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Low-level quantitation of oxycodone and its oxidative metabolites, noroxycodone, and oxymorphone, in rat plasma by high-performance liquid chromatography–electrospray ionization–tandem mass spectrometry

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Abstract

A method was developed for quantification of oxycodone, noroxycodone, and oxymorphone in small volumes (50 μl) of rat plasma by high-performance liquid chromatography–electrospray ionization–tandem mass spectrometry using turbo ion-spray. Deuterated (d_3) opioid analogues acted as internal standards. Sample preparation involved protein precipitation with acetonitrile, centrifugal evaporation, and reconstitution in mobile phase; analyte separation was performed on a C18 (5 μm, 2.1 mm × 50 mm) column using a linear gradient program. Lower limits of quantitation (ng/ml) and their between-day accuracy and precision were—oxycodone, 0.9 (−0.2 and 7.8%); noroxycodone, 1.0 (0.6 and 6.2%); oxymorphone 1.0 (−1.8 and 9.5%).

Keywords: Oxycodone; Noroxycodone; Oxymorphone; Rat; High-performance liquid chromatography (HPLC); Electrospray ionization (ESI); Tandem mass spectrometry (MS-MS)

1. Introduction

The semisynthetic opioid, oxycodone (OXY), has been in clinical use since 1917, and for the past decade it has been used in controlled-release preparations for the treatment of chronic pain [1]. Oxycodone undergoes oxidative metabolism to form noroxycodone and oxymorphone (Fig. 1). In human liver microsomes, the predominant oxidative metabolic pathway for OXY is via *N*-demethylation to noroxycodone (NOR), with *O*-demethylation to oxymorphone (OXM) accounting for only 13% [2]. However, following administration of a 15 mg oral dose of OXY to human volunteers, maximal plasma concentrations (C_{max}) of OXY, and NOR were 38 and 26 ng/ml, respectively, while C_{max} for OXM was only 1.1 ng/ml [3]. This finding is consistent with other studies, where oxycodone's pharmacodynamic effects in humans were shown to be produced by the parent drug, rather than its *O*-demethylated metabolite, oxymorphone [4,5]. Additionally, recent *in vivo* studies in the rat have found evidence for the active influx of OXY across the blood–brain barrier [6].

OXY has been reported to have a high oral bioavailability (60–87%) in humans [7,8]. However, there are no studies that have investigated the oral bioavailability of OXY in the rat, despite this species being widely used to study the *in vivo* pharmacology of OXY. Hence, the purpose of the present study was to develop and validate a highly sensitive and specific method for the quantitation of OXY and its oxidative metabolites, NOR and OXM, in small volumes of rat plasma.

We have previously reported a method employing high-performance liquid chromatography (HPLC) combined with electrospray ionization–tandem mass spectrometry for low-level quantitation of both OXY and MOR, and their respective primary metabolites NOR and morphine-3-glucuronide (M3G) in rat plasma [9]. This method which involved the use of a standard nebulizer ion-spray source was subsequently utilized to simultaneously quantify plasma OXY, NOR, and OXM concentrations in diabetic and non-diabetic rats [10]. In the improved method described herein, turbo ion-spray was used in concert with a linear gradient program for solvent delivery to achieve greater

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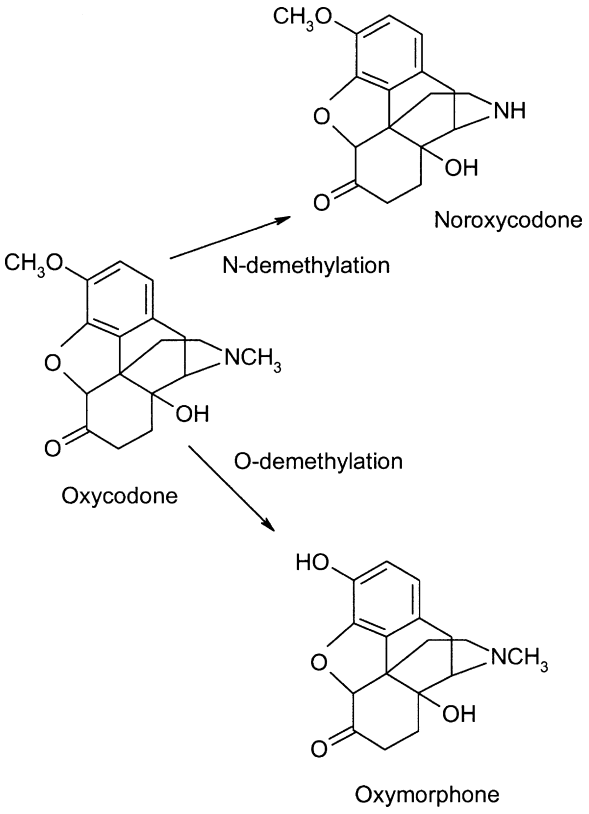

Fig. 1. Structures of oxycodone and its oxidative metabolites, noroxycodone, and oxymorphone.

analytical sensitivity for the simultaneous quantitation of OXY, NOR, and OXM in small volumes of rat plasma. Deuterated (d_3) analogues of each analyte were used as internal standards. Sample preparation comprised deproteination with acetonitrile, followed by centrifugal evaporation to dryness and reconstitution in mobile phase, prior to analysis by HPLC–ESI–MS-MS.

