Quantitative determination of the enantiomers of methadone in human plasma and saliva by chiral column chromatography coupled with mass spectrometric detection

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Print: Black and white

Abstract

Methadone is a potent lipophilic synthetic opioid that is effective in the treatment of cancer pain and perceived benefit in difficult pain control scenarios (especially in cases of neuropathic pain). The use of methadone in clinical practice is challenging however, due to the narrow therapeutic window and large inter- and intra- individual variability in therapeutic response. Quantitation of the enantiomers d- and l-methadone (d- and l-MTD) in plasma and saliva provides a basis for studying its pharmacokinetics in patients with cancer and for monitoring efficacy, toxicity and side-effects. This assay involves quantitation of the enantiomers of methadone using their respective deuterated internal standards, in plasma and saliva matrices with no impact of ion suppression in either matrix. The analytical recoveries of d- and l-MTD from the saliva collection devices (Salivette®) are optimised in this novel method with an accurate and simple extraction method employing dichloromethane. Optimal enantioselective separations were achieved using an α 1-acid glycoprotein chiral stationary phase and triple quadrupole tandem mass spectrometer. Linearity was demonstrated over 0.05 – 1000 μ g/L for both enantiomers in plasma and in saliva with correlation coefficients greater than 0.998. The lower limit of quantitation (LLOQ) was determined to be 0.1 μ g/L in plasma and saliva for d- and l-MTD. Accuracy of the method ranges from 100% - 106% even at the LLOQ and total precision, expressed as the coefficient of variation, was between 0.2 and 4.4% for both analytes in both matrices. A simple one step extraction procedure resulted in recoveries greater than 95% for both analytes, at concentrations as low as 0.5 μ g/L, from the Salivette®. The validated method was applied successfully in 14 paired plasma and saliva samples obtained from adult patients with cancer pain receiving methadone.

Key words: Methadone, Enantiomers, HPLC–MS/MS, Plasma, Saliva, Adsorption

Abbreviations: ANOVA-analysis of variance; CDB-cotton dental bud; Conc-concentration; CV-coefficient of variation; d,l-MTD-racemic methadone; d,l-MTD-D3-deuterated racemic methadone; ESI-electrospray ionization; HPLC-MS/MS-liquid chromatography coupled with tandem mass spectrometry; IS-internal standard; LLOQ-lower limit of quantitation; MMT-methadone maintenance treatment; MRM-multiple reaction monitoring; MTD-methadone; PCI-post column infusion; PK-pharmacokinetics; QC-quality control

1. Introduction

Moderate to severe pain in cancer is common and affects 70-80 % of patients with advanced malignancy. Methadone (6-(dimethylamino)-4, 4-diphenylheptan-3-one) is a synthetic μ opioid receptor agonist that is effective in treating cancer pain [1]. It is administered as a racemic mixture of two enantiomers with distinct actions and elimination profiles. The (R)- or l-methadone enantiomer is more potent than the (S)- or d-isomer by a factor of eight to 50 and is believed to be almost entirely responsible for the analgesic properties [2]. Clinical studies have indicated that methadone metabolism and disposition is enantioselective [3, 4]. This suggests that the d- and l- enantiomers of methadone have different pharmacokinetic (PK) profiles. Studies on the CYP involvement in stereo-selective metabolism of methadone suggest that CYP2B6 is the main determinant of enantioselective metabolism [5, 6]. The free fraction of l-methadone is greater, yet has a slower clearance rate relative to d-methadone [3]. It is, therefore, useful to quantify both enantiomers individually when investigating the PK of methadone [7].

In addition to cancer pain, methadone is also used in the management of other forms of acute and chronic pain scenarios including neuropathic pain and in opioid replacement therapy. The doses used in methadone maintenance treatment (MMT) are generally much higher than those used for pain management. A narrow therapeutic window between pain control and toxicity as well as large inter-individual variation in the pharmacokinetics and pharmacodynamics of methadone challenge the management of severe pain with this drug [8, 9]. If opioid dose individualisation, based on PK modelling from saliva or plasma concentrations, without the need for dose titration was feasible, it would represent a major breakthrough in the use of methadone in cancer pain. This would be a significant step forward in improving pain control in cancer patients on a worldwide basis, as methadone is one of the least expensive opioids currently available. In addition, personalised therapy would facilitate effective methadone dosing and pain control without compromising the patient's safety. Moreover, a convenient assay could facilitate effective and safe individualised conversion schedules for methadone.

