Quantitative Analysis of Naltrexone and 6β-Naltrexol in Human, Rat, and Rabbit Plasma by Liquid Chromatography–Electrospray Ionization Tandem Mass Spectrometry with Application to the Pharmacokinetics of Depotrex® in Rabbits

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Abstract

To improve the analysis of naltrexone and its primary metabolite 6β-naltrexol, a sensitive and specific method for the analysis of subnanogram-per-milliliter concentrations of these analytes in human, rat, and rabbit plasma was developed utilizing liquid chromatography (LC) coupled to electrospray ionization (ESI) tandem mass spectrometry (MS–MS). Plasma samples were extracted utilizing a liquid–liquid extraction technique. Chromatographic separation was achieved using an isocratic solvent system consisting of dilute formic acid and methanol pumped through an ODS-AQ HPLC column. ESI-MS–MS was in the positive ion mode followed by collision-induced dissociation of the protonated molecular ions for naltrexone, 6β-naltrexol, and their deuterated analogues. This method was validated using Good Laboratory Practice approved methods and was compared to an existing gas chromatography (GC)–MS method by analyzing plasma samples collected from a clinical study. Specificity determined from comparing blank plasma fortified with internal standard to samples fortified with internal standard and analyte at the lower limit of quantitation (LLOQ) from six different human, rat, and rabbit sources demonstrated sufficient signal-to-noise to set the LLOQ at 0.1 ng/mL. This assay has a quantitative range of 0.1–100 ng/mL. The inter- (human only) and intra-assay precision and accuracy in plasma varied by less than 13, 11, and 16% at the LLOQ for both analytes and by less than 10, 10, and 9% at higher concentrations for human, rat, and rabbit plasma, respectively. No loss of analyte was observed after 24 h of room temperature storage in human, rat, and rabbit plasma or three cycles of freezing and thawing of human plasma prior to extraction. Human samples that had been extracted were stable for at least five days when stored frozen at −20°C or for at least two days when stored at room temperature on an autosampler. The GC–MS and LC–MS–MS methods correlated in the measured plasma concentrations of both naltrexone and 6β-naltrexol. This method has been validated and subsequently used in the determination of the pharmacokinetics of Depotrex in rabbits. In rabbits, the parent compound shows dose-dependent pharmacokinetics as seen in humans, but rabbits have much lower unconjugated metabolite, 6β-naltrexol, than that seen in humans.

Introduction

Naltrexone is a potent mu opioid receptor antagonist first synthesized in 1965. It is approved for the treatment of both alcohol and opioid dependence. When administered orally, naltrexone is rapidly metabolized in the liver to its primary metabolite, 6β-naltrexol (Figure 1). The metabolite has weaker pharmacological properties but a longer half-life after oral administration (~ 9 h compared to ~ 2.5 h for naltrexone) (1). Oral administration of naltrexone has been associated with poor compliance; therefore, recent research has focused on the development of sustained-release forms of naltrexone, either as an injectable pellet (2–6) or a surgical implant (7).

Human studies must often be accompanied by studies in experimental animals to gain a better understanding of pharmacodynamic mechanisms and potential toxicities. These are best accompanied by pharmacokinetic studies. Much of what is known of the pharmacokinetics of naltrexone and its metabolites in experimental animals comes from earlier studies using radiolabeled naltrexone (8–11) or specific quantitation of drug and metabolite in urine (12–14). In experi-
mental animals, the plasma pharmacokinetics of naltrexone alone has been studied after intravenous (i.v.) (14-16), oral (14), and sustained-release (11,17,18) administration. Information on 6β-naltrexol plasma concentrations is currently lacking.

