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Simultaneous determination of oxymorphone and its active metabolite 6-OH-oxymorphone in human plasma by high performance liquid chromatography–tandem mass spectrometry

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ABSTRACT

A selective high performance liquid chromatography–tandem mass spectrometric (LC–MS/MS) method for the simultaneous determination of oxymorphone and its active metabolite 6-OH-oxymorphone in human plasma was developed and validated using oxymorphone-d_3 as the internal standard. Chromatographic conditions were optimized to separate oxymorphone from the other metabolite, oxymorphone-3-glucuronide, which may convert to oxymorphone in MS ion source, resulting in inaccurate quantitation of oxymorphone. Solid phase extraction (SPE) was used to extract oxymorphone and 6-OH-oxymorphone from plasma. SPE offered the advantage of being able to remove the unwanted metabolite, oxymorphone-3-glucuronide, through the wash step during the extraction. The developed method was precise and reproducible as shown by good linearity of calibration curves (correlation coefficients ≥0.9968 for oxymorphone and ≥0.9967 for 6-OH-oxymorphone) with high intraday assay and interday assay precision (CV% ≤11.0% for oxymorphone and ≤12.6% for 6-OH-oxymorphone) over a range of 35/25 – 5000/5000 pg/mL for oxymorphone/6-OH-oxymorphone. The method has been successfully applied to analyze oxymorphone and 6-OH-oxymorphone in plasma from 19 healthy volunteers in a bioequivalence study. A total of 1026 samples were analyzed. Good linearity (average correlation coefficient 0.9988 for oxymorphone and 0.9966 for 6-OH-oxymorphone) was achieved with calibration curves and high precision (CV% ≤5.9% for oxymorphone and ≤10.9% for 6-OH-oxymorphone) was obtained with QCs.

1. Introduction

Oxymorphone, 14-hydroxydihydromorphinone, is a semisynthetic μ-opioid agonist that is mainly used for the relief of moderate to severe pain [1]. It transfers faster across the blood–brain barrier when compared to other opioids such as oxycodone and morphine because it is more lipid soluble [2,3]. Therefore, it has a more rapid onset of action [4–6]. Studies also indicate that oxymorphone has a much higher analgesic potency than morphine and oxycodone [6,7]. The structure of oxymorphone is shown in Fig. 1(a).

Oxymorphone currently has various types of formulations available including suppository, parenteral injection, oral IR (immediate release) tablets and oral ER (extended release) tablets [6]. This provides patients more options for the drug administration. Oxymorphone IR has a duration of action for ca. 4–6 h while oxymorphone ER has been shown to be effective for over 12 h [6].

Oxymorphone is extensively metabolized to oxymorphone-3-glucuronide and analgesic active 6-OH-oxymorphone in the liver; only 2% is excreted unchanged in urine [6,8]. The structure of 6-OH-oxymorphone is shown in Fig. 1(b).

Different assays have been reported for the quantitation of oxymorphone together with other opioids in different matrices using LC–MS/MS [9–13], GC–MS [14] and HPLC with electrochemical detection [15]. The lower limits of quantifications (LLOQs) are usually higher than 0.5 ng/mL. To our knowledge, there is presently no LC–MS/MS method published for the simultaneous analysis of oxymorphone and its active metabolite 6-OH-oxymorphone. Here we describe a selective and sensitive LC–MS/MS method for the simultaneous determination of oxymorphone and 6-OH-oxymorphone in human plasma. The developed and validated method was successfully applied to the bioequivalence studies of oxymorphone tablets in healthy volunteers.

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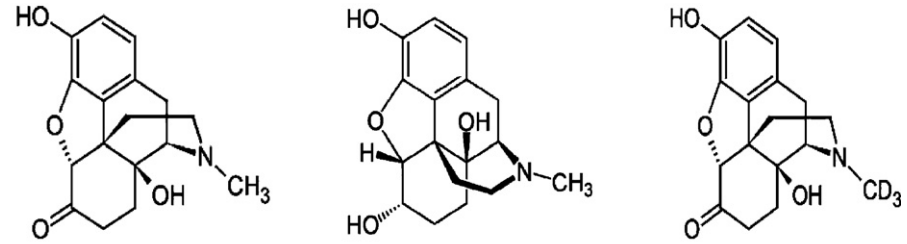


Fig. 1. Structure of oxymorphone (a), 6-OH-oxymorphone (b) and oxymorphone-d_3 (c).

