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# Simultaneous determination of morphine, oxycodone, morphine-3-glucuronide, and noroxycodone concentrations in rat serum by high performance liquid chromatography–electrospray ionization–tandem mass spectrometry☆

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**Abstract**

An assay using high performance liquid chromatography (HPLC)–electrospray ionization–tandem mass spectrometry (ESI–MS–MS) was developed for simultaneously determining concentrations of morphine, oxycodone, morphine-3-glucuronide, and noroxycodone, in 50 μl samples of rat serum. Deuterated ($d_3$) analogues of each compound were used as internal standards. Samples were treated with acetonitrile to precipitate plasma proteins; acetonitrile was removed from the supernatant by centrifugal evaporation before analysis. Limits of quantitation (ng/ml) and their between-day accuracy and precision (%deviation and %CV) were—morphine, 3.8 (4.3% and 7.6%); morphine-3-glucuronide, 5.0 (4.5% and 2.9%); oxycodone, 4.5 (0.4% and 9.3%); noroxycodone, 5.0 (8.5% and 4.6%).


*Keywords:* Morphine; Morphine-3-glucuronide; Oxycodone; Noroxycodone; High performance liquid chromatography; Electrospray ionization; Tandem mass spectrometry

## 1. Introduction

In comparative studies, the analgesic potency of oxycodone has been shown to be ∼1.5 times higher than that of morphine, following intravenous (i.v.) administration for the management of postoperative pain [1], and also after oral administration for the management of chronic cancer-related pain [2,3]. Subsequent behavioural studies by our group in laboratory rodents have shown that the antinociceptive effects of oxycodone appear to be mediated by putative κ-opioid receptors, whereas morphine acts via μ-opioid receptors [4]. Additionally, in other work by our group co-administration of sub-analgesic doses of morphine (MOR) and oxycodone (OXY), via either intracerebroventricular (i.c.v.), intraperitoneal (i.p.), or subcutaneous routes (s.c.), was found to produce marked antinociceptive synergy in the rat [5]. To examine the possible contribution of pharmacokinetic effects to this synergistic interaction between MOR and OXY in rats, it is necessary to be able to quantify these two opioids in serum. Previously, our group has used separate assays, employing solid-phase extraction (SPE) followed by quantitation by high performance liquid chromatography (HPLC) with electrochemical detection, for the analysis of MOR, morphine-3-glucuronide (M3G) [6] and OXY [7] concentrations in plasma.

A number of methods employing HPLC combined with electrospray ionization–tandem mass spectrometry (HPLC–ESI–MS–MS) for low level quantitation of MOR and its glucuronide metabolites have recently been described [8–12]. These methods have employed SPE to co-extract both MOR and its hydrophilic glucuronide metabolites from

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aliquots of plasma or serum prior to their analysis. Analysis of OXY in plasma by HPLC–ESI–MS–MS following liquid–liquid extraction has also recently been reported [13]. Here, we document an HPLC–ESI–MS–MS procedure for the simultaneous quantitation of MOR and OXY, in addition to their respective primary metabolites M3G and noroxycodone (NOR), in rat serum, to enable pharmacokinetic investigations of combined oxycodone and morphine administration in rats. Deuterated ($d_3$) analogues of each analyte were used as internal standards. Rather than employing SPE, samples were deproteinated by the addition of acetonitrile, and the acetonitrile was subsequently removed in an evaporative centrifuge, prior to analysis by HPLC–ESI–MS–MS.

## 2. Experimental

### 2.1. Drugs and reagents

MOR hydrochloride BP (Macfarlan Smith Ltd, Edinburgh, UK), M3G (Sigma-Aldrich, Sydney, NSW, Australia), OXY hydrochloride USP (Macfarlan Smith Ltd), and NOR (Du Pont Merck, Wilmington, DE, USA) were used for the preparation of standard solutions. Deuterated ($d_3$) analogues of MOR, OXY, M3G, and NOR (Cambridge Isotope Laboratories, Andover, MS, USA) were used as internal standards. HPLC grade acetonitrile, methanol, formic acid, and hydrochloric acid (AR grade, BDH Chemicals) were obtained from local suppliers.

### 2.2. HPLC–ESI–MS–MS instrumentation and analytical procedures

The LC system configuration for delivery of mobile phase and sample injection consisted of dual Shimadzu LC-10AT pumps, with a SCL-10A system controller, and an Agilent series 1100 autoinjector. Sample analysis was performed on a PE Sciex API 3000 triple quadrupole mass spectrometer, operating in electrospray mode, with a standard nebulizer ionspray source.