Recently published methods for the analysis of naltrexone and 6β-naltrexol utilize gas chromatography coupled to mass spectrometry (GC–MS) (19-21), liquid chromatography (LC)–electrochemical detection (7), LC–MS (22), or GC–tandem mass spectrometry (GC–MS–MS) (23). Though providing excellent sensitivity, these methods typically require extensive sample cleanup steps and derivatization prior to analysis. We sought to develop and validate a method utilizing LC coupled to electrospray ionization (ESI)-MS–MS. Use of LC–MS–MS to determine naltrexone and 6β-naltrexol has been described in a few clinical papers, but no validation experiments have accompanied these descriptions (4,5). Our current method has been extensively validated in human plasma under Good Laboratory Practice (GLP) guidelines (24) with cross-validation to the rat and rabbit plasma matrices. The goal of this work was to take advantage of the increased specificity offered by MS–MS. In doing so, sample preparation could be simplified, increasing the overall efficiency of the analysis. To further demonstrate the utility of this method, plasma samples collected from a recently published study (3) were used to compare the LC–MS–MS method with our previously published GC–MS method (20). The method has now been used to study the dose-dependent pharmacokinetics of naltrexone and 6β-naltrexol in rabbits administered a single dose of the currently available 30-day sustained-release naltrexone formulation, Depotrex.

Materials and Methods

Chemicals

Naltrexone hydrochloride was purchased from Sigma (St. Louis, MO). Naltrexone-d₃, 6β-naltrexol, and 6β-naltrexol-d₇ were prepared by Research Triangle Institute (Research Triangle Park, NC) and provided by the National Institute on Drug Abuse. HPLC-grade butyl chloride, acetonitrile, and methanol were purchased from J.T. Baker (Phillipsburg, NJ). Concentrated ammonium hydroxide and concentrated (88%) formic acid were purchased from Fisher (Fair Lawn, NJ). All aqueous solutions and LC mobile phase were prepared in Milli-Q grade water (Millipore, Bedford, MA). The Depotrex 30-day naltrexone microcapsule formulation was prepared by Biotek (Woburn, MA). The microcapsules ranged from 105 to 150 microns in diameter and consisted of naltrexone base and poly L-lactide polymer.

Preparation of stock solutions, calibrators, and quality control samples

Stock solutions containing naltrexone and 6β-naltrexol (1 mg/mL calculated as the free base) were prepared in methanol and stored at -20°C. Tenfold serial dilutions of the 1 mg/mL stock solution were used to prepare spiking solutions at concentrations of 10, 1, 0.1, 0.01, and 0.001 μg/mL of naltrexone and 6β-naltrexol. These spiking solutions were used daily to prepare calibration samples. Calibration curves were obtained by analyzing drug-free human plasma (preserved with 1% NaF) fortified with naltrexone and 6β-naltrexol at 0.1, 0.5, 1, 5, 10, 40, 75, and 100 ng/mL (n = 2 at each concentration). Lower limit of quantitation (LLOQ) samples (0.1 ng/mL) and quality control (QC) samples (0.3, 5, and 75 ng/mL) were prepared from working solutions (prepared in a similar manner as described) made with reference materials with lot numbers that were different from the reference materials used to prepare the calibration standards. QC samples were prepared in a single batch, and 1-mL aliquots were prepared and stored at -20°C until use. All calibrators, QCs, and samples were prepared in silanized glass screw-cap culture tubes. Human plasma was obtained from the local blood bank. Rat and rabbit plasma for QCs and calibrators were purchased from Biochemed Pharmacologicals (Winchester, VA).

LC–MS–MS method

Sample preparation and extraction. One milliliter of calibrator, QC, or plasma sample was pipetted into labeled, silanized glass tubes and allowed to equilibrate to room temperature prior to extraction. Internal standard (5 ng/mL of naltrexone-d₃ and 6β-naltrexol-d₇; 50 μL of a 0.1 μg/mL solution) was added to each sample. Samples were vortex mixed.
analyte concentrations were calculated by dividing the average peak area at each concentration from the extracted samples by the average peak area for each concentration from the unextracted samples and multiplying by 100.

