, within subjects or among occasions (Between
Occasion Variability, BOV) caused, for example, by disease progression, along with the residual ‘unexplained’ variability (RUV) [264-266].

A number of different software programs using different methods to estimate population PK parameters are available. However, the NONMEM software package developed by Beal & Sheiner in 1979 is the package most commonly used in academia and industry [267-270]. NONMEM is an abbreviation for Non-linear Mixed Effects Modelling and is a computer program, written in FORTRAN 77, designed to fit general statistical (non-linear) models to a wide variety of data. NONMEM fits the data for all subjects simultaneously and estimates the parameters, their variances and residual error using a maximum likelihood method [271].

PK models are developed by fitting the model to the observed concentration-time data. The software estimates the set of parameter values that produced the smallest difference between the actual observations and those predicted by the model. It provides a powerful and flexible means of obtaining the (typical) population PK parameter estimate, as well as estimates for BSV and BOV around the typical PK parameter estimate. The individuality of each subject is preserved such that an individual’s set of parameters, called empirical Bayesian estimates, can be obtained. This approach is well suited to clinical situations, such as in palliative care, as it can accommodate sparse sampled data of individual patients, as well as unbalanced and unstructured dosing and sampling protocols. Additionally, as mentioned above, a very important aspect of population modelling is that the sources of variability can be screened and quantified [262]. Variability in the pharmacokinetics may be due to any number of patient characteristics, such as body size, age, sex, other medications and disease state. Identifying the sources of PK variability can reduce the variability in drug exposure when doses are individualised according to the patient’s characteristics and supports decision-making when performing therapeutic drug monitoring. Any characteristic or factor can be tested for significance by incorporating it into a covariate model to explain variability.

Several steps are involved in finding the most appropriate model to describe a particular set of concentration-time data. Typical steps in building a PK model are [272]:

a) structural model development
b) stochastic model development
1. residual unexplained error model development
2. estimation of BSV
3. estimation of BOV

c) covariate model-building
d) full covariate model finalisation and final model interpretation
e) model evaluation and diagnostics
f) model validation
g) model application and simulation.

7.1.2 Structural model
To describe the transfer of a drug from the absorption site to the blood, as well as the distribution within, and elimination from, the body, single or multi-compartment systems can be used. The processes and transfer between the compartments are often governed by first-order kinetics (passive diffusion) but can also involve more complex saturable relationships. A simple one-compartment, linear elimination PK model describing the relationship between drug concentrations in the body and an intravenous (iv) administered dose can be written as:

\[ c(t) = \text{Dose}/V \times \exp\left(\frac{CL}{V} \times t\right) \]  

Eq- 7.1

This model states the relationship between the independent variable, time (t), and the dependent variable, concentration (c). The notation \( c(t) \) suggests that concentration depends on \( t \). Dose is a known constant, while CL and Vd are parameters that do not change with different values of \( t \) [273]. As mentioned above, fixed effects represent the typical population PK parameters (unknown constants, eg. CL, Vd, \( k_a \)) and their relationship to covariates (e.g. weight, age, creatinine clearance). Fixed effects are represented by the Greek letter THETA (\( \Theta \)) in the software NONMEM and have the same value for every subject, if no covariate is attached to the parameter; otherwise they have the same value for every subject with the same covariate value. Often more complex models need to be used to describe the PK properties of a drug. The model selection process is important in being able to describe the PK relationships of the drug as accurately as possible for further estimations and predictions. NONMEM has an inbuilt library where different models are automatically selected using model subroutines. For example, selecting ADVAN 1, TRANS 2 from the library builds a one-compartment distribution model after iv dosing and estimates CL and Vd.
7.1.3 Residual unexplained variability (RUV)

RUV is the variance of the error between observations and predictions. It is random and essentially unidentifiable with respect to various causes, accounting for variability arising from inaccurate recording of sampling and dosing times, variability in the dose (e.g. manufacturing tolerances), model misspecification and measurement (drug assay) errors. The difference between observed and predicted concentrations of the model may be described by the RUV in three different structures as required, i.e. constant variance or additive (Eq-6.2), an exponential error model (Eq-6.3) or a combined error model (Eq-6.4), as shown in the equations:

\[ C_{ij} = C_{pred,ij} + \varepsilon_{ij} \]  \hspace{1cm} \text{Eq- 7.2}  \\
\[ C_{ij} = C_{pred,ij} \times \exp^{\varepsilon_{ij}} \]  \hspace{1cm} \text{Eq- 7.3}  \\
\[ C_{ij} = C_{pred,ij} \times \exp^{\varepsilon_{ij}} + \varepsilon_{ij} \]  \hspace{1cm} \text{Eq- 7.4}

where

\( C_{ij} \) is the \( j^{th} \) observed concentration of the \( i^{th} \) individual, \( C_{pred,ij} \) is the predicted concentration from the model, and \( \varepsilon_{ij} \) is the difference between the observed and predicted concentrations.

The exponential error model is often preferred over the constant variance model in population PK modelling because observed and predicted measurements can only be positive, and as measured values increase, the value is often less precise, and their variance is proportional to the square of the predicted response [274].

7.1.4 Between subject and between occasion variability

BSV and BOV are referred to as random effects. Population models provide a means of characterising the extent of between-subject variability, i.e. the differences in exposure between one patient and another, and between-occasion variability (the differences in the same patient from one dose to the next) that a drug exhibits for a specific dose regimen in a particular patient population [273]. BOV is sometimes also referred to as within patient variability. Variability is an important concept in the development of safe and efficacious dosing; if a drug has a relatively narrow therapeutic window but extensive variability, the probability of both sub-therapeutic and/or toxic exposure may be higher [275], making the quantitation of variability an important objective for population modelling. The knowledge of BSV and BOV is essential in decision-making around TDM during clinical care. TDM is performed to optimise dosing regimens to
achieve a specific target concentration aimed at an optimal therapeutic response in the individual patient. If the estimation of BOV is greater than BSV, not much information will be gained from TDM due to the wide variability within an individual between dosing occasions [276]. TDM is recommended for drugs which show a large BSV to ensure each patient achieves concentrations within the target range by adjusting individual doses.

Random effects are represented by the Greek letter ETA (η) in the software NONMEM and are reflecting the difference between an individual’s parameter value and the population value. ETA is assumed to be normally, or log-normally, distributed across the population being evaluated, to be centred on zero and summarised by its variance, termed as OMEGA (Ω) in NONMEM. OMEGA describes the distribution of ETAs representing the BSV for the parameter across the population being studied [273]. An exponential error model representing BSV is given by equation 6.5 below:

$$\Theta_i = \Theta_{\text{pop}} \times e^{\eta_i} \quad \text{Eq- 7.5}$$

where

- $\Theta_i$ is the individual parameter value,
- $\Theta_{\text{pop}}$ is the typical population value and
- $\eta$ is the vector of the difference between the $i^{\text{th}}$ individual’s parameter values and the mean population value.

Similarly, BOV can be incorporated into the model as shown in Equation 6.6 below:

$$\Theta_{ik} = \Theta_{\text{pop}} \times e^{(\eta_i + \kappa_{ik})} \quad \text{Eq- 7.6}$$

where

- $\Theta_{ik}$ represents the PK parameter for the $i^{\text{th}}$ individual on the $k^{\text{th}}$ occasion and
- $\kappa$ is the within patient random effect (BOV).

‘Occasion’ is commonly defined by the analysis and can vary from one study to the next.

### 7.1.5 Covariate model-building

The covariate model identifies the relationship between a parameter such as clearance and a covariate, such as body weight in the population being studied. Included covariates should improve the model fit statistically, but also inform about the clinical importance of a covariate [277]. Questions such as “How much does drug exposure vary with age and in which direction?” or “What is the influence of weight on...
exposure?” – or any other covariates that are included – are answered using the covariate model [273]. Covariates such as body weight, age, renal or liver function, route of administration, co-administration of enzyme inducer, inhibitor and pain score could be included in the model to observe any influence on the exposure variability in the studied population. Each covariate is introduced into the structural model separately and the pattern of residuals and changes in the BSV, RUV and the OFV are examined, guiding decision-making on the importance of the relationship.

7.1.6 Full covariate model and final model interpretation
This step of model building incorporates the complete population model, consisting of structural, stochastic and covariate models. It encompasses the interpretation of the population model, with, assessment of the assumptions made and the outcomes for clinical decision-making.

7.1.7 Model evaluation and diagnostics
The modelling evaluation is performed at several steps throughout the modelling process to discern whether the constructed models improve the fit to the data. The objective function value (OFV) produced by NONMEM is the most common criteria used to access models. Other model diagnostics and evaluation criteria include an assessment of the model to demonstrate reduced RUV, including covariate relationships explaining BSV and graphical diagnostics such as ‘goodness-of-fit’ plots and VPC performed to assess predictive performance of the model.

7.1.7.1 Objective function value
There are several different mathematical ways of evaluating the best fit of the predictions to the actual observations. NONMEM uses a Least Squares type criterion and expresses it as the OFV. The OFV is minus twice the log likelihood [271] Since the computation of the OFV depends on the differences between observations and model predictions, the lower the OFV value, the better the fit to the data. Negative OFV values are also possible to obtain but have no special significance in relation to positive OFV values. Comparison between two competing models can be done by calculating the difference of two log-likelihoods ($\Delta$OFV), which is asymptotically and approximately chi-squared distributed. For a model to be considered a significant improvement at $p<0.05$ or 0.01 with one degree of freedom (one parameter difference), the $\Delta$OFV needs to be at least 3.84 or 6.63 respectively [278].
**7.1.7.2 Graphical diagnostics**

Goodness-of-fit plots are performed to visually assess the relationship between observations and population on the one hand and individual predictions and weighted residual distributions on the other. The visual predictive check (VPC) is a model diagnostic that can be used to: (a) allow comparison of alternative models, (b) suggest model improvements, and (c) support the appropriateness of a model while assessing its predictive performance. It is rapidly becoming one of the most important diagnostic tools available. The VPC is constructed from stochastic simulations from the model; therefore, all model components contribute, and a VPC can diagnose both structural and stochastic model specifications. In a typical VPC, the model is used to repeatedly simulate observations, usually \( n \geq 1000 \), according to the original design of the study. Based on these simulations, percentiles of the simulated data are plotted over against an independent variable. It is then desirable that the same percentiles be calculated and plotted for the observed data in order to aid comparison of predictions with observations. The percentiles chosen for plotting are often the 5\(^{th}\), 50\(^{th}\) and 95\(^{th}\) percentiles. However, for small data sets, less extreme percentiles (e.g. 25\(^{th}\) and 75\(^{th}\)) may be more appropriate [279].

**7.1.8 Validation of model**

There are two types of validation: (a) external and (b) internal. External validation is the application of the developed model to a new data set (validation data set). External validation provides the most stringent method for testing a developed model; however, is not commonly used due to limitations on resources.

