A Rapid and Sensitive High-Performance Liquid Chromatography–Electrospray Ionization–Triple Quadrupole Mass Spectrometry Method for the Quantitation of Oxycodone in Human Plasma

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Abstract

A sensitive method for the determination of oxycodone concentrations in plasma by high-performance liquid chromatography (HPLC)–electrospray ionization–triple quadrupole mass spectrometry is described. The method is rugged, reliable, selective, and rapid with a run time of 2 min. One milliliter of plasma is made basic and extracted with 2-mL duplicate portions of 2% isoamyl alcohol in *n*-butyl chloride. The combined extracts are then evaporated to dryness, reconstituted in 100 μL of the mobile phase (15% methanol–85% water containing 0.1% acetic acid), and injected onto the HPLC. The limit of quantitation is 1 ng/mL, and the estimated limit of detection is 33 pg/mL (signal-to-noise = 3). Standard curves are linear over the range of 1 to 100 ng/mL with all correlation coefficient values greater than 0.9989. The method is used to determine the concentration of oxycodone in human plasma following the intravenous infusion of doses ranging from 5 to 15 mg in which the analysis of over 3000 plasma samples is required.
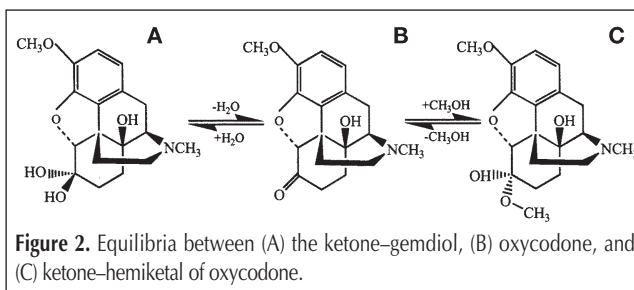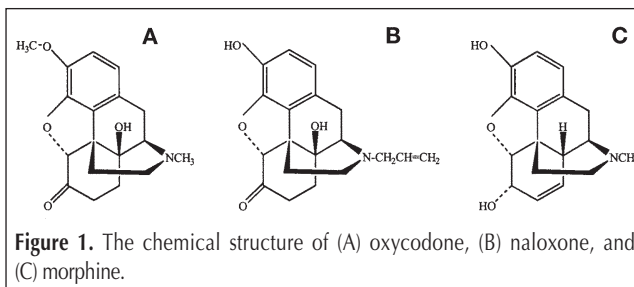
Introduction

Oxycodone (4,5-epoxy-14-hydroxy-3-methoxy-17-methylmorphinan-6-one) (Figure 1) is a potent opioid agonist with a dose-dependent analgesic effect (1) comparable with morphine (Figure 1) (2) and has been available for clinical use since 1915 (3). Clinically, oxycodone is administered alone as immediate or modified release products or in combination with mild analgesics (2) for the management of moderate to severe pain.

The quantitation of oxycodone concentrations in biological fluids by gas chromatography (GC) is not straightforward because of the need to derivatize the 14-hydroxyl group (3–6), and none of the derivatives evaluated by us exhibited satisfactorily on machine stability. Similarly, the fronting and tailing of the oxycodone peak when high-performance liquid chromatography (HPLC) is used causes reductions in sensitivity and selectivity. Broglé (7) investigated the causes of the peak fronting of oxycodone under a number of HPLC conditions. It was concluded that the cause of peak fronting was not the coexistence of keto–enol tautomers in the solutions analyzed, but rather the presence of ketone–gem-diol and ketone hemi-ketal equilibria species resulting from reactions with water and methanol, respectively, in the acidic mobile phase (Figure 2). These equilibria are temperature dependent, and fronting can be minimized by raising the column temperature.

Literature methods for the determination of oxycodone also include radioimmunoassay and thin-layer chromatography

Figure 1. The chemical structure of (A) oxycodone, (B) naloxone, and (C) morphine.

Figure 2. Equilibria between (A) the ketone–gemdiol, (B) oxycodone, and (C) ketone–hemiketal of oxycodone.

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(4,8–10); however, these methods are usually only used for screening purposes because they lack either the required degree of sensitivity or selectivity for use in pharmacokinetic studies.