2. Experimental

2.1. Chemicals

Oxymorphone (1 mg/mL in methanol, 100%), 6-OH-oxymorphone (powder, 98%) and oxymorphone-d_3 (1 mg/mL in methanol, 99.9%) were all purchased from Cerilliant (Round Rock, TX, USA). Sodium phosphate, dibasic, heptahydrate (100%) was supplied by Mallinckrodt Baker (Phillipsburg, NJ, USA). Methanol and acetic acid were both HPLC grade from EMD Chemicals Inc. (Gibbstown, NJ, USA). Deionized water was produced in-house using a Millipore ultrapure water purification system (Billerica, MA, USA). Bond Elut C18 (3 mL) cartridges were bought from Agilent (Santa Clara, CA, USA).

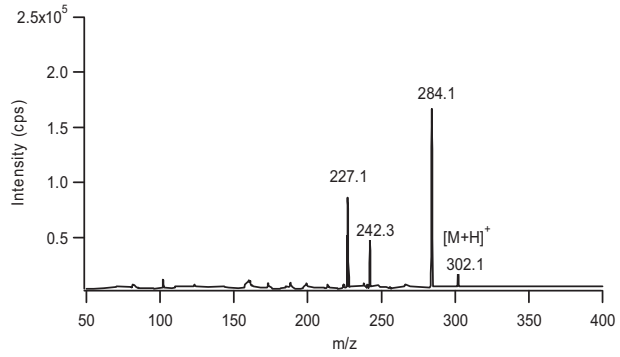
2.2. High performance liquid chromatography and mass spectrometry

Chromatographic separation was obtained under isocratic conditions using a Shimadzu LC-10AD *vp* LC system (Columbia, MD, USA) with a Phenomonex (Torrance, CA, USA) Synergi Polar-RP column (75 mm × 2.0 mm, 4 μm). The mobile phase consisted of 62% A (water with 0.1% acetic acid) and 38% B (methanol) by volume. The flow rate for the mobile phase was set at 0.18 mL/min.

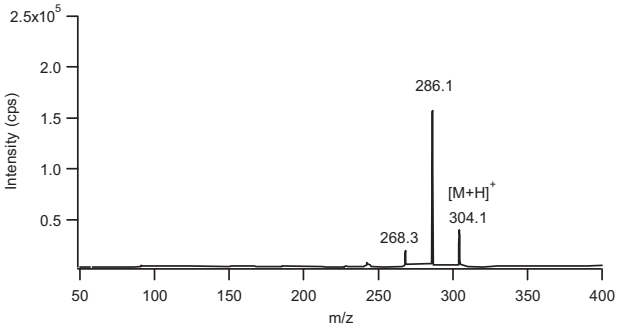
Detection was performed by an AB/Sciex (Concord, ON, Canada) API 4000 triple quadrupole mass spectrometer with a turbo-ion spray interface in the positive ion mode. Oxymorphone-d_3 was used as the internal standard (IS). The structure of oxymorphone-d_3 is shown in Fig. 1(c). The MS/MS transitions (m/z) used for quantification were: oxymorphone, 302.1 → 227.1; 6-OH-oxymorphone, 304.1 → 286.1; and oxymorphone-d_3 (IS), 305.1 → 230.1 (shown in Fig. 2). The ion source was heated to 700 °C and the spray voltage was set at 4000 V. The nebulizer gas, auxiliary gas, collision-activated dissociation (CAD) gas and curtain gas were all ultra-high purity nitrogen gas and applied at 60, 60, 8 and 20 psi (413,685, 413,685, 55,158 and 137,895 Pa), respectively. The collision energy was set at 37 V for oxymorphone, 29 V for 6-OH-oxymorphone and 37 V for oxymorphone-d_3. The Software Analyst 1.4.2 (Sciex, Concord, ON, Canada) was used to control the LC–MS/MS system and acquire the data.