The most robust way to ensure model appropriateness and validate a model prior to its application is external model validation with new validation data. Validation is evaluation of the predictability of the model developed (i.e., the model together with the model parameter estimates) using a new data set not used for model building and parameter estimation. Validation also depends on the objective of the analysis. A model may be valid for one purpose and invalid for another. There is no right or wrong model, nor is there a right or wrong method of fitting. The choice of a validation approach depends on the objective of the analysis. Not all population models need to be validated [280]. However, validation methods are still being evaluated and may require the model to give an appropriate power.
Internal validation is the use of data-splitting and re-sampling techniques. Data-splitting is a useful internal validation technique for creating a validation dataset to test the predictive performance of a model when it is not practical to collect new data to be used as a validation data set. Another technique of internal validation is re-sampling. There are two ways to perform re-sampling: (a) cross-validation and (b) bootstrapping. Cross-validation is the use of repeated data-splitting, which may have the advantage that the size of the model development data set can be much larger than with alternative internal validation methods. This way, fewer data are discarded from the estimation process, and the variability is reduced by not relying on a single sample split. Bootstrapping, another way to perform internal evaluation via re-sampling, also has the advantage of using the entire data set for model development [281]. Because the sample size is limited on some occasions e.g. in the palliative care settings, where ethical or medical concerns prevent recruitment into studies, bootstrapping can be especially useful for evaluating the performance of a population model if there are no test data set, but it is also to obtain non-parametric confidence intervals of the parameter estimates [282].

7.1.9 Model application and simulation
Simulation is an important component of population PK model evaluation and inference. For the purpose of evaluation, the model may be used to simulate data that are suitable for direct comparison with the index data. This can be done by employing either a subset of the original database used in deriving the model (internal validation) or a new data set (external validation). For the purpose of inference, the model is generally used to simulate data other than observed data [273]. Simulating a planned study or potential alternative dosing regimens offers a potentially useful tool for evaluating and understanding the consequences of different choices. Shortcomings in study design might otherwise result in the collection of uninformative data. Simulation can reveal the effect of input variables and assumptions on the results of a planned population PK study [280]. Furthermore, simulations can be used to demonstrate the benefits in target attainment of different doses or dosing regimens and to develop more adequate dosage regimens to achieve target therapeutic concentration. Simulating the stochastic models is more complex than simulation from simple fixed-effect models. In theory, a larger number of simulation replicates is recommended, although the number is limited by considerations of computation time and data size. A common “rule of thumb” is that at least 200 simulations are needed when summarizing simulated data as mean values, and at least 1,000 are needed to obtain confidence intervals [273].
Hence, from the final population model it is possible to evaluate different dosing strategies for patients to try to improve treatment, at the same time recognising the comparative treatment costs. Nevertheless in some drugs the therapeutic target is still doubtful, and the potential risks of these dosing schedules need to be assessed on an individual basis. Further investigation should be performed in an attempt to improve the treatment.

7.1.10 Fentanyl population pharmacokinetics

This chapter describes the fentanyl population PK of cancer patients in plasma in order to determine the average PK behaviour of fentanyl in this patient population – the BSV and BOV – and identify any subject-specific factors (covariates) that influence it, with the aim of supporting dose individualisation. As mentioned in earlier chapters, fentanyl dosing is based upon a hit-and-trial mechanism with dose titration starting at a lower dose and increasing until analgesia is achieved. Due to large variability in patients, dosing is challenging. A population pharmacokinetic model for fentanyl to identify covariates influencing the dose-exposure relationship among and within cancer patients aims to support initial dose selection as well as dose adjustment in the future. A sound understanding of the influence of covariates such as body weight, age, renal/hepatic function, and effect of enzyme inhibitor/inducer on the drug dose (exposure) is important for dose individualisation, in order to improve the safety and efficacy of a drug by appropriately controlling variability in drug exposure [273].

7.2 Methods

7.2.1 Patient and sampling

Patients in this study were receiving regular care through the oncology and palliative care units. They were selected only on the basis that they were being given Durogesic® patches for pain control. Blood sampling and details about the collection procedure are explained in Chapter 5 of this thesis. Additionally, a range of demographic data and information on concomitant medications and organ function, as measured by routine biochemical and haematological tests were taken. A range of baseline information including age, height, weight, tumour-characteristics, renal function [283], liver enzymes (aspartate transaminase (AST) and alanine transaminase (ALT)), fentanyl dose, duration of treatment, site of patch placement, timing of samples in relation to timing of fentanyl patch change and concomitant medications inhibiting or inducing CYP 3A4/5 enzymes were collected from each participant. Specifically,
Cloramphenicol (n=1), diltiazem (n=1), fluconazole (n=2), tamoxifen (n=2) and fluoxetine (n=1) were identified as enzyme inhibitors and dexamethasone (n=28) as an enzyme inducer for n number of patients. Body mass index and body surface area were determined at the first observation. At each time of sampling, participants were asked to complete a numerical rating scale for “pain right now” as mentioned in Chapter 5. Similarly, at each fentanyl sampling time, a visual and descriptive 5-point scoring system for adhesion of transdermal patches was used to grade the degree of patch adhesion as mentioned in Chapter 5. Blood collection methods and storage have been described in Chapter 5. Plasma fentanyl concentrations were measured by HPLC-MS/MS detection (see Chapter 2). A previous population PK modelling study in the same target population \[141\] indicated that 40 subjects is the minimum number necessary to provide satisfactory estimates of the structural parameters (e.g. clearance, volume of distribution) and the variance parameters (e.g. BSV and BOV). In this study, 56 patients were approached to obtain plasma and saliva samples.

7.2.1.1 PK data set
The data was entered in chronological order by patient to enable population pharmacokinetic analysis. A visual inspection of the data was undertaken by creating plots of the concentration versus time to identify outliers and mistakes due to incorrect data entry. One outlier in the data set was identified where plasma concentration was almost 300 % higher than the population average after repeated analysis. All the missing data for the covariates included were replaced by the averaged value of the entire population being studied.

7.2.2 Parameter estimation methods
The population analysis was performed in NOMMEM (Version 7.3). NONMEM (http://www.iconplc.com/technology/products/nonmem/) was run with double precision with a tan Intel FORTRAN compiler. NONMEM \[284\] runs were executed using PsN (version 3.76), which is available free of charge at http://wfn.sourceforge.net. The software was accessed through a windows server via remote desktop connection. The software licence was supported by the Australian Centre for Pharmacometrics at the time of modelling.

To estimate the population PK model parameters, the first order conditional estimation (FOCE) method with INTERACTION was used. Using INTERACTION allows interaction
between BSV (\(\eta\)) and RUV (\(\varepsilon\)). It makes it possible to account for different individuals having different residual variability i.e. having different expected concentration with the same dose given at any time. The FOCE method with INTERACTION is preferred over the FOCE method for parameter estimation in NONMEM [278]. Plots utilising empirical Bayes estimates as diagnostics were produces only when the shrinkage [285] was below 30%.

7.2.3 Pharmacokinetic model building and model selection
The pharmacokinetic model was developed using the following steps in order to define the model providing the best fit with the observed concentration-time data.

a) A model was considered a better fit if a statistically significant reduction in OFV in comparison to other models was seen. The difference in OFV between a full and reduced model is assumed to be asymptotically and approximately \(\chi^2\)-distributed for nested models. The degree of freedom equals the difference in the number of parameters between different models. The significant level was set to a \(p\)-value of <0.05. It corresponds to a drop in OFV by 3.84 units when one parameter is added to the model.

b) A visual examination of standard ‘goodness of fit’ diagnostic scatter plots was undertaken: These were: a population and individual predicted versus observed concentrations, b) residual and conditional weighted residual plots.

c) Prior to covariate model building, potential parameter-covariate relationships were investigated graphically, and only those that correlated and were physiologically plausible were included in the base model.

d) Total body weight was included \textit{a priori} into the model on clearance and volume of distribution, as the weight range in the obtained dataset was limited and the relationship was believed to be true [286].

e) Afterwards a newly introduced covariate was considered to improve the model if a reduction in OFV by 3.84, with a preferable decrease in BSV on the parameter of interest, was observed.

7.2.4 Structural and statistical error model
The model building started with the simplest model and proceeded towards more complexity as required, stopping when the model failed to further improve the fit to the data. One- and two-compartment models with linear elimination were fitted to the data using the library model subroutines ADVAN1 TRANS2, ADVAN3 TRANS4
and ADVAN4 TRANS4. Further model extension was limited due to the limited amount of data collected.

Three different types of residual error models – additive, exponential and combined – were tested in order to describe the residual error model. The stochastic model was developed by adding etas (η) in a stepwise manner to each parameter of the compartment model. The covariance between subject variability was initially set to zero. Using the ‘BLOCK’ option in NONMEM, the estimated covariance between PK parameters (non-zero value) was then evaluated. The stochastic model was re-evaluated in the final model.

7.2.5 Covariate screening and modelling
After defining the best structural and statistical error model, screening for potential covariates was performed. Scatter plots between predicted PK parameter values and laboratory results, including different covariates such as patch adhesion, pain score, enzyme inducer/inhibiter, clearance and AST/ALT were completed and visually examined. Their influence on the model was determined by adding the covariate to the model.

7.2.6 Predictive performance of final PK model
Visual predictive checks were used to establish the predictive performance of the developed PK model. The estimates of the PK parameters were fixed, and concentration profiles were simulated using NONMEM and PSN software and the study design from the original data set. The simulated profiles were plotted on top of the raw data, with the 10th, 50th and 90th percentiles calculated for both, and graphically displayed. A model was considered to appropriately fit the raw data if the data points were evenly distributed around the 50th percentile of the simulated data and 80% was within the 10th and 90th percentile range over all aspects of the concentration time curve, i.e., input, disposition and elimination.

7.2.7 Bootstrapping
The stability and robustness of the final model was assessed via an internal evaluation method which involved bootstrapping with replacement to assess how accurately the value of the population parameters of a sampled distribution can be estimated in the wider population and to assess stability and predictive performance of the
pharmacokinetic model. Five hundred bootstrap replicates were performed to obtain parameter estimates with 90 % CI [281].

7.3 Results and Discussion

7.3.1 Patients
Patients who provided samples in this study had malignant disease primarily of the breast, prostate, cervical, pulmonary and ovary. There were 163 venous blood samples drawn from 56 patients with a median (range) of 2 (1–10) samples per patient. Patient demographics and measured drug concentrations are shown in Tables 5.1 and 5.2 of Chapter 5. Observed concentrations were collated with time since last patch administration to identify outliers and errors due to incorrect data entry (Figure 7.1). The data set was reliable, with only one concentration considered an outlier and removed from the data set.

