Development, validation and application of an HPLC-MS/MS method for the determination of fentanyl and nor-fentanyl in human plasma and saliva

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Highlights:

HPLC-MS/MS method to quantify fentanyl and nor-fentanyl was developed

The method was validated in human saliva and plasma matrix.

A method to extract analytes directly from cotton dental buds is presented.

The extraction method overcomes adsorption of drug occurring in Salivettes®

The method also overcomes small saliva sample volumes obtained from patients.

Abstract:

Monitoring fentanyl concentration in saliva and plasma may be useful in pharmacokinetic /pharmacodynamic studies. Salivettes® have been used widely for collecting saliva samples. However due to its lipophilicity, fentanyl adsorbs to the cotton dental bud (CDB) used in this device. Furthermore, due to dry mouth being a common adverse effect seen in patients treated with opioids, obtaining enough saliva for analysis is often a challenge. Hence, a simple simultaneous method to quantify fentanyl and its metabolite in both human plasma and saliva was developed and validated. A novel extraction method was also developed and validated to recover fentanyl in saliva directly from the CDB. This extraction method utilises acetonitrile to recover the fentanyl directly from the CDB rather than recovery by centrifugation, which is not always possible. Reverse phase chromatographic separation was performed on a Shimadzu LC 20A HPLC system using gradient elution. The electrospray ion source (ESI) was operated in positive ion mode using an Applied Biosystems API 3200 LC/MS/MS as detector. Deuterated fentanyl (D_5) and nor-fentanyl (D_5) were used as internal standards (IS). The retention times for fentanyl and nor-fentanyl were 3.70 mins and 3.20 mins respectively. The lower limit of quantitation (LLOQ) was determined to be 0.030 µg/L in plasma and 0.045 in saliva for fentanyl and nor-fentanyl. Acceptable linearity for fentanyl and nor-fentanyl in both plasma and saliva was demonstrated from 0.02 to 10 µg/L (R^2 0.9988 to 0.9994). Accuracy for fentanyl and nor-fentanyl in both plasma and saliva samples was between 96 % and 108 %. Total imprecision expressed as the co-efficient of variation was between 1.0 – 15.5 % for both analytes in both matrices. The validated method was applied successfully in 11 paired plasma and saliva samples obtained from patients with cancer pain receiving transdermal fentanyl (Duragesic®) at doses from 25 µg to 100 µg.

Key words: Fentanyl, HPLC-MS/MS, Plasma, Saliva, Salivette®, Adsorption

1. Introduction

Fentanyl [N-(1-phenethyl-4-piperidyl) propionanilide] is a synthetic, highly selective opioid agonist that acts primarily at the mu-opioid receptor, with minor activity at the delta and kappa receptors [1]. The oral (enteral) bioavailability of fentanyl is poor [2] and hence the routes of administration include intravenous, subcutaneous, transdermal, transmucosal and spinal. The transdermal patch is the most common means of delivery of fentanyl to patients with cancer. Fentanyl is primarily metabolized in the liver by cytochrome P450 (CYP) 3A4. N-dealkylation is the primary pathway of metabolism in humans [3]. The main metabolite is norfentanyl; minor metabolites are hydroxypropionyl-fentanyl and hydroxypropionyl-norfentanyl that do not have relevant pharmacological activity [4]. These metabolites have been shown to appear in plasma [3] stool and urine [5].

Moderate to severe pain in cancer is common and affects 70-80 % of patients with advanced cancer. The skilled use of opioid analgesics is crucial to the relief of cancer pain [6]. Fentanyl, being a potent opioid, is used widely for cancer pain management, however due to the inter- and intra-individual variability seen with fentanyl, there is a considerable challenge in maintaining analgesia [7]. Hence pharmacokinetic/pharmacodynamic (PK/PD) studies would provide some insight into how each individual responds to fentanyl. Furthermore patients with cancer are by definition frail and of poor performance status. There is therefore considerable reluctance on the part of health professionals to subject them to non-essential tests and investigations including repeated venepuncture that has been necessary in pharmacokinetic (PK) studies to date [8].