**Precision and accuracy experiments.** Intra-assay precision and accuracy were evaluated by analyzing QC samples at each concentration (n = 5) and determining the % of target (calculated concentration/target concentration x 100) and the % coefficient of variation (%CV, standard deviation/mean concentration x 100) for each analyte at each concentration. Interassay precision and accuracy were determined from five analytical runs. This included data from the intra-assay precision and accuracy experiment and four others with QCs at n = 3 for each concentration. The mean QC values for each concentration, in each run, were used to determine % target and %CV among the five analytical runs. For human plasma, the LLOQ results were from QCs prepared at 0.1 ng/mL and handled as described. For rat and rabbit plasma, intrarun precision and accuracy at the LLOQ was determined from the six different sources of plasma per species used in the specificity experiments that were fortified at the LLOQ.

**Stability experiments.** For freeze-thaw stability experiments, human QC samples at 0.3 and 75 ng/mL were stored at −20°C for at least 12 h. Samples were removed and allowed to thaw unassisted at room temperature. This cycle was repeated two more times prior to the day of extraction and analysis. Samples were analyzed and quantified using a freshly prepared calibration curve and compared to target concentrations to determine stability in the freeze-thaw cycled samples.

**QC samples used for determining bench-top stability at room temperature were thawed at 24, 5, or 3 h (human plasma) and at 24 h (rat and rabbit plasma) prior to extraction. Quantitative results for the 24, 5, and 3 h time points were compared to target concentrations to determine any loss of analyte.**

**Human QC samples at 0.3 and 75 µg/mL were used for processed sample stability and prepared as described. The analyzed extracts were then stored at either −20°C or at room temperature on the autosampler to determine processed sample storage limits. Samples were re-injected with a freshly extracted calibration curve and quantified using that curve to
evaluate stability in the stored samples.

To determine stock solution stability, stock solutions prepared at the beginning of method development were compared to stock solution prepared at the conclusion of validation experiments (total time: 115 days). Stability of stock solutions at room temperature was also evaluated by comparing aliquots of freshly prepared stock solution held at room temperature for up to 22 h with freshly prepared stock solution prepared on the day of analysis. The methanolic stock solutions were appropriately diluted and analyzed directly on the LC–MS–MS without extraction.

**Ion suppression.** Aliquots of drug-free human plasma from five individuals were extracted according to the method described above. The LC–MS–MS was plumbed to allow for a post LC column infusion of 1 µg/mL naltrexone and 6β-naltrexol and their deuterated internal standards (28). The infusion rate was 10 µL/min. The extracted samples were injected into the instrument under analytical conditions described. Any ion suppression is indicated by a depression in the baseline generated by the infused naltrexone/6β-naltrexol mixture. Any observed ion suppression at the retention times of the analytes of interest may warrant further evaluation of chromatographic conditions, internal standards, or limits of detection.

**Clinical sample experiments**

**Human sustained-release naltrexone.** The samples used for methods comparison in the present study were collected from an IRB-approved clinical protocol designed to evaluate the safety, time course, and effectiveness of a sustained-release formulation of naltrexone (Depotrex) in antagonizing the effects of intravenously administered heroin (3). Twelve heroin-dependent individuals participated in an eight-week inpatient study. After a one-week detoxification period, six participants received 192-mg naltrexone base and six participants received 384-mg naltrexone base. For safety, the low dose of sustained-release naltrexone was tested before the high dose. Further details of the experimental protocol are available in the primary publication (3). Blood was collected in heparinized (green top) Vacutainer tubes. Plasma was separated by centrifugation, transferred to sterile vials, and stored frozen until shipped to our laboratory by overnight courier. Samples were then stored in our laboratory at −20°C until analysis. Plasma samples collected from three participants who received the 384-mg dose of naltrexone were re-analyzed using the LC–MS–MS method described here. The results obtained using the LC–MS–MS method were compared to the results obtained using our previously published GC–MS method (20).

**Rabbit sustained-release naltrexone.** The Depotrex 30-day naltrexone formulation was tested at two dose levels (40 mg/kg and 80 mg/kg) in a group of five adult female SPF New Zealand White rabbits per dose. Each rabbit weighed at least 3.0 kg. The required amount of suspending medium was added to the vials containing the microcapsules. The suspension was vortex mixed for 15 s to ensure uniformity and then injected subcutaneously in two sites in each rabbit using an 18-gauge 1-in. needle. Blood collection tubes were heparinized prior to use. Four-milliliter blood samples were collected just prior to injection and on days 1, 3, 6, 9, 13, 16, 20, 23, 27, 30, 34, 37, 41, 44, 48, and 51. The blood was collected from the marginal ear vein of the rabbit, centrifuged at 2500 rpm for 10–15 min, and the plasma collected. Plasma samples were stored frozen until tested.