2.3. Preparation of stock solution and standards

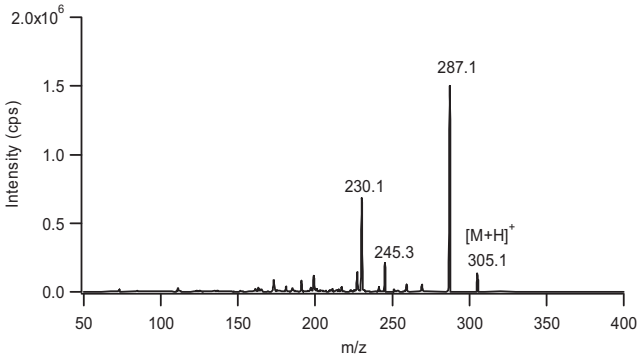
Oxymorphone (1.00 mg/mL in methanol) and IS (1.00 mg/mL in methanol) purchased from Cerilliant were used as stock solutions. 6-OH-oxymorphone stock solution, 1.00 mg/mL, was prepared in methanol. All stock solutions were stored at −20 °C. Two independent stock solutions for each analyte were used for the standard and QC preparation (one for standards and the other one for QCs). Two-in-one working solutions of oxymorphone/6-OH-oxymorphone (200/200 ng/mL, 20/20 ng/mL and 7.0/5.0 ng/mL) were prepared



(a) Product ion mass spectra of $[M+H]^+$ of oxymorphone



(b) Product ion mass spectra of $[M+H]^+$ of 6-OH-oxymorphone



(c) Product ion mass spectra of $[M+H]^+$ of oxymorphone-d_3

Fig. 2. Product ion mass spectra of $[M+H]^+$ of oxymorphone (a), 6-OH-oxymorphone (b) and oxymorphone-d_3 (c).

by dilution of stock solutions with methanol/deionized water, 1/1 (v/v). The IS working solution containing 5.0 ng/mL oxymorphone-d_3 was diluted from the IS stock solution with deionized water/methanol, 9/1 (v/v).

The plasma used for spiking was examined to make sure it was free of interferences from oxymorphone, 6-OH-oxymorphone and the IS before preparing the standard calibration curve. A standard curve was prepared daily, in duplicate. Appropriate amounts of oxymorphone/6-OH-oxymorphone two-in-one working solutions were spiked into the plasma to achieve the calibration curve points equivalent to 35/25, 70/50, 200/200, 400/400, 2000/2000, 3000/3000 and 5000/5000 pg/mL of oxymorphone/6-OH-oxymorphone. Quality control (QC) samples were prepared in a similar way to calibration standards at LLOQ (35/25 pg/mL), low level (105/75 pg/mL), mid-level (1000/1000 pg/mL) and high level (4000/4000 pg/mL). QC samples were stored at −20 °C after their preparation.

2.4. Extraction procedure

Solid phase extraction (SPE) was used for sample extraction and clean-up. A 500 μL aliquot of human plasma was transferred into a 16 mm × 100 mm culture tube, and 2.0 mL of 25 mM sodium phosphate, dibasic, and 100 μL of the IS working solution were added. The mixture was vortexed for 10 s and loaded into a Bond Elut C18 (3 mL) cartridge previously conditioned with 1.0 mL of methanol and 1.0 mL of 25 mM sodium phosphate, dibasic. The cartridge was washed with 2.0 mL of deionized water and 2.0 mL of methanol/deionized water, 2/8 (v/v). The elution of analytes was performed with 1.0 mL of methanol. The eluate was evaporated under a stream of nitrogen and redissolved in 100 μL of mobile phase. An aliquot of 5.0 μL was injected into the LC–MS/MS system.

2.5. Method validation

The developed method has been validated for linearity and sensitivity, precision and accuracy, selectivity, matrix effects, recovery and stability according to the US Food and Drug Administration (FDA) bioanalytical method validation guidance [16].

3. Results and discussion

3.1. Optimization of chromatographic conditions and sample extraction

Mobile phase was first prepared with methanol and 5.0 mM ammonium acetate and the separation was conducted on a C18 column, but poor peak shape was observed for all analytes due to tailing. The pH of the buffer, 5.0 mM ammonium acetate, was lowered to pH 5.0 by adding acetic acid, and the peak tailing was reduced. When water with 0.1% acetic acid was used to replace the buffer, peak shape for all the analytes became much better and the analytes sensitivities also increased. Water with 0.1% acetic acid and methanol were finally used in the mobile phase.