2. Experimental

2.1. Drugs and reagents

OXY hydrochloride USP (Mcfarlan Smith Ltd., Edinburgh, UK), NOR (Du Pont Merck, Wilmington, DE, USA), OXM (Sigma–Aldrich, Sydney, Australia) were used for the preparation of standard solutions. Deuterated (d_3) analogues of OXY, NOR, and OXM (Cambridge Isotope Laboratories, Andover, MS, USA) were used as internal standards. The following items were obtained from local suppliers: acetonitrile and methanol (HPLC grade), formic acid, and hydrochloric acid (AR grade). Deionized water (18 MΩ cm) was obtained from a Millipore® water purification system.

2.2. HPLC–ESI–MS-MS instrumentation and operating conditions

The HPLC system configuration consisted of an Agilent 1100 Series Binary Pump and an Agilent 1100 Series Well-plate Sampler. Chromatographic separations were performed on an Agilent Zorbax SB-C18 (5 μm, 2.1 mm i.d. × 50 mm) column, with Phenomex C18 (4.0 mm × 2.0 mm) SecurityGuard™ cartridges acting as the pre-column. The mobile phase comprised two components, and was delivered at a flow rate of 200 μl/min. Component A was 0.1% (v/v) aqueous formic acid; component B was 90% (v/v) methanol:water containing 0.1% (v/v) formic acid. The following gradient program was used for solvent delivery: 0–0.05 min, 100% A; linear gradient to 100% B at 4.5 min; 100% B until 6.0 min; 6.1–10.5 min, 100% A (solvent delay, 2.5 min; re-equilibration interval, 1.9 min). The sample injection volume was 10 μl.

Sample analysis was performed on an Applied Biosystems API 3000 triple quadrupole mass spectrometer using a TurboIonSpray® source operating in positive ion mode. High purity nitrogen acted as the nebulizer and drying gas, as well as the collision gas. Multiple reaction mode (MRM) operating parameters were optimized for highest sensitivity by direct infusion of mobile phase solution (components A:B, 90:10) containing 1 μg/ml concentrations of OXY, NOR, and OXM into the turbo ion-spray source, at 20 μl/min using a Harvard 11 syringe pump. The ion-spray voltage was 4500 V, while the declustering, focusing, and entrance potentials were set to 30, 225, and 14 V, respectively. The collision cell energy was 40 V using at a collision activated dissociation (CAD) gas setting of 4, and the collision cell exit potential was 10 V. The turbo ion-spray probe temperature was set to 350 °C, and the drying gas was delivered at 8 l/min. Optimal nebulizer and curtain gas flow rates of 10 and 12 l/min, respectively, were subsequently determined by infusion of the 1 μg/ml solution of OXY, NOR, and OXM at 20 μl/min into mobile phase (components A:B, 90:10), delivered at 200 μl/min into the turbo ion-spray source.

The following ion transitions were monitored using a dwell time of 200 ms for each compound and its deuterated analogue: OXY, 316.2/241.1; d_3-OXY, 319.2/244.2; NOR and OXM, 302.2/227.2; d_3-NOR and d_3-OXM, 305.2/230.2. Chromatograms were integrated using the Analyst®1.4 software package IntelliQuan Algorithm (Applied Biosystems).

2.3. Preparation of standard solutions

Stock standard solutions of OXY, NOR, and OXM were prepared in cetrimide-treated volumetric flasks and stored at 4 °C. All solutions and serial dilutions were prepared using 18 MΩ cm deionized water. The stock OXY solution was prepared by dissolving OXY hydrochloride into deionized water to give a 449.0 μg/ml solution of OXY (as the free base). NOR and OXM were dissolved into 0.1 M HCl to give individual stock solutions of NOR and OXM at concentrations at 501.0 and 102.3 μg/ml, respectively.