The use of saliva as opposed to blood has been shown to be an attractive alternative for therapeutic drug monitoring (TDM) in children because its collection is painless, simpler and cheaper than venesection [9]. In a previous study [8] the majority of patients voiced a preference for saliva sampling over venesection. It has also been used widely for forensic and toxicological investigations [10]. A review of the relationship between saliva and plasma concentrations suggests that salivary concentrations may relate better to efficacy and/or toxicity than plasma levels [11]. Strong correlations between plasma and saliva concentrations for the analgesics paracetamol [12] and

hydromorphone [13] have been described. Other opioids including codeine [14], diamorphine [15], methadone [16], morphine [17], and dihydrocodeine [18] have also been analysed to investigate saliva/plasma (S/P) ratios. Mixed results have been reported with marked variability in S/P ratios between studies.

Despite the advantages of saliva sampling, there are problems when quantifying the drug/s of choice in saliva. The major problems reported are: low volume obtained after centrifugation [19]; adsorption of drug to collection devices, and; variation in recovery depending upon the specific product used [20]. Therefore this study aimed to develop a method that allows extraction of the drug of choice directly from the cotton dental bud (CDB) used to collect saliva in the Salivette® system.

A simple simultaneous HPLC-MS/MS method to quantify fentanyl and nor-fentanyl in plasma and saliva was also developed and validated. Koch *et al.* have reported a method to quantify fentanyl and its metabolite in rat plasma [21]; likewise, Verplaetse and Tytgat [22] have also developed a method to quantify fentanyl and nor-fentanyl in whole blood and urine for forensic studies; however saliva was not included in their assessment. Further, Verplaetse and Tytgat have used solid phase extraction cartridges, which are expensive and time consuming. Hence a method was developed for simultaneous assay of both fentanyl and nor-fentanyl in both human plasma and saliva utilizing protein precipitation.

2. Material and methods

2.1 Chemicals and reagents

Fentanyl, nor-fentanyl, deuterated (D₅) fentanyl and deuterated (D₅) nor-fentanyl were obtained from Cerilliant®, Round Rock, Texas, USA. HPLC grade acetonitrile, dichloromethane, methanol, sodium chloride (NaCl) and formic acid were obtained from Merck, New Jersey, USA. NaH₂PO₄ and Na₂HPO₄ were obtained from ChemSupply, Gillman, South Australia. Salivettes® were obtained from Sarstedt, Nümbrecht, Germany. 18 MΩ water was obtained from a Labmate water purification system (Aquacure, Brisbane, Australia).

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 Autosampler) (Nakagyo-Ku, Koyoto, Japan) coupled to 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 and handling. The electrospray ion source (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) as reported in Table 1. Nitrogen was used as the source gas. Curtain gas pressure was maintained at 10 psi, collision gas at 10 psi, ion source gas 1 (GS-1) and ion source gas 2 (GS-2) were maintained at 25 and 20 psi respectively. Ion spray voltage 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 HPLC 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 MOhm 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 minutes, held at 100 % MP-B for 1.5 minutes 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 autosampler operated at 10 °C. Total flow rate of the mobile phase was 0.3 mL/min. Run time was set to 8 minutes with a retention time of 3.70 minutes for fentanyl and 3.20 minutes for nor-fentanyl.

2.3 Preparation of calibrators, internal standard and spiked controls.

The fentanyl and nor-fentanyl storage solution (100 µg/L) was prepared from the stock solution in blank plasma and saliva respectively and stored at -70 °C. Fentanyl and nor-fentanyl calibrators were prepared in both blank plasma and saliva from the storage solution in concentrations of 0.02, 0.1, 0.5, 2 and 10 µg/L and stored at -20 °C. The internal standard (IS) storage solution (100 µg/L) was prepared from the fentanyl D-5 and nor-fentanyl D-5 stock solution and stored at -70 °C. The IS (fentanyl D-5 and nor-fentanyl D-5) working solution (1.5 µg/L) for both plasma and saliva samples

was prepared in acetonitrile and stored at -20 °C. The quality control (QC) samples with concentrations of 9µg/L as high control (HC) for both plasma and saliva, and 0.03µg/L and 0.045 µg/L as low control (LC) for plasma and saliva respectively, were prepared and stored at -20 °C.