Chromatographic separations were performed on an Agilent Zorbax SB-C18 (5 μm, 2.1 mm i.d. × 50 mm) column, with Phenomenex C18 (4.0 mm × 2.0 mm) security-guard cartridges acting as the pre-column, using a sample injection volume of 50 μl. The mobile phase comprised two components, and was delivered in a stepwise gradient fashion at a flow rate of 0.15 ml/min. Component A was a 0.1% (v/v) solution of formic acid, while component B consisted of 90% (v/v) methanol:water containing 0.1% (v/v) formic acid. The gradient run time was 8.1 min (component A for 0.5 min; component B:A (11:89) for 1.6 min; component B:A (50:50) for 2.6 min; component A for 3.4 min). There was an additional 2 min period until the next injection; thus the re-equilibration interval was 5.4 min. The LC effluent was split and 10% introduced into the MS system.

Signal sensitivity was optimized by infusing a solution containing MOR hydrochloride, M3G, OXY hydrochloride, and NOR at concentrations of ∼1 μg/ml, in mobile phase components B:A (11:89), directly into the electrospray source at 20 μl/min using a Harvard 11 syringe pump. Running conditions were optimal when the nebulizer gas ($N_2$) flow rate was 10 l/min, and the ion spray source voltage was 4.6 kV, while orifice and ring voltages were set at 60 V and 200 V, respectively. The collision cell energy was 50 V, and $N_2$ was used as the collision gas. The following ion transitions for each compound and its deuterated analogue were monitored with a dwell time of 200 ms: MOR, 286.1/165.1; $d_3$-MOR, 289.2/165.1; M3G, 462.2/286.1; $d_3$-M3G, 465.3/289.2; OXY, 316.2/241.1; $d_3$-OXY, 319.2/244.2; NOR, 302.2/227.2; $d_3$-NOR, 305.2/230.2. The limits of detection were investigated by on-column injections of known amounts of each analyte dissolved in a solvent mixture comprising mobile phase components B:A (11:89).

Chromatograms were integrated using PE Sciex software (Mac Quan version 1.6). Weighted linear regression ($1/x^2$) was used to generate standard curves ($y = mx + b$), which related the analyte's concentration ($x$) to the peak area ratio ($y$) of the analyte and its respective internal standard. Inverse predicted values for sample analyte concentrations were subsequently determined from the ratio of the peak areas for the analyte and its deuterated analogue, using the values for slope ($m$) and $y$-intercept ($b$) that were derived from the analytes standard curve.

### 2.3. Preparation of assay calibration standard solutions and assay quality control standard solutions

All solutions and serial dilutions were prepared using 18.0 MΩ de-ionized water. Stock assay calibration standard solutions and quality control (QC) standard solutions of MOR hydrochloride, M3G, OXY hydrochloride, and NOR were prepared in cetrimide-treated volumetric flasks and stored at 4 °C. MOR hydrochloride, OXY hydrochloride, and M3G were dissolved in de-ionized water, while NOR was dissolved in 0.1 M HCl. Serial dilutions of each stock assay calibration standard solution and QC standard solution were prepared in 1.5 ml polypropylene tubes, on the day the assay was performed, to produce working stock assay calibration standard solutions and working stock assay QC standard solutions that contained MOR hydrochloride, M3G, OXY hydrochloride, and NOR at concentrations of ∼1000 ng/ml. Serial dilutions were subsequently prepared to produce the working assay calibration standard solutions, required for an eight-point standard curve, that contained the following range of concentrations for each analyte: MOR 3.8–760.0 ng/ml, M3G 5.0–1005 ng/ml, OXY 4.5–897.1 ng/ml, and NOR 5.0–1001 ng/ml (see Table 1 for individual standard concentrations). In an analogous manner, the working assay QC standard solutions were prepared to contain concentrations of each analyte at the lower, middle, and upper limits of

Table 1
Accuracy and precision data for inverse predicted values of the assay calibration standards from standard curves ($n = 4$) in the assay validation studies