Serial dilutions of each stock standard solution were prepared in 1.5 ml polypropylene tubes on the day that each assay procedure was performed, and combined to give a stock assay calibration standard solution containing OXY, NOR, and OXM at concentrations of 898.0, 1002.0, and 1023.0 ng/ml, respectively. Serial dilutions were subsequently prepared to give the assay calibration standard solutions required for 9-point standard curves over the following range of concentrations for each

analyte: OXY, 0.9–449.0 ng/ml; NOR, 1.0–500.4 ng/ml; OXM, 1.0–511.5 ng/ml.

Individual stock solutions of d_3-OXY (100 μg/ml), d_3-NOR (10 μg/ml), and d_3-OXM (10 μg/ml), were stored at −20 °C. A stock internal standard solution was prepared in a 15 ml polypropylene tube to contain 1000 ng/ml concentrations of d_3-OXY, d_3-NOR, and d_3-OXM, in deionized water and stored at −20 °C. The working internal standard solution was subsequently prepared in a 15 ml polypropylene tube to contain 100 ng/ml concentration of each deuterated analogue in deionized water and stored at 4 °C.

2.4. Preparation of assay calibration standards and quality control samples for analysis

Aliquots (50 μl) of each working assay calibration standard solution were transferred to 1.5 ml polypropylene tubes. Working internal standard solution (50 μl) and blank pooled rat plasma (50 μl) was then added to each assay tube, and the assay tubes were briefly vortex-mixed. After addition of acetonitrile (300 μl) to each assay tube, the samples were then vortex-mixed, and left to stand for 30–60 min at 4 °C to facilitate protein precipitation and flocculation. Following centrifugation at 14,000 rpm for 5 min, the supernatants were transferred to fresh 1.5 ml polypropylene tubes, placed in a Savant evaporative centrifuge, and evaporated to dryness. The residues were subsequently reconstituted in 0.1% (v/v) formic acid (100 μl), briefly vortex-mixed, and sonicated for 2 min. Each assay tube was then centrifuged at 3000 rpm for 2 min, and the reconstituted solution transferred to 250 μl polypropylene inserts for analysis.

Nine point calibration curves ($y = mx + b$) were generated by weighted linear regression ($1/x^2$) relating the analyte's concentration to the peak area ratio of the analyte and its internal standard. Inverse predicted values for sample analyte concentrations were subsequently determined from the ratio of the peak areas for the analyte and its internal standard, using the values for slope (m) and y-intercept (b) that were derived from the analyte's standard curve. Quality control (QC) samples containing OXY, NOR, and OXM concentrations at the lower (0.9, 1.0, and 1.0 ng/ml, respectively), middle (44.9, 50.0, and 51.2 ng/ml, respectively), and upper (449.0, 500.4, and 511.5 ng/ml) ranges of the standard curve were prepared as described above.

2.5. Assay validation

The on-column limit of detection (LOD) was defined as the amount of analyte that could be detected at a minimum signal-to-noise ratio of 3:1. Accuracy and precision were determined using the QC samples. Between-day accuracy and precision were determined from three assays performed on separate days. Each assay contained the assay calibration standards, and the QC samples were analyzed in triplicate (i.e. $n = 3 \times 3$). Within-day accuracy and precision were determined in an additional assay containing the assay calibration standards and nine replicates of each QC sample (i.e. $n = 3 \times 9$). Accuracy was expressed as the percent deviation (%Dev) of the mean value for each analyte in the QC sample from the nominal concentration of the analyte. Precision was expressed as the percent coefficient of variation for the analyte (%CV). A lower limit of quantitation (LLOQ) with values of %Dev and %CV of ≤20% was considered acceptable; values for %Dev and %CV of ≤15% were acceptable at the middle and upper range of the standard curve.

The recovery of OXY, NOR, and OXM following protein precipitation, centrifugal evaporation, and reconstitution was determined for concentrations of OXY, NOR, and OXM at the lower (0.9, 1.0, and 1.0 ng/ml, respectively), middle (44.9, 50.0, and 51.2 ng/ml, respectively), and upper (449.0, 500.4, and 511.5 ng/ml) ranges of the standard curve. Triplicate aliquots (50 μl) of aqueous standard solutions containing the aforementioned concentrations of OXY, NOR, and OXM were transferred to 1.5 ml polypropylene tubes. Blank pooled rat plasma (50 μl) and deionized water (50 μl) were then added to each tube, and the recovery samples were prepared for analysis as described in Section 2.4. Recovery was subsequently determined by comparing the analyte's peak area following on-column injection of the reconstituted recovery sample to the analyte's peak area following on-column injection of the relevant aqueous assay standard solution.