2.4 Sample preparation

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 respective, appropriately labelled Eppendorf tubes. Samples were vortex mixed for 30 seconds and centrifuged at 2000 g for 10 minutes. Supernatants were transferred to respective 12 mL glass culture tubes 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 then centrifuged at 2000 g for 10 minutes. The supernatants (150 µL) were again transferred to respective autosampler vials and 50 µL was injected into the HPLC-MS/MS.

2.4.2 Extraction of fentanyl and nor-fentanyl from saliva samples

Preparation of saliva samples for validation of the method was undertaken using two separate procedures to mimic: (1) the assay of saliva directly, and; (2) direct extraction from the CDB used in the Salivette® system. Procedure (1) was the same as for section 2.4.1, except saliva was used in place of plasma and procedure (2) is described in section 2.4.3 below.

2.4.3 Extraction of fentanyl and nor-fentanyl in saliva directly from CDB

To validate the method to be applied to saliva samples in practice – that is, extraction of analytes from saliva directly from CDBs, the method involved recording the initial weight of individual Salivette® devices before adding spiked saliva samples. Once the initial device weights were taken, calibrators prepared in saliva were pipetted into respective CDBs using the five spiked calibrators as per section 2.3 and the final weights were recorded. From these data a calibration curve was plotted. Similarly, HC and LC in saliva were pipetted onto respective preweighed CDBs and the final weights taken. The differences in the initial and final weights were used to normalise the concentrations, as per actual samples collected from patients/subjects. Similar procedures were followed for blank and double blank samples. Further, 600 µL of IS working solution was added to respective CDBs in Salivettes®.

All Salivettes® were allowed to stand for approximately 10 minutes. The CDB was removed from the Salivette® device using clean forceps and placed into a glass beaker. Approximately 4 mL of acetonitrile was poured onto the CDB and the beaker contents mixed well. The acetonitrile was then decanted into a 12 mL glass culture tube. This extraction was repeated with acetonitrile three times. The acetonitrile from each sample was combined in respective 12 mL glass culture tubes. The acetonitrile was evaporated under nitrogen gas and 200 µL of MP-A was added and vortex mixed for 30 seconds. The samples were then transferred to respective 1.5 mL Eppendorf tubes and centrifuged at 2000 g for 10 minutes. 150 µL of each supernatant was transferred into respective autosampler vials ready for assay. 50 µL of each sample was then injected into the HPLC-MS/MS.

2.5. Bioanalytical method validation for plasma

Validation was based on the European Medicine Agency (EMA) guidelines [23].

2.5.1 Specificity

Specificity was demonstrated by comparing chromatograms of blank plasma and blank saliva from each of six subjects, before and after seeding with fentanyl, nor-fentanyl and IS.

2.5.2 Linearity

For the purpose of determining linearity, four calibration curves were generated using five concentrations (0.02, 0.1, 0.5, 2 and 10 µg/L) for both plasma and saliva.

2.5.3 Inaccuracy and Imprecision

Inaccuracy and imprecision were determined using results from the assay of five replicates of the low and high controls assayed on each of three days in intra- and inter-day assay. Inaccuracy was then calculated as percent deviation of measured concentration from the original value, and imprecision determined using ANOVA [24].

2.5.4 Matrix effect (Ion suppression)

The assay was tested for matrix effects, including ion suppression, in plasma and saliva using the post column infusion (PCI) approach [27] and as indicated in the EMA guidelines (IS normalised matrix factor). Ion suppression using PCI was assessed in both plasma and saliva matrix for each of six different subjects. 9 µg/L fentanyl and nor-fentanyl in 18 MΩ water was prepared and infused

using the syringe driver pump at the “T” piece between the column and ion source at a flow rate of 3 $\mu\text{L}/\text{min}$. Blank plasma and blank saliva samples were extracted as per section 2.4, and injected during the constant post-column infusion. Additionally, plasma and saliva samples obtained from each of six subjects were spiked with either 0.045 or 9.00 $\mu\text{g}/\text{L}$ of fentanyl and nor-fentanyl respectively. For each analyte and IS, the matrix factor (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.