Most new bioanalytical methods for drug assays utilise high performance liquid chromatography coupled with mass spectrometry (HPLC–MS/MS) which permits simultaneous analysis of multiple, non-volatile, polar and/or high molecular weight compounds in various biological matrices even with low sample volume. The use of saliva in therapeutic drug monitoring has increased over the last four decades for anticonvulsants, analgesics and opioids [10-12]. Strong correlations between plasma and saliva concentrations for analgesics such as paracetamol [10] and hydromorphone [13] have been described. Other opioids including codeine [14], diamorphine [11], methadone (for methadone maintenance treatment) [12], morphine [15], dihydrocodeine [16], oxycodone [17], and fentanyl [18], have also been analysed to investigate saliva/plasma (S/P) ratios of drug concentration. Saliva sampling is non-invasive and painless and does not require specially trained personnel. In studies involving patients with advanced malignant disease, this method of sample collection, avoids reluctance on the part of health professionals to subject their patients to

venesection. Though Shiran *et al* [10] reported insignificant correlation for saliva and plasma concentrations of methadone among a MMT patient population, Wolff *et al* [19] reported a positive correlation ($r=0.81$) between paired saliva and plasma methadone concentrations in 21 patients receiving MMT. The enantiomeric ratio of methadone in saliva has been found to have good correlation with the d/l ratio in serum [20]. Methadone's lipophilicity could contribute to passive diffusion into saliva and better reflect either free or total plasma concentration. Despite the advantage of saliva sampling, there are challenges involved with sample analysis, including low sample volumes (associated with xerostomia in cancer patients) and adsorption of methadone to sample collection devices. The nonspecific adsorption of methadone to the cotton dental bud (CDB) has resulted in recoveries of $\leq 70\%$ [21, 22]. To date, there have been no studies addressing this problem. For quantitative purposes, accurate determination of concentration is essential [23]. In a recent study, involving quantitation of fentanyl in saliva samples, adsorption to Salivette® was addressed by an extraction procedure involving multiple steps of extraction [18].

Several hyphenated analytical methods have been proposed for the analysis of methadone enantiomers in matrices including plasma, serum, saliva, whole blood, liver microsomes and urine [24-34]. All of these methods were developed to assay methadone enantiomers in plasma for methadone maintenance patients or toxicological studies. HPLC-MS/MS methods to quantitate methadone enantiomers in cancer patients especially in both matrices (plasma and saliva) samples are lacking. The aim of this study was therefore to develop and validate a simple, simultaneous HPLC-MS/MS method to quantify the d- and l- enantiomers of methadone in plasma and saliva to study the pharmacokinetics of methadone in cancer patients. Absolute chromatographic separation was achieved by isocratic elution and an extended run time assured repeatable results and ruggedness of the assay. Multiple reaction monitoring (MRM) mode was used in mass spectrometer which added specificity to the assay as compared to previous methods, without any loss in sensitivity (lower limit of quantitation (LLOQ) = $0.1 \mu\text{g/L}$). Deuterated internal standards for each analyte were used to correct for any loss in sensitivity to ion suppression. However, the evaluation of ion suppression in both the matrices with multiple samples confirmed no interference throughout the entire chromatographic run. This assay method utilised protein precipitation for plasma samples, and a simple, direct and validated extraction of the analytes from the saliva collection device (Salivette®), overcoming the adsorption of methadone to the Salivette®, including the cotton dental bud (CDB) with recoveries approaching 100% even with very low sample volumes.

2. Materials and methods

2.1 Chemicals and reagents

Racemic methadone (d,l-MTD) and deuterated racemic methadone (d,l-MTD-D3) were obtained from Cerilliant®, Round Rock, Texas, USA. The pure enantiomers, d- and l-MTD, were obtained from Ultrafine Chemicals (Manchester, UK) by custom synthesis. HPLC grade acetonitrile, dichloromethane and isopropyl alcohol were obtained from Merck, New Jersey, USA. Ammonium acetate and Ammonium hydroxide were

obtained from Scharlau Chemie, Spain and Sigma Aldrich Chemie, Germany. 18 M Ω water was obtained from a Labmate water purification system (Aquacure, Brisbane, Australia). Salivettes® were obtained from Sarstedt, Nümbrecht, Germany. The blank plasma samples were obtained from the Pathology Services, Mater Health Services, Brisbane and the blank saliva samples were obtained from volunteer staff within the laboratory.