2.6. Application of the assay

Ethical approval for the study was obtained from The University of Queensland's Animal Ethics Committee. Indwelling cannula lines were implanted into the jugular vein and femoral artery of a male Sprague–Dawley rat 24 h prior to the study procedure. A single 5 mg/kg i.v. bolus dose of oxycodone was subsequently administered via the jugular cannula on the day of the study. Blood samples (0.15 ml) were collected from the femoral cannula pre-dose, and at the following post-dosing times: 5, 15, 30, 45, 60, 90, 120, 180, 240, 360, and 480 min. Following collection, blood samples were transferred to chilled heparinized tubes, and left to stand on ice until centrifugation at 6500 rpm for 10 min at 4 °C. The plasma was transferred to pre-labelled 0.5 ml polypropylene tubes and stored at −20 °C until analysis.

Prior to commencing the assay, plasma samples were thawed then vortex-mixed, and centrifuged at 6000 rpm for 2 min. Aliquots (50 μl) of each plasma sample were transferred to 1.5 ml polypropylene tubes. The internal standard solution (50 μl) and deionized water (50 μl) were subsequently added. The samples were then prepared for analysis as described in Section 2.4. Samples containing analyte concentrations that exceeded the upper limit of the assay standard curve were re-analyzed following appropriate dilution with pooled blank rat plasma. The concentration of OXY (C_0) at time 0 min was estimated by linear regression from the natural log transformed values for the concentrations of OXY between 5 and 45 min. Values of C_{max} for NOR and OXM and the times when these values occurred (T_{max}) were derived by visual inspection of the data.

3. Results and discussion

In previous HPLC–ESI–MS-MS methods used by our group for the quantification of OXY, NOR, and OXM in small volumes

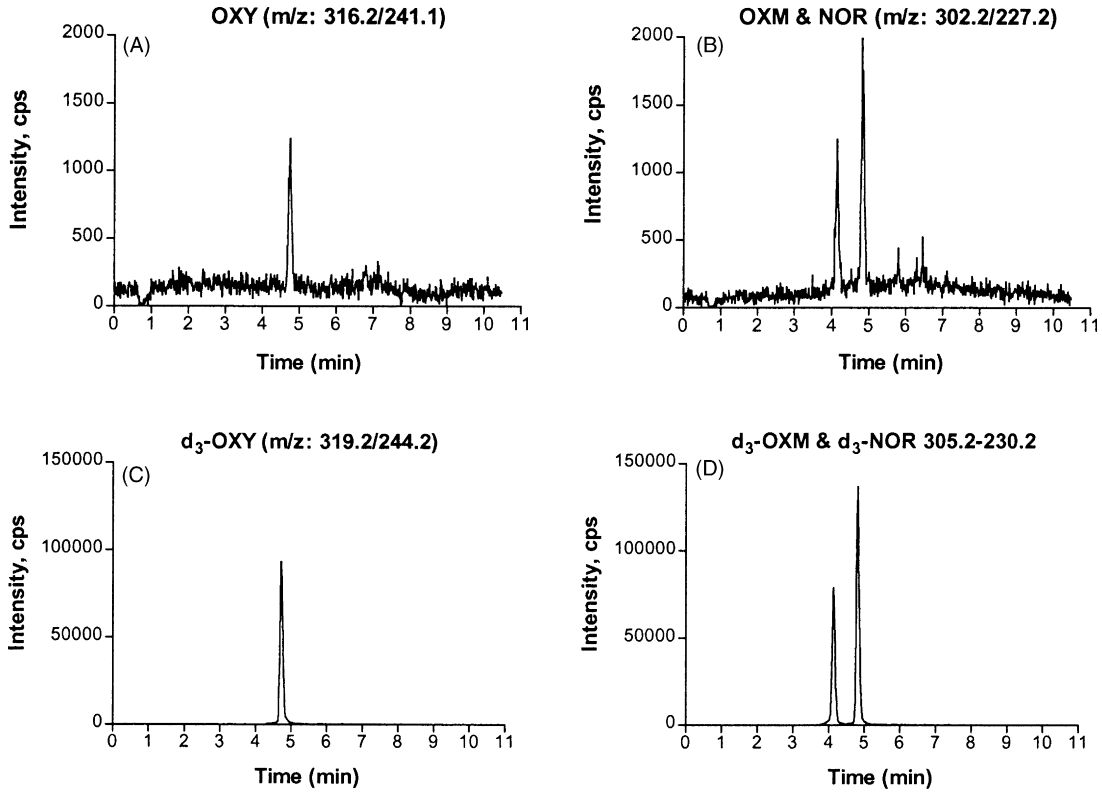

Fig. 2. Representative chromatograms from an assay calibration standard at the LLOQ prepared with assay calibration standard solution (0.9 ng/ml OXY, 1.0 ng/ml OXM, and 1.0 ng/ml NOR), blank pooled rat plasma, and internal standard solution, as described in Section 2.4. (A) OXY (4.8 min); (B) OXM (4.2 min), and NOR (4.8 min); (C) d_3-OXY (4.7 min); (D) d_3-OXM (4.1 min) and d_3-NOR (4.8 min). Retention times are shown in parentheses.