Figure 7.1 Raw data showing plasma fentanyl concentration versus time since last patch change. The outlier (9.78 µg/L at 36 hr) was excluded.

7.3.2 Covariates and parameters
Several covariates–parameter relationships were tested in the analysis. Information on patient demographics specific to the covariates included in this study is given in Table 7.1 below. Table 7.2 shows a list of covariates and parameters along with the reasons for their inclusion and their impact on the study.
Table 7.1 Patient demographics for covariates tested in this analysis

<table>
<thead>
<tr>
<th>Covariate</th>
<th>Median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (kg)</td>
<td>69.9</td>
<td>41.8–110</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.67</td>
<td>1.52–1.93</td>
</tr>
<tr>
<td>Pain score (scale of 0-10)</td>
<td>2</td>
<td>0–10</td>
</tr>
<tr>
<td>Patch adhesion (score of 0-4)</td>
<td>0</td>
<td>0–3</td>
</tr>
<tr>
<td>Enzyme inhibitor/inducer (number of patients)</td>
<td>7/28</td>
<td></td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>35</td>
<td>8.0-153.0</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>40.7</td>
<td>15.0-169.0</td>
</tr>
</tbody>
</table>

ALT= Alanine aminotransferase; AST= Aspartate aminotransferase

Table 7.2 List of covariates tested in the analysis

<table>
<thead>
<tr>
<th>Covariates</th>
<th>Parameter</th>
<th>Reason for including in the study</th>
<th>Impact</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight</td>
<td>CL/Vd</td>
<td>Patients with higher body mass have greater Vd and CL</td>
<td>Altered CL and Vd with weight</td>
</tr>
<tr>
<td>Height</td>
<td>CL/Vd</td>
<td>Taller patients have greater Vd and CL</td>
<td>Altered CL and Vd with height</td>
</tr>
<tr>
<td>Patch adhesion</td>
<td>F/kₐ</td>
<td>Greater patch adhesion results in faster kₐ and/or increased F</td>
<td>Reduced adhesion results in reduced F</td>
</tr>
<tr>
<td>Pain score</td>
<td>F</td>
<td>Improved pain management with higher F</td>
<td>Reduced F decreases drug exposure and increases pain intensity</td>
</tr>
<tr>
<td>Enzyme inducer</td>
<td>F,CL, Vd</td>
<td>Enzymatic induction causes increased (first-pass) metabolism</td>
<td>Decreased drug exposure</td>
</tr>
<tr>
<td>Enzyme inhibitor</td>
<td>F,CL, Vd</td>
<td>Enzyme inhibition decreases (first-pass) metabolism</td>
<td>Increased drug exposure</td>
</tr>
<tr>
<td>ALT/AST</td>
<td>CL</td>
<td>Altered liver function decreases CL</td>
<td>Increased drug exposure</td>
</tr>
</tbody>
</table>

F=bioavailability, CL=clearance, Vd=volume of distribution, kₐ=absorption rate, ALT= Alanine aminotransferase; AST= Aspartate aminotransferase

7.3.3 Structural PK and statistical error model

The steps performed to establish the basic structural and stochastic error model are presented in Table 7.3. A proportional residual error model was shown to best describe the RUV model. A 2-compartment model was constructed to describe the data; however, it failed to improve the fit over the 1-compartment model, so no further compartmental models were tested. The stochastic model is best described as adding BSV to CL. Adding BSV on Vₜ and kₐ did not improve the model fit to the data or reduce the OFV,
possibly because of the very limited number of samples drawn early after patch administration and in non-steady-state conditions. Setting the covariance to a non-zero value between CL and Vd and/or ka by using the BLOCK option did not improve the model, as shown by a non-significant change to OFV.

Table 7.3 Results of PK base model building

<table>
<thead>
<tr>
<th>No</th>
<th>Model description</th>
<th>OFV</th>
<th>Δ OFV</th>
<th>Compared to model</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>1 COMPT; BSV CL, PROP RUV</td>
<td>−185.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1 COMPT; BSV CL, ADD RUV</td>
<td>−76.0</td>
<td>109.6</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>1 COMPT; BSV CL, COMB RUV</td>
<td>−185.6</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>1 COMPT; BSV CL &amp; Vd, PROP RUV</td>
<td>−186.0</td>
<td>−0.35</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>1 COMPT; BSV CL &amp; ka, PROP RUV</td>
<td>−185.6</td>
<td>−0.004</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>1 COMPT; BSV CL, Vd, BLOCK (CL, Vd), PROP RUV</td>
<td>−187.3</td>
<td>−1.6</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>2 COMPT; BSV on CL &amp; Vd, PROP RUV</td>
<td>−185.6</td>
<td>0.0421</td>
<td>1</td>
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<tr>
<td>9</td>
<td>1 COMPT; BSV CL, BOV CL, PROP RUV</td>
<td>−190.5</td>
<td>−4.85</td>
<td>1</td>
</tr>
</tbody>
</table>

*Base model; COMPT=compartment; PROP=proportional; RUV=residual unexplained variability; ADD=additive; COMB=combined; BSV=between subject variability; BOV=between occasion variability

7.3.4 Covariate model building

Once the base model had been developed, various covariates were added to it to identify the influence of the covariates (Table 7.4). Prior to model building, parameters were also plotted against covariates to identify any significant relationships (Figures 7.2, 7.3 and 7.4). Such initial plots were designed to allow those covariates to be identified that would have more influence and impact on the PK of the drug of interest.
Figure 7.2  Relationship of volume of distribution and clearance to creatinine clearance

Figure 7.3  Relationship of various covariates to volume of distribution
Figure 7.4 Relationship showing various covariates against clearance

Table 7.4 Results of PK covariate model building

<table>
<thead>
<tr>
<th>No.</th>
<th>Model description</th>
<th>OFV</th>
<th>Δ OFV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>Base Model</td>
<td>−185.6</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Model 1 + PA on F</td>
<td>−185.8</td>
<td>−0.2</td>
</tr>
<tr>
<td>3</td>
<td>Model 1 + CL&lt;sub&gt;Cr&lt;/sub&gt; (mL/min) on CL</td>
<td>−185.8</td>
<td>−0.2</td>
</tr>
<tr>
<td>4</td>
<td>Model 1 + inducer effect on CL</td>
<td>−186.6</td>
<td>−1</td>
</tr>
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<td>5</td>
<td>Model 1 + inhibitor effect on CL</td>
<td>−186.0</td>
<td>−0.4</td>
</tr>
<tr>
<td>6</td>
<td>Model 1 + AST on CL</td>
<td>−185.8</td>
<td>−0.2</td>
</tr>
<tr>
<td>7</td>
<td>Model 1 + ALT on CL</td>
<td>−185.8</td>
<td>−0.2</td>
</tr>
<tr>
<td>8#</td>
<td>Model 1 + BOV on CL</td>
<td>−190.5</td>
<td>−4.9</td>
</tr>
</tbody>
</table>

* Base model = 1 compartment distribution model; BSV CL, proportional residual error, weight included on CL & V<sub>d</sub> (CL/F=θ(1) • [weight ÷ 70]<sup>0.75</sup>, V<sub>d</sub> /F=θ(2) • [weight ÷ 70]), θ(1) = estimated typical value of CL, θ(2) = fixed to 350 for 70 kg. 
# Final model including BOV on CL 
Δ OFV = change in OFV from model 1

BSV: between subject variability; BOV: between occasion variability; CL: clearance; V<sub>d</sub>: Volume of distribution; PA: patch adhesion; F: bioavailability; CL<sub>Cr</sub>: Creatinine clearance; AST: Aspartate transaminase; ALT: Alanine transaminase; OFV: objective function value obtained from NONMEM.
7.3.5 Final model evaluation

The diagnostic scatter plots for the final pharmacokinetic model are given in Figure 7.5. Individual and population prediction against observed concentration gives an overall impression of how well the model can predict the data. Larger or uneven deviations from the line of identity suggest that there are problems with the fit. The plots (Figure 7.5A and B) showed that the model could well predict the population and individual data. Plots of the weighted residual versus model predicted concentrations, and conditional residual versus time post dose, as well as individual and population predictions versus observed concentrations are also shown. Weighted residuals versus individual predictions are used for assessing the stochastic model, with no trend in the magnitude, thus presenting a well-fitted model (Figure 7.5C). Similarly, conditional weighted residuals were plotted to diagnose the structural model. When the residuals are small and scattered evenly around the “0” line, the model was assumed to be acceptable (Figure 7.5D).

A prediction-corrected visual predictive check of the final pharmacokinetic model is shown in Figure 7.6. The 10th and 90th percentile are summarised and graphed, along with the median (50th) of the raw data and the simulated data from the model. A visual inspection of the results of the predictive check shows that the final pharmacokinetic model describes the data well, with about 80% of the observed fentanyl concentrations within the 10-90th percentile lines and symmetrically distributed around the median.

The mean estimates resulting from the bootstrap procedure were very similar to the population estimates of the final model, indicating that the estimates for the population PK parameters in the final model are robust and the final model was stable. The results of bootstrap analysis also revealed that population pharmacokinetic parameters have a high level of internal validity, with all 500 runs converging and 462 finishing with successful minimisation. The results of the 500 bootstrap replicates are summarised in Table 7.5.
Figure 7.5  Diagnostic scatter plots ('goodness-of-fit' plots) for the final pharmacokinetic model
This study investigated the population pharmacokinetics of fentanyl in cancer patients, particularly in relation to the effects of patch adhesion, as well as several other covariates. Results from the covariance screening and modelling are shown in Table 7.5.
### Table 7.5 Final parameter estimates from the basic and the final model with their RSE and the median parameter estimates from 500 bootstrap replicates with the 90% CI

<table>
<thead>
<tr>
<th>Pharmacokinetic parameters</th>
<th>Base model Mean (RSE %)</th>
<th>Final model Mean (RSE %)</th>
<th>500 Bootstrap replicates Mean (90 % CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clearance (CL/F) (L/h/70 kg)</td>
<td>108.0 (8.7)</td>
<td>122.0 (9.4)</td>
<td>122.2 (104.9 – 142.7)</td>
</tr>
<tr>
<td>Volume of distribution (Vd/F) (L/70 kg)</td>
<td>350*</td>
<td>350*</td>
<td>350*</td>
</tr>
<tr>
<td>Absorption rate constant (ka) (hr⁻¹)</td>
<td>0.014 (19.9)</td>
<td>0.013 (21.1)</td>
<td>0.013 (0.008 – 0.018)</td>
</tr>
<tr>
<td>BSV CL (CV %)</td>
<td>39.9 (16.5)</td>
<td>38.5 (19.5)</td>
<td>37.2 (24.9 – 50.0)</td>
</tr>
<tr>
<td>BOV CL (CV %)</td>
<td>-</td>
<td>22.5 (36.1)</td>
<td>21.5 (0.2 – 43.6)</td>
</tr>
<tr>
<td>Residual unexplained variability Proportional error (%)</td>
<td>41.5 (6.7)</td>
<td>36.3 (10.2)</td>
<td>36.0 (21.9 – 40.8)</td>
</tr>
</tbody>
</table>