**Pharmacokinetics.** Noncompartmental pharmacokinetics were used to evaluate the rabbit plasma results. The maximal plasma concentration (C_max) and the time to maximal plasma concentration (T_max) were determined by inspection. The area under the plasma concentration versus time curve (AUC) were determined using the trapezoidal rule.

[Image of ion chromatograms]

**Figure 2.** Ion chromatograms for an extracted plasma sample fortified with 5 ng/mL of deuterated internal standard. The x-axis represents retention time in minutes; the y-axis represents signal intensity. The “NL” value gives absolute intensity counts which can be used to estimate and compare signal-to-noise ratios. Note: Although naltrexone and 6β-naltrexol were not added, peaks at their retention time did have sufficient shape to be partially integrated. This was much lower than peak areas for LLOQ (see Specificity experiments).

**Results and Discussion**

**Mass spectra and chromatography**

Fragment ions monitored correspond to a loss of water from the protonated molecular ions of naltrexone and 6β-naltrexol (Figures 1A and 1B). Adequate chromatographic separation of naltrexone and 6β-naltrexol was achieved using the mobile phase presented. Some slight elevation of the baseline was observed preceding the elution of naltrexone and naltrexone-d3 but this did not compromise the ability to properly validate the method. Figure 2 shows an extracted ion chromatogram from an extracted blank human plasma sample demonstrating that no discernable interference is attributable to the plasma matrix. Figure 3 shows an extracted ion chromatogram from a human sample fortified at the LLOQ, 0.1 ng/mL. All chromatograms show gaussian peak shape and an estimated
An ODS-AQ column was selected because it was able to more efficiently resolve both analytes within a reasonable time frame compared to other C18 bonded phases tested.

Although it may be argued that chromatographic separation is not needed when analytes can be separated by their MS–MS transitions, distinct retention times give the assay an increased reliability and quality control. Retention factors (k) greater than 2 for analytes (naltrexone, k = 2.6; 6β-naltrexol, k = 3.8) also helps ensure that all analytes are being separated from the unretained fraction, thus minimizing the potential for ion suppression, which is common in this fraction. Chromatographic separation coupled with SRM MS–MS analysis also minimizes the potential for interferences from co-administered drugs or endogenous compounds that may increase in disease states. We have shown previously that the coupling of chromatographic separation and mass analysis by SRM produce analytical methods free of interference from a number of drugs commonly co-administered with anti-abuse medications like naltrexone (25,26,29).

It is noteworthy that the monitoring of a single MS–MS transition (SRM) was selected in this study to optimize reliability and signal-to-noise ratio of the targeted, quantitative analytical nature of this study. If a qualitative identification of unknown or undocumented drug use is desired, then this method should be modified to collect additional MS–MS product ion data (i.e., qualifier ions) in order to reliably identify the unknown drug. While this type of approach is commonly used in forensic drug testing situations, the goals of this study were different.

Ion suppression was not a problematic factor in the development and validation of this method. Experiments designed explicitly to rule out ion suppression were performed, and no deleterious suppression was observed in plasma from five sources (Figure 4). The use of a deuterated internal standard for both analytes also mitigates any possible ion suppression effects in individual samples. Although the absolute response may be diminished in a sample with ion suppressing co-eluents, the ratio of analyte to internal standard would not be expected to change, thus negating any possible deleterious effect of ion suppressing co-eluents. In the case where a significant, unexpected ion suppression event occurs, a depression in internal standard signal would likely be also be seen and thus an investigation into the cause of the suppression could occur. No examples of this were observed in the experiments of this study.