of rat plasma, a standard nebulizer ion-spray source was used that required the column effluent to be split, so that only 10% of the effluent was introduced into the ion-spray source [9,10]. The use of a turbo ion-spray source in the method reported herein enabled the on-column injection volume used for sample analysis to be reduced to 10 μl, whereas previously an on-column injection volume of 50 μl was used [9,10]. The on-column LOD for OXY, NOR, and OXM, in the method reported herein was 1 pg at a signal-to-noise ratio of between 3–4 and 1, while the LLOQs for OXY, NOR, and OXM were 0.9, 1.0, and 1.0 ng/ml, respectively. Our previously reported LLOQs for OXY, NOR, and OXM were 1.8, 2.2, and 2.3 ng/ml, respectively [10].

Representative chromatograms are shown for assay calibration standards that were prepared to contain the following: the assay calibration standard solution at the LLOQ (0.9 ng/ml OXY, 1.0 ng/ml NOR, and 1.0 ng/ml OXM) plus internal standard solution added to blank pooled rat plasma (Fig. 2), internal standard solution added to blank pooled rat plasma and deionized water (Fig. 3), and blank pooled rat plasma and deionized water only (Fig. 4). The recoveries for OXY, NOR, and OXM are shown in Table 1. Mean recoveries of OXY and NOR were higher than those of OXM following protein precipitation.

Accuracy, precision, and linearity data from the assay calibration standard curves for OXY, NOR, and OXM in the validation study are shown in Table 2. For each analyte, values of %Dev and %CV were <7% for all points on the assay calibration standard curves, and the standard curves were highly linear ($R^2 > 0.991$). Accuracy and precision data for OXY, NOR, and OXM from the assay QC samples are shown in Table 3. Between-day and within-day %Dev and %CV were within the following values, respectively, for each analyte: OXY, 6.6 and 7.8%; NOR, 6.8 and 6.6%; OXM, 7.7 and 9.5%. Stability data shown in Table 4 for QC samples kept at room temperature in the autosampler demonstrate that each analyte was stable under these conditions over a 12 h time period.

Plasma OXY, NOR, and OXM concentrations in a single male Sprague–Dawley rat administered a bolus 5 mg/kg i.v. dose of OXY are shown in Fig. 5. The estimated value of C_0 for OXY was 2104.9 ng/ml; respective values of C_{max} (and T_{max}) for NOR and OXM were 142.5 ng/ml (30 min), and 16.6 ng/ml (15 min). Thus, consistent with previous findings [2,3], NOR was the major oxidative metabolite of OXY. In a previously reported study by our group, plasma OXM concentrations in laboratory rodents were found to be <2.1 ng/ml after s.c. administration of a 2 mg/kg bolus dose of OXY [10]. However, administration of a 5 mg/kg bolus dose of OXY via the i.v. route resulted in mea-

Table 1
Analyte recovery following protein precipitation with acetonitrile

OXY (ng/ml)	4.5	44.9	449.0
% Recovery	85.0 ± 2.2	85.7 ± 2.0	96.9 ± 1.9
NOR (ng/ml)	5.0	50.0	500.4
% Recovery	81.0 ± 2.3	84.4 ± 0.7	91.4 ± 1.1
OXM (ng/ml)	5.1	51.2	511.5
% Recovery	69.1 ± 3.4	74.5 ± 1.9	83.6 ± 2.7

Data are shown as the mean ± S.D. ($n = 3$).