| | MOR (ng/ml) | | | | | | | | Slope | $R^2$ |
|---|---|---|---|---|---|---|---|---|---|---|
| | 3.8 | 7.6 | 15.2 | 38.0 | 76.0 | 152.0 | 379.9 | 760.0 | | |
| Mean | 3.77 | 7.79 | 14.95 | 37.14 | 78.29 | 153.70 | 377.18 | 750.21 | 0.0047 | 0.9999 |
| %Dev | 0.81 | −2.56 | 1.63 | 2.25 | −3.02 | −1.12 | 0.72 | 1.29 | | |
| %CV | 1.90 | 4.80 | 2.50 | 2.72 | 1.20 | 2.08 | 0.50 | 0.89 | 1.32 | 0.0067 |
| | M3G (ng/ml) | | | | | | | | | |
| | 5.0 | 10.1 | 20.1 | 50.3 | 100.5 | 201.0 | 502.5 | 1005 | | |
| Mean | 4.94 | 10.23 | 20.43 | 50.56 | 100.27 | 201.18 | 503.95 | 976.41 | 0.0036 | 0.9997 |
| %Dev | 1.10 | −1.32 | −1.66 | −0.51 | 0.23 | −0.40 | −0.29 | 2.84 | | |
| %CV | 1.40 | 1.76 | 3.01 | 1.40 | 1.63 | 0.99 | 0.46 | 2.38 | 1.48 | 0.041 |
| | OXY (ng/ml) | | | | | | | | | |
| | 4.5 | 9.0 | 17.9 | 44.9 | 89.7 | 179.4 | 448.5 | 897.1 | | |
| Mean | 4.57 | 8.59 | 18.16 | 46.03 | 90.08 | 179.98 | 447.25 | 883.35 | 0.0054 | 0.9998 |
| %Dev | −1.63 | 4.53 | −1.45 | −2.52 | −0.42 | −0.32 | 0.28 | 1.53 | | |
| %CV | 0.77 | 1.15 | 3.35 | 1.63 | 1.87 | 1.39 | 2.34 | 1.45 | 1.35 | 0.0164 |
| | NOR (ng/ml) | | | | | | | | | |
| | 5.0 | 10.0 | 20.0 | 50.1 | 100.1 | 200.2 | 500.5 | 1001 | | |
| Mean | 4.96 | 10.14 | 20.10 | 49.96 | 101.28 | 203.53 | 497.33 | 971.62 | 0.0045 | 0.9998 |
| %Dev | 0.86 | −1.37 | −0.49 | 0.27 | −1.18 | −1.67 | 0.63 | 2.94 | | |
| %CV | 1.51 | 3.31 | 1.28 | 2.41 | 0.94 | 1.22 | 1.19 | 0.85 | 1.03 | 0.0084 |

Linearity data for the standard curves of each analyte are also shown.

quantitation (see Table 2 for individual standard concentrations).

The stock internal standard solution was prepared in a 15 ml polypropylene tube to contain $d_3$-MOR, $d_3$-M3G, $d_3$-OXY, and $d_3$-NOR each at concentrations of 1000 ng/ml. The working internal standard solution was subsequently prepared in a 15 ml polypropylene tube to contain 200 ng/ml concentrations of each deuterated analogue. Both stock and working internal standard solutions were stored at 4 °C.

Table 2
Between-day ($n = 3 \times 3$) and within-day ($n = 9$) accuracy and precision data for MOR, M3G, OXY, and NOR from the assay QC standards in the validation procedure

| Analyte | Between-day | | | Within-day | | |
|---|---|---|---|---|---|---|
| | Mean | %Dev | %CV | Mean | %Dev | %CV |
| MOR (ng/ml) | | | | | | |
| 3.8 | 4.0 | 4.3 | 7.6 | 3.6 | −4.2 | 6.4 |
| 76.0 | 77.0 | 1.3 | 2.6 | 77.8 | 2.4 | 2.0 |
| 760.0 | 757.8 | −0.3 | 2.3 | 766.1 | 0.8 | 2.7 |
| M3G (ng/ml) | | | | | | |
| 5.0 | 5.2 | 4.5 | 2.9 | 5.2 | 4.4 | 5.8 |
| 100.7 | 101.5 | 0.0 | 1.4 | 104.2 | 3.5 | 1.7 |
| 1007 | 998.5 | 1.8 | 1.9 | 985.9 | −2.1 | 3.1 |
| OXY (ng/ml) | | | | | | |
| 4.5 | 4.5 | 0.4 | 9.3 | 4.7 | −10.0 | 4.9 |
| 89.7 | 90.1 | 0.5 | 1.4 | 92.4 | 3.0 | 1.4 |
| 897.1 | 883.4 | −1.5 | 1.5 | 893.7 | −0.4 | 4.9 |
| NOR (ng/ml) | | | | | | |
| 5.0 | 5.4 | 8.5 | 4.6 | 5.7 | 13.2 | 3.3 |
| 100.0 | 102.1 | 2.1 | 1.7 | 102.3 | 2.3 | 1.7 |
| 1000 | 987.2 | −1.3 | 1.8 | 982.0 | −1.8 | 1.9 |

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

Aliquots (50 μl) of each working assay calibration standard solution and each working assay QC standard solution were transferred to 1.5 ml polypropylene tubes, prior to the addition of working internal standard solution (50 μl) and blank rat serum (50 μl). The tubes were then briefly vortex-mixed. Acetonitrile (300 μl) was subsequently added to each tube, and the tubes were then briefly vortex-mixed again before being left to stand for 30 min at 4 °C, to facilitate protein precipitation and flocculation. After 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 the volume was reduced to ∼90 μl. Formic acid (1%, v/v, 10 μl) was subsequently added to each tube, and the tubes were then briefly vortex-mixed, before centrifugation again at 14,000 rpm for 5 min. The supernatants were then transferred to 250 μl polypropylene inserts for analysis.

