Journal of Chromatography B, 874 (2008) 33–41

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

An automated, highly sensitive LC-MS/MS assay for the quantification of the opiate antagonist naltrexone and its major metabolite 6β-naltrexol in dog and human plasma

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ARTICLE INFO

Article history:
Received 30 January 2008
Accepted 13 August 2008
Available online 5 September 2008

Keywords:
Naltrexone
6β-Naltrexol
LC-MS/MS
Column switching
Dog plasma
Human plasma

ABSTRACT

To support animal studies and clinical pharmacokinetic trials, we developed and validated an automated, specific and highly sensitive LC-MS/MS method for the quantification of naltrexone and 6β-naltrexol in the same run. In human plasma, the assay had a lower limit of quantitation of only 5 pg/mL. This was of critical importance to follow naltrexone pharmacokinetics during its terminal elimination phase. The assay had the following key performance characteristics for naltrexone in human plasma: range of reliable quantification: 0.005–100 ng/mL ($r^2 > 0.99$), inter-day accuracy (0.03 ng/mL): 103.7% and inter-day precision: 10.1%. There were no ion suppression, matrix interferences or carry-over.

1. Introduction

Naltrexone, a pure competitive antagonist at opioid receptor sites [1], has been used in the treatment of addiction, including heroin [2], alcohol [3] and gambling [4]. It has also been used for rapid and ultra-rapid opiate withdrawal [5] with success attributed to its antagonistic potency of 2.5 and 12 times that of naloxone and nalorphine, respectively [6].

6β-Naltrexol is the major metabolite in human plasma (Fig. 1). Although it has weaker opioid antagonistic properties, 6β-naltrexol makes a significant contribution to the overall naltrexone effects after oral administration, potentially due to its 10-fold higher systemic exposure compared to its parent.

Several HPLC assays in combination with UV, electrochemical or mass spectrometry detection have been reported for the quantification of naltrexone and, in some cases, its metabolite 6β-naltrexol [7–12]. All of these assays had either similarly complex multi-step offline extraction procedures and/or relatively high lower limits of quantitation of approximately 0.1 ng/mL (LC-MS) or worse (HPLC-UV). This sensitivity may not be sufficient since naltrexone pharmacokinetics is probably best described by a three-compartment model [13] and naltrexone concentrations during the terminal elimination phase are significantly lower than 0.1 ng/mL. In addition, it has been described that naltrexone plasma concentrations below 0.1 ng/mL are still efficacious [14,15].

Recently, an LC-MS/MS assay with a lower limit of quantitation of 10 pg/mL for naltrexone was reported [16]. However, this assay was only developed for dog plasma and did not include the major metabolite 6β-naltrexol that, as mentioned above, may contribute to the clinical activity of naltrexone in humans. This method also used a liquid–liquid extraction in combination with an evaporation step.

To support animal studies and clinical pharmacological trials, we developed and validated an automated LC-MS/MS method for the quantification of naltrexone and its major metabolite 6β-naltrexol within the same run that was sensitive enough to allow for measuring naltrexone plasma concentrations during the terminal elimination phase and after intra-muscular injection of naltrexone sustained release formulations in humans and dogs, the major

Abbreviations: APCI, atmospheric pressure chemical ionization; EDTA, ethylene diamino tetra acetylic acid; LLOQ, lower limit of quantitation; MRM, multiple reaction monitoring; *m/z*, mass/charge; QC, quality control; R.S.D.%, residual standard deviation in %.