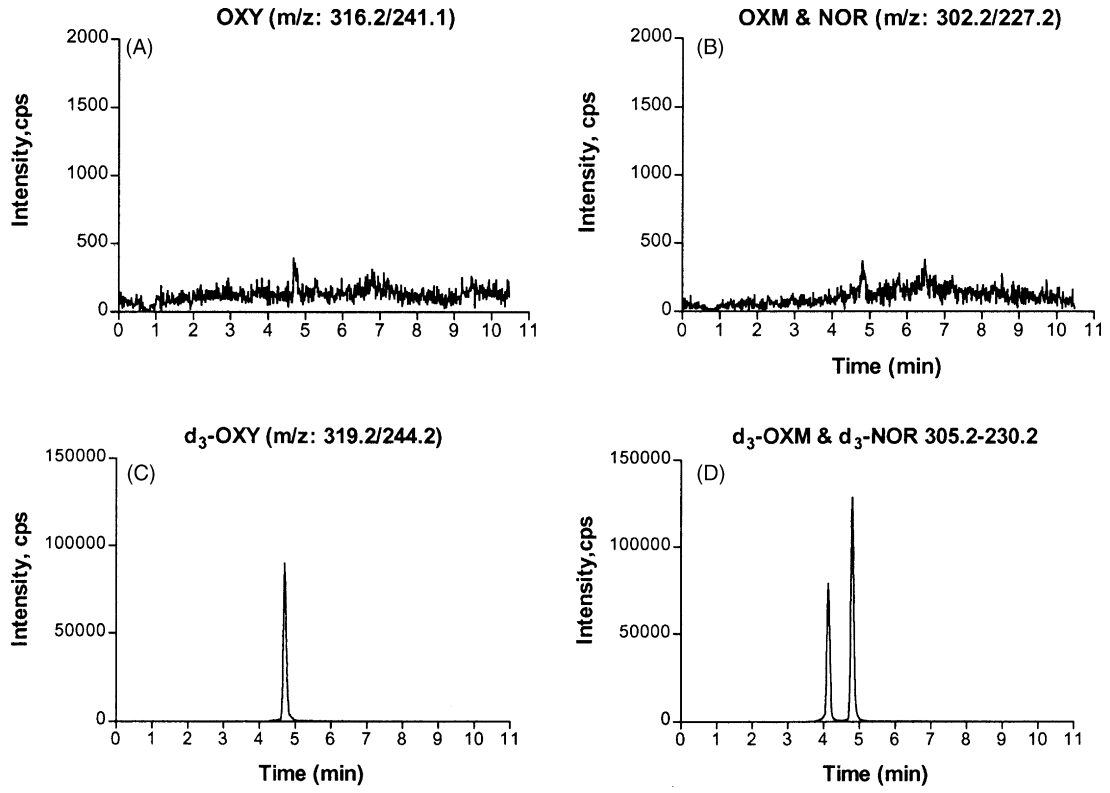

Fig. 3. Representative chromatograms from an assay calibration standard prepared with blank pooled rat plasma and internal standard solution, as described in Section 2.4. (A) OXY; (B) OXM and NOR; (C) d_3-OXY (4.7 min); (D) d_3-OXM (4.1 min), and d_3-NOR (4.8 min). Retention times are shown in parentheses.