2.5.5 Stability

The stability study for the analytes in plasma was performed using low and high controls.

- a) Autosampler stability at 4 $^{\circ}\text{C}$: This was performed by extracting controls and storing them in the autosampler for 72 h prior to assay against freshly prepared calibrators.
- b) Bench-top stability: This was achieved by thawing the controls and allowing them to stand for 6 h at laboratory ambient temperature (22 ± 2 $^{\circ}\text{C}$) before extraction and assay against freshly thawed calibrators.
- c) Long term stability: This was performed by storing the controls for 60 days at -20 $^{\circ}\text{C}$. After 60 days the controls were extracted and analysed against calibrators stored at -70 $^{\circ}\text{C}$.
- d) Freeze-thaw stability: This was performed by freezing and thawing the controls prepared in plasma on each of three days respectively and then analysing them against calibrators stored at -70 $^{\circ}\text{C}$.

2.5.6 Limits of quantification

Upper and lower limits of quantitation were determined based on the highest and lowest concentrations for which acceptable linearity, accuracy and precision were demonstrated.

2.6 Bioanalytical method validation for saliva

Validation of the assay for saliva was also performed as for plasma, as set out in section 2.5, except inaccuracy and imprecision data were based on quadruplicate assays on each of four days. The validation was performed for both sample preparation protocols as mentioned in sections 2.4.2 and 2.4.3.

2.7 Collection and preparation of plasma and saliva samples from patients

The HPLC-MS/MS method developed and validated as mentioned above was applied to quantify fentanyl and nor-fentanyl from 11 paired plasma and saliva samples from subjects receiving fentanyl for cancer pain via transdermal patches of different doses. The samples were collected randomly irrespective of any specified time period. 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. Saliva samples were obtained by having the participant chew a CDB from a Salivette®. The Salivettes® were pre-weighted before taking the sample and post weighted immediately after taking the sample and stored at -70 °C until analysis. The analysis for plasma and saliva was performed as specified in section 2.4.

3 Results and Discussion

3.1 Bioanalytical method validation

3.1.1 Specificity

Fig. 1 to 4 show blank and spiked chromatograms for both analytes and both internal standards in plasma and saliva typical of the six obtained. These chromatograms demonstrate the absence of any chromatographic and/or mass spectrometric interference. The retention times for fentanyl and nor-fentanyl were 3.70 mins and 3.20 mins respectively.

3.1.2 Linearity

Calibration curves were plotted for fentanyl and nor-fentanyl prepared from both human plasma and saliva respectively at concentrations of 0.02, 0.1, 0.5, 2 and 10 µg/L, using a $1/x^2$ weighting factor. This weighting factor was used as it was found to produce optimal accuracy and imprecision data. The linearity in plasma was demonstrated by an average (n=4) $R^2 (\pm \text{SEM})$ 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) $R^2 (\pm \text{SEM})$ 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 CDBs was plotted for fentanyl and nor-fentanyl respectively. Linearity was demonstrated by an average (n=4) $R^2 (\pm \text{SEM})$ value of 0.9991 ± 0.0003 for fentanyl and 0.9971 ± 0.0007 for nor-fentanyl.

3.1.3 Inaccuracy and Imprecision

Intra- and inter-day inaccuracy and imprecision data are presented in Tables 2a, 2b and 3. 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 %.