A good chromatographic separation between oxymorphone and the other metabolite, oxymorphone-3-glucuronide, is needed because the glucuronide metabolite may convert to oxymorphone by deconjugation in the ion source of MS [17]. The conversion may result in inaccurate quantitation of oxymorphone. To achieve the separation, C18 columns and other stationary phases were tested. It was found that the Polar-RP was the best type of column for the separation. A Polar-RP column of 75 mm was eventually chosen for the chromatographic separation. The mobile phase was optimized with the selected column. Different percentages of methanol in mobile phase were examined. The results showed that the separation increased while the sensitivity decreased with lower percentage of methanol in the mobile phase. Considering both the separation and the sensitivity, 38% of methanol and 62% of water with 0.1% acetic acid were finally used as the mobile phase. The flow rate was set at 0.18 mL/min and the run time was 3.0 min. A solution containing all the analytes and oxymorphone-3-glucuronide was analyzed with these optimized conditions. The in-source conversion of a small portion of oxymorphone-3-glucuronide to oxymorphone was observed, but the peak of converted oxymorphone was clearly separated from oxymorphone.

Oxymorphone and 6-OH-oxymorphone had been previously extracted by a liquid–liquid extraction method in our lab. Two-step extraction with different solvents and buffers for each step had been used. Here we used solid phase extraction (SPE) method to treat the sample. SPE not only offers extensive matrix clean-up compared to other extraction techniques, but also can be used to eliminate the unwanted glucuronide metabolite in this case. Because the glucuronide metabolite is more polar than the analytes of interest, an appropriate wash solution can be used to remove the glucuronide metabolite while retaining the analytes of interest on the cartridge during the wash step. The wash solutions containing different percentages of methanol in water were tested, and it was found that the solution of methanol/water, 20/80 (v/v), was the best one for the wash. The final optimized SPE method was described in Section 2.4. A sample spiked with all the analytes and glucuronide metabolite (100 ng/mL oxymorphone-3-glucuronide) was then extracted using the optimized SPE method and analyzed. Only a small peak of oxymorphone-3-glucuronide was observed and the recovery of oxymorphone-3-glucuronide with this extraction method was 1.2%, i.e. more than 98% of the glucuronide metabolite was removed by SPE. This final SPE extraction method and the optimized chromatographic separation completely removed the possibility of interference to oxymorphone resulting from the glucuronide metabolite in-source conversion.

3.2. Product ion selection

The product ion of 284.1 (m/z) was first chosen for the MRM mode detection of oxymorphone because it has the highest intensity. However, when the extracted samples were tested, an interference peak appeared and partially overlapped with oxymorphone. The product ion was then switched to 227.1 (m/z), and the interference peak disappeared. The peak height of oxymorphone decreased, but the noise also lowered and the signal-to-noise ratio (S/N) did not change. The product ion of 227.1 (m/z) was finally selected.

3.3. Method validation

3.3.1. Linearity and sensitivity

Standard calibration curves of seven points that correspond to oxymorphone/6-OH-oxymorphone concentrations ranging from 35/25 to 5000/5000 pg/mL were extracted and analyzed. A blank plasma sample (containing no drug or IS) and a zero sample (only IS added) were also processed to ensure the absence of interferences. Another blank sample was used for assessing the level of carryover if any by following the highest concentration sample. The calibration curve was plotted based on the peak area ratio of oxymorphone (or 6-OH-oxymorphone) to the IS versus oxymorphone (or 6-OH-oxymorphone) concentration. The weighted least squares regression analysis with a weighting factor of $1/x^2$ was performed for calibration curves.