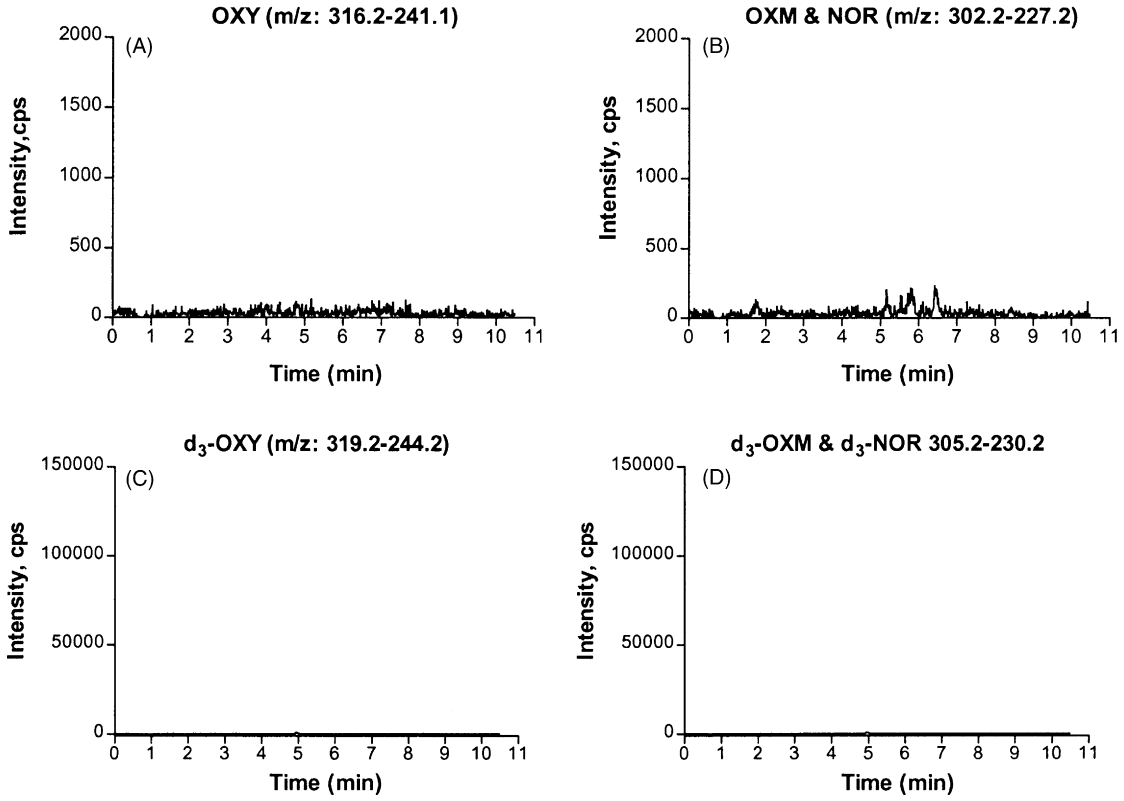

Fig. 4. Representative chromatograms from a blank assay calibration standard prepared with only blank pooled rat plasma and deionized water, as described in Section 2.4. (A) OXY; (B) OXM and NOR; (C) d_3-OXY; (D) d_3-OXM and d_3-NOR.

Table 2
Accuracy, precision, and linearity data for inverse predicted values of the assay calibration standards from the standard curves ($n = 4$) in the assay validation studies

	OXY (ng/ml)									Slope	*y*-Intercept
	0.9	1.8	4.5	9.0	17.9	44.9	89.8	179.6	449.0		
Mean	0.9	1.9	4.6	9.3	18.9	46.0	90.2	179.6	421.5	0.0104	0.00266
%Dev	−0.7	4.0	2.1	3.7	5.6	2.5	0.4	0.0	−6.1		
%CV	5.4	5.2	5.3	3.4	1.5	1.2	4.2	4.3	3.8	3.2	28.2
	NOR (ng/ml)									Slope	*y*-Intercept
	1.0	2.0	5.0	10.0	20.0	50.0	100.1	200.2	500.4		
Mean	1.0	2.0	5.0	10.2	20.2	50.9	100.7	198.2	472.4	0.0100	0.00295
%Dev	−1.5	2.4	0.5	1.9	1.2	1.7	0.6	−1.0	−5.6		
%CV	2.1	4.3	2.4	1.8	2.9	1.5	2.4	1.2	1.2	2.0	13.9
	OXM (ng/ml)									Slope	*y*-Intercept
	1.0	2.1	5.1	10.2	20.5	51.2	102.3	204.6	511.5		
Mean	1.0	2.0	5.2	10.4	20.6	52.6	102.7	206.8	493.9	0.0107	0.00176
%Dev	−1.3	−6.1	2.1	1.9	0.7	2.6	0.4	1.1	−3.4		
%CV	6.8	4.4	2.1	2.8	3.7	1.4	0.8	1.0	1.7	1.3	28.0

Table 3
Between-day ($n = 3 \times 3$) and within-day ($n = 9$) accuracy and precision data for OXY, NOR, and OXM derived from QC samples in the validation study

Analyte	Between-day			Within-day		
	Mean	%Dev	%CV	Mean	%Dev	%CV
OXY (ng/ml)						
0.9	0.9	−0.2	7.8	0.9	3.2	6.6
44.9	45.7	1.7	5.0	45.7	1.7	2.0
449.0	419.2	−6.6	5.6	422.4	−5.9	2.3
NOR (ng/ml)						
1.0	1.0	0.6	6.2	1.0	−2.2	6.6
50.0	51.0	2.1	1.4	50.5	1.1	1.1
500.4	466.3	−6.7	1.6	466.4	−6.8	0.6
OXM (ng/ml)						
1.0	1.0	−1.8	9.5	1.1	7.7	4.0
51.2	52.0	1.5	2.2	51.5	0.5	1.3
511.5	492.2	−3.8	1.9	486.0	−5.0	1.0

Table 4
Stability data for QC samples kept at room temperature in the autosampler over a 12 h assay period

	Initial	Final
OXY nominal concentration (ng/ml)		
0.9	0.9 ± 0.1	0.9 ± 0.1
44.9	44.8 ± 2.0	44.8 ± 1.7
449.0	417.4 ± 6.3	411.5 ± 13.5
NOR nominal concentration (ng/ml)		
1.0	0.9 ± 0.1	1.0 ± 0.1
50.0	50.7 ± 1.1	50.4 ± 1.0
500.4	476.5 ± 5.6	465.1 ± 4.9
OXM nominal concentration (ng/ml)		
1.0	0.9 ± 0.1	1.0 ± 0.1
51.2	51.8 ± 0.3	51.8 ± 0.9
511.5	485.2 ± 8.2	483.4 ± 8.8

Data are shown as the mean ± S.D. ($n = 6$). Initial *vs.* final: not significantly different (Mann–Whitney test).

surable OXM concentrations in plasma of 1.6–16.6 ng/ml, using the method reported herein.