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 (AB SCIEX, Mount Waverly, Victoria, Australia). Analyst software version 1.4.2 from AB SCIEX was used for data acquisition and analysis. The electrospray ion source (ESI) was operated in positive ion mode, employing MRM with optimised declustering potential, entrance potential, collision energy and collision cell exit potential as reported in Table 1. Nitrogen was used as the source gas. Curtain gas pressure was maintained at 30 psi, collision gas at 5 psi, ion source gas 1 (GS-1) and ion source gas 2 (GS-2) were maintained at 60 and 70 psi respectively. Ion spray voltage was maintained at 5500 V and drying gas temperature at 750°C. For chromatographic separation of the enantiomers, a chiral α -acid glycoprotein (AGP), 5 μ , 100 x 4.0 mm ID column (ChromTech, Hagersten, Sweden) and a 10 x 4.0 mm precolumn containing the same stationary phase were used. The mobile phase comprised 85:10:5 % v/v of 20 mM ammonium acetate buffer (pH adjusted to 7.4), isopropyl alcohol and acetonitrile respectively. An isocratic flow of 0.9 mL/min, at ambient room temperature (22 $\pm$ 2°C) resulted in adequate separation of the d- and l- enantiomers at 8.4 and 11.8 minutes with a total run time of 20 minutes.

2.3 Preparation of standards, internal standard and spiked controls

The racemic mixture of methadone (100 μ g/mL) was stored at -70°C. Standards were prepared in both blank plasma and saliva from the storage solution in a concentration range of 0.05 - 1000 μ g/L and stored at -20°C. A wide analytical range was used in the development of this assay to encompass both the lower doses administered for pain management in cancer patients, and higher doses administered to methadone-maintenance patients, and the subsequent plasma and saliva concentrations. From the internal standard (IS) stock solution of 100 μ g/mL, a working solution was prepared in acetonitrile at 125 μ g/L and stored at -70°C. The quality control (QC) samples were prepared at concentrations of 0.1, 10, 500 and 900 μ g/L for each analyte in plasma and saliva and stored at -20°C.

2.4 Sample preparation

2.4.1 Extraction of d- and l-methadone from plasma samples

Sample preparation involved simple protein precipitation. To 200 μ L of sample (standard, control or unknown), 600 μ L of IS working solution (125 μ g/L in acetonitrile) was added and vortex mixed for 30 s

followed by centrifugation at 12000 x g for 8 min. 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 100 µL of mobile phase, vortex mixed for 30 seconds and 10 µL was injected from the autosampler vials.

2.4.2 Extraction of d- and l-methadone from saliva samples

A direct extraction procedure was developed for saliva samples. As this method is intended to assay the saliva samples collected using a Salivette®, a direct extraction of analytes from the CDB was developed to minimise error and loss of analytes associated with multiple extraction processes/techniques. 400 µL of sample (standard, control or unknown) was added to CDB (of Salivette®) followed by 400 µL of IS working solution (125 µg/L in acetonitrile). The Salivettes® were allowed to stand for approximately 5 min and 1.0 mL of dichloromethane was pipetted onto the CDB and allowed to stand for further 5 min. The addition of dichloromethane was repeated and after 5 mins the Salivette® was centrifuged at 700 x g for 15 mins. The extract was decanted into 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 mobile phase and vortex mixed for 30 seconds. The samples were then transferred to respective 1.5 mL Eppendorf tubes and centrifuged at 12000 x g for 10 min followed by injection of 10 µL of supernatant from autosampler vials into the HPLC–MS/MS. In application to the actual patient samples, the actual weight of the samples (difference between pre- and post-sampling) was used to normalise for sample volume.

2.5 Bioanalytical method validation

2.5.1 Specificity

The assay selectivity was determined by analysing extracts of multiple samples (n=6) of plasma and saliva and no co-eluting peaks were observed at the retention time of the analytes of interest. Optimal chromatographic separation of the enantiomers was achieved by isocratic elution with baseline separation. The retention time of the enantiomers was confirmed using pure solutions of each enantiomer. The MRM transition “cross-talk” was evaluated by the analysis of multiple plasma and saliva samples with each enantiomer and its respective deuterated IS.