### 2.5. Preparation and analysis of stability standards

A working stock QC standard solution was prepared in a 1.5 ml polypropylene tube, as described in Section 2.3. Serial dilutions were subsequently prepared, and 0.1 ml of the appropriate dilution was added to 0.9 ml of blank rat serum to produce each stability standard. The following respective concentrations of MOR, M3G, OXY, and NOR were present in each stability standard: stability standard 1–7.6, 10.1, 9.0, and 10.0 ng/ml; stability standard 2–76.0, 100.7, 89.7, and 100.0; stability standard 3–760.0, 1007, 897.1, 1000 ng/ml. Aliquots (50 μl) of each stability standard were transferred to 1.5 ml polypropylene tubes and stored frozen at −20 °C until analysis. After thawing, individual aliquots of each stability standard were prepared for analysis, as outlined in Section 2.4, following the addition of working internal standard (50 μl) and de-ionized water (50 μl) to each 1.5 ml tube.

### 2.6. Assay validation

Between-day accuracy and precision were determined from three assays that were performed on separate days. Each assay contained eight-assay calibration standards, and the assay QC standards were analysed in triplicate (ie $n = 3 \times 3$). Within-day accuracy and precision were determined from an additional single assay containing eight assay calibration standards, in which nine replicates of each assay QC standard were analysed (i.e. $n = 3 \times 9$). Accuracy was determined as the percent deviation (%Dev) of the mean values for each analyte in the assay QC standards from their nominal concentrations, while precision was determined as the percent coefficient of variation (%CV) for the analyte. A lower limit of quantitation (LLOQ) having values for %Dev and %CV that were ≤20% was considered acceptable, whereas at the middle and upper limits of quantitation values for %Dev and %CV of ≤15% were acceptable.

Analyte stability was examined by assaying triplicate aliquots of each stability standard within the first week of preparation, and after storage for 6 and 12 weeks. In addition, triplicate aliquots of each stability standard were analysed following three successive freeze–thaw cycles, within the first week of storage. Stability standards assayed within the first week of storage and following three successive freeze–thaw cycles were included in the third and fourth assays of the validation procedure for accessing between-day and within-day accuracy and precision. An additional two assays of stability standards following 6 and 12 weeks of storage were subsequently performed.

The measured concentrations of each analyte from the stability standards, assayed following 1, 6, and 12 weeks of storage, and following three successive freeze–thaw cycles within the first week of storage, were subtracted from their nominal values, and the differences between the nominal and measured values were subsequently normalised by conversion to their respective natural log values. The 90% confidence intervals for the differences between the nominal and measured values of the analytes were then derived from the natural log-normalised data. Since the acceptability criterion for assay performance was a %Dev of ≤20% at the lower limit of quantitation, confidence intervals for the differences between the nominal and measured values for each analyte within the range of 0.8–1.2 were considered acceptable.

### 2.7. Application of the assay

Ethical approval for the study was obtained from The University of Queensland's Animal Experimentation Ethics Committee. A combined bolus dose of MOR (25 mg/kg) and OXY (20 mg/kg) was administered by oral gavage to a single rat. Blood samples (0.2 ml) were collected from an indwelling femoral arterial cannula pre-dose and at the following post-dosing times: 5, 15, 30, 45, 60, 90, 120, 180, 240, and 360 min. Following collection, the samples were placed into a small esky containing ice. Once the final sample had been taken, the samples were centrifuged in a refrigerated centrifuge (4 °C) at 4000 × g for 20 min. The resulting serum samples were subsequently transferred into 1.5 ml polypropylene tubes and stored frozen at −20 °C until analysis. On the day of analysis, serum samples were initially thawed and vortex-mixed. Aliquots (50 μl) were then transferred to 1.5 ml polypropylene tubes prior to the addition of internal standard (50 μl) and de-ionized water (50 μl). The samples were then prepared for analysis as outlined in Section 2.4. Samples containing analyte concentrations that exceeded the upper limit of quantitation were re-analysed using an appropriate dilution with blank rat serum. The maximum serum concentrations ($C_{max}$) and the time at which $C_{max}$ was achieved ($T_{max}$) were derived by visual inspection of the data.

## 3. Results and discussion

Representative chromatograms for MOR, $d_3$-MOR, M3G, $d_3$-M3G, OXY, $d_3$-OXY, NOR, and $d_3$-NOR derived from blank serum and an assay calibration standard at the lower limit of quantitation are shown in Figs. 1 and 2, respectively. Typical retention times for each analyte under the conditions described herein were 4.1, 4.5, 5.4, and 5.4 min for M3G, MOR, OXY, and NOR, respectively. Since a small amount of fragmentation of M3G to MOR at the HPLC-MS interface was found to occur, chromatographic resolution of MOR and M3G was necessary to prevent M3G being falsely detected as MOR. Chromatographic resolution of OXY and NOR was not necessary for accurate quantitation of each analyte, owing to their unique mass transitions. Signal to noise ratios found for the following on-column amounts of each analyte were: MOR 8 pg, 5:1; M3G 10 pg, 29:1; OXY 9 pg, 12:1; NOR 10 pg, 17:1. The following mean recoveries ($n = 4$) were associated with MOR, M3G, OXY, and NOR concentrations from the medium QC: morphine 76.0 ng/ml, 92%; M3G 100.7 ng/ml, 84%; oxycodone 89.7 ng/ml, 95%; noroxycodone 100.0 ng/ml, 100%.