BSV: between subject variability; BOV: between occasion variability. *fixed

Although some research showed that fentanyl pharmacokinetics can be described with a three-compartment distribution model [51] passing through enterohepatic circulation [287], this study has constructed a one-compartment model, including the first-order absorption. The basis for approximating the pharmacokinetics after fentanyl patch administration in such a manner is the limited data per patient and the non-steady-state conditions. All patients were terminally ill and receiving continuous pain medication. Furthermore, in order to describe the pharmacokinetics of fentanyl with the incorporation of the enterohepatic circulation, the extent of reabsorption needs to be much higher and requires many more measurements to estimate the higher number of parameters [51, 287]. As in the research of McClain and Hug, Murphy et al. and Ariano et al., the elimination of fentanyl from the general circulation is here described by linear kinetics [51, 288, 289]. Furthermore, in agreement with Kokubun et al., collecting multiple samples from cancer patients in practice to build a three compartment model analysis, including enterohepatic circulation [290], is difficult due to logistical and procedural limitations. Additionally, the dose-concentration relationship in this study was linear within the observed data set. The typical population CL/F for fentanyl (BSV, CV%) was estimated at 122 L/h/70kg (38.5%, shrinkage of 18.6%), Vd/F was fixed to 350 L/70 kg [61], and the absorption rate was 0.013 h⁻¹. BOV estimation was supported for CL/F (22.0%) in which the OFV was reduced by 4.8 points and the RUV reduced from 41.5% to 36.3%.
A further aim of this study was to investigate the effects of patch adhesion, as well as several other covariates on the population PK of fentanyl in cancer patients. No significant change in OFV was observed with added patch adhesion score in the covariate model. Patch adhesion was scored at the time of each blood sampling point. A relationship between predicted PK parameter values and adhesion was noted in scatter plots prior to covariate model building. Though patch adhesion was scored in 5 categories, here it was included as just 2 categories, with one of the categories being perfect patch adhesions measured at each sampling point, and the other category including all less than perfect attached patches as insufficient data was available to estimate the impact for each category separately. The patch adhesion model estimated that almost all the patient had 90 % or greater patch adhesion over the entire 72 hr administration time. The low patch adhesion seen in 10 % of cases could be due to patch lifting only on day three or close to the 72 hr patch change time, and patch adhesion on days one and two was appropriate and potentially the reasons why incomplete patch adherence did not significantly impact on overall bioavailability. The absorption rate (kₐ) was estimated to be 0.013 h⁻¹, similar to reports from previous studies [289, 290] and showed that within 72 hr of the fentanyl patch administrations, >50-75 % of the drug from the patch reached the systemic circulation, given an approximate absorption half-life of 50 hours. No concentration data could be obtained before the first patch administration as all patients’ pain had been managed with fentanyl chronically. This limited the estimation of a previous reported lag time [290] and simultaneous estimation of Vd/F and absorption rate.

As described in earlier chapter, fentanyl is released from transdermal fentanyl at a constant rate. The concentration reaches a steady state within 24 hours, and a constant serum concentration of the drug can be maintained through repeated patch application at 72 hr intervals [61]. However, a study has reported that fentanyl serum concentrations declined between 24 and 72 hr after application of the Durotep® Patch, a reservoir fentanyl patch. The findings indicate that at steady state, fentanyl serum concentrations may not be maintained at a constant level for three days following application of transdermal fentanyl, and that the analgesic effect of the drug may be reduced by 72 hr after applying the patch [291]. In that study, however, the level of patch adhesion was not accounted for. The study also excluded data from patients who had severe organ failure and high fever. The variation in fentanyl concentration may therefore be due to insufficient patch adhesion within the 72 hr time frame. In our study patients did not
required a patch change prior to 72 hours and we observed only a marginal decline within a dosing interval. Findings from this study show that at 72 hr post-patch administration, sufficient drug still remains in the patch for absorption.

Several other covariates were tested and included in the model to identify sources of variability; however, no or only minimal changes in OFV were seen. In screening, the influence of patient characteristics, as well as the effect of pain score, enzyme inhibitor/inducer, ALT/AST and estimated CrCL on fentanyl clearance and volume of distribution did not strongly correlate. Testing it in the model did not improve the fit to the data. Moreover, none of these characteristic resulted in any significant reduction in the interpatient variability in clearance and volume of distribution. The relationship between weight and CL/F and Vd/F was included a priori based on theoretical allometric scaling, to focus the investigation on identifying other influences on CL/F than patient’s size. This relationship between weight and CL/F has been included in other studies similarly [155], allowing the possibility of dosing by weight. Some trends were noted, where patients who were being concomitantly treated with an enzyme inducer had a CL/F of 128.5 ± 43.1 L/hr/70kg compared with a CL/F of 122.2 ± 46.2 L/hr/70kg for all patients. The identification of a significant relationship between co-medications and CL/F of fentanyl was challenging due to multidrug administration commonly seen in this patient population resulting in several drug-drug interactions. The data set had only a small number of patients in one of the categories (inhibitor/inducer) (Table 7.1), meaning that the basis for appropriately testing these covariates was inadequate. Patients with AST values >100 IU/L, had individual CL/F estimates >100 L/h/70 kg. Perhaps this should be investigated further in a larger data set including more patients.

Kokubun et al. [290] performed a population PK study on cancer patients receiving transdermal fentanyl. The study concluded that Child-Pugh Score, as a measure of chronic liver disease, and use of a cytochrome P450 3A4 (CYP3A4) inducer were the most significant factors with relation to variations in serum fentanyl concentration. The Child–Pugh Classification is a broadly based (three categories) scale for chronic liver disease based collectively on bilirubin, albumin, prothrombin time, encephalopathy, and ascites data, but it is not commonly applied to liver function in cancer patients [155]. However, in this study no significant influence on liver function or an enzyme inhibitor or inducer was detected, possibly due to the limited number of patients. Overall, the
data collected from this vulnerable patient population were sparse and it is not unexpected that covariate relationships were not identified [155].

The relation between pain score and individual CL/F was investigated to evaluate if patients with a faster individual CL/F had higher pain scores due to an overall lower fentanyl exposure. However, this no such relationship was found probably due to most patients being on break through pain mediation (other than fentanyl) at the same time and the subjectivity of the score. The factors leading to variability in exposure and pain management still remain largely unknown. A more comprehensive study with more patients is needed in the future.

BSV estimated for CL was 38.5%, reflective of the heterogeneity in this patient cohort. Estimation of BSV on other parameter estimates was not supported by the data. However as mentioned above, TDM is recommended for drugs that show a large BSV, in order to ensure that each patient achieves drug exposure as close as possible to the target by adjusting individual doses. However, if BOV is greater than BSV, not much information would be gained from TDM due to the wide variability within an individual between dosing occasions [276]. In this study, as shown in Table 7.5, BOV on clearance was found to be smaller (22.5 %) than BSV on clearance for fentanyl. This confirms that TDM is useful if performed and supported by dose individualisation. Future studies should investigate whether the application of TDM to improve dosing can lead to more effective pain control in cancer patients receiving transdermal fentanyl.

7.4 Conclusion

In this study a model was successfully developed that was capable of describing fentanyl exposure in cancer patients. Besides a priori included weight, no patient characteristic could be identified that significantly influenced fentanyl pharmacokinetics in a predictive manner. The overall degree of patch adhesion within the study cohort was high (>90% patients scored 0) and potentially the reason why incomplete patch adherence did not significantly impact on overall bioavailability. Therefore, patch adhesion, while not identified as a significant covariate, is likely to influence fentanyl exposure and should be monitored in clinical practice. Changes in fentanyl CL/F within the same patient from one patch to the next are small and TDM of fentanyl plasma concentration might be useful in the future to achieve target concentrations.
The relationship between WT and CL/V was allometrically scaled, since the weight range in the data set was not broad enough to estimate this relationship. However, other lipophilic drugs have previously included WT as a relationship [155], allowing the possibility of dosing by weight for future initiatives. The factors leading to variability in exposure and pain management still remain largely unknown. A more extensive study is needed due to the heterogeneity of the population. Future models could also include the saliva fentanyl concentrations to identify any subject-specific factors (covariates) that could influence the average PK behaviour of fentanyl in this patient population, with the aim of supporting dose individualisation.

The results from this study have been published [292] and presented at various conferences and other professional forums.
Chapter 8. Conclusion and Future Directions

In this study, extensive research was performed on fentanyl as it is used for pain management in cancer patients. Many studies have reported intra- and interpatient variability with fentanyl. In the case of cancer patients, such variability tends to obstruct the better management of pain. One reason for this is a lack of knowledge of the effect of various factors on the PKPD of opioid analgesics. This study has specifically addressed some of the knowledge gaps and could thus prove helpful in understanding the PKPD of the drug. Many patient-related factors were studied and modelled to identify if such factors contribute to dose response variability including pain, liver function, kidney function, effect of enzyme inducer/inhibitor and patch adhesion. No significant patient characteristic could be identified that significantly influenced fentanyl PK in a predictive manner. However, incorporation of patch adhesion scores in the PKPD study was novel in that no reports were available elsewhere investigating the significance of patch adhesion in PKPD studies.

One of the crucial achievements of this study was the validation of a patch adhesion scoring system for use in clinical settings. The FDA has a recommended scoring system for the adhesion of transdermal patches that is used by the pharmaceutical industry when designing new patches; however, this tool had not yet been validated in a clinical setting. The validation study found that the FDA scoring tool was reliable for use in routine clinical practice for assessing patch adhesion and should be used as a standard for any future PK studies. This study suggests that patch adhesion should be taken into account whenever a PKPD study is undertaken with a transdermal delivery system.

Another aspect of this study was to analyse saliva fentanyl concentration to determine if it could be used as surrogate for plasma fentanyl concentration. Cancer patients are very fragile and reluctant to provide repeated blood samples, so if saliva and plasma correlation could be demonstrated; it could be a significant contribution to the welfare of such fragile patient groups. In acknowledgement of previous studies that have demonstrated that plasma drug concentration better predicts saliva concentration, fentanyl plasma concentration was correlated with saliva fentanyl concentration. This study did not support the correlation between fentanyl plasma and saliva concentration. However the detection of higher fentanyl concentrations in saliva than plasma, with a good correlation to dose, may allow saliva to be used as an alternative to plasma in
PK/PD studies of fentanyl in cancer patients. Similarly, pain scores were also assessed in order to determine whether saliva concentration could better predict pain control. The study found that pain was adequately managed, but that no correlation was evident between plasma and saliva fentanyl concentrations and pain scores. The study also assumed that adequate pain management was not solely dependent on fentanyl concentration, as all of the patients were on breakthrough medication which could impact pain scores.