3.1.4 Matrix effect (Ion suppression)

Ion suppression using PCI was also assessed in blank plasma (n = 6) and saliva samples (n=6) from different subjects. PCI was performed using chromatographic conditions described in section 2. Example chromatograms of PCI of fentanyl and nor-fentanyl into chromatograms of mobile phase, blank plasma and saliva are shown in Fig. 5, Fig. 6 and Fig. 7 respectively. The change in the organic component of the mobile phase due to gradient elution influenced the signal produced (see Fig. 5), with an increase in organic component in the mobile phase resulting in increased ionisation. This behaviour is likely due to improved volatilisation of the mobile phase in the ion source. PCI of fentanyl and nor-fentanyl in plasma and saliva resulted in similar behaviour with a background matrix effect due to the changing mobile phase composition. Allowing for this, the matrix effect due to ion suppression in the presence of both plasma and saliva was as much as 50 %, as shown in Fig. 5. For this reason matrix factors and normalised matrix factors were also determined as described, to assess the impact of the stable isotope internal standards in normalising for the ion suppression. Matrix factors and IS normalised matrix factors are summarised in Table 4. While the matrix factors determined support the PCI experiments in suggesting the presence of ion suppression, the normalised matrix factors (and their CV's) indicate the stable isotopes are adequately correcting for this phenomenon.

3.1.5 Stability

Results of the stability studies are presented in Tables 5a and 5b. No significant loss of analytes or IS was observed, with all recoveries being better than 90 %. Stability of prepared samples in the autosampler for 72 h at 4 °C and at bench top for 6 h at 22 ± 2 °C indicated no issues. Similarly, three freeze/thaw cycles did not affect assay results for either analyte in either matrix. Likewise, no effect on analyte was observed during long term storage at -20 °C.

3.1.6 Limits of quantification

Based on acceptable imprecision, accuracy and linearity at 0.030 µg/L for plasma and 0.045 µg/L for saliva, for both analytes, these values have been set as the respective 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 quantitation (ULOQ).

3.2 Application

Fentanyl and nor-fentanyl from 11 paired plasma and salivary samples are presented in Fig. 8. Mean fentanyl concentration in plasma and saliva was 0.785 and 3.335 µg/L respectively. Similarly mean nor-fentanyl concentration in plasma and saliva was 0.531 and 0.517 µg/L respectively. These data show salivary fentanyl concentrations exceeding plasma concentrations, suggesting the possibility of active transport into saliva is likely, as previously demonstrated for oxycodone [8]. It has been previously described that drugs enter saliva through passive diffusion [25] due to the concentration gradient between plasma and saliva. Furthermore, if the drug exhibits low plasma protein binding, the free (unbound) concentration is available to diffuse from plasma to saliva [8]. However, as fentanyl is almost 85-90 % protein bound [26], there is less free fentanyl available in plasma. Since our results showed that salivary concentration exceeded plasma concentration, some active transport may be likely. Further, these preliminary data do not show any obvious relationship between plasma and saliva concentrations (Fig. 8) with the R^2 value for fentanyl and nor-fentanyl in plasma and saliva being 0.230 and 0.397 respectively. A larger sample size is perhaps needed to draw any strong conclusions regarding the relationship. These data may also reflect the variable sample collection times with respect to time of dose, as distribution mechanisms will likely alter the saliva/plasma concentration ratio across the dosing interval.

In contrast to previous reports, our method uses a simple one-step sample preparation. Additionally, this method was successful to quantify fentanyl and nor-fentanyl in both human saliva and plasma samples for PK studies at concentrations as low as 0.045 and 0.03 µg/L respectively. Simple protein precipitation generated good results without any significant impact of ion suppression. Such simple