2.5.2 Linearity

To demonstrate linearity, seven standards were prepared in a concentration range of 0.05 –1000 µg/L for both enantiomers of methadone in plasma and saliva. Although an extended calibration range was required to include a wide range of concentration in plasma and saliva, linearity was demonstrated over the entire range and the possibility of carryover was also tested and negated. Calibration data were fitted by least-square $1/x^2$ -weighted linear regression.

2.5.3 Accuracy and Precision

Each accuracy and precision run included calibration standards, low, medium and high QC samples in quadruplicate on each of four days (n=16). The data were subjected to analysis of variance (ANOVA) to estimate the inter-day, intra-day, and total imprecision, estimated as the relative standard deviation (coefficient of variation, CV%) [35].

2.5.4 Recovery from collection device (Salivettes®) for saliva sample

The recovery of analytes from the cotton dental buds in Salivettes® was determined by comparing the absolute peak areas of extracts obtained as per section 2.4.2. Saliva samples fortified with analytes (500 µg/L and 0.5 µg/L) were extracted from Salivettes® with CDB in quadruplicate, and analysed against the saliva samples extracted from Salivettes® without the CDB.

2.5.5 Carry over effects

Carry over was assessed by injecting blank samples after the high concentration standard (1000 µg/L) in both matrices in three sequences.

2.5.6 Matrix effect (ion suppression)

The method was evaluated for ion suppression in both saliva and plasma extracts in multiple sample matrices (plasma and saliva n=6). The assay was tested for matrix effects, including ion suppression, in plasma and saliva by the post column infusion (PCI) approach. A syringe pump was connected via a “tee” to the column effluent, and a test solution of the d- and l-enantiomers of methadone (125 µg/L) was introduced into the mass detector at a constant rate of 2 µL/min to obtain a constant ESI response. For testing matrix effects, blank plasma and saliva samples were extracted (Section 2.4) and injected into the HPLC system.

2.5.7 Stability

The stability of the analytes in plasma and saliva was tested using low and high quality control samples.

- a) Autosampler stability at 4°C: This was tested by extracting controls and storing them in the autosampler for 72 h prior to assay against freshly prepared standards.
- b) Bench-top stability: The quality control sample was allowed to stand at the ambient temperature of the laboratory for 24 hrs before extraction and assay against freshly prepared standards.
- c) Long term stability: Quality control samples stored for 120 days at –20°C were analysed against standards prepared from stock on the day of analysis.
- 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 standards stored at –70°C.
- e) Stability of saliva samples in Salivettes®: Quality control samples spiked into the CDB were stored at –70°C for 60 days prior to analysis against fresh samples extracted from Salivettes® on the day of analysis (performed to investigate the stability of methadone in Salivettes® used in the pilot study).

The stability of stock solutions of analytes and IS stored at –70°C was also performed.

2.6 Collection and preparation of plasma and saliva samples from patients

This study was granted ethical approval by the Mater Human Research Ethics Committee, Mater Health Services, South Brisbane, Australia and Human Research Ethics Committee, Griffith University, Australia. The HPLC–MS/MS method developed and validated as mentioned above was applied to quantify d- and l-MTD in 14 paired plasma and saliva samples from subjects receiving methadone for cancer pain. Blood samples (3–4 mL) were collected in standard 5 mL EDTA tubes without a serum separator plug. The samples were centrifuged within 1 h of collection and the plasma stored at -70°C until analysis. Saliva samples were obtained by having the patient chew a CDB from a Salivette®. The Salivettes® were pre-weighted before taking the sample and post weighted after sampling and stored at -70°C until analysis. The analysis for plasma and saliva was performed as specified in Section 2.4.

3. Results

3.1 Evaluation of assay performance

3.1.1 Specificity

Assay selectivity is demonstrated by the absence of interfering peaks at the retention times of d-MTD and l-MTD and their deuterated internal standards in extracted blank plasma and saliva samples. Fig. 1 and Fig. 2 depicts chromatograms of a blank matrix (plasma and saliva), and matrix with internal standard. The retention times for d-MTD and l-MTD were 8.4 min and 11.8 min respectively. These chromatograms demonstrate the absence of any chromatographic and/or mass spectrometric interference.

3.1.2 Linearity and detection limits

Calibration curves for both enantiomers were generated by weighted ($1/x^2$) least squares linear regression with standards prepared in the concentration range of 0.05–1000 $\mu\text{g/L}$ in both matrices. The degree of linear correlation was calculated as coefficient of determination $R^2 > 0.998$ for standard curves based on four replicates of a 7-point calibration curve. With the stated assay conditions, extracted analyte samples (with IS in acetonitrile) at a concentration of 0.050 $\mu\text{g/L}$ of d- and l-MTD could be reliably distinguished from baseline/zero with a signal to noise ratio ≥ 3 .