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doi:10.1016/j.jchromb.2008.08.021

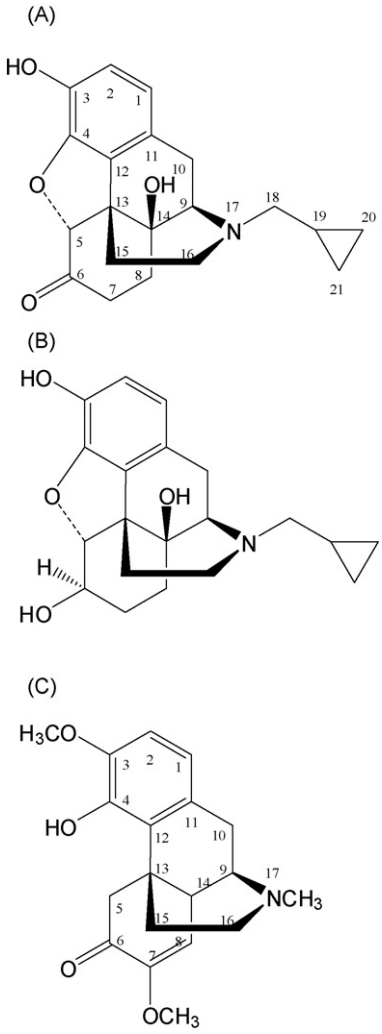

Fig. 1. Structures of (A) naltrexone, (B) 6β-naltrexol and (C) the internal standard sinomenine.

animal model for studying pharmacokinetics after injection of naltrexone sustained release formulations. This assay involved one step-protein precipitation in 96-well plates and a fast online column switching extraction step [17].

2. Materials and methods

2.1. Chemicals and reagents

Solvents and reagents (HPLC-grade methanol and water, formic acid 88%, zinc sulfate) used for sample extraction and as mobile phase were from Fisher Scientific (Fair Lawn, NJ) and used without further purification. Naltrexone and 6β-naltrexol were purchased from Tyco-Mallinckrodt Chemical (St. Louis, MO). The structurally similar opioid sinomenine was used as the internal standard and was purchased from Sigma–Aldrich. The structures of naltrexone, 6β-naltrexol and the internal standard sinomenine are shown in Fig. 1.

2.2. Calibrators and quality control samples

Naltrexone, 6β-naltrexol and the internal standard stock solutions were prepared after three independent weightings. Working solutions for quality control samples, standard curve and the internal standard solution were prepared by dilution of the stock solution in methanol.

Human EDTA plasma samples were obtained from healthy volunteers. Collection of human blood samples from outdated blood bank samples for assay validation and quality control was considered exempt by the Colorado Multi-Institutional Review Board (COMIRB), Denver, Colorado. EDTA dog plasma was obtained from MPI Research (Mattawan, MI) and blank plasma collection was part of an approved animal protocol. The protein precipitation/internal standard solution (methanol/0.2 M $ZnSO_4$, 7:3, v/v, 8 ng/mL sinomenine) was prepared freshly before every extraction. The expiration time for the protein precipitation solution was set to 12 h and the solution was discarded hereafter.

Calibration and quality control samples were prepared by enriching EDTA plasma samples with naltrexone and 6β-naltrexol.

2.3. Sample extraction

The extraction procedure consisted of two steps: a protein precipitation and subsequent online column extraction. The only manual step during the extraction of samples was protein precipitation. Online extraction was based on automated column switching after injection of the supernatant into the HPLC system. Four hundred microliters of protein precipitation solution containing the internal standard (*vide supra*) was added to 100 μL plasma in 96-well plates with 1 mL wells (Agilent Technologies, Santa Clara, CA). Hereafter, the 96-well plates were covered, vortexed (5 min) and centrifuged (4 °C, 13,000 × *g*, 5 min). The 96-well plates were placed into the HPLC autosampler. The online extraction step is described below.

2.4. Equipment

The extracts were analyzed using an LC-MS/MS system in combination with online extraction (LC/LC-MS/MS). The system consisted of the following components: Two G1312A binary pumps, two G1322A vacuum degassers, and a G1316A thermostatted column compartment (all Agilent 1100 series, Agilent Technologies, Santa Clara, CA) in combination with a CTC/PAL thermostatted autosampler (Zwingen, Switzerland) and a 6-port Rheodyne column switching valve (EHMA 055-1431V) mounted on a remote control step motor (Rheodyne, Cotati, CA). The loop volume was 500 μL. The connections of the switching valve are shown in Fig. 2. A Sciex API 5000 triple-stage quadrupole mass spectrometer was used as detector (Applied Biosystems, Foster City, CA). The HPLCs, the switching valve, and the mass spectrometer were controlled by the Analyst software (version 1.4.1).