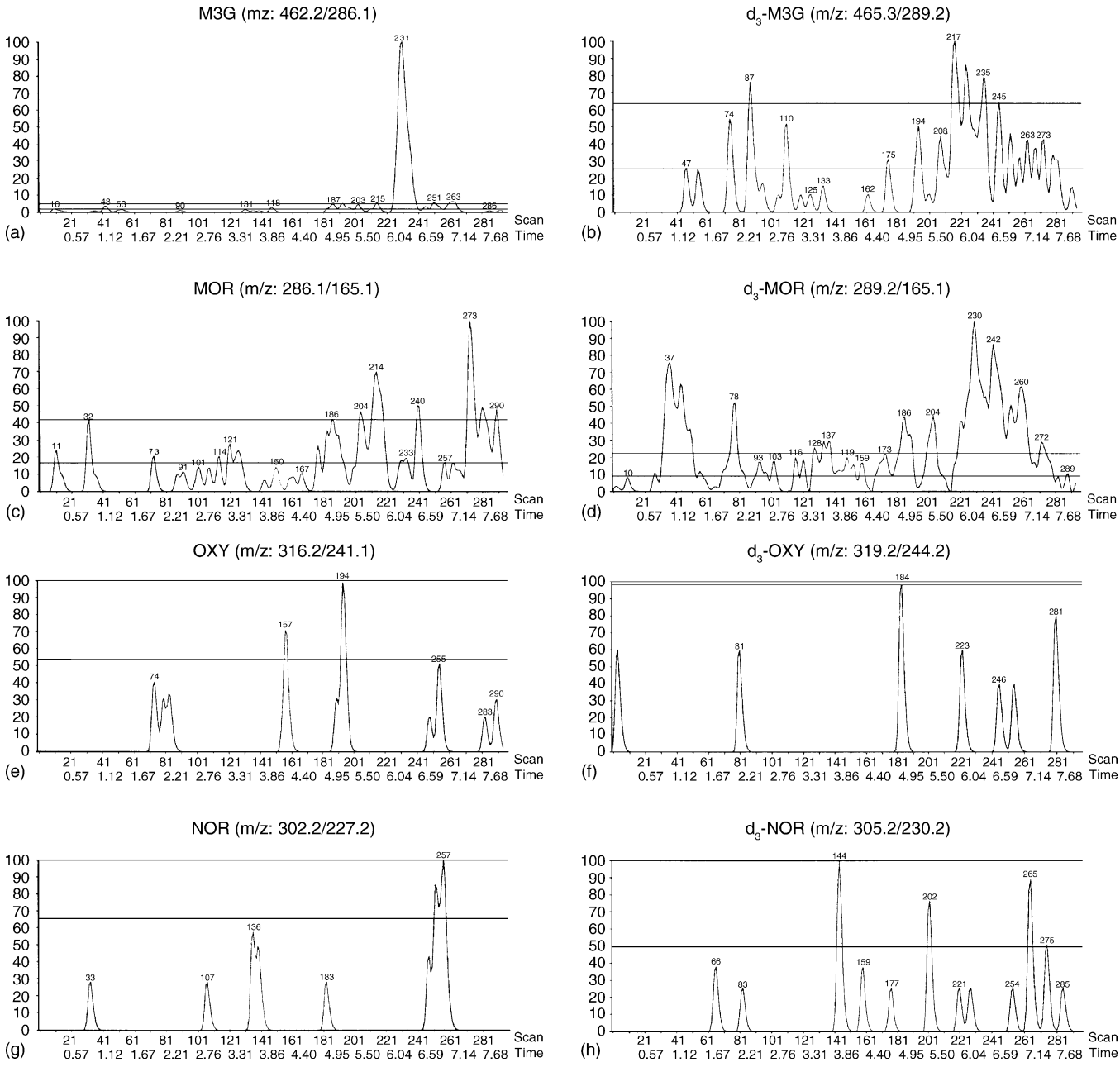


Fig. 1. Representative chromatograms for each ion transition derived from blank rat serum.