HPLC–electrochemical detection (ECD) methods have been reported (11–13). Although these have limits of quantitation in the low nanograms-per-milliliter range, they require extensive work-up. GC–nitrogen–phosphorus detection (NPD) methods have been reported (14–16). These have limits of detection in the range of 1.8 to 3 ng/mL, but no data on the limit of quantitation have been provided. A GC–ECD method, which included a derivatization step with heptafluorobutyrlimidazole, had the reported lowest point on the standard curve of 10 ng/mL (17).

Various GC–mass spectrometry (MS) methods report similar sensitivity to HPLC–ECD and GC–NPD methods. One GC–MS method for measuring oxycodone in urine had a limit of quantitation of 25 ng/mL after extensive sample preparation, which included sequential derivatization with 2% methoxyamine in pyridine followed by propionic anhydride (5). Another described a method based on the GC–MS analysis of the trimethylsilyl ether derivatives of oxycodone that was linear over the range of 150 to 1500 ng/mL (4). Others have reported methods in which sample preparation included derivatization with acetic anhydride–pyridine. The linear ranges of the methods were 50–1000 ng/mL (6) and 0.2–100 ng/mL (2).

Although many analytical methods have been reported in the literature for the determination of oxycodone in biological fluids, none were suitable for the purposes of our study, which required the rapid analysis of over 3000 plasma samples. Reported methods either lacked the desired degree of sensitivity or were excessively time consuming with respect to work-up and HPLC run time.

The method described in this study has comparable limits of quantitation to the more sensitive methods previously reported in the literature, but has significant advantages (such as the sample preparation was simple and the run time very short). Sample preparation consisted of the addition of an internal standard (naloxone) followed by the liquid–liquid extraction of the plasma sample and a subsequent concentration of the extract prior to injection onto the liquid chromatograph (LC)–MS–MS. Triple quadrupole MS provided unsurpassed selectivity in which the analyte and internal standard were separated by mass and not time. Retention times were 1.30 min for naloxone and 1.41 min for oxycodone, and the run time was 2 min, thus allowing for very high throughput of samples.

The mobile phase consisted of 15% methanol–85% water (v/v) with 0.1% acetic acid, and the column temperature was 60°C. These conditions produced minimal fronting and tailing with almost symmetrical peaks.

A highly sensitive assay was required for pharmacokinetic studies (dose range from 5 to 15 mg), and in order to generate the required data it was necessary to follow the time course of oxycodone for as long as possible after the administration of each dose. The method developed and reported in this study is more sensitive, more selective, less complicated, and has a significantly shorter analysis time than other published assays, allowing for the rapid and accurate determination of very low levels of oxycodone in plasma.

Experimental

Materials and reagents

Reference samples of oxycodone hydrochloride and naloxone hydrochloride were obtained from Boots (Sydney, Australia) and Sigma (St. Louis, MO), respectively. HiPerSolv-grade n-butyl chloride was obtained from BDH Chemicals (Melbourne, Australia); SurfaSil was obtained from Pierce (Rockford, IL); and all other chemicals used were analytical-reagent grade. Water was purified by a Milli-Q system obtained from Millipore (Sydney, Australia).

All glassware was supplied by Alltech (Sydney, Australia). A Searle vortex mixer (Buchler Instruments, Ft. Lee, NJ) and a Medos JAK vacuum evaporator (Dynavac Engineering, Sydney, Australia) were used in the sample preparation.

Preparation of glassware

Glassware was silanized by immersion in a 5% solution of SurfaSil in n-hexane for 10 min and then rinsed with acetone and dried in an oven at 100°C for 1 h.

Instrumentation

Analyses were performed using a PerkinElmer (Melbourne, Australia) Series 200 autosampler, micro PerkinElmer series 200 LC pump, vacuum degasser, and PerkinElmer SCIEX API 365 LC–MS–MS with Turbo IonSpray. The Zorbax SB-C18 5-µm column (50- × 2.1-mm i.d.) (Agilent, Melbourne, Australia) was maintained at 60°C. The mobile phase consisted of 15% methanol–85% water containing 0.1% acetic acid, the flow rate was 0.2 mL/min, and the injection volume was 10 µL.