A simple and cost-effective method was developed and validated for plasma and saliva matrices. This should be useful in the analysis of multiple samples in routine laboratory tests. Lipophilic opioids such as fentanyl have the tendency to adsorb to the CDB in saliva collecting devices (Salivette®). Additionally, saliva samples obtained from fragile cancer patients are not always of sufficient volume for regular assay. Hence this study has also developed a novel method to successfully analyse saliva samples in the presence of the challenges of adsorption and small sample volume. This extraction method should be useful in the case of any lipophilic drug that displays adsorption problems in saliva collecting devices.

Yet another significant finding from this study was the protein binding behaviour of fentanyl. Surprisingly for a basic drug such as fentanyl, ALB was the primary binding plasma protein, with AAG contributing to a minor proportion of the binding in plasma. Although this study showed total plasma protein binding of fentanyl to be close to that reported in the literature (80–85 %), protein binding in plasma samples from cancer patients receiving transdermal fentanyl was much higher (average: 95.1 %).

Hence an extensive study on fentanyl for pain management in cancer patients was undertaken, and it has provided insight about the use of the drug in the palliative care setting. Findings from this study could be successfully incorporated into future PKPD studies, as well as into day-to-day clinical practice, to improve pain management.
8.1 Future directions

Several important findings were achieved from this study that will contribute to improved use of fentanyl in pain management of cancer patients. However, there is still much more that can be done to further elucidate the intra- and interpatient variability seen with transdermal fentanyl in this population and to further enhance the use of fentanyl in a more predictive manner. Examples of suggested prospective work for continuing this research are detailed below:

1) PK modelling was performed only on plasma fentanyl concentrations. Future models could include the saliva fentanyl concentrations to identify any subject-specific factors (covariates) that influence the average PK behaviour of fentanyl in this patient population, with the aim of supporting dose individualisation.

2) In the PK model building, the relationship between WT and CL/Vd was allometrically scaled, since the weight range in the data set was not broad enough to estimate this relationship. A larger data set that includes a wider weight range may allow for the possibility of dosing fentanyl by weight.

3) In this study BOV on clearance was found to be smaller than BSV on clearance for fentanyl. This confirms that TDM will be useful and support dose individualisation to improve dosing for more effective pain control in cancer patients receiving transdermal fentanyl. A predictive pharmacokinetic program could be developed for dose individualisation to improve dosing protocols for effective clinical and pharmacoeconomic outcomes.

4) For future PG studies, a larger sample size is needed that also incorporates the important polymorphisms in genes not included in this study, to allow for a better understanding of any potential variability due to genetic polymorphisms in cancer patients receiving transdermal fentanyl at varying doses.
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Appendix 11. CRF – Clinical Study
Appendix 1. MHS HREC approval HREC/13/MHS/18 – Patch Adhesion Study

4 July 2013

Mr Sudeep Bista
Australian Centre for Paediatric Pharmacokinetics
Level 3
Mater Children’s Hospital

Dear Mr Bista

HREC Ref No: HREC/13/MHS/18
Project title: Optimising pain management with fentanyl in adult patients with cancer

I refer to your application dated 23 May 2013.

I write to advise that the Mater Health Services Human Research Ethics Committee reviewed this research project on 28 June 2013 and recognises that the project meets the requirement for Low and Negligible Risk Research as set out in the National Statement on Ethical Conduct in Human Research (2007) (Section 5.1.18 - 5.1.21) and has granted ethical approval for your research project. Please accept our very best wishes for the success of this research project. In all future correspondence with the Research Ethics and Governance Office please quote the Mater reference number.

Documents reviewed and approved include:

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<th>Document</th>
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This HREC is constituted and operates in accordance with the National Health and Medical Research Council’s (NHMRC) National Statement on Ethical Conduct in Human Research (2007). The processes used by this HREC to review multi-centre research proposals have been certified by the National Health and Medical Research Council.

Mater Medical Research Institute

Mater Research HREC Office
Room 270 Level 2 Aubigny Place  Ph: 07 3163 1585  Fax: 07 3163 2278

Mater Medical Research Institute
Level 3, Aubigny Place, Raymond Terrace, South Brisbane, Queensland 4101 Australia
Phone 07 3163 2555  Fax 07 3163 2555
www.mmi.mater.org.au
This approval is valid until 28 June 2016. You are reminded that this letter constitutes ethical approval only. You must not commence this research project until authorisation from the Research Governance Office has been obtained.

Please note the following conditions of approval.

- The Principal Investigator has responsibility for ensuring that the project is conducted in accordance with the National Statement, with relevant legislation and with Mater Health Services and responsibility for monitoring compliance rests with your Head of Department.
- Any departure from the protocol detailed in your proposal must be reported immediately to the Human Research Ethics Committee.
- When you propose a change to an approved protocol, which you consider to be minor, you are required to submit a written request for approval to the Chairperson, through the Research Ethics and Governance Office. Such requests will be considered on a case by case basis and interim approval may be granted subject to ratification at the next meeting of the Human Research Ethics Committee.
- Where substantial changes to any approved protocol are proposed, you are required to submit a full, new proposal for consideration by the Human Research Ethics Committee.
- You are required to advise the Research Ethics Coordinator immediately of any complaints made, or expressions of concern raised, in relation to the study, or if any serious or unexpected adverse events occur.
- To access medical records, for the purpose of this study, please provide a copy of this approval letter to the Health Information Services and Privacy Office (if applicable).
- The Research Ethics and Governance Office may choose to conduct an interim audit of your research project.

Should you have any queries, please do not hesitate to contact the Research Ethics office on 07 3163 1585 for assistance.

Yours sincerely

[Signature]

A/Professor Andrew Crowden
Chairperson
Mater Health Services Human Research Ethics Committee
Appendix 2. GU HREC approval PHM/18/13/HREC – Patch Adhesion Study

GRiffith University Human Research Ethics Committee

28-Nov-2013

Dear Mr Bista

I write further to your application for ethical clearance for your project PR: Optimising pain management with fentanyl in adult cancer patients / Adhesion of fentanyl patches in adult patients" (GU Ref No: PHM/18/13/HREC). This project has been considered by Human expedited review 1.

The Chair resolved to grant this project conditional ethical clearance, subject to you resolving the following matters:

As per the expectations articulated in the National Statement on Ethical Conduct in Human Research (2007) and Booklet 8 of the Griffith University Research Ethics Manual, because of the prior review by another HREC, this research has been subject to a special administrative review.

Please provide an assurance that the Manager, Research Ethics, Griffith University will be promptly notified if any adverse events occur or if any concerns or complaints are received about the ethical conduct of this research, or if the project is suspended or discontinued for any reason.

This decision was made on 29-Nov-13. Your response to these matters will be considered by Office for Research.

The ethical clearance for this protocol runs from 29-Nov-13 to 01-Jun-14.

Please forward your response to Rick Williams, Manager, Research Ethics, Office for Research, as per the details below.

Please refer to the attached sheet for the standard conditions of ethical clearance at Griffith University, as well as responses to questions commonly posed by researchers.

It would be appreciated if you could give your urgent attention to the issues raised by the Committee so that we can finalise the ethical clearance for your protocol promptly.

Regards

Rick Williams
Manager, Research Ethics
Office for Research
Bray Centre, N54 Room 0.15 Nathan Campus
Griffith University
ph: 07 3735 4375
fax: 07 373 57994
email: rick.williams@griffith.edu.au
Researchers are reminded that the Griffith University Code for the Responsible Conduct of Research provides guidance to researchers in areas such as conflict of interest, authorship, storage of data, & the training of research students. You can find further information, resources and a link to the University's Code by visiting http://policies.griffith.edu.au/pdf/Code%20for%20the%20Responsible%20Conduct%20of%20Research.pdf

PRIVILEGED, PRIVATE AND CONFIDENTIAL
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Appendix 3. PICF (Patient) – Patch Adhesion Study

PATIENT INFORMATION AND CONSENT FORM

<table>
<thead>
<tr>
<th>Protocol Title:</th>
<th>Adhesion of fentanyl patches in adult patients with pain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lay Title:</td>
<td>Fentanyl patch adhesion study</td>
</tr>
<tr>
<td>Principal Investigator:</td>
<td>Mr Sudeep Bista</td>
</tr>
<tr>
<td>Address:</td>
<td>Mater Health Services</td>
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<td></td>
<td>Raymond Terrace</td>
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<td>South Brisbane, QLD, 4101      Australia</td>
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<td>Telephone Number:</td>
<td>07 3163 8881</td>
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<tr>
<td></td>
<td>07 3163 3884</td>
</tr>
<tr>
<td>Research Nurse:</td>
<td>Angela Tapuni</td>
</tr>
</tbody>
</table>

Introduction
Your doctor has asked you if you would be willing to take part in this study called Adhesion of fentanyl patches in adult patients with pain. Before you decide to participate, it is important for you to understand why the research is being done and what it will involve. The following information explains all the procedures and any risks involved. Please read this Information and Consent Form carefully and feel free to discuss it with friends, relatives or your doctor, if you wish. Your doctor and the research team will answer any questions you may have about this Consent Form and about the study.

Purpose of the study
If we can prove that the tool for measuring how well your fentanyl patch is attached to your skin is accurate, then we will have identified a simple way of increasing our knowledge of fentanyl patches and how they work. The adhesion (sticking) of your patch to your skin is essential for the medicine to be completely absorbed through the skin. We will also be able to determine if patients are having problems with patches sticking to their skin. This in turn may allow us to manage pain control in individual patients more effectively.

Why have you been chosen to participate?
You are currently taking fentanyl to control your pain. The purpose of this study is to determine how well your patch is sticking to your skin. To do this we will need to take a photo of your patch, which we will later score.

Do you have to take part in this study?
Participation is entirely voluntary. If you decide to take part, you will be asked to sign the attached consent form, indicating that you understand the information and that you give your consent to participate in the research project. You will be given a copy of the Participant Information Sheet and Consent Form to keep as a record. If you decide not to take part, or to take part and then withdraw, the treatment you receive now or in the future will not be affected in any way. You also have the right to withdraw from the research project at any time. If you do withdraw your consent during the study, the study doctor and relevant study staff will not collect additional photos from you, although the results from the photos and personal information already collected will be retained, to ensure the results of the study can be measured properly. You should be aware that data collected by the researchers up to the time you withdraw will form part of the study results. You will be unable to have your data already collected removed.
What will happen if I take part in this study?
Photographs of your patch will be taken at a time convenient for you. The photograph will be a close-up of the patch and will not include your face or other parts of the body other than the immediate area to which the patch is attached. The camera is an ordinary camera, similar to one encountered in everyday use. A maximum of 3 photos will be collected. We will also collect some information from your notes about you (age, sex, weight) and your patch (dose, length of use, day of application) so we can ensure we have photos that cover a range of situations.