The results showed a good linear relationship between the peak area ratio of oxymorphone (or 6-OH-oxymorphone) to the IS and oxymorphone (or 6-OH-oxymorphone) concentration over the range of 35 (25 for 6-OH-oxymorphone) to 5000 pg/mL. The correlation coefficients were higher than 0.9968 for oxymorphone

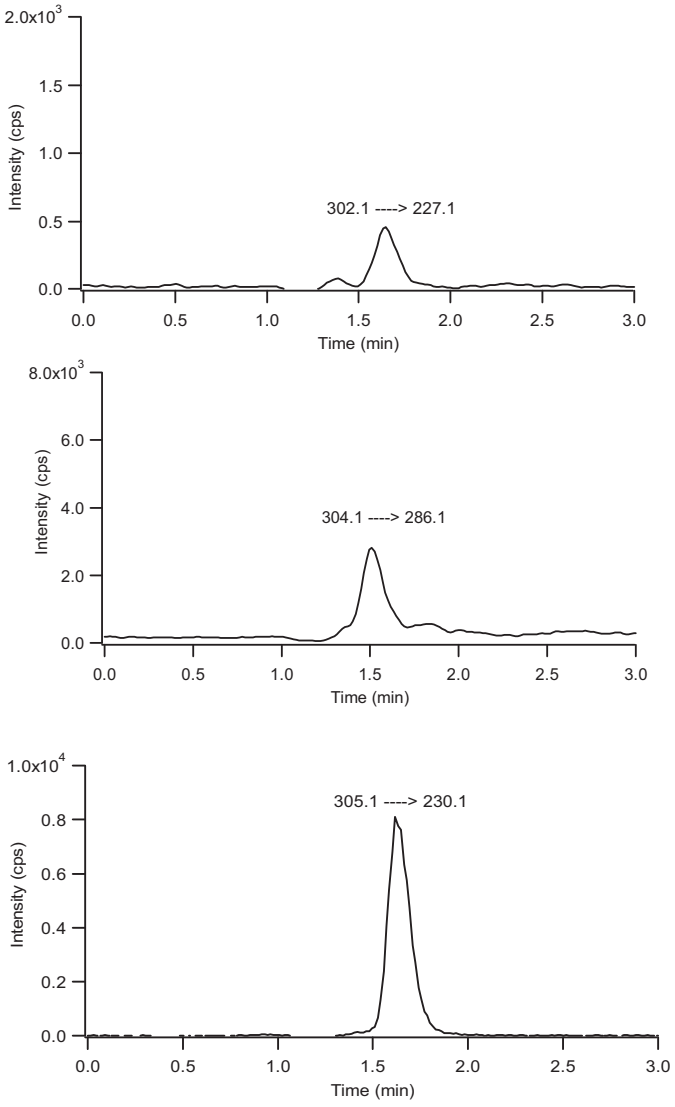


Fig. 3. Chromatogram of a blank plasma spiked at LLOQ (35 pg/mL oxymorphone and 25 pg/mL 6-OH-oxymorphone).