3.1.3 Accuracy and Precision

Accuracy was estimated as the mean assayed concentration expressed as a percentage of the spiked concentration. Intra- and inter-day accuracy and precision data are presented in Tables 2 and 3. The accuracy for d-MTD and l-MTD in plasma was 100–103% and precision expressed as % CV was $< 3.5\%$. In saliva, accuracy ranged from 100–106% and precision $< 4.4\%$. The lowest concentration of the analytes at which adequate linearity, specificity, accuracy and precision has been demonstrated (LLOQ) was 0.1 $\mu\text{g/L}$ for both d- and l-MTD in both matrices.

3.1.4 Recovery from Salivettes®

The recoveries of d- and l-MTD from saliva samples collected using the Salivette® was investigated. The peak area of enantiomers following extraction (n=4) and duplicate analysis were compared to the peak area of the enantiomers in the same control sample following the same extraction method but without the CDB. The recoveries for d- and l-MTD at 0.5 µg/L were 96% and 97%, and at 500 µg/L 99% and 98% respectively.

3.1.5 Carry over effects

Injection of blank sample following the high concentration standards resulted in blank chromatograms assuring accuracy and precision of analysis.

3.1.6 Matrix effect (ion suppression)

Ion suppression using the PCI approach was assessed in plasma and saliva samples (n=6) from different origins. Matrix associated suppression was observed at around 2.6 minutes in the chromatographic run. However this interference is significantly isolated from the analyte/s retention time (Fig. 3 and Fig.4). These results confirm the validity of the extraction procedure for sample preparation before chromatography to obtain reproducible and reliable quantitative results for d- and l-MTD, without interference of matrix components.

3.1.7 Stability

Results of the stability studies are presented in Tables 4 and 5. No significant loss of analytes or IS was observed, in all the conditions as described in Section 2.5.6. As per the stability conditions described in section 2.5.7, the recovery in plasma ranged from 94-100% and 95-100% for d- and l-MTD respectively. In saliva samples, the recoveries were 99-103% for both d- and l-MTD.

3.1.8 Application

In a pilot study, 14 paired plasma and saliva samples from adult cancer patients who were prescribed methadone for pain management were analysed using the above method. Evaluation of the correlation of saliva and plasma drug concentrations of d- and l-MTD was primarily based on visual inspection of the scatter plots as presented in Fig.5. The mean concentrations of d- and l-MTD were 57.6 and 48.6 µg/L in plasma, and 64.1 and 104.3 µg/L in saliva respectively. The more recent and sensitive method [24] reports an LLOQ of 25 µg/L in plasma samples. Concentrations less than 5 µg/L were observed for both enantiomers in plasma and saliva samples. Further, Spearman's rank correlation was used to evaluate associations between saliva and plasma concentrations of the enantiomers of methadone. A statistical probability of $p < 0.001$ was considered statistically significant for these comparisons. The S/P ratio exhibited a linear correlation ($r_s = 0.89$ and 0.92 for d- and l-MTD respectively) with an average S/P ratio of 1.5 and 0.8. However a larger

sample size would be essential to establish and conclude a linear relationship between saliva and plasma to validate the use of saliva in pharmacokinetic studies and therapeutic monitoring of methadone.

