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Development of a sensitive method for the determination of oxycodone and its major metabolites noroxycodone and oxymorphone in human plasma by liquid chromatography–tandem mass spectrometry

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ABSTRACT

Oxycodone is an opioid agonist largely prescribed for the treatment of moderate to severe pain. Variability in analgesic efficacy could be explained by inter-subject variations in plasma levels of parent drug and its active metabolite, oxymorphone. For this purpose it is necessary to develop and validate a sensitive and selective analytical method for the quantification of oxycodone and its major metabolites, noroxycodone and oxymorphone, in human plasma. The analytical method consisted of a liquid–liquid extraction procedure followed by a high performance liquid chromatography with heated assisted electrospray ionization mass spectrometry (HPLC–HESI–MS/MS). The chromatographic separation was achieved using gradient elution with a mobile phase consisting of ethanol and 10 mM ammonium acetate on a Synergi MAX-RP analytical column (150 × 2 mm, 4 μm) protected by a security guard cartridge (C12 4 × 2 mm) at a flow rate of 300 μL/min.The calibration functions are linear in the range of 300–50,000 pg/mL for oxycodone and noroxycodone and 50 to 10 000 pg/mL for oxymorphone. Intra- and inter-day relative standard deviations are less than 5.5% and 6.4%, respectively for all analytes. The limit of detection was 30 pg/mL for all analytes. We introduce a new HPLC–HESI–MS/MS sensitive and specific analytical method capable to simultaneously quantify oxycodone, noroxycodone and oxymorphone, in human plasma, and suitable for the conduct of pharmacokinetic studies after a single dose administration of the parent compound.

1. Introduction

Oxycodone is a potent semi-synthetic μ-opioid agonist widely used for the treatment of moderate to severe pain including post-operative pain, cancer-related pain and chronic pain [1–5]. Oxycodone is extensively metabolized by Cytochromes P450 (CYP450) with less than 10% of the oral dose excreted unchanged in the urine [6,7]. CYP3A4 and CYP2D6 constitute the major drug-metabolizing enzymes involved in the oxidation pathways of oxycodone in humans [8]. Mainly, oxycodone is *N*-demethylated by CYP3A4/5 to noroxycodone. On the other hand, the *O*-demethylation of oxycodone to oxymorphone is mediated by CYP2D6 and accounts for only 10% of the urinary metabolites [9,10]. Oxymorphone confers an increased analgesic potency compared to oxycodone [11,12]. Finally, noroxymorphone is a secondary metabolite mostly formed by *O*-demethylation of noroxycodone by CYP2D6 [8]. A wide inter-subject variability in activity of these enzymes, CYP2D6 and CYP3A4/5, explained by genetic polymorphisms and drug–drug interactions is well-known. Indeed, important variations in the levels and activities of CYP3A and CYP2D6 have been reported to affect the circulating plasma concentrations of oxycodone and its metabolites [13,14]. Hence, it has been proposed that these factors could partly explain inter-individual variability observed in the analgesic response of oxycodone [15–17]

The role of genetically-determined polymorphic CYP2D6 activity in the *O*-demethylation of codeine to morphine for their analgesic efficacy is now well recognized. The same relationship could be expected with oxycodone which is dependent on the same

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CYP450 pathway for the formation of oxymorphone. Despite the large clinical experience with oxycodone, the role of the CYP2D6-mediated effects remains unclear. In fact, conflicting results have been reported on the associations between oxycodone and its metabolites on the observed pharmacodynamic effects [17–22].

Various LC-based methods were developed for the determination of oxycodone and its major metabolite, noroxycodone [23–25]. As well, several assays have been published for the quantification of oxycodone and its major metabolites including noroxycodone and oxymorphone [26–28]. However, the limit of quantification for most of them bordered the circulating levels of oxymorphone and did not allow a proper characterization of the drug and metabolites half-life. Furthermore, HPLC-based methods lack the selectivity of LC–MS/MS methods and can consequently hinder our ability to detect adequately trace levels of the drug and metabolites. The objective of this study was to develop and validate a selective and sensitive LC–MS/MS method capable of quantifying oxycodone and its major metabolites noroxycodone and oxymorphone in human plasma in accordance with generally accepted criteria in bioanalysis [29].

2. Material and methods

2.1. Reagents

Oxycodone, noroxycodone, oxymorphone, $^{2}H_3$-oxycodone, $^{2}H_3$-noroxycodone and $^{2}H_3$-oxymorphone were purchased from Cerilliant (Round Rock, TX, USA) and received in vials containing 1.0 or 0.1 mg/mL dissolved in methanol. Drug free human plasma containing EDTA as anticoagulant was purchased from Bioreclamation (Westbury, NY, USA). Ammonium acetate and sodium hydroxide were purchased from J.T. Baker (Phillipsburg, NJ, USA). Ethanol was purchased from Commercial Alcohols (Brampton, ON, Canada). Other chemicals, including methanol and ethyl acetate were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

2.2. Instrumentation

The HPLC system consisted of a Shimadzu Prominence series UFLC pump and auto sampler (Kyoto, Japan). The tandem MS system used was a Thermo TSQ Quantum Ultra (San Jose, CA, USA). Data were acquired on a Dell Precision desktop computer (Round Rock, TX, USA) equipped with operation Windows XP professional. Data acquisition and analysis were performed using Xcalibur 2.0.7 (San Jose, CA, USA). Calibration curves were calculated from the equation $y = ax + b$, as determined by weighted $(1/x)$ and $(1/x^2)$ linear regressions of the calibration lines constructed from the peak-area ratios of the drug or metabolites to the internal standard.