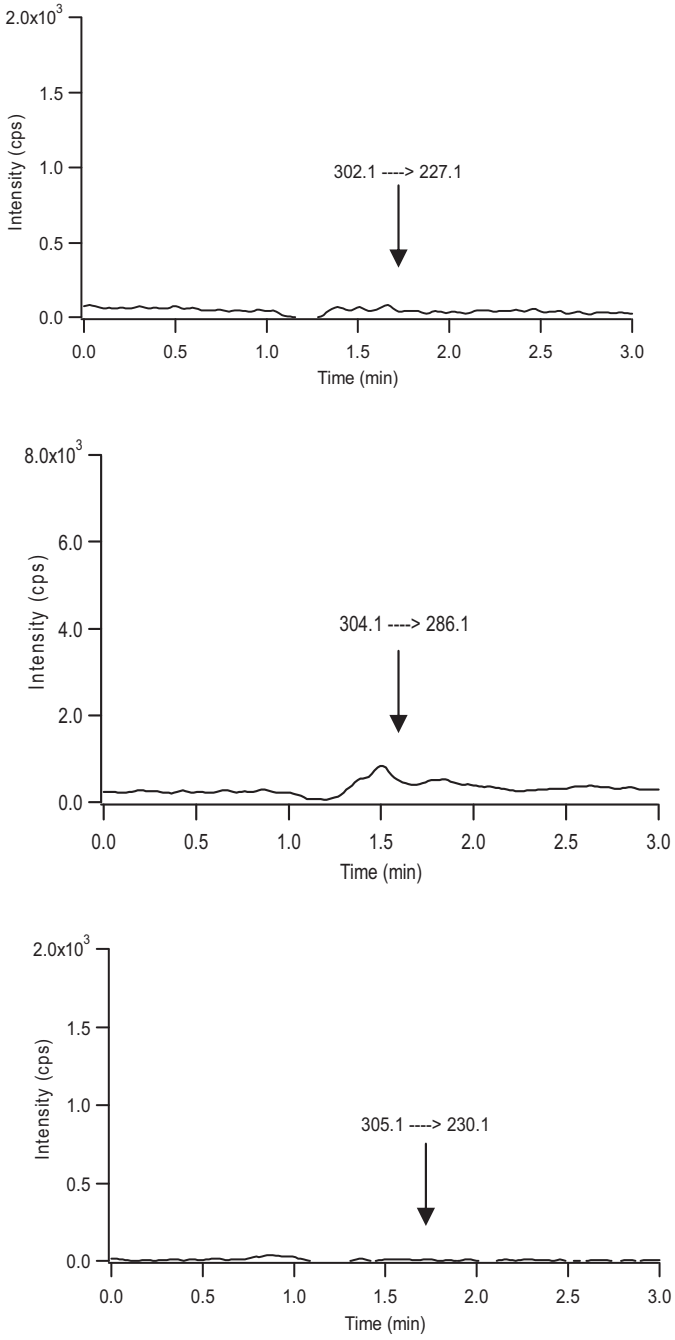


Fig. 4. Chromatogram of an extracted blank plasma sample showing the absence of interference with oxymorphone, 6-OH-oxymorphone and IS.

and 0.9967 for 6-OH-oxymorphone, with an average of 0.9976 for oxymorphone and 0.9970 for 6-OH-oxymorphone.

The LLOQ was set at the lowest standard concentration in the calibration curve, 35 pg/mL for oxymorphone and 25 pg/mL for 6-OH-oxymorphone. Fig. 3 presents the chromatogram of an extracted LLOQ sample.

The blank plasma sample and zero sample indicated that there were no interferences for oxymorphone, 6-OH-oxymorphone and IS, and the blank plasma sample following the highest standard showed no carryover for oxymorphone, 6-OH-oxymorphone and IS.

3.3.2. Precision and accuracy

The intraday assay precision and accuracy were determined by analyzing six replicates of the QC samples at three concentration levels (105/75 pg/mL, 1000/1000 pg/mL and 4000/4000 pg/mL) and at the LLOQ. Three intraday assays were performed. For oxymorphone, the precision (CV%) was between 1.1% and 11.2%, and the accuracy ranged from 96.6% to 103.7% for QC levels. The precision was 5.3–11.0% and the accuracy was 89.8–101.8% at the LLOQ. For 6-OH-oxymorphone, the precision was between 1.4% and 7.6%, and the accuracy ranged from 98.6% to 114.5% for QC levels. The precision was 5.6–12.6% and the accuracy was 92.3–96.8% at the LLOQ.

The interday assay precision and accuracy were measured by three repeated analyses of the QC samples and the LLOQ on three different days. The sample concentrations were determined by the standard calibration curve prepared and analyzed on the same day. For oxymorphone, the interday assay precision (CV%) of the QC samples was 1.9–7.1% and accuracy was between 98.4% and 103.1%. The precision at the LLOQ was 7.9% and the accuracy was 105.7%. For 6-OH-oxymorphone, the interday assay precision of the QC samples was 4.9–6.4% and accuracy was between 100.8% and 109.9%. The precision at the LLOQ was 9.9% and the accuracy was 94.7%.