The MS was run in multiple reaction monitoring mode. The nebulizing gas flow rate of the Turbo IonSpray was 1.25 L/min. The electrospray ion source voltage was 5000 V, the orifice voltage was +30 V, and the source temperature was 300°C. The ring voltage was +220 V, the Q0 energy was –10 V, and RO2 was –35 V in order to give a collision energy of 25 V for both oxycodone and naloxone. The curtain gas was nitrogen at a flow rate of 1.25 L/min. The ions monitored were m/z 316.1 (Q1) → 298.1 (Q3) for oxycodone and m/z 328.1 (Q1) → 310.1 (Q3) for naloxone. The dwell time was 200 ms for both oxycodone and naloxone.

Sample preparation

Blood samples obtained from healthy human subjects before and after receiving oxycodone were placed in heparinized blood-collecting tubes and centrifuged at 1500 g for 10 min. The plasma was separated and stored at –20°C until analysis.

A stock solution of 10 mg/mL oxycodone (as the hydrochloride) was prepared in Milli-Q water. Standards in plasma were then prepared by adding volumes of stock solution to drug-free plasma to give final concentrations covering the range of 1 to 100 ng/mL. The working internal standard solution (670 ng/mL naloxone hydrochloride) was prepared in Milli-Q water.

One-milliliter aliquots of the plasma samples and the plasma oxycodone standard solutions were pipetted into 4-mL glass vials containing the internal standard (naloxone hydrochloride, 10 µL, 6.7 ng) and made alkaline to pH 9 with 200 µL carbonate buffer. The carbonate buffer was prepared from a saturated sodium

hydrogen carbonate solution, which was adjusted to pH 9 with a saturated sodium carbonate solution. The extraction solvent, 2% isoamyl alcohol in *n*-butyl chloride (2 mL), was added, and the mixture was vortex mixed for 10 min followed by centrifugation at 2000 g for 5 min. The samples were frozen at –80°C for 20 min and the organic layer decanted into a 2-mL glass vial and evaporated to dryness under vacuum at 40°C. The sample was re-extracted, and the extraction residues were combined and reconstituted in a 100-µL mobile phase, vortex mixed (30 s), centrifuged (1 min), and then transferred into a 250-µL silanized glass insert. An aliquot (10 µL) was injected onto the HPLC.

Results and Discussion

Sample preparation considerations

In order to increase the sensitivity of this assay, several factors were addressed. First, all glassware was deactivated with a silanizing reagent to minimize the loss of oxycodone through binding to active sites on the glassware. Second, ultrahigh-purity solvents were used to minimize solvent impurity interferences. Third, 2% isoamyl alcohol in *n*-butyl chloride was used as the extraction solvent because it caused no emulsification with

plasma, which was noted with other extraction solvents evaluated.

Chromatography

Chromatographic conditions were optimized to provide both short retention times and an adequate peak shape in order to allow for the quantitation of oxycodone and naloxone. Factors were taken into account when selecting the final conditions such as our observation that increasing concentrations of methanol in the mobile phase reduced the sensitivity of oxycodone because of mobile phase ion suppression. Naidong (18) reported that mobile phases with little organic component resulted in poor spray conditions because of the high surface tension of the aqueous droplets, thus adversely affecting sensitivity. The methanol content selected in this study was that which gave short retention times without adversely affecting sensitivity.

It was also found that the addition of 0.1% acetic acid not only improved the retention time stability of both analytes, but also provided an increase in sensitivity. The mobile phase selected for this assay provided the required sensitivity with both short retention times and nearly symmetrical peaks.

Peak symmetry was calculated using the 4.4% peak height/peak asymmetry factor. Five replicate chromatograms of extracts from plasma containing 100 ng/mL oxycodone gave an asymmetry value of 1.4, which indicated that there was only slight tailing associated with these peaks.

The sensitivity of the assay was excellent as shown in Figure 3, which shows the chromatograms of an extract from 1 mL of plasma spiked with 1 ng oxycodone (Figure 3C) and 6.7 ng naloxone (Figure 3D) and a representative blank for each transition (Figures 3A and 3B).