2.5. LC/LC-MS/MS analysis

One hundred microliters of the sample supernatant was injected onto the online extraction column (12.5 mm × 4.6 mm, 5 μm particle size, C18, Zorbax XDB, Agilent Technologies). The needle of the autosampler was adjusted not to aspire any of the precipitated proteins. Samples were washed with a mobile phase of 20% methanol (containing 0.1% acetic acid) and 80% 0.1% acetic acid in 2 mmol/L ammonium acetate. The flow was 5 mL/min and the temperature for the extraction column was set to 65 °C. After 1 min, the switching valve was activated and the analytes were eluted in the backflush mode from the extraction column onto a 30 mm × 4.6 mm analytical column filled with CN material of 5 μm particle size (Luna, Phenomenex, Torrance, CA). The mobile phase consisted of acetic acid in methanol and 0.1% acetic acid in 2 mmol/L ammonium acetate. The following gradient was run: time 0 min: 40% 0.1% acetic acid + methanol, 4.5 min: 95% 0.1%

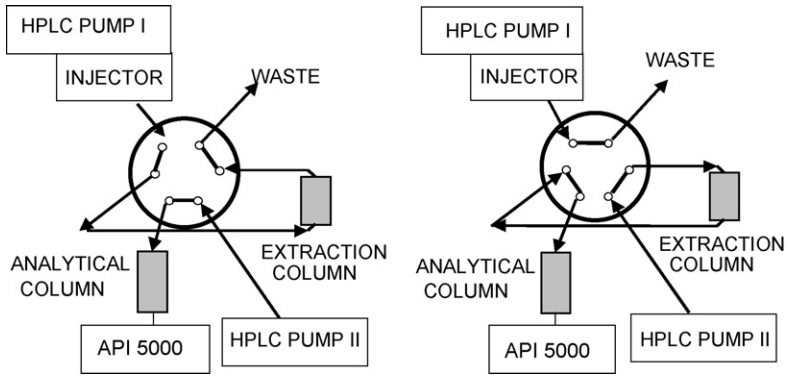

Fig. 2. Connections and positions of the column switching valve.