4. Discussion

Although enantioselective HPLC–MS/MS assays for the quantitation of methadone in plasma and saliva have been previously reported [20, 31, 36], simultaneous analysis of d- and l-MTD in plasma and saliva using deuterated internal standards is sparse. This assay describes the validation of a highly sensitive, accurate and simplified method for the quantitation of methadone enantiomers in human plasma and saliva. This assay reports improved sensitivity over any of the previously reported methods [24, 33, 34] with LLOQ of 0.1 µg/L per enantiomer in both matrices and LOD of 0.05 µg/L per enantiomer. Compared to the most recent report [24], optimal chromatographic separation of the enantiomers with distinctive baseline separation was achieved. Deuterated internal standards for each enantiomer were used in quantitation. The method is reproducible and accurate and was successfully used in the analysis of real patient samples. A review of the relationship of saliva/plasma drug concentrations of methadone suggests that saliva concentrations may relate better to efficacy and/or toxicity than plasma levels [37]. Study with 60 MMT patients [12] report a significant, but weak S/P correlation. With the variability owing to pH variation in saliva samples and correction for plasma binding, the variation in S/P ratios is still unexplained [12]. Increased saliva concentration suggests the possibility of an active transport mechanism which is influenced by several factors including the physicochemical properties of the analyte [38] and genetic variation between individuals. Protein binding of methadone to α_1 -acid glycoprotein significantly determines the unbound fraction of methadone [39]. The inter-individual variation in the relative expression of the genetic variant of α_1 -acid glycoprotein [39] could contribute to variation in protein binding and subsequent bound/free concentrations of methadone. Pharmacokinetic modelling with saliva or plasma concentrations would represent a major breakthrough in the use of methadone in cancer pain. This assay will allow assessment of the concentration relationship of paired plasma and saliva samples. This method also addresses the limitations encountered with saliva sampling, namely; (a) *low sample volume* by normalising the actual concentration in relation to the actual weight of the sample and (b) *adsorption of methadone to Salivettes®* by developing a validated, simple and accurate extraction method. Ion suppression studies using PCI demonstrated the absence of ion suppression due to endogenous substances at the relevant retention times of the analyte s of interest. The use of a stable isotope internal standard will also guarantee that any ion suppression in individual samples is normalised, allowing for accurate quantitation, even in these unlikely circumstances.

5. Conclusion

A highly sensitive, selective and precise HPLC–MS/MS method for simultaneous quantitation of methadone enantiomers in plasma and saliva without any ion suppression effect was developed and validated. Further, this method validates the use of Salivettes® for saliva collection in studies of methadone, especially in

cancer patients, where low sample volumes due to xerostomia are challenging in sample analysis. This method describes accurate measurement of the enantiomers in both plasma and saliva, and could be used in the analysis of methadone in cancer patients towards personalising therapy with improved efficacy and maximum safety.

Acknowledgements

We thank Mater Palliative Care, Mater Research Institute and Griffith University for partially funding this study.

Accepted manuscript

Table 1. Optimal multiple reaction monitoring parameters used for the development of the method for quantitation of d- and l-methadone in plasma and saliva samples.

Analyte	Transition monitored (Q1/Q3)	DP(v)	EP(v)	CE(v)	CXP(v)
d-MTD	310.3/265.1	31	3.5	23	6
l-MTD	310.3/265.1	31	3.5	23	6
d-MTD-D3	313.3/268.4	31	3.5	23	6
l-MTD-D3	313.3/268.4	31	3.5	23	6

DP = declustering potential; EP = entrance potential; CE = collision energy; CXP = collision cell exit potential

Table 2. Intra- and inter-day accuracy and precision of d- and l-methadone in plasma samples.

	Nominal Conc (µg/L)	Accuracy (%) (n=16)	Intra-day Precision (%CV)	Inter-day Precision (%CV)	Total Precision (%CV)
d-MTD	0.1	103	1.6	1.6	2.2
	10	103	2.2	1.4	2.7
	500	103	0.3	0.6	0.7
	900	100	0.3	0.3	0.5
l-MTD	0.1	103	1.6	2.9	3.4
	10	104	1.8	1.1	2.1
	500	102	0.8	0.8	1.1
	900	100	1.1	0.4	1.2

CV = Coefficient of variation

Table 3. Intra- and inter-day accuracy and precision of d- and l-methadone in saliva samples extracted from cotton dental bud (Salivette®).

	Nominal Conc (µg/L)	Accuracy (%) (n=16)	Intra-day Precision (%CV)	Inter-day Precision (%CV)	Total Precision (%CV)
d-MTD	0.1	103	2.9	0.7	2.9
	10	100	1.4	1.7	2.1
	500	100	0.6	0.5	0.8
	900	100	0.2	0.2	0.3
l-MTD	0.1	106	4.1	1.5	4.4
	10	102	2.3	0.5	2.3
	500	100	0.7	0.4	0.8
	900	100	0.3	0.1	0.3

CV = Coefficient of variation

Table 4. Stability of d- and l-methadone in plasma.