Electrospray mass spectra

The fragmentation of the product ions of oxycodone and naloxone are shown in Figures 4A and 4B, respectively. The predominant peaks for oxycodone (Figure 4A) were m/z 316.1 (corresponding with MH+) and m/z 298.1 (corresponding with MH+–H_2O). The predominant peaks for naloxone (Figure 4B) were m/z 328.1 (corresponding with MH+) and m/z 310.1 (corresponding with MH+–H_2O). Q3 scan conditions were optimized by infusing
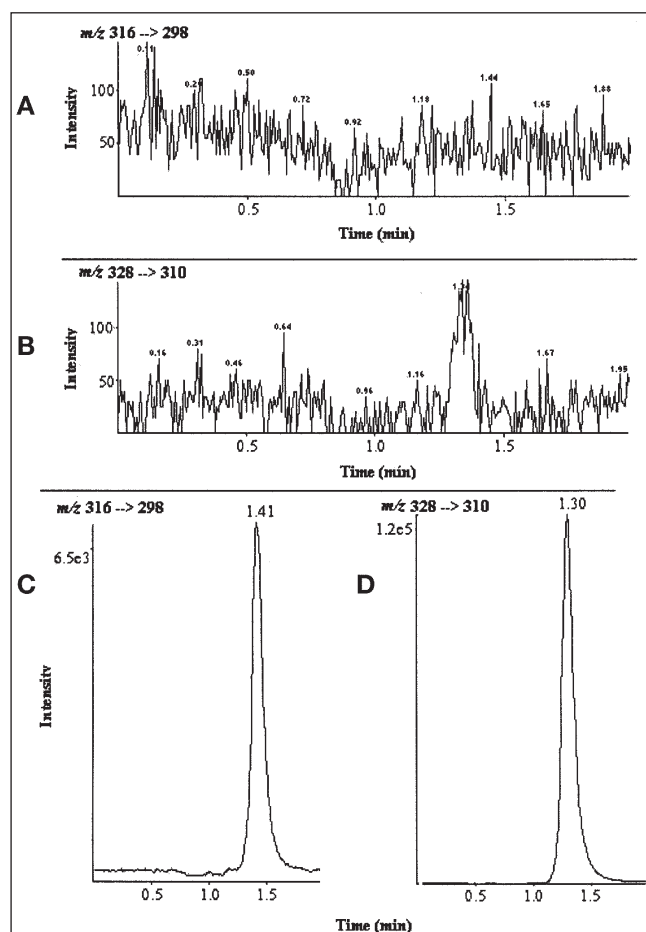
Figure 3. Chromatograms of an extract from (A and B) 1 mL blank plasma and (C) 1 mL plasma spiked with 1 ng oxycodone and (D) 6.7 ng naloxone. The transitions were oxycodone (m/z 316 → 298) and naloxone (m/z 328 → 310).
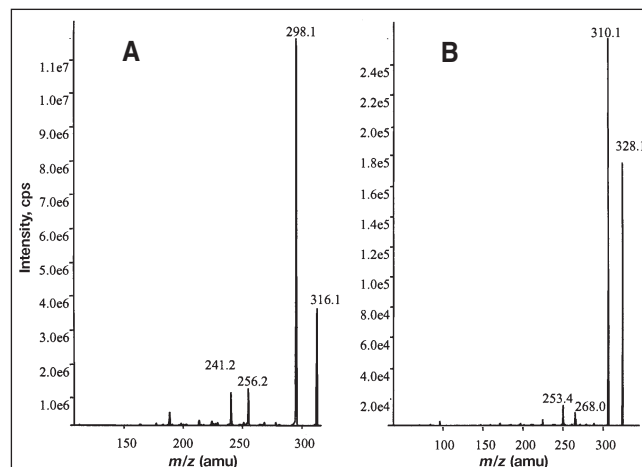
Figure 4. The collision activated decomposition spectrum of the protonated molecule of (A) oxycodone (m/z 316.1) and (B) naloxone (m/z 328.1).

a solution of analyte (oxycodone or naloxone) in the mobile phase via a syringe pump (2.5 mL/h) and using Autotune Optimisation software.