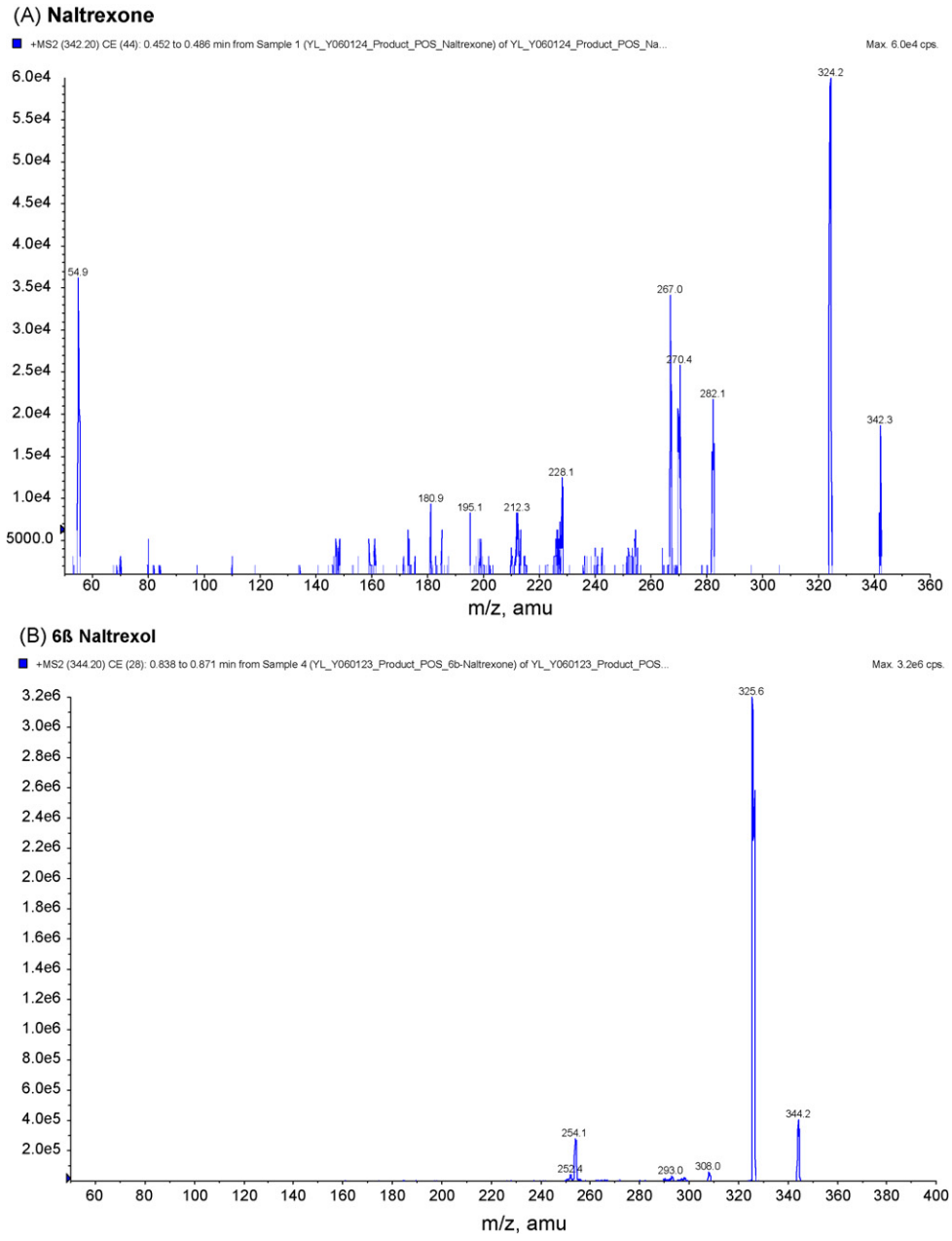

Fig. 3. Product ion scans of naltrexone (A), 6β-naltrexol (B) and the internal standard sinomenine (C). An APCI interface was used.

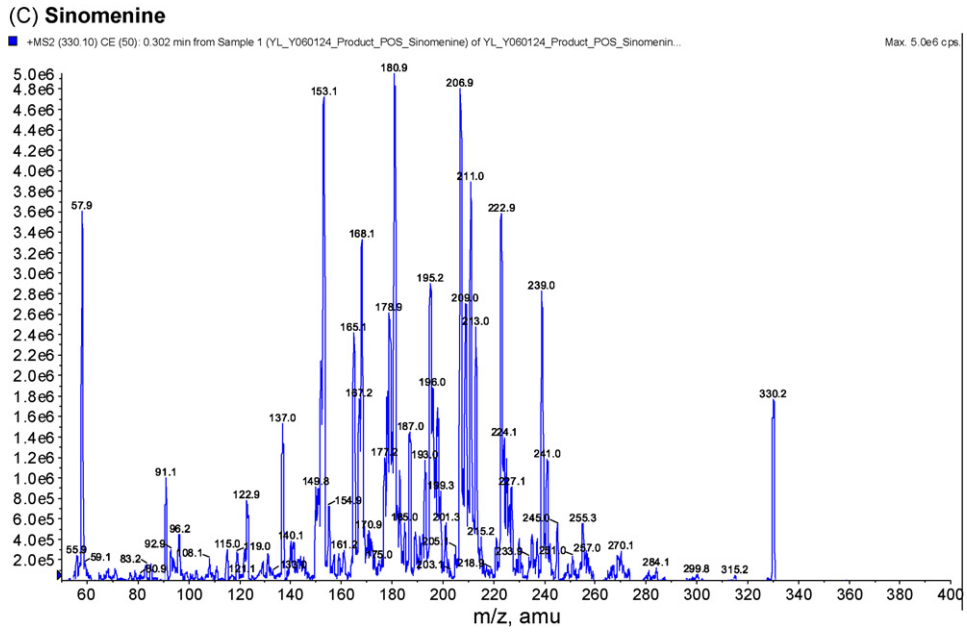

Fig. 3. (*Continued*)