Earlier studies of oxycodone's pharmacokinetics and pharmacodynamics in healthy human volunteers used gas chromatography–mass spectrometry (GC–MS) and negative chemical ionization to quantify OXY, NOR, and OXM, with a LLOQ of 0.2 ng/ml for each analyte [4,5]. This procedure required solid phase extraction (SPE) and subsequent derivatization prior to sample analysis by GC–MS [4]. Although this method achieved high levels of sensitivity, lengthy sample preparation procedures were required prior to analysis. The HPLC–ESI–MS method reported herein employs a small sample volume, and uses a simpler sample preparation procedure. In addition to the application of our method to the analysis of small volumes of rat plasma following sequential sampling in pharmacokinetic studies, the method is also ideally suited to the analysis of plasma samples from pediatric patients, as well as other patients where larger sample volumes are not available.

The use of HPLC and ESI single quadropole MS (HPLC–ESI–MS) for the analysis of OXY and a number of

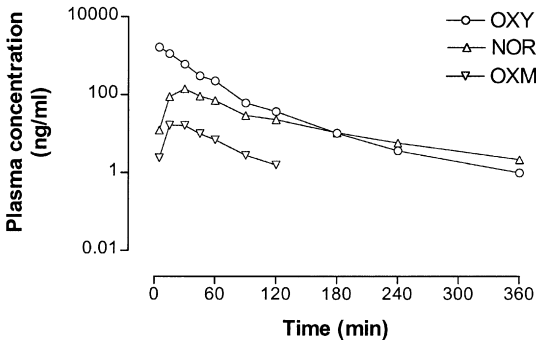

Fig. 5. Plasma concentrations of OXY, NOR, and OXM in a single male Sprague–Dawley rat, following i.v. administration of a 5 mg/kg bolus dose of OXY.

its major and minor metabolites in plasma and urine has also recently been reported [3]. In this method, SPE employing a C2/C18 mixed resin sorbent was required for sample preparation prior to analysis, and the run time for the analysis was 28 min with retention times for OXY, NOR, and OXM, of 18, 16, and 6 min, respectively [3]. In the method reported herein, the simpler procedure of protein precipitation with acetonitrile, followed by solvent evaporation and reconstitution in mobile phase, was successfully used for sample preparation prior to analysis. Using the gradient program reported herein, OXM and NOR which share the same mass transition (*m/z*: 302.2/227.2) were successfully separated with respective retention times of 4.2 and 4.8 min, using a run time of only 10.5 min. It is therefore likely that the method reported herein could be easily adapted for the quantitation of OXY's other oxidative and reductive metabolites as well, by incorporation of the appropriate standards and selective mass transitions into the assay.

A method for the analysis of OXY, NOR, and OXM in Ringer's solution, rat plasma, and rat brain tissue by HPLC–ESI–MS-MS in concert with column switching has also recently been reported, with a LLOQ of 0.5 ng/ml for each analyte [11]. Ringer's solution samples were successfully analyzed following 1 in 2 dilution by direct injection, using an on-column injection volume of 16 μl. However, poor peak shape was reported for noroxycodone when plasma samples were prepared for analysis by protein precipitation with acetonitrile, and a C18 SPE procedure was subsequently used for sample preparation. Moreover, the volume of rat plasma required for that method was 100 μl, and the on-column injection volume used was 30 μl following reconstitution into 100 μl of mobile phase [11].

LLOQs of 50 ng/ml were reported for OXY and OXM in an HPLC–ESI–MS-MS method for the analysis of opioids in urine by direct injection, after the initial preparation of urine samples by centrifugation to remove particulate matter [12]. An HPLC–MS-MS screening method for opioids in plasma has also been recently reported with LLOQs of 5.3 and 2.9 ng/ml, respectively, for OXY and OXM, following extraction from 1 ml of plasma by an automated SPE extraction procedure [13].

In the method reported herein, excellent peak shape was maintained for OXY, NOR, and OXM over the course of the validation study, when protein precipitation with acetonitrile was used for sample preparation prior to analysis. The initial aqueous component prior to the linear gradient in the gradient program described herein allows OXY, NOR, and OXM to be retained on the HPLC column, and separated from more polar compounds prior to entry of the analytes into the ion-spray source. Additionally, the linear increase to maximum organic modifier content in the mobile phase minimizes the build up of lipophilic compounds on the column that may result in deterioration of peak shape and the production of ion suppression. Furthermore, use of a small plasma volume (50 μl) and a small on-column injection volume (10 μl) has the additional advantage of minimizing the amount of salts and non-volatile material entering the MS, and thereby avoids the necessity of using column switching to regulate the entry of column effluent into the MS. Thus, the high selectivity and sensitivity of HPLC–ESI–MS-MS enables small volumes of plasma to be prepared by a simple work-up procedure for low-level quantitation with high accuracy and precision.

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