Previously published work by a co-author of this study reported that isotope-labeled internal standards may actually suppress the response of unlabelled analogues and vice versa under electrospray ionization (30). The authors conclude that this phenomenon could influence sensitivity, reproducibility, linearity, etc. The compiled validation data presented here...
would indicate this phenomenon was not a problem in the present study.

Method validation

Specificity and recovery. Experiments designed to evaluate the potential for quantitative interference due to endogenous material in the plasma indicated that no substantial interference at the retention times of the analytes was observed. When ion current signals at the retention times of naltrexone and 6β-naltrexol were manually integrated and the resulting peak area ratios were compared to that of the aqueous LLOQ sample (human), no signal greater than 11.6% and 6.5% of the naltrexone and 6β-naltrexol LLOQs, respectively, was observed in the six individual plasma lots examined (Table I). Figure 2 shows a representative extracted blank chromatogram fortified with internal standards only. Similar experiments performed using rat and rabbit plasma compared against plasma from the same sources fortified with naltrexone and 6β-naltrexol at the LLOQ had even smaller peaks at the retention time of the analytes (Table I).

Average recovery for naltrexone and 6β-naltrexol at three different concentrations from human plasma was 83.3% and 45.7%, respectively. Although the extraction recovery of 6β-naltrexol would not be considered ideal (likely due to a change in polarity of the metabolite), sufficient signal-to-noise ratios were obtained from the MS–MS analysis to permit a reproducible LLOQ of 0.1 ng/mL. This underscores the value of MS–MS in trace analysis in biological specimens in that sensitive and specific analytical instruments can overcome certain limitations within the sample preparation process.

Precision and accuracy. QC concentrations were selected based upon recommendations given in the FDA guidelines for bioanalytical method validation (24). The low QC was selected because its target concentration is less than 3× the LLOQ; the high QC was selected because its target concentration is less than 3× the LLOQ; the high QC was selected because its target concentration is within

<table>
<thead>
<tr>
<th>Source Number</th>
<th>Mean peak-area ratio as % of LLOQ</th>
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<tr>
<td>Human plasma</td>
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<td>6β-Naltrexol</td>
<td>5.13</td>
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Table I. Specificity for Naltrexone and 6β-Naltrexol in Human, Rat, and Rabbit Plasma

Table II. Intra-assay Accuracy and Precision for Determination of Naltrexone and 6β-Naltrexol in Human, Rat, and Rabbit Plasma and Interassay in Human Plasma

Table III. Stability of Naltrexone and 6β-Naltrexol in Human Plasma and Processed Human Samples and Rat and Rabbit Plasma

* Values are from the mean of three replicates of the matrix source, which were fortified with internal standard and had peak areas determined for any signal within the retention time of respective -d0 analyte peak width. Values were compared to the mean peak-area ratio of six samples, one from each source, fortified with analyte at the LLOQ and internal standard.

* Intra-assay values are the from the mean of five replicates for QCs; for the LLOQ they are from the mean of six differences sources of matrix each fortified at the LLOQ. Interassay values are from the means for 5 analytical runs, the first run at N = 5 (N = 6 for LLOQ), the others at N = 3.
30% of the upper limit of the calibration curve. No specific guidelines are presented for the selection of the middle QC (24); 5 ng/mL was selected as this was the same concentration as the median calibrator. Intra-assay precision and accuracy is summarized in Table II. The mean measured concentrations of both analytes are within 2, 11, and 1% of the target values at the human, rat, and rabbit LLOQs, respectively, and within 8, 7, and 8% of the target value at the human, rat, and rabbit higher QC concentrations, respectively. Respective coefficients of variation were less than 13, 6, and 16% for the LLOQ and less than 10, 6, and 7% for the higher QC concentrations (Table II). Inter-assay precision and accuracy in human plasma were also acceptable (Table II). Both analytes were within 4% of target and had %CV less than 12% at the LLOQ. Higher QC concentrations were within 9% of target and had %CV values that were within 7%.