acetic acid + methanol. Hereafter, the column was washed with 95% methanol 0.1% acetic acid for 1.5 min and re-equilibrated to starting conditions during injection and extraction of the next sample. The flow rate was 1 mL/min and the analytical column was kept at 65 °C. The mass spectrometry signal was recorded starting after injection and the total run time was 6 min including the time for online extraction.

The HPLC system was interfaced with the mass spectrometer with an atmospheric pressure chemical ionization (APCI) source. Nitrogen (purity: 99.999%) was used as collision-activated dissociation gas. The mass spectrometer was run in the positive multiple reaction monitoring (MRM) mode. The declustering potential was set to 150 V, the entrance potential (EP) to 10 V, the interface to 300 °C, and the collision energy to 35 eV. The following ion transition was monitored: $m/z = 342.3$ $[M+H]^+ \rightarrow 324.1$, for 6β-naltrexol $m/z = 344.3$ $[M+H]^+ \rightarrow 326.1$ and for the internal standard sinomenine $m/z = 330.2$ $[M+H]^+ \rightarrow 180.9$.

Calibration curves were constructed using non-weighted linear regression by plotting nominal concentration versus analyte area/IS area ratios. Naltrexone and 6β-naltrexol concentrations were quantified using the calibration curves that were included in each batch.

Calibration standards and quality control samples were prepared by spiking blank dog or human plasma with naltrexone and 6β-naltrexol. In addition to blank and zero samples, the calibration curves, were constructed by enriching blank plasma samples with the following naltrexone and 6β-naltrexol concentrations: 0.005, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 25, 50, and 100 ng/mL. During pre-study validation, six calibration curves were analyzed in six different runs. Quality control (QC) samples were prepared at four concentration levels (0.03, 0.3, 3 and 30 ng/mL).

2.6. Validation procedures

The assay was validated using enriched EDTA plasma samples from dogs and healthy humans following the guidelines for bioanalytical method validation by the FDA Center for Drug Evaluation and Research [18]. While the method was primarily validated for human plasma, an abbreviated validation strategy was used for dog plasma (determination of lower limit of quantitation, range of reliable response, intra-day precision and accuracy as well as exclusion of ion suppression) to demonstrate that the change in matrix did not affect assay performance.

2.6.1. Predefined acceptance criteria

The assay was considered acceptable if precision at each concentration with the exception of the lower limit of quantitation (*vide infra*) was ≤15% for intra-day variability. The accuracy had to be within ±15% of the nominal value for both intra- and inter-day variability. The calibration curve had to have a correlation coefficient $r^2 \geq 0.99$.

2.6.2. Lower limit of quantitation (LLOQ)

The LLOQ was determined as the lowest concentration of the calibration curve consistently achieving accuracy ≤±20% of the nominal concentration and a precision ≤20%.

2.6.3. Precision and accuracy

The method was validated using human and dog plasma. The intra-day precision and accuracy were determined based on the quality control (QC) samples containing 0.03, 0.3, 3 and 30 ng/mL naltrexone and 6β-naltrexol ($n = 6$/concentration). Determination of intra-day and inter-day accuracies and precisions was also based on QC samples. Samples were extracted and analyzed on five different days ($n = 6$/concentration and day). Precision is reported as relative standard deviations in % (as estimated by analysis of variance) and accuracy in % of the nominal concentration. The SPSS software (version 15.0, SPSS Inc., Chicago, IL) was used for statistical analyses.

2.6.4. Absolute recoveries

Absolute recoveries were determined by comparing the signals for naltrexone and 6β-naltrexol after extraction of the quality control samples ($n = 6$/concentration) with the signals of extracted blank matrix spiked with the respective concentration of naltrexone and 6β-naltrexol after the extraction procedure.