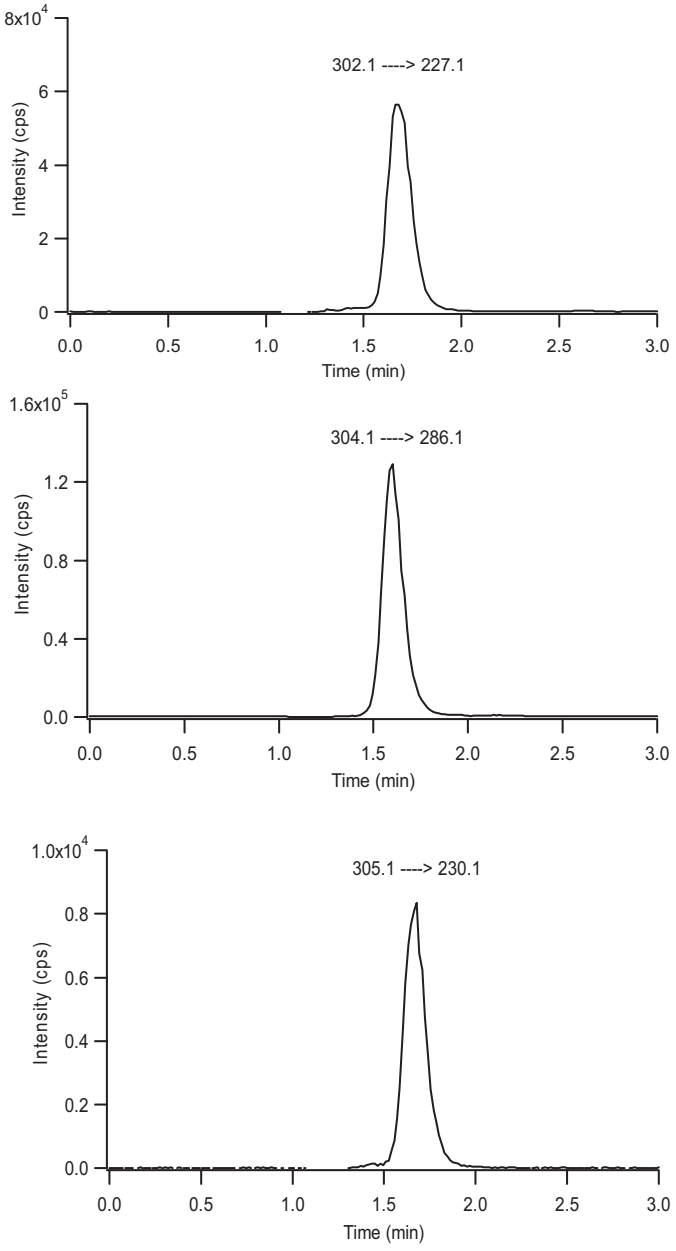


Fig. 5. Chromatogram of an extracted incurred plasma sample (determined concentrations: 4500 pg/mL oxymorphone and 1700 pg/mL 6-OH-oxymorphone; IS added: 5000 pg/mL).

3.3.3. Selectivity

Six different lots of blank human plasma samples from different donors were extracted to assess the selectivity of the method. The results revealed that the method was selective for oxymorphone, 6-OH-oxymorphone and the IS. No endogenous interference was found at the retention times of oxymorphone, 6-OH-oxymorphone and the IS as illustrated in the chromatogram of an extracted blank plasma sample shown in Fig. 4.

3.3.4. Matrix effects

In order to confirm that this method would not be affected by the matrix constituents from various donors, the consistency of matrix effects was evaluated by analyzing plasma samples from six different donors spiked with oxymorphone and 6-OH-oxymorphone at the concentrations of 105 pg/mL and 75 pg/mL. Results showed the MS responses of oxymorphone, 6-OH-oxymorphone and the IS