**Stability.** These experiments were designed to evaluate the handling conditions that mimic normal laboratory processing. Results of these experiments are summarized in Table III. Naltrexone was found to be stable in human plasma at room temperature for up to 24 h. Less than an 8% loss of analyte was observed in the 0.3-ng/mL sample-set after 24 h. For 6β-naltrexol, the 0.3-ng/mL sample-set quantitated to within 87% of the target concentration. After three freeze-thaw cycles, naltrexone quantitated to within 92% of target at 0.3 ng/mL and 6β-naltrexol quantitated to within 88% of the target concentration. The 75 ng/mL stability samples quantitated even closer to target (Table III). Extracted sample residues were also shown to be stable for at least five days when stored at -20°C or at least two days at ambient temperature on the autosampler (Table III). Rat and rabbit low and high QCs stored at room temperature for 24 h also had results within 14% of target concentrations (Table III). Stability of the stock solutions was determined to be acceptable for at least 22 h at room temperature and at least 115 days when stored at -20°C.

**Clinical samples**  
**Humans treated with sustained-release naltrexone.** The validated method presented here was compared with an existing GC–MS method (20) to determine the cross validity between
analytical techniques. Samples used for this comparison were plasma samples collected from a clinical protocol investigating the use of a sustained-release formulation of naltrexone (Depotrex) as a treatment for heroin dependence (3). Samples had been initially analyzed by GC–MS (20), and a subset from three participants receiving high-dose naltrexone was re-analyzed by this LC–MS–MS method. Reanalysis was performed within 12 months, a time period for which samples had been collected to investigate the pharmacokinetics of a currently available Depotrex formulation.

Rabbits treated with sustained-release naltrexone. To further explore the pharmacokinetics of the Depotrex formulation, female rabbits (N = 5 per dose) were injected with either 40 or 80 mg/kg. The time profile of naltrexone and 6β-naltrexol are shown in Figures 6A and 6B. Mean naltrexone plasma concentrations were highest at the first blood collection (day 1), but that varied with rabbits, more so with the higher dose (Table IV). Naltrexone concentrations declined slowly with fairly high concentrations maintained out to 20 days, at which time a more rapid decline occurred with concentrations dropping below 1.0 ng/mL at approximately 34 days (Figure 6A). 6β-Naltrexol showed a similar pattern, but at concentrations only 1/30th of the parent compound (Figure 6B). The pharmacokinetic parameters of both naltrexone and 6β-naltrexol were dose-dependent, with significant increases in the Cmax and AUC (Table IV). When comparing the graphed plasma concentrations seen in humans (3), similar dose-dependent increases in plasma naltrexone were found in our current study of rabbits. A major difference between rabbits and humans is the much lower concentration of the metabolite 6β-naltrexol in rabbits. As shown here (Figure 5) and described by others (3–5,7), 6β-naltrexol concentrations are higher than naltrexone concentrations in humans given the injected or implanted sustained-release formulations of naltrexone. The smaller formation of 6β-naltrexol from naltrexone in rabbits is consistent with earlier studies (10,12).

### Conclusions

In summary, this report describes a sensitive and specific method for the analysis of naltrexone and its principle metabolite, 6β-naltrexol, in plasma using LC–MS–MS. The assay has an LLOQ of 0.1 ng/mL for both analytes with good quantitative precision, accuracy, and stability under a number of storage and processing conditions intended to resemble actual sample handling. This method has the advantage of no derivatization requirement as is the case with GC–MS methods. Also the limits of quantitation are low enough to detect trace concentrations of both drug and metabolite making it useful in low dosage studies or long-term single-dose sustained-release studies involving naltrexone. The simple extraction scheme and relatively short analysis time allow for a fairly high throughput of samples. This method has been validated and used in the analysis of both human clinical and experimental animal samples collected to investigate the pharmacokinetics of naltrexone and 6β-naltrexol and the use of naltrexone as an effective therapeutic agent in the treatment of opioid and alcohol dependence. The studies in rabbits represent the first study in experimental animals to follow both naltrexone and 6β-naltrexol plasma concentrations and the only study in experimental animals on the pharmacokinetics of a currently available Depotrex formulation.

### Acknowledgment

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