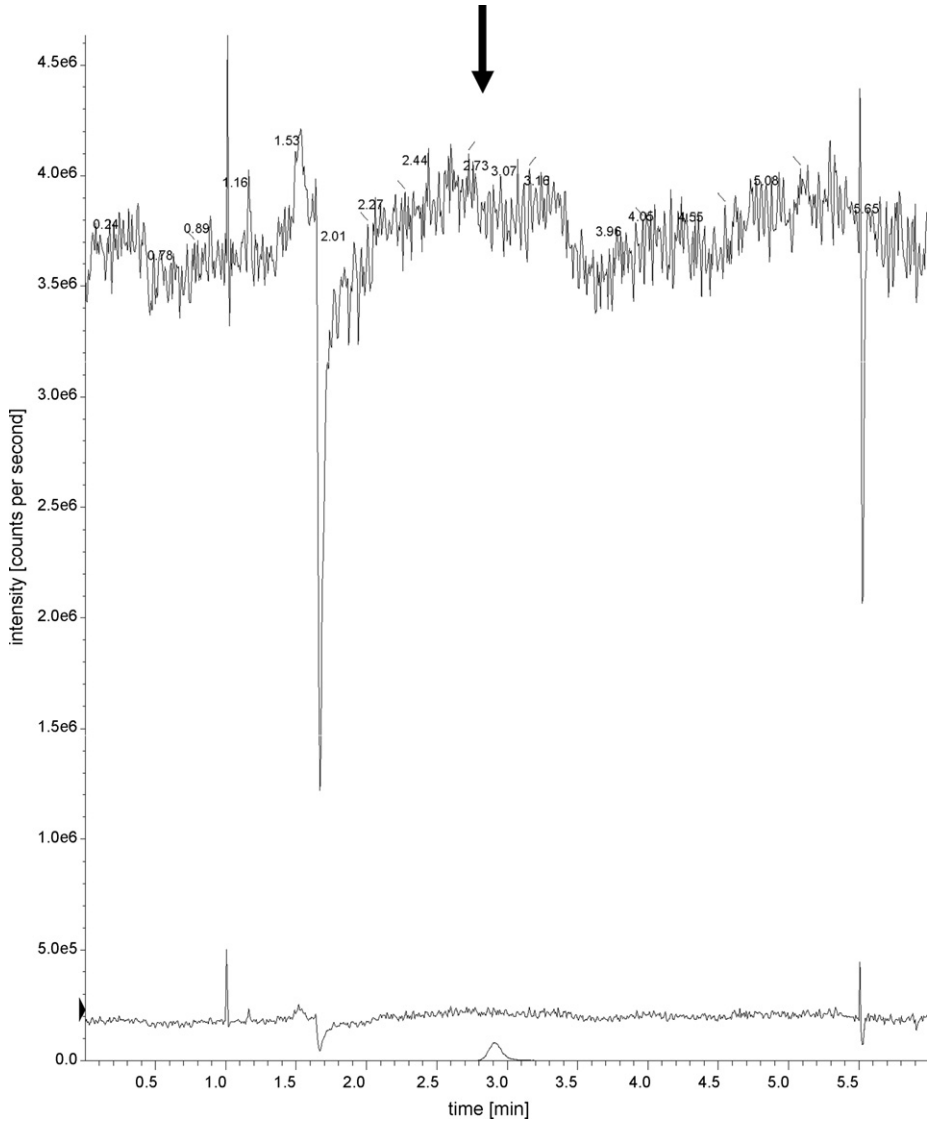

Fig. 4. Lack of ion suppression. A representative experiment of a total of $n = 10$ is shown. After protein precipitation samples were extracted online and back-flushed onto the analytical column. Naltrexone or 6β-naltrexol (10 μg/mL dissolved in 0.1 acetic acid/methanol, 1:4, v/v) was infused post-column *via* T-piece at 10 μL/min using a syringe pump (Harvard Apparatus, Holliston, MA). The lack of ion suppression at the time of elution was established by monitoring the intensity of the ion currents in MRM-mode at the retention times of the analyte and internal standard following the procedure described by Müller et al. [19]. The representative ion chromatograms show the ion suppression experiment on top. In the absence of ion suppression, the continuous post-column infusion of naltrexone should result in a constant signal. Significant "dips" indicate ion suppression. The arrow marks the retention time of the naltrexone peak. The bottom ion chromatograms show the same blank sample after injection without constant naltrexone infusion and a blank sample enriched with 0.03 ng/mL naltrexone.