Assay calibration standard curve accuracy, precision, and linearity data for each analyte are shown in Table 1. The standard curves were highly linear ($R^2 > 0.9996$) over the concentration range of each analyte. For all analytes, values for %Dev and %CV were <2% at the lower limit of quantitation, and <5% for all other points on the analyte assay standard calibration curves. Accuracy and precision data for the assay QC standards are shown in Table 2. Between-day and within-day %Dev and %CV were below the following respective values for each analyte: MOR, <5% and <8%; M3G, <5% and <6%; Oxy, <11% and <10%; NOR, <14% and <9%.

Stability standard data are shown in Table 3. All of the values for confidence limits, derived from the stability standard data, fell within the range of acceptability (0.8–1.2). Additionally, Mann–Whitney-U comparisons found there were no significant differences ($p > 0.05$) between data from stability standards assayed within the first week of storage, and following three successive freeze–thaw cycles within the first week of storage. Hence, the analytes were all stable over the 12-week study period, when stored at −20 °C.

Serum drug and metabolite concentration–time data from the pilot study, in which a single rat was co-administered MOR (25 mg/kg) and OXY (20 mg/kg) by oral gavage, are

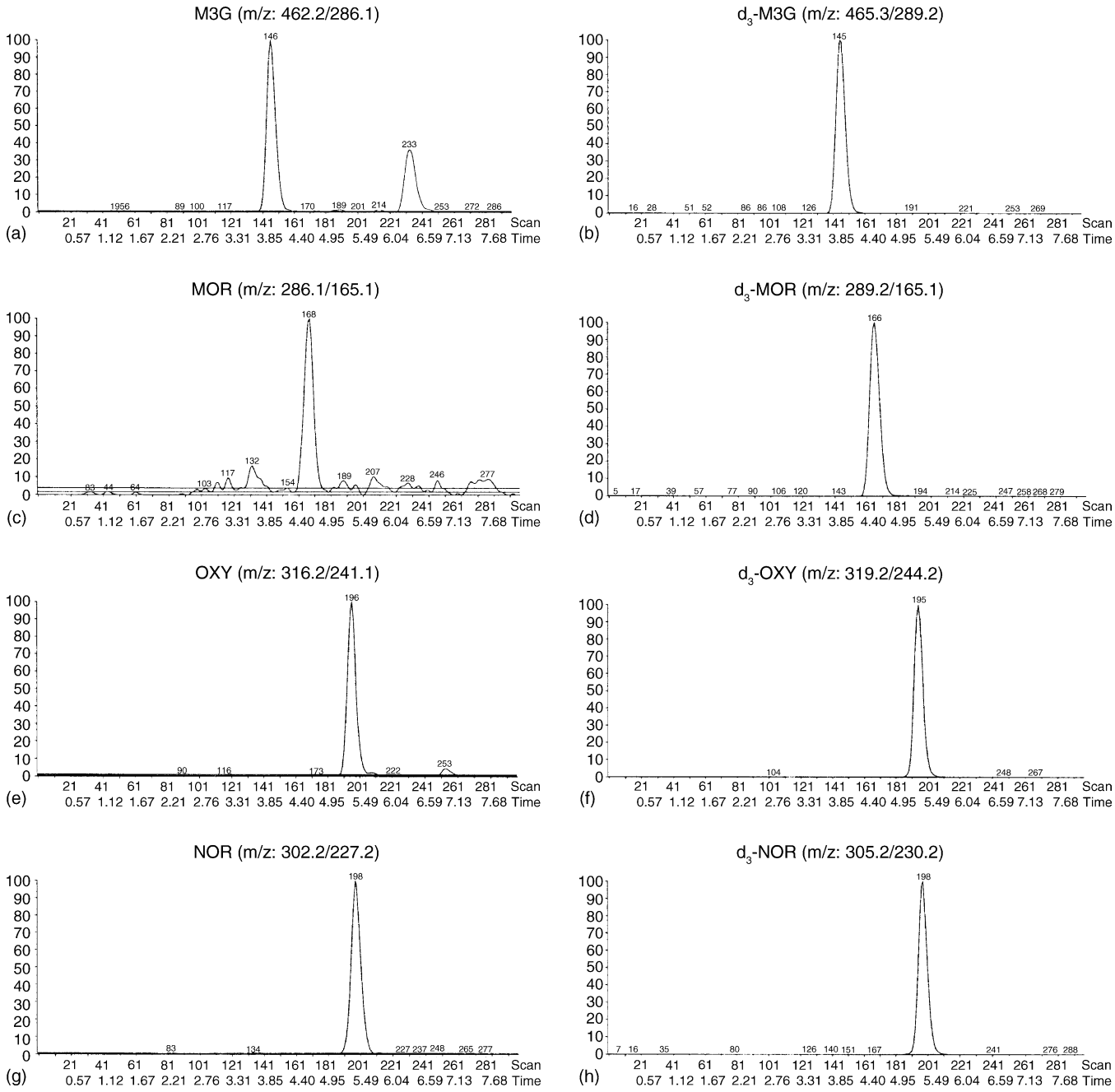


Fig. 2. Representative chromatograms for each ion transition derived from the assay standard at the lower limit of quantitation.