and cost effective methods are very useful when analysing multiple samples in routine laboratory tests. Preliminary studies showed that patients were often unable to provide adequate saliva volumes to allow collection from the CDB by centrifugation. In preliminary studies, significant adsorption to the CDB was demonstrated when tested using spiked samples. Fentanyl and nor-fentanyl at two different concentrations (0.045 and 9 µg/L) in duplicate was prepared in saliva and passed through the CDB. After centrifuging, the samples were assayed and compared to the solution without passing through the CBD. The results showed that fentanyl and nor-fentanyl was adsorbed approximately 50% and 70% in the CDB respectively. Hence this study also demonstrated a practical approach to using Salivettes® to collect saliva even when drugs bind to the CDB, or only low saliva volumes can be obtained, overcoming two major drawbacks with this collection method. Moreover there are also various devices commercially available which could collect saliva at a volume as small as 0.03 mL [19] but such small volumes are not sufficient for some HPLC assay methods where samples as high as 200-300 µL are required. Ion suppression can have a significant deleterious impact on HPLC-MS/MS assays [27] and previously reported assay methods have not considered the issue of ion suppression and/or other matrix effects on plasma and saliva [21-22]. Our data demonstrate that this has limited, if any, impact on our method.

4 Conclusions

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

Acknowledgements

The study to conduct research on plasma and saliva from patients has research ethics approved by Mater Health Services Human Research Ethics Committee and Human Research Ethics Committee, Griffith University. We thank Mater Palliative Care, Mater Research Institute and Griffith University for partially funding this study.

Table 1

MRM parameters set up for quantifying fentanyl, nor-fentanyl, D₅ fentanyl and D₅ nor-fentanyl in plasma and saliva samples.

	Q1 Mass	Q3 Mass	Time (mSec)	DP (V)	EP (V)	CE (V)	CXE (V)
fentanyl	337	188	70	40	12	30	4
D ₅ Fentanyl	342	188	70	40	12	30	4
Nor-fentanyl	233	84	70	46	11	25	6
D ₅ Nor-fentanyl	238	84	70	46	11	25	6

Table 2a

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

	Spiked Conc. (µg/L)	Mean assayed Conc. (µg/L)	Inaccuracy (%)	Intra-day Imprecision (% CV)	Inter-day Imprecision (% CV)	Total Imprecision (% CV)
Fentanyl	0.030	0.030	100.8	4.39	7.21	8.44
	9.0	8.84	98.2	3.10	1.00	3.25
Nor-fentanyl	0.030	0.029	96.2	9.64	12.4	15.5
	9.0	8.80	97.8	4.02	6.87	7.69

Table 2b

Inaccuracy and imprecision data for fentanyl and nor-fentanyl in human saliva.

	Spiked Conc.(µg/L)	Mean assayed Conc.(µg/L)	Inaccuracy (%)	Intra-day Imprecision (% CV)	Inter-day Imprecision (% CV)	Total Imprecision (% CV)
Fentanyl	0.045	0.044	98.6	2.22	2.71	3.50
	9.0	8.97	99.7	2.15	0.94	2.34
Nor-fentanyl	0.045	0.046	102.8	3.65	2.77	4.58
	9.0	9.14	101.6	2.59	1.14	2.83

Table 3

Intra- and inter-day inaccuracy and imprecision of fentanyl and nor-fentanyl in human saliva extracted from CDB (Salivette®).

	Spiked Conc. (µg/L)	Mean assayed Conc. (µg/L)	Inaccuracy (%)	Intra-day Imprecision (% CV)	Inter-day Imprecision (% CV)	Total Imprecision (% CV)
Fentanyl	0.045	0.045	100.7	2.85	2.89	4.20
	9.0	9.42	104.7	3.79	1.79	4.06
Nor-fentanyl	0.045	0.047	104.1	4.83	4.25	6.44
	9.0	9.71	107.9	5.65	6.76	7.38

Table 4

Mean matrix factors (n = 6) and imprecision for fentanyl and nor-fentanyl in plasma and saliva.

		Plasma			Saliva		
		MF (Analyte)	MF (IS)	MF (IS Normalised)	MF (Analyte)	MF (IS)	MF (IS Normalised)
Fentanyl	HC	1.11	1.04	1.07	1.03	0.95	1.09
	(% CV)	(15.3)	(15.3)	(1.9)	(20.8)	(21.5)	(1.25)
	LC	1.86	1.85	1.02	1.05	1.05	0.991
	(% CV)	(9.8)	(10.0)	(2.1)	(23.7)	(23.8)	(7.66)
Nor-fentanyl	HC	1.24	1.13	1.09	0.993	0.965	1.03
	(% CV)	(11.4)	(10.9)	(2.30)	(22.9)	(24.4)	(4.69)
	LC	1.19	1.20	0.99	1.08	1.05	1.03
	(% CV)	(10.6)	(10.9)	(1.60)	(26.5)	(26.0)	(5.93)

Table 5a

Stability studies of fentanyl and nor-fentanyl in human plasma.