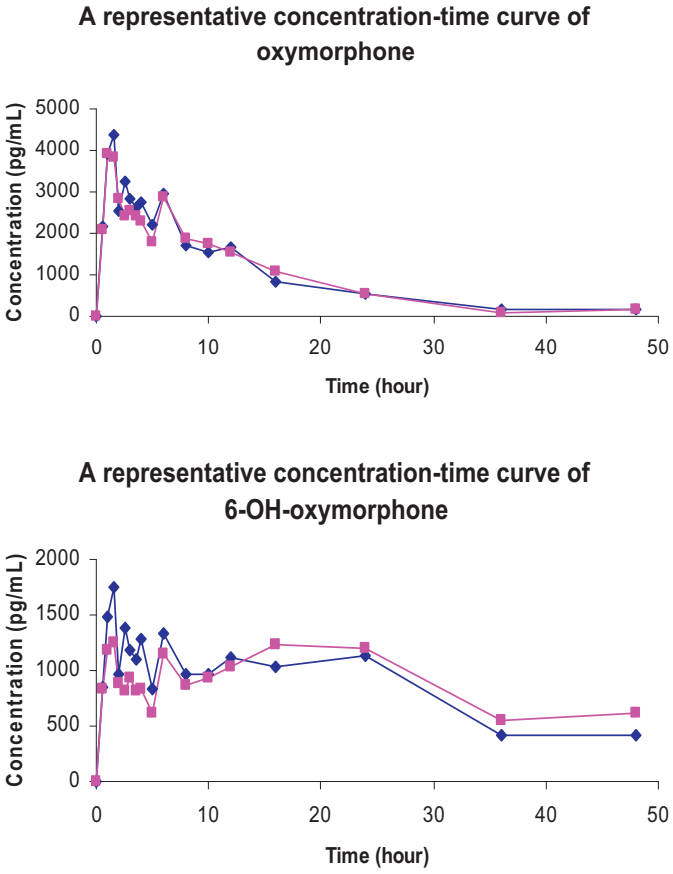


Fig. 6. A representative concentration–time curve of oxymorphone and 6-OH-oxymorphone.

were consistent with the six different plasma sources. Reproducible and accurate results were obtained.

3.3.5. Recovery

The matrix effect factor was considered in obtaining the extraction recovery. Reference solutions were prepared in mobile phase and mixed with the evaporated residue of a blank plasma sample to mimic the matrix of extracted samples. The recovery of oxymorphone, 6-OH-oxymorphone and the IS was then determined by comparing the peak areas of the extracted samples at three different concentrations (35/25 pg/mL, 400/400 pg/mL and 5000/5000 pg/mL) with those of reference solutions (the same theoretic concentrations as extracted samples).

The overall extraction recovery was 88.9% for oxymorphone, 92.2% for 6-OH-oxymorphone and 86.2% for the IS.

3.3.6. Stability

The stability of oxymorphone and 6-OH-oxymorphone in human plasma under different conditions including temperature and time was evaluated. To determine the stability, the QC samples together with standard calibration curves were prepared freshly, and then they were analyzed with the stability samples (six replicates) at the same time. The acceptance criterion is that precision and accuracy deviation values must be less than 15% for at least 67% stability samples at each concentration level.

For freeze–thaw stability evaluation, each cycle included thawing QC samples unassisted at room temperature until they were completely thawed, and re-freezing them at −20 °C for at least 12 h (24 h for the first cycle). Results showed that oxymorphone and 6-OH-oxymorphone in human plasma were stable for at least three freeze–thaw cycles.

For short term stability determination, QC samples were left on bench for a certain time and then analyzed. Results indicated that oxymorphone and 6-OH-oxymorphone were stable at room temperature for at least 4.0 h.

Oxymorphone and 6-OH-oxymorphone were stable at −20 °C for at least 89 days for long-term stability.

Oxymorphone and 6-OH-oxymorphone stock solution stabilities were also evaluated and they were found to be stable at −20 °C for at least 44 days.

3.4. Application to bioequivalence study

This validated method was applied to analyze plasma samples from 19 healthy volunteers in a bioequivalence study. Each volunteer was administered an oral dose of extended release 40 mg tablet and the blood samples were collected over a period of 48 h. The blood samples were immediately centrifuged and plasma was separated from blood cells and stored at −20 °C until the analysis was complete. Good linearity (average correlation coefficient 0.9988 for oxymorphone and 0.9966 for 6-OH-oxymorphone) was achieved with calibration curves and high precision (CV% ≤5.9% for oxymorphone and ≤10.9% for 6-OH-oxymorphone) was obtained with QCs. A representative chromatogram of an incurred sample is shown in Fig. 5 and an example of plasma concentration–time curves of oxymorphone and 6-OH-oxymorphone from one volunteer is shown in Fig. 6.

4. Conclusion

A highly selective and sensitive LC–MS/MS method for the simultaneous determination of oxymorphone and 6-OH-oxymorphone concentrations in human plasma was developed and validated. The chromatographic conditions and sample extraction were optimized to ensure the absence of interference to oxymorphone resulting from the in-source conversion of the glucuronide metabolite. The method was proven to be simple, fast and reproducible for the analysis of oxymorphone and 6-OH-oxymorphone, and was successfully applied to bioequivalent studies.

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