shown in Fig. 3. Values of $C_{max}$ (and $T_{max}$) for MOR and M3G were 321.2 ng/ml (30 min) and 2239 ng/ml (60 min), while $C_{max}$ (and $T_{max}$) for OXY and NOR were 244.4 (30 min) and 448.4 ng/ml (30 min). In other work, values of $C_{max}$ (and $T_{max}$) for MOR and M3G, following administration of morphine (4 mg/kg, i.p.), were 302.1 ng/ml (8 min) and 1341 ng/ml (28 min) [8]. Currently, there is very little information available on the pharmacokinetics of oxycodone in rats.

Previously reported HPLC–ESI–MS–MS methods for the analysis of morphine and its glucuronide metabolites have generally employed SPE on C2 [8] or C18 [9–11] sorbents, whereas the method described herein simply used deproteination with acetonitrile for sample workup prior to analysis. A highly automated procedure for sample preparation and transfer, to use in concert with a 96 well plate C18 SPE format, has also recently been developed, and applied to the analysis of MOR and its glucuronide metabolites by HPLC–ESI–MS–MS [12]. Although this procedure allows a fast turnaround time and increases sample throughput, a Packard MultiprobeTM II robotic liquid handler is required for the preparation and transfer of samples during the extrac-

Table 3
Mean (±S.D.) concentrations and the 90% confidence intervals (90% CI) for MOR, M3G, OXY, and NOR following analysis of stability standards

| | Week 1 | Week 6 | Week 12 | Three freeze–thaw cycles |
|---|---|---|---|---|
| MOR (ng/ml) | | | | |
| 7.6 | 7.7 ± 0.2 | 7.9 ± 0.3 | 7.6[a] | 7.4 ± 02 |
| 76.0 | 76.6 ± 0.5 | 76.7 ± 2.5 | 71.5 ± 1.8 | 79.8 ± 1.4 |
| 760.0 | 752.7 ± 10.7 | 745.2 ± 45.7 | 696.2 ± 16.1 | 766.0 ± 18.4 |
| 90% CI | 0.98–1.01 | 0.97–1.02 | 1.02–1.09 | 0.96–1.01 |
| M3G (ng/ml) | | | | |
| 10.1 | 10.3 ± 0.3 | 10.1 ± 0.1 | 10.2 ± 0.2 | 10.3 ± 0.2 |
| 100.7 | 102.9 ± 2.3 | 101.8 ± 2.1 | 103.2 ± 0.3 | 105.7 ± 1.4 |
| 1007 | 984.4 ± 20.9 | 968.0 ± 32.2 | 982.9 ± 25.2 | 970.8 ± 10.3 |
| 90% CI | 0.98–1.01 | 1.00–1.02 | 0.97–1.01 | 0.96–1.01 |
| OXY (ng/ml) | | | | |
| 9.0 | 9.0 ± 0.2 | 8.7 ± 0.1 | 9.4 ± 0.3 | 9.4 ± 0.4 |
| 89.7 | 93.4 ± 0.9 | 88.4 ± 2.0 | 89.9 ± 1.3 | 94.6 ± 2.3 |
| 897.1 | 894.7 ± 12.8 | 857.2 ± 26.2 | 850.8 ± 19.7 | 880.6 ± 8.3 |
| 90% CI | 0.97–1.00 | 1.03–1.06 | 0.96–1.01 | 0.95–1.00 |
| NOR (ng/ml) | | | | |
| 10.0 | 10.2 ± 0.3 | 10.1 ± 0.2 | 9.9 ± 0.1 | 10.5 ± 0.2 |
| 100.0 | 102.2 ± 0.7 | 100.3 ± 2.8 | 98.9 ± 2.5 | 104.5 ± 1.2 |
| 1000 | 978.7 ± 17.0 | 964.8 ± 27.7 | 947.8 ± 4.7 | 973.9 ± 4.0 |
| 90% CI | 0.98–1.01 | 0.99–1.02 | 1.00–1.03 | 0.95–1.00 |

[a] $n = 2$.