Retention times

The retention times for oxycodone and naloxone (internal standard) were 1.41 and 1.30 min, respectively.

Linearity

Standard curves covering the concentration range of 1 to 100 ng/mL oxycodone in plasma were found to be linear. Six standard curves had correlation coefficient values ranging from 0.9989 to 0.9999.

Limit of quantitation and limit of detection

The limit of quantitation was 1 ng/mL and the limit of detection was 33 pg/mL (signal-to-noise = 3).

Within-run precision

A measure of within-run precision was estimated from the coefficient of variation of samples ($n = 6$) processed in the same batch and analyzed on the same day. An assay of six replicate samples at 1, 10, and 100 ng/mL gave coefficients of variation of 8.6%, 1.9%, and 1.9%, respectively.

Between-run precision

A measure of between-run precision was estimated by averaging the coefficients of variation from the analysis of six replicate samples run over 3 days ($n = 18$). The average between-run precision was 11.2%, 4.4%, and 3.0% for samples at concentrations of 1, 10, and 100 ng/mL, respectively.

Accuracy

Accuracy was determined as a percentage of calculated concentration over the actual concentration. An assay of six replicate samples at 1, 10, and 100 ng/mL gave calculated accuracy values of 126.5%, 98.6 %, and 100.1%, respectively.

Selectivity

An analysis of blank plasma samples showed there were no interfering peaks at the elution time of either oxycodone or the internal standard.

Stability

Smith (11) reported that oxycodone was stable in plasma samples stored at –20°C for up to 6 months.

Extracted oxycodone samples were stable for at least 24 h at room temperature. There was no significant difference ($\alpha = 0.05$) for samples reanalyzed after storage in the autosampler carousel for 24 h at concentrations of 1, 10, and 100 ng/mL ($n = 6$ at each concentration).

Samples were stable following three freeze/thaw cycles. There was no significant difference ($\alpha = 0.05$) for samples reanalyzed after three freeze/thaw storage cycles at concentrations of 1, 10, and 100 ng/mL ($n = 6$ at each concentration).

Application of the method

Clinical studies were carried out in which an intravenous infusion dose of oxycodone was given for 60 min (dose concentrations ranged from 5 to 15 mg). Samples were taken over a period of 3 h after the dose. A blank sample was taken prior to dosing to determine whether there were any interfering ions. None of the blank samples for the subjects enrolled in the study contained interfering ions at the two transitions studied (i.e., m/z 316.1 → 298.1 and m/z 328.1 → 310.1). It was possible to quantitate oxycodone levels over the period for which respiratory data were gathered for all subjects enrolled in the study. A typical oxycodone concentration-time profile following a single intravenous infusion of 5 mg is shown in Figure 5.

Conclusion

The LC–electrospray ionization–MS–MS method described has been used to process more than 3000 plasma samples. All calibration curves had a correlation coefficient greater than 0.9989 over the plasma concentration range of 1 to 100 ng/mL. Although some previously reported methods have achieved similar limits of quantitation, the method reported in this study requires significantly less sample preparation, and the very short run time allows for very high sample throughput.

Acknowledgments

Figure 5. The oxycodone plasma concentration versus time profile following an intravenous dose of 5 mg oxycodone.

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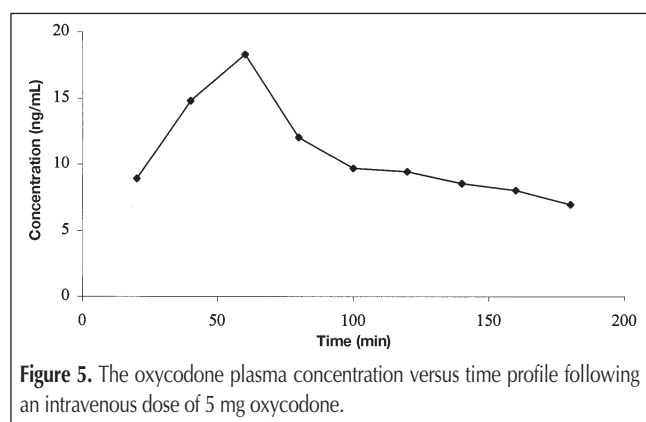
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