What are the risks in taking part in this study?
It is not anticipated that there are any risks associated with participating in this study. There are side effects associated with fentanyl however, that should have been explained to you when you were first prescribed the drug. These include drowsiness, constipation, a reduction in the rate at which you breath, nausea, bad dreams and hallucinations. Most of these side-effects (apart from constipation and nausea), generally only occur in over-dose situations. Fentanyl patches sometimes cause irritation to the skin that resolves once the patch is removed.

What are the benefits in taking part in this study?
We cannot guarantee or promise that you will receive any direct benefits of this project, however the information we get from this study may help us treat patients in the future.

How will confidentiality be maintained?
All data collected during the study will be regarded as confidential. All photographs will be labeled by code numbers only. All records containing personal information will remain confidential and no information which could lead to identification of any individual will be released. You should be aware that the results from this study may be processed by computer, but your name will not be used in the data entered on the computer.

Information from this study will also be used by Mr Sudeep Bista, as part of his PhD study. Mr Bista will conduct his research under the direction of Prof Janet Hardy and will be bound by the confidentiality arrangements of the Mater Health Services. It is anticipated that the results of this study will be published and or presented in a variety of forums. In any publication and/or presentation, information will be provided in such a way that you cannot be identified, except with your express permission.

Representatives from the Mater Health Services Human Research Ethics Committee and Government Regulatory Authorities may need to access your medical record for information related to the study. You are asked to consent to this access should it be required.

All research records will be stored for at least 5 years after which time all written documentation will be shredded and electronic records wiped clean. Photographs will only be used for this research study and will be destroyed at the end of the study period.

What should you do if you want to discuss this study further before you decide?
When you have read this information, the researcher will discuss it with you and any queries you may have. If you would like to know more at any stage, including during the research study, please do not hesitate to contact Angela Tapuni on 07 3163 3884.

Before deciding to take part you may discuss this matter with your family, a friend or your local doctor, if you feel this would help you make a decision.

Who should you contact if you have concerns about the conduct of this study?
This research has been approved by the Mater Health Services Human Research Ethics Committee, which is responsible for ensuring that the rights of human patients are protected at this institution, have given approval this study to be conducted at the Mater Adult Hospital.
If you have any complaints about any aspect of the project, the way it is being conducted or any questions about your rights as a research participant; please be advised that you may contact the Research Coordinator on (07) 3163 1585. The Research Coordinator may contact the Patient Representative or Hospital Ethicist at its discretion.

THANK YOU FOR CONSIDERING THIS STUDY.
**PATIENT CONSENT FORM**

<table>
<thead>
<tr>
<th>Protocol Title:</th>
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</table>

I have:
- Read and understood the information sheet;
- Had any questions or queries answered to my satisfaction;
- Been informed of the possible risks or side effects of the tests being conducted;
- Understood that the project is for the purpose of research and not for treatment;
- Been informed that the confidentiality of the information will be maintained and safeguarded;
- Given permission for access to my medical records, for the purpose of this research;
- Given permission for medical practitioners, other health professionals, hospitals or laboratories outside this hospital, to release information concerning my disease and treatment which is needed for this study and understand that such information will remain confidential;
- Been assured that I am free to withdraw at any time without comment or penalty; and
- Agreed to participate in the project.

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<tr>
<th>Name (please print)</th>
<th>Signature</th>
<th>Date &amp; Time</th>
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<tr>
<td>Participant</td>
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| Member of research team | | |
|-------------------------| | |

In my opinion, consent was freely given and the participant understands what is involved in this research.
Appendix 4. PICF (Volunteer) – Patch Adhesion Study

VOLUNTEER INFORMATION AND CONSENT FORM

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<th>Protocol Title:</th>
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<td>Research Nurse:</td>
<td>Angela Tapuni</td>
</tr>
</tbody>
</table>

Introduction
Your colleague has asked you if you would be willing to take part in this study called Adhesion of placebo fentanyl patches in adults. Before you decide to participate, it is important for you to understand why the research is being done and what it will involve. The following information explains all the procedures and any risks involved. Please read this Information and Consent Form carefully and feel free to discuss it with friends, relatives or your doctor, if you wish. Prof Hardy and the research team will answer any questions you may have about this Consent Form and about the study.

Purpose of the study
If we can prove that the tool for measuring how well fentanyl patches are attached to the skin is accurate, we will have identified a simple way of increasing our knowledge of fentanyl patches and how they work. The adhesion (sticking) of patches to the skin is essential for the medicine to be completely absorbed through the skin. We will also be able to determine if patients are having problems with patches sticking to their skin. This in turn may allow us to manage pain control in individual patients more effectively.

Why have you been chosen to participate?
You are being asked to participate in this study as we need a range of skin types to assess how well placebo fentanyl patches adhere.

What will participation in this study involve?
If you agree to participate in this study, we will apply a placebo fentanyl patch to your skin and leave it there for 1 to 4 days. We will take a series of photos of the patch over that time when convenient for you. The photographs will later be scored for adhesion. It will not be possible to identify you from the photos. We ask you to undertake all your normal activities (eg bathing, swimming, exercising) while the patch is in place. We will also ask for some basic demographic data (age, sex, weight) and will document how long you have had the patch on at which site.

Do you have to take part in this study?
Participation is entirely voluntary. If you decide to take part, you will be asked to sign the attached consent form, indicating that you understand the information and that you give your consent to participate in the research project. You will be given a copy of the Participant Information Sheet and Consent Form to keep as a record. If you decide not to
take part, or to take part and then withdraw, the treatment you receive now or in the future will not be affected in any way. You also have the right to withdraw from the research project at any time. If you do withdraw your consent during the study, the study doctor and relevant study staff will not collect additional photos from you, although the results from the photos and personal information already collected will be retained, to ensure the results of the study can be measured properly. You should be aware that data collected by the researchers up to the time you withdraw will form part of the study results. You will be unable to have your data already collected removed.

What are the risks in taking part in this study?
Fentanyl patches occasionally cause skin irritation that resolves once the patch has been removed. It is possible that the placebo patches might cause irritation in some people.

What are the benefits in taking part in this study?
We cannot guarantee or promise that you will receive any direct benefits of this project, however the information we get from this study may help us treat patients in the future.

How will confidentiality be maintained?
All data collected during the study will be regarded as confidential. All records containing personal information will remain confidential and no information which could lead to identification of any individual will be released. You should be aware that the results from this study may be processed by computer, but your name will not be used in the data entered on the computer.

Information from this study will also be used by Mr Sudeep Bista, as part of his PhD study. Mr Bista will conduct his research under the direction of Prof Janet Hardy and will be bound by the confidentiality arrangements of the Mater Health Services. It is anticipated that the results of this study will be published and or presented in a variety of forums. In any publication and/or presentation, information will be provided in such a way that you cannot be identified, except with your express permission.

Representatives from the Mater Health Services Human Research Ethics Committee and Government Regulatory Authorities may need to access your medical record for information related to the study. You are asked to consent to this access should it be required.

All research records will be stored for at least 5 years after which time all written documentation will be shredded and electronic records wiped clean. Photographs will only be used for this research study and will be destroyed at the end of the study period.

What should you do if you want to discuss this study further before you decide?
When you have read this information, the researcher will discuss it with you and any queries you may have. If you would like to know more at any stage, including during the research study, please do not hesitate to contact Angela Tapuni or Prof Hardy on 07 3163 3884.

Before deciding to take part you may discuss this matter with your family, a friend or your local doctor, if you feel this would help you make a decision.

Who should you contact if you have concerns about the conduct of this study?
This research has been approved by the Mater Health Services Human Research Ethics Committee, which is responsible for ensuring that the rights of human patients are protected at this institution, have given approval this study to be conducted at the Mater Adult Hospital.

If you have any complaints about any aspect of the project, the way it is being conducted or any questions about your rights as a research participant; please be advised that you may contact the Research Coordinator on (07) 3163 1585. The Research Coordinator may contact the Patient Representative or Hospital Ethicist at its discretion.
THANK YOU FOR CONSIDERING THIS STUDY.

VOLUNTEER CONSENT FORM

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I have:
- Read and understood the information sheet;
- Had any questions or queries answered to my satisfaction;
- Been informed of the possible risks or side effects of the tests being conducted;
- Understood that the project is for the purpose of research and not for treatment;
- Been informed that the confidentiality of the information will be maintained and safeguarded;
- Given permission for access to my medical records, for the purpose of this research;
- Given permission for medical practitioners, other health professionals, hospitals or laboratories outside this hospital, to release information concerning my disease and treatment which is needed for this study and understand that such information will remain confidential;
- Been assured that I am free to withdraw at any time without comment or penalty; and
- Agreed to participate in the project.

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<tr>
<th>Name (please print)</th>
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<th>Date &amp; Time</th>
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<tbody>
<tr>
<td>Participant</td>
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<td>Investigator</td>
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</tbody>
</table>

In my opinion, consent was freely given and the participant understands what is involved in this research.
Appendix 5. CRF (Assessment) – Patch Adhesion Study

Case Report Form

Protocol: ADHESION OF FENTANYL PATCHES IN ADULT PATIENTS

<table>
<thead>
<tr>
<th>INVESTIGATOR NAME:</th>
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<tr>
<th>CO-INVESTIGATORS:</th>
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<th>RESEARCH NURSE:</th>
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<table>
<thead>
<tr>
<th>PARTICIPANT NUMBER:</th>
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<thead>
<tr>
<th>Date commenced:</th>
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</table>
Visit questionnaire

The following questionnaire consists of a series of photographs of fentanyl and placebo patches on adult patient skin. Please provide a score of 0 – 4 for each of the photos according to the following scoring tool:

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>≥ 90% adhered</td>
<td>Essentially no lift off the skin</td>
</tr>
<tr>
<td>1</td>
<td>≥ 75% to &lt; 90% adhered</td>
<td>Some edges only lifting off the skin</td>
</tr>
<tr>
<td>2</td>
<td>≥ 50% to &lt; 75% adhered</td>
<td>Less than half of the patch lifting off the skin</td>
</tr>
<tr>
<td>3</td>
<td>&gt; 0% to &lt; 50% adhered but not detached</td>
<td>More than half of the patch lifting off the skin without falling off</td>
</tr>
<tr>
<td>4</td>
<td>0% adhered - patch detached</td>
<td>Patch completely off the skin</td>
</tr>
</tbody>
</table>

Please answer the following questions to provide us with demographic detail:

1. Gender: ☐ M  ☐ F
2. Occupation: ☐ Registered nurse  ☐ Doctor  ☐ Other
3. Years experience in this occupation: ______ yrs
4. Age(yrs)  <20  21-40  41-60  >60
   ☐ ☐ ☐ ☐

THANK YOU FOR CONSIDERING THIS STUDY
Appendix 6. CRF (Patient) – Patch Adhesion Study

Case Report Form

Protocol: ADHESION OF FENTANYL PATCHES IN ADULT PATIENTS

INVESTIGATOR NAME:

_____________

CO-INVESTIGATORS:

_____________

_____________

_____________

RESEARCH NURSE:

_____________

PARTICIPANT NUMBER:

_____________

Date commenced:

_____________
### TRIAL PERIOD

<table>
<thead>
<tr>
<th>Participant Number:</th>
<th>Visit Date:</th>
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### Demographic details

<table>
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<tr>
<th>Gender: M or F</th>
<th>Age: ____ yrs</th>
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<th>Performance Status: ________</th>
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### Fentanyl patch details

<table>
<thead>
<tr>
<th>Date &amp; time of patch change</th>
<th>Date &amp; time photo taken</th>
<th>Patch or placebo</th>
<th>Dose of patch</th>
<th>Site of patch placement</th>
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### Visit questionnaire

1. Have you had any problems with your patch sticking properly?
   - [ ] Yes
   - [ ] No
   - [ ] Sometimes

2. Have you noticed anything that might interfere with your patch sticking properly?

   .........................................................................................................................................................
Case Report Form

Protocol: ADHESION OF PLACEBO FENTANYL PATCHES IN ADULTS

INVESTIGATOR NAME: 

CO-INVESTIGATORS: 

RESEARCH NURSE: 

PARTICIPANT NUMBER: 

Date commenced:
# TRIAL PERIOD

Participant Number: ___________________  Visit Date: ____________

## Demographic details

- Gender: M or F
- Age: ____ yrs
- Height: _____
- Weight: _____

## Fentanyl patch details

<table>
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<tr>
<th>Date &amp; time of patch change</th>
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## Visit questionnaire

1. Have you had any problems with your patch sticking properly?
   - [ ] Yes
   - [ ] No
   - [ ] Sometimes

2. Have you noticed anything that might interfere with your patch sticking properly?
   ........................................................................................................................................................................................................................................................................................................
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Appendix 8. MHS HREC approval 1909A – Clinical Study

MATER HEALTH SERVICES HUMAN RESEARCH ETHICS COMMITTEE

12th July 2012

Professor Janet Hardy
City Ms Angela Tapuni
Palliative Care
Level 10
Mater Adult Hospital

Dear Professor Hardy

Re: Protocol Ref No. 1909A Optimising Pain Management with Fentanyl in Adult Patients with Cancer

I write to advise that the Mater Health Services Human Research Ethics Committee considers the above study to meet the requirements of the National Statement on Ethical Conduct in Human Research (2007) and has granted ethical approval for your research proposal. Please accept our very best wishes for the success of this study. In all future correspondence with the Committee please quote the Mater reference number.

Documents reviewed and approved include:

- NEAF Version 2008-V2.0 dated 6th June 2012
- Research Protocol Version 1.0 dated 6th June 2012
- Patient Information Sheet and Consent Form Version 1.0 dated 6th June 2012
- Staff Information Sheet Version 1.0 dated 6th June 2012
- CRF’s Version 1.0 dated 6th June 2012

This approval is valid until 12th June 2015.

Please note the following conditions of approval.

- Any departure from the protocol detailed in your proposal must be reported immediately to the Committee.
- When you propose a change to an approved protocol, which you consider to be minor, you are required to submit a written request for approval to the Chairperson, through the Secretary. Such requests will be considered on a case by case basis and interim approval may be granted subject to ratification at the next meeting of the Committee.
- Where substantial changes to any approved protocol are proposed, you are required to submit a full, new proposal for consideration by the Human Research Ethics Committee.
- You are required to advise the Research Ethics Coordinator immediately of any complaints made, or expressions of concern raised, in relation to the study, or if any serious or unexpected adverse events occur.
- Under the NH&MRC National Statement on Ethical Conduct in Research Involving Humans, research ethics committees are responsible for monitoring approved research to ensure continued compliance with ethical standards, and to determine the method of monitoring appropriate to each project. You are required to provide written reports on the
progress of the approved project annually, the first report being due on and finally on completion of the project. (The Progress Report is located at http://www.mater.org.au/Home/Research/Human-Research-Ethics-Committee.aspx or can be accessed through the Mater Intranet, Applications, Research Register then under the project name or alternately can be emailed to you.) Please inform the Committee of publications, presentations at Conferences, education and quality improvement outcomes from this study. The Committee may also choose to conduct an interim audit of your research.

- Please be aware that all study procedures including follow up of participants and data analysis should be completed within the approval time frame or an extension should be requested.

You are reminded that this letter constitutes ethical approval only. You must not commence this research project until authorisation from the Research Governance Office has been obtained.

Please contact the Executive Director in the participating hospital/hospitals prior to commencing of the study. To access medical records, for the purpose of this study, please provide a copy of this approval letter to the Corporate Health Information Manager. I would also be grateful if you could confirm the date of commencement. (All correspondence should be directed to the Mater Research Ethics Coordinator.)

Yours sincerely

[Signature]

A/Prof Andrew Crowden
Chairperson
Mater Health Services Human Research Ethics Committee
Dear Mr Bista

I write further to your application for ethical clearance for your project PR: OPTIMISING PAIN MANAGEMENT WITH FENTANYL IN ADULT PATIENTS WITH CANCER" (GU Ref No: PHM/16/13/HREC). This project has been considered by Human expedited review 1.

The Chair resolved to grant this project conditional ethical clearance, subject to you resolving the following matters:

As per the expectations articulated in the National Statement on Ethical Conduct in Human Research (2007) and Booklet 8 of the Griffith University Research Ethics Manual, because of the prior review by another HREC, this research has been subject to a special administrative review.

Please provide an assurance that the Manager, Research Ethics, Griffith University will be promptly notified if any adverse events occur or if any concerns or complaints are received about the ethical conduct of this research, or if the project is suspended or discontinued for any reason.

This decision was made on 28-Nov-13. Your response to these matters will be considered by Office for Research.

The ethical clearance for this protocol runs from 28-Nov-13 to 12-Jun-15.

Please forward your response to Rick Williams, Manager, Research Ethics, Office for Research, as per the details below.

Please refer to the attached sheet for the standard conditions of ethical clearance at Griffith University, as well as responses to questions commonly posed by researchers.

It would be appreciated if you could give your urgent attention to the issues raised by the Committee so that we can finalise the ethical clearance for your protocol promptly.

Regards

Rick Williams
Manager, Research Ethics
Office for Research
Bray Centre, N54 Room 0.15 Nathan Campus
Griffith University
ph: 07 3735 4375
fax: 07 373 57994
email: rick.williams@griffith.edu.au
web:
Cc:

Researchers are reminded that the Griffith University Code for the Responsible Conduct of Research provides guidance to researchers in areas such as conflict of interest, authorship, storage of data, & the training of research students. You can find further information, resources and a link to the University's Code by visiting http://policies.griffith.edu.au/pdf/Code%20for%20Responsible%20Conduct%20of%20Research.pdf

PRIVILEGED, PRIVATE AND CONFIDENTIAL
This email and any files transmitted with it are intended solely for the use of the addressee(s) and may contain information which is confidential or privileged. If you receive this email and you are not the addressee(s) [or responsible for delivery of the email to the addressee(s)], please disregard the contents of the email, delete the email and notify the author immediately.
Appendix 10. PICF – Clinical Study

**Patient Information Sheet and Consent Form**

<table>
<thead>
<tr>
<th>Protocol Title:</th>
<th>Optimising Pain Management with Fentanyl in Adult Patients with Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lay Title:</td>
<td>Fentanyl and Saliva</td>
</tr>
<tr>
<td>Principal Investigator:</td>
<td>Mater Health Services Raymond Terrace South Brisbane, QLD, 4101 Australia</td>
</tr>
<tr>
<td>Address:</td>
<td>07 3163 8881 or 07 3163 2775</td>
</tr>
<tr>
<td>Telephone Number:</td>
<td>Angela Tapuni Phone: 07 3163 3884 Mobile: 0466 206 213</td>
</tr>
</tbody>
</table>

**Introduction**

Your doctor has asked you if you would be willing to take part in this study called *Optimising Pain Management with Fentanyl in Adult Patients with Cancer* because you have been prescribed a fentanyl patch to manage your pain. Before you decide to participate, it is important for you to understand why the research is being done and what it will involve. The following information explains all the procedures and any risks involved. Please read this Information and Consent Form carefully and feel free to discuss it with friends, relatives or your doctor, if you wish. Your doctor and the research team will answer any questions you may have about this Consent Form and about the study.

**Purpose of the study**

If we can prove that saliva sampling is as good as blood sampling for measuring fentanyl levels, we will have identified a simple non-invasive means of greatly increasing our knowledge of fentanyl and how it acts. Very little is known about how much the drug is processed in the body or how its ability to control pain is affected by other drugs, age or organ function. This in turn may allow us to tailor drug doses according to the unique features of each patient and to optimise pain control.

**Why have you been chosen to participate?**

You are currently taking fentanyl via a patch on your skin to control your pain. The purpose of this study is to measure the fentanyl levels in your body by testing both your saliva and blood. Cancer patients are often frail and unwell and we are reluctant to expose these patients to invasive tests such as repeated blood sampling. We want to see if we can avoid blood tests by measuring drug levels in saliva.
Do you have to take part in this study?
Participation is entirely voluntary. If you decide to take part, you will be asked to sign the attached consent form, indicating that you understand the information and that you give your consent to participate in the research project. You will be given a copy of the Participant Information Sheet and Consent Form to keep as a record.

If you decide not to take part, or to take part and then withdraw, the treatment you receive now or in the future will not be affected in any way.
You also have the right to withdraw from the research project at any time. Your doctor may also withdraw you from the study if he or she feels it appropriate to do so for any reason. If you do withdraw your consent during the study, the study doctor and relevant study staff will not collect additional blood or saliva tests or personal information from you, although the results from the saliva and blood tests and personal information already collected will be retained, to ensure the results of the study can be measured properly. You should be aware that data collected by the researchers up to the time you withdraw will form part of the study results. You will be unable to have your data already collected removed.

What will happen if I take part in this study?
Blood and saliva samples will be taken at the same time by the research nurse at times convenient for you. Wherever possible we will use blood samples leftover from your routine blood testing. If you are not having routine blood samples taken, we may ask you to provide an extra blood test. A maximum of 6 pairs of samples both blood and saliva will be collected but you may only provide 3 or 4 if you so wish. There is no set number of samples that need to be taken from each person and no set times. Saliva samples will be obtained either by having you chew a dental bud (a dental swab in a plastic tube designed for saliva collection), or by expectorating directly into a small plastic container. Thorough rinsing of the mouth will be required prior to collecting of the saliva as remnants of orally administered medicines may contaminate the saliva specimens.