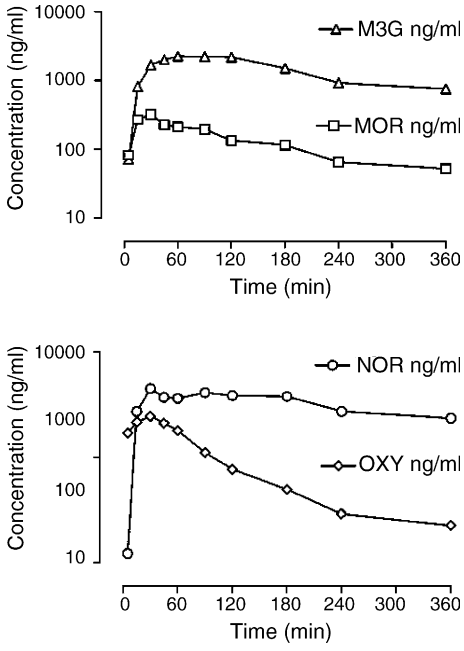


Fig. 3. Serum concentrations of MOR, M3G, OXY, and NOR in a single rat, following the bolus administration of morphine(25 mg/kg) and oxycodone (20 mg/kg) by oral gavage.

tion procedure. The limit of quantitation by this automated procedure was 0.5 ng/ml for MOR and 10 ng/ml for M3G, using a 0.25 ml sample volume.

A number of methods employing HPLC with ESI single quadropole MS (HPLC–ESI–MS) for low level quantitation of MOR and its glucuronide metabolites have also been reported [14–18]. These methods have employed SPE with C2 [15], C18 [14], and Oasis MCX® [16] sorbent types. However, deproteination of small plasma samples (40 μl) with acetonitrile has also been used successfully with HPLC–ESI–MS, and the lower limits of quantitation for MOR and M3G reported in this latter method were 0.7 ng/ml and 2.3 ng/ml, respectively [17].

SPE using Oasis MCX® 96 well plates for the extraction of MOR and its glucuronide metabolites, prior to analysis by HPLC–ESI–MS, has recently been used to reduce sample preparation time and increase assay throughput [18]. Oasis MCX® extraction cartridges use a mixed-mode polymeric sorbent, which is unaffected by dryness during the sample extraction procedure. Although modification of the generic Oasis MCX® extraction method to increase the recovery of MOR, was reported to result in interference build up, this was successfully resolved by flushing the column after every 16 samples. A gradient program was used to flush the col-

umn that increased mobile phase acetonitrile concentrations from 2.5% to 85% over 4 min, and maintained acetonitrile concentrations at 85% for a further 4 min. Using a 0.5 ml sample volume, this procedure was able to reach lower limits of quantitation of 0.5 ng/ml for MOR and 5 ng/ml for M3G.

An earlier method for the analysis of OXY in plasma by HPLC–ESI–MS–MS used liquid–liquid extraction, but a sample volume of 1 ml was required to achieve a limit of quantitation of 1 ng/ml [13]. A comparative strength of the method reported herein is that OXY, NOR, MOR, and M3G were analysed concomitantly. Although OXY and NOR both had the same retention times under the chromatography conditions used in the present investigation, their unique mass transitions permitted accurate resolution and quantitation by HPLC–ESI–MS–MS, without chromatographic resolution. Peak fronting has been reported to be a problem in HPLC chromatographic procedures used for the quantitation of OXY [19], but it was not observed under the chromatographic conditions used in this investigation.

Since morphine-6-glucuronide (M6G) is not formed in detectable quantities in the rat following administration of MOR [20], it was not included in the assay documented herein. However, under the chromatographic conditions described in the present investigation M3G and M6G were resolved, with retention times of 4.1 min and 4.5 min, respectively. A second peak corresponding to the retention time of M6G was never apparent in the *m*/*z*: 462.2/286.1 chromatograms derived from serum samples in the pilot study reported herein, in which a single rat was administered morphine and oxycodone. The method described herein could be relatively easily adapted to the analysis of blood samples taken from humans, where M3G, M6G, and MOR are present concomitantly after the administration of MOR [21], by modification of the gradient elution profile reported herein.

Recent studies with human liver microsomes and recombinant human cytochrome P450s have now established that *N*-demethylation of oxycodone to noroxycodone represents the predominant oxidative pathway for oxycodone, with *O*-demethylation to oxymorphone representing an eight-fold lesser pathway [22]. Consistent with this finding, very low plasma oxymorphone concentrations (<0.8 ng/ml) have been previously reported in human studies following oral, intramuscular, or parentral administration of oxycodone [23–26]. We have also found that serum oxymorphone concentrations were very low (<2.1 ng/ml) in rats following subcutaneous administration of oxycodone [27]. Consequently, oxymorphone was not included in the method described herein.

The very high selectivity and sensitivity of HPLC–ESI–MS–MS, permits simultaneous low level quantitation of serum MOR, M3G, OXY, and NOR concentrations in small sample volumes (50 μl), using minimal sample preparation and a short run time. Because the sample volume is small, serum protein precipitation by treatment with acetonitrile can be used for the sample work-up. Additionally, by monitoring unique mass transitions for OXY and NOR chromatographic resolution of these two analytes is not required, but chromatographic resolution is still required for analytes with identical mass transitions such as M3G and M6G. Thus, the use of HPLC–ESI–MS–MS in concert with analysis of small sample volumes enables a simple work-up procedure to be used, while allowing high levels of accuracy and precision to be achieved.

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