Stability condition		Nominal Conc ($\mu\text{g/L}$)	Precision (% CV) (n=3)	Accuracy (%)
d-MTD	Autosampler (4°C) for 72 h	0.5	2.13	94
		500	0.12	100
	Bench-top (24°C) for 12 h	0.5	1.17	99
		500	0.23	100
	Freezer (−70°C) for 120 days	0.5	1.21	95
		500	0.35	100
	Freeze/thaw 3 cycles	0.5	1.21	95
		500	0.31	99
	Autosampler (4°C) for 72 h	0.5	4.36	95
		500	0.20	99
l-MTD	Bench-top (24°C) for 12 h	0.5	2.42	95
		500	0.20	100
	Freezer (−70°C) for 120 days	0.5	3.50	98
		500	0.23	101
	Freeze/thaw 3 cycles	0.5	1.19	97
		500	1.33	100

CV = Coefficient of variation

Table 5. Stability of d- and l-methadone in saliva.

Analyte	Stability condition	Nominal Conc µg/L	Precision (% CV) (n=3)	Accuracy (%)
d-MTD	Autosampler (4°C) for 72 h	0.5	0.96	103
		500	0.12	100
	Bench-top (24°C) for 12 h	0.5	0.92	100
		500	0.12	100
	Freezer (−70°C) for 120 days	0.5	0.60	100
		500	1.83	99
	Freeze/thaw 3 cycles	0.5	1.03	100
		500	0.92	101
	Stability in Salivette®	0.5	0.69	101
		500	0.12	100
	Autosampler (4°C) for 72 h	0.5	0.57	100
		500	0.81	100
l-MTD	Bench-top (24°C) for 12 h	0.5	0.92	100
		500	1.04	100
	Freezer (−70°C) for 120 days	0.5	1.78	100
		500	0.87	100
	Freeze/thaw 3 cycles	0.5	1.65	102
		500	2.24	104
	Stability in Salivette®	0.5	1.10	100
		500	2.46	102

CV = Coefficient of variation

Fig. 1. Specificity in plasma: (A) Chromatogram of extract of blank plasma at d.l-MTD transition; (B) extract of blank plasma at d.l-MTD-D3 transition; (C) an extract of blank plasma with d.l-MTD at a spiked concentration of 0.1 µg/L monitored at d.l-MTD transition. Chromatography as described in text. [Unsmoothed chromatograms; offset for display purposes].

Fig. 2. Specificity in saliva: (A) Chromatogram of extract of blank saliva at d.l-MTD transition; (B) extract of blank saliva at d.l-MTD-D3 transition; (C) an extract of blank saliva with d.l-MTD at a spiked concentration of 0.1 $\mu\text{g/L}$ monitored at d.l-MTD transition. Chromatography as described in text. [Unsmoothed chromatograms; offset for display purposes].

Fig. 3. Ion suppression in plasma: Chromatograms of blank plasma extracts with post column infusion of d- and l- methadone at a concentration of 125 $\mu\text{g/L}$. Chromatography as described in text. [Response not to scale, plasma samples 2-6 offset for display purposes].

Fig. 4. Ion suppression in saliva: Chromatograms of blank saliva extracts with post column infusion of d- and l- methadone at a concentration of 125 $\mu\text{g/L}$. Chromatography as described in text. [Response not to scale, saliva samples 2-6 offset for display purposes].

Fig.5. d- and l-MTD concentrations in paired plasma and saliva samples (n=14) from patients prescribed methadone for chronic cancer pain.

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Highlights

A highly sensitive HPLC–MS/MS method for quantitation of methadone enantiomers. Validated in plasma and saliva matrices, using Salivettes® for saliva collection. Applicable for studies in cancer patients with very low saliva sample volume. Novel one step extraction of methadone from saliva samples collected in Salivettes®. Nonspecific adsorption of methadone to Salivettes® is addressed.

graphical abstract

Author's Accepted Manuscript

Quantitative determination of the enantiomers of methadone in human plasma and saliva by chiral column chromatography coupled with mass spectrometric detection

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PII: S0039-9140(15)30498-7
DOI: <http://dx.doi.org/10.1016/j.talanta.2015.11.044>
Reference: TAL16140

To appear in: *Talanta*

Received date: 16 September 2015
Revised date: 17 November 2015
Accepted date: 17 November 2015

Cite this article as: Rani George, Michael Lobb, Alison Haywood, Sohil Khan, Janet Hardy, Phillip Good, Stefanie Hennig and Ross Norris, Quantitative determination of the enantiomers of methadone in human plasma and saliva by chiral column chromatography coupled with mass spectrometric detection *Talanta*, <http://dx.doi.org/10.1016/j.talanta.2015.11.044>

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