2.3. Standard solutions

A series of standard working solutions of oxycodone, noroxycodone and oxymorphone were obtained by mixing the standard stock solutions and further diluting with methanol. Calibration standards were prepared by fortifying blank human plasma with the standard working solutions at 2% (v/v) to enable concentrations spanning the following analytical ranges: 300–50 000 pg/mL for oxycodone and noroxycodone and 50–10,000 pg/mL for oxymorphone. The internal standard working solution was prepared at 5.0 ng/mL for $^{2}H_3$-oxycodone, $^{2}H_3$-noroxycodone and $^{2}H_3$-oxymorphone in water.

2.4. Sample preparation

Using a liquid–liquid extraction procedure, oxycodone, noroxycodone and oxymorphone were extracted from human plasma. One hundred μL of internal standard solution (5.0 ng/mL of $^{2}H_3$-oxycodone, $^{2}H_3$-noroxycodone and $^{2}H_3$-oxymorphone in water) was added to an aliquot of 500 μL of plasma and subsequently alkalinized using 500 μL 1N sodium hydroxide solution in a 16 × 100 mm borosilicate screw cap tube. 6 mL of ethyl acetate was added to the sample and gently mixed by rotation for 20 min. The sample was then centrifuged at approximately 3500 × *g* for 10 min and the organic layer was transferred into a clean 16 × 100 mm borosilicate tube and evaporated to dryness at 50 °C under a gentle stream of nitrogen. The dried extract was resuspended with 100 μL of water and transferred to an injection vial for analysis.

2.5. Chromatographic conditions

A gradient mobile phase was used with a Phenomenex Synergi MAX-RP column (150 × 2 mm I.D., 4 μm) and C12 (4 × 2.0 mm) security guard cartridge operating at 50 °C. The initial mobile phase condition consisted of ethanol and 10 mM ammonium acetate in water at a ratio of 20:80, respectively, and this ratio was maintained for 1 min. From 1 to 6 min a linear gradient was applied up to a ratio of 75:25 and maintained from for 6 min. At 12.1 min, the mobile phase composition was reverted to 20:80 and the column was allowed to equilibrate for 5 min for a total run time of 17 min. The flow rate was fixed at 300 μL/min and oxymorphone, noroxycodone and oxycodone eluted at 8.5, 9.4 and 11.6 min, respectively. The eluent was diverted to the waste for the first five minutes. Ten microliters of the extracted sample was injected and the acquisition time was set to 15 min.

2.6. Mass spectrometry conditions

The mass spectrometer was interfaced with the HPLC system using a pneumatic assisted heated electrospray ion source. MS detection was performed in positive ion mode, using selected reaction monitoring (SRM). The SRM transitions selected for the analytes were set at 316 → 241, 302 → 187 and 302 → 227 for oxycodone, noroxycodone and oxymorphone, respectively. The SRM transitions selected for the internal standards were set at 319 → 244 for $^{2}H_3$-oxycodone and 305 → 230 for both $^{2}H_3$-noroxycodone and $^{2}H_3$-oxymorphone. In order to optimize the MS/MS parameters, standard solutions of the analytes were infused into the mass spectrometer and the following parameters were set: nitrogen was used for the sheath and auxiliary gases and was set at 35 and 20 arbitrary units; the HESI electrode was set to 3500 V; the capillary temperature was set at 350 °C and its voltage offset at 35 V; argon was used as collision gas at a pressure of 1.5 mTorr; the collision energy was set at 20 eV for all compounds; scan width for SRM was 0.5 m/z; and scan time 0.2 s; peak width of Q1 and Q3 were both set at 0.7 FWHM.

2.7. Validation procedures

The validation procedures were conducted to meet the Bioanalytical Method Validation guidelines published by the US FDA [29]. The selectivity of the assay was investigated by processing and analyzing six independent blank (drug-free) samples using our extraction procedure. Each blank sample was tested for interference, and selectivity was ensured at the lower limit of quantification (LLOQ). The LLOQ was the lowest calibration standard giving an analyte response of at least 5 times the blank signal response with acceptable precision and accuracy (within 20%). Each calibration curve consisted of eight calibration points. The intra-batch precision was evaluated as relative standard deviation (RSD) and the accuracy as relative error (RE). The determination of intra-batch precision and accuracy was performed by replicate analyses ($n = 6$) of the QC samples at three different concentrations. Similarly,