You will also be asked just prior to the blood and saliva testing to indicate to the research nurse your level of pain at the time of the testing. This information will be used to match a patient's pain level against their level of fentanyl in their body. You will also be interviewed at the end of the study for the purpose of identifying your preference for either saliva or venipuncture (blood sampling).

What are the risks in taking part in this study?
A sample of venous blood will be required and the risk associated with this test is no greater than usual blood sampling, that is, a small risk of pain at the sampling site, bruising and infection. All blood collection will be undertaken by a trained member of staff. The collection of saliva may cause discomfort to some patients especially those with a dry mouth.

What are the benefits in taking part in this study?
We cannot guarantee or promise that you will receive any direct benefits of this project, however the information we get from this study may help us treat patients in the future. Your doctors will monitor you carefully during and after the study to ensure you are getting adequate pain relief.

How will confidentiality be maintained?
All data collected during the study will be regarded as confidential. All records containing personal information will remain confidential and no information which could lead to identification of any individual will be released.

Optimising Pain Management With Fentanyl In Adult Patients With Cancer
Participant Information Sheet and Consent Form
Version: 1.1 Date: 10th July, 2012
Page 2 of 5
You should be aware that the results from this study may be processed by computer, but your name will not be used in the data entered on the computer.

Information from this study will also be used by Mr Sudeep Bista, as part of his PhD study. Mr Bista will conduct his research under the direction of Dr Ross Norris and will be bound by the confidentiality arrangements of the Mater Health Services. It is anticipated that the results of this study will be published and/or presented in a variety of forums. In any publication or presentation, information will be provided in such a way that you cannot be identified, except with your express permission.

Representatives from the Mater Health Services Human Research Ethics Committee and Government Regulatory Authorities may need to access your medical record for information related to the study. You are asked to consent to this access should it be required.

All research records will be stored for at least 5 years after which time all written documentation will be shredded and electronic records wiped clean. Your blood and saliva samples will only be used for this research study and any leftover samples will be destroyed at the end of the study period.

What if something goes wrong?
Every reasonable precaution will be taken to ensure your safety during the course of the study. If you are injured as a direct result of participating in this study, medical treatment will be provided to you free of charge, at any Australian public hospital. Your participation in this study will not affect any other right to compensation that you may have under statute or common law.

What should you do if you want to discuss this study further before you decide?
When you have read this information, the researcher will discuss it with you and any queries you may have. If you would like to know more at any stage, including during the research study, please do not hesitate to contact Angela Tapuni on 07 3163 3884.

Before deciding to take part you may discuss this matter with your family, a friend or your local doctor, if you feel this would help you make a decision.

Who should you contact if you have concerns about the conduct of this study?
This research has been approved by the Mater Health Services Human Research Ethics Committee, which is responsible for ensuring that the rights of human patients are protected at this institution, have given approval this study to be conducted at the Mater Adult Hospital.

If you have any complaints about any aspect of the project, the way it is being conducted or any questions about your rights as a research participant; please be advised that you may contact the Research Coordinator on (07) 3163 1585. The Research Coordinator may contact the Patient Representative or Hospital Ethicist at its discretion.

THANK YOU FOR CONSIDERING THIS STUDY.

Optimising Pain Management With Fentanyl In Adult Patients With Cancer
Participant Information Sheet and Consent Form
Version: 1.1 Date: 10th July, 2012
Page 3 of 5
**Patient Consent Form**

<table>
<thead>
<tr>
<th>Protocol Title:</th>
<th>Optimising Pain Management with Fentanyl in Adult Patients with Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lay Title:</td>
<td>Fentanyl and Saliva</td>
</tr>
<tr>
<td>Principal Investigator:</td>
<td>Prof. Janet Hardy</td>
</tr>
<tr>
<td>Address:</td>
<td>Mater Health Services</td>
</tr>
<tr>
<td></td>
<td>Raymond Terrace</td>
</tr>
<tr>
<td></td>
<td>South Brisbane, QLD, 4101</td>
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<tr>
<td></td>
<td>Australia</td>
</tr>
<tr>
<td>Telephone Number:</td>
<td>07 3163 8881</td>
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<tr>
<td></td>
<td>or</td>
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<tr>
<td></td>
<td>07 3163 3894</td>
</tr>
<tr>
<td>Research Nurse:</td>
<td>Angela Tapuni</td>
</tr>
<tr>
<td></td>
<td>Helen Anderson</td>
</tr>
<tr>
<td></td>
<td>Phone: 07 3163 5889</td>
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<tr>
<td></td>
<td>Mobile: 0496 206 213</td>
</tr>
</tbody>
</table>

I have:
- Read and understood the information sheet;
- Had any questions or queries answered to my satisfaction;
- Been informed of the possible risks or side effects of the tests being conducted;
- Understood that the project is for the purpose of research and not for treatment;
- Been informed that the confidentiality of the information will be maintained and safeguarded;
- Given permission for access to my medical records, for the purpose of this research;
- Given permission for medical practitioners, other health professionals, hospitals or laboratories outside this hospital, to release information concerning my disease and treatment which is needed for this trial and understand that such information will remain confidential;
- Been assured that I am free to withdraw at any time without comment or penalty; and
- Agreed to participate in the project.

Signatures: (Please turn to next page)
Appendix 11. CRF – Clinical Study

Case Report Forms

OPTIMISING PAIN MANAGEMENT WITH FENTANYL IN ADULT PATIENTS WITH CANCER

INVESTIGATOR NAME:

__________________________

CO-INVESTIGATORS:

__________________________

__________________________

RESEARCH NURSE:

__________________________

PARTICIPANT NUMBER:

__________________________

Date commenced:

__________________________
### Inclusion Criteria

<table>
<thead>
<tr>
<th>Inclusion criteria fulfilled?</th>
<th>Yes</th>
<th>No*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Male or Female, at least 18 years or older</td>
<td></td>
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<tr>
<td>2. A diagnosis of malignant disease</td>
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<tr>
<td>3. Receiving, about to receive or about to discontinue Transdermal fentanyl patch</td>
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<tr>
<td>4. Understand the patient information sheet and provide written consent</td>
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<tr>
<td>5. Willing to provide up to 6 blood samples</td>
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<tr>
<td>6. Willing to provide up to 6 saliva samples</td>
<td></td>
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<tr>
<td>7. Willing to describe the experience of saliva sampling</td>
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<td></td>
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</tbody>
</table>

* No leads to an exclusion of the participant in the study.

### Exclusion Criteria

<table>
<thead>
<tr>
<th>Exclusion criteria fulfilled?</th>
<th>Yes*</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Oral mucositis, infection and/or xerostomia that is painful or not possible to collect a saliva sample</td>
<td></td>
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<tr>
<td>2. Patients using fentanyl for breakthrough medication</td>
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</tbody>
</table>

* Yes leads to an exclusion of the participant in the study.
### Trial Period

<table>
<thead>
<tr>
<th>Participant Number:</th>
<th>Visit Date:</th>
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</table>

<table>
<thead>
<tr>
<th>Demographic details</th>
</tr>
</thead>
</table>

| Date of Birth: / / | Age: ___ yrs |
|---------------------|

<table>
<thead>
<tr>
<th>Gender: M or F</th>
</tr>
</thead>
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| Height: ___ | BSA ___ | Weight: ___ |
|-------------|

<table>
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<tr>
<th>Performance Status:</th>
</tr>
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</table>

| Diagnosis: | Sites of Mets: |
|------------|

<table>
<thead>
<tr>
<th>Current fentanyl patch dose:</th>
</tr>
</thead>
</table>

| Commencement of current dose: | Start date: ___ | Stop date: ___ |
|-------------------------------|

<table>
<thead>
<tr>
<th>Date of initial fentanyl commencement:</th>
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</thead>
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<table>
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<tr>
<th>Type and dose of breakthrough medication:</th>
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<tbody>
<tr>
<td>Date/time of patch change</td>
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Number of samples taken from participant:

> Se = blood sample
Sa = saliva sample
## Blood Results

<table>
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<tr>
<th>Biochemistry and haematology screen</th>
<th>Date:</th>
<th>Date:</th>
<th>Date:</th>
<th>Date:</th>
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<th>Date:</th>
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<td>ALP</td>
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<td>GGT</td>
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<td>LDH</td>
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<td>Ca</td>
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<td>Ca corr</td>
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<td>Urea</td>
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<td>Alb</td>
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<td>eGFR</td>
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<td>Hb</td>
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<td>Creat Cl</td>
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</table>
### All concomitant medications:

<table>
<thead>
<tr>
<th>Name of Drug/dose</th>
<th>route</th>
<th>Sample 1 Date:</th>
<th>Sample 2 Date:</th>
<th>Sample 3 Date:</th>
<th>Sample 4 Date:</th>
<th>Sample 5 Date:</th>
<th>Sample 6 Date:</th>
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<tbody>
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Instructions for completion of medication table:
- Be sure to enter all medications and exact time taken on the day of each sample
- If there has been a dose change, enter new dose and date
- If the drug has been stopped, enter a cross and the stop date
The recommended scoring system for adhesion of transdermal patches (FDA guidelines):

0 = ≥ 90% adhered (essentially no lift off the skin)
1 = ≥ 75% to < 90% adhered (some edges only lifting off the skin)
2 = ≥ 50% to < 75% adhered (less than half of the patch lifting off the skin)
3 = > 0% to < 50% adhered but not detached (more than half of the patch lifting off the skin without falling off)
4 = 0% adhered - patch detached (patch completely off the skin)

Please circle the adhesion score above at time of collecting samples

_____

(Score)

Source:
FDA Guidelines:
### Brief Pain Inventory

**Brief Pain Inventory**

1. Please rate your pain by circling the one number that best describes your pain at its **WORST** in the last 24 hours?

| No Pain at all | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | Pain as bad as you can imagine |

2. Please rate your pain by circling the one number that best describes your pain at its **LEAST** in the last 24 hours?

| No Pain at all | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | Pain as bad as you can imagine |

3. Please rate your pain by circling the one number that best describes your pain at its **AVERAGE**?

| No Pain at all | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | Pain as bad as you can imagine |

4. Please rate your pain by circling the one number that tells how much pain you have **RIGHT NOW**?

| No Pain at all | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | Pain as bad as you can imagine |
References

12. Smith, J., Ellershaw, J., Improvement in pain control by change of fentanyl patch after 48 hours compared with 72 hours., in EAPC Congress, Poster PO1/13761999: Geneva.


Accessed: 20/02/2015.