		Stability condition	Spiked conc.(µg/L)	Mean Assayed Conc. (n=3) (µg/L)	Imprecision (% CV)	Inaccuracy (%)
Fentanyl	Autosampler (4 °C) for 72 h		0.045	0.043	1.80	95.6
			9.0	8.73	1.08	97.0
	Bench-top (24 °C) for 6 h		0.045	0.045	3.62	99.1
			9.0	8.77	0.46	97.4
	Freezer (-80 °C) for 60 days		0.045	0.045	0.95	100.0
			9.0	8.61	2.32	95.7
	Freeze-thaw for three cycles		0.045	0.045	4.01	99.8
			9.0	8.81	1.43	97.9
Nor-fentanyl	Autosampler (4 °C) for 72 h		0.045	0.042	1.08	92.9
			9.0	8.55	4.20	95.0
	Bench-top (24 °C) for 6 h		0.045	0.041	4.98	91.6
			9.0	8.45	1.60	93.9
	Freezer(-80 °C) for 60 days		0.045	0.043	7.02	96.2
			9.0	8.32	0.87	92.4
	Freeze-thaw for three cycles		0.045	0.044	5.99	97.8
			9.0	8.40	2.15	93.3

Table 5b

Stability studies of fentanyl and nor-fentanyl in human saliva.

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

Fig. 1

Specificity in plasma (fentanyl and nor-fentanyl). Chromatograms of extracts of blank plasma and analyte at LLOQ from bottom to top: a) blank plasma at nor-fentanyl transition b) blank plasma at fentanyl transition c) nor-fentanyl d) fentanyl. Retention times for fentanyl and nor-fentanyl, 3.7 and 3.2 mins respectively.

Fig. 2

Specificity in plasma (fentanyl IS and nor-fentanyl IS). Chromatograms of extracts of blank plasma and IS at LLOQ from bottom to top: a) blank plasma at D₅-nor-fentanyl transition b) blank plasma at D₅-fentanyl transition c) D₅-nor-fentanyl d) D₅-fentanyl. Retention times for D₅-fentanyl and D₅-nor-fentanyl, 3.7 and 3.2 mins respectively.

Fig. 3

Specificity in saliva (fentanyl and nor-fentanyl). Chromatograms of extracts of blank 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 d) fentanyl. Retention times for fentanyl and nor-fentanyl, 3.7 and 3.2 mins respectively.

Fig. 4

Specificity in saliva (fentanyl IS and nor-fentanyl IS). Chromatograms of extracts of blank saliva and IS at LLOQ from bottom to top: a) blank saliva at D₅-nor-fentanyl transition b) blank saliva at D₅-fentanyl transition c) D₅-nor-fentanyl d) D₅-fentanyl. Retention times for D₅-fentanyl and D₅-nor-fentanyl, 3.7 and 3.2 mins respectively.

Fig. 5

Ion Suppression (matrix effect) in mobile phase: Example chromatograms of mobile phase injection with fentanyl and nor-fentanyl added by post column infusion. Chromatography as described in text.

Fig. 6

Ion Suppression in plasma: Example chromatograms of blank plasma with fentanyl and nor-fentanyl added by post column infusion. Chromatography as described in text.

Fig. 7

Ion Suppression in saliva: Example chromatograms of blank saliva with fentanyl and nor-fentanyl added by post column infusion. Chromatography as described in text.

Fig. 8

Fentanyl and nor-fentanyl concentrations in paired plasma and saliva samples from cancer patients receiving fentanyl via a transdermal patch.

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