2.6.5. Matrix interferences, ion suppression, and carry-over effect

To exclude interferences from the matrices and changes in ionization efficiency by co-eluting matrix compounds, human EDTA plasma samples from 10 different healthy individuals were extracted and analyzed. After protein precipitation samples were extracted online and back-flushed onto the analytical column. Naltrexone and 6β-naltrexol (10 μg/mL dissolved in 0.1 acetic acid/methanol, 1:4, v/v) was infused post-column *via* T-piece at 10 μL/min using a syringe pump (Harvard Apparatus, Holliston, MA). The lack of ion suppression at the time of elution was established by monitoring the intensity of the ion currents in MRM-mode at the retention times of the analyte and internal standard following the procedure described by Müller et al. [19]. Potential sample carry-over was assessed by analyzing plasma samples spiked with concentrations of naltrexone and 6β-naltrexol at the upper limit of quantitation (100 ng/mL, $n = 6$ each) followed by blank samples.

2.6.6. Stability studies

Stability studies were based on quality control samples at four different concentration levels (0.03, 0.3, 3 and 30 ng/mL). Stability of non-extracted enriched EDTA plasma samples during up to three freeze/thaw cycles was tested ($n = 6$/concentration level per cycle). Samples were kept frozen at -80 °C and thawed at room temperature. Stability of naltrexone, 6β-naltrexol and the internal standard in extracted samples was tested at 0.03, 0.3, 3 and 30 ng/mL ($n = 6$/concentration and experiment). To establish in-process stability of samples after protein precipitation, samples were kept at either −80, −20, and +4 °C or at room temperature. After 12, 24 and 48 h samples ($n = 6$ per temperature group and time point) were extracted, analyzed and compared with freshly extracted samples. Samples were considered stable when the concentrations fell within a range of ±15% of the concentrations measured in reference samples of the same concentration and from the same QC lot measured immediately after preparation.

2.6.7. Dilution integrity

Dilution integrity was established using freshly prepared EDTA plasma samples enriched with 25ng/mL naltrexone and 6β-naltrexol. Dilutions (1:2, 1:4, 1:8, 1:16, 1:32 and 1:64, $n = 6$) were made using blank EDTA plasma before protein precipitation. Deviations from the nominal concentrations after dilution were calculated. Results falling into a range of 85–115% of the nominal concentration were considered acceptable.

3. Results

As a first step, MS and MS/MS spectra were recorded after direct infusion of naltrexone, 6β-naltrexol or sinomenine into the APCI source *via* a syringe pump (Harvard Scientific, Holliston, MA). The analytes were dissolved at a concentration of 10 μg/mL in methanol/0.1 acetic acid 80/20, v/v and were delivered at a rate of 20 μL/min. Fig. 3 shows representative full scan and product ion scan spectra of naltrexone (Fig. 3A), 6β-naltrexol (Fig. 3B) and its internal standard sinomenine (Fig. 3C). Based on these studies, the ion transitions mentioned above were monitored.

To exclude the possibility that ion suppression compromised quantification of naltrexone and 6β-naltrexol, the effect of EDTA plasma samples from 10 different individuals for each species was tested following the recommendations by Müller et al. [19]. When using an APCI interface, ion suppression in human and dog EDTA plasma was detected only during the time of the injection peak (Fig. 4) and did not interfere with the detection of naltrexone (average retention time: 3.0 min), 6β-naltrexol (2.8 min) or the internal standard (3.0 min).

The absolute recoveries of naltrexone during extraction of human plasma ranged from 82.1 to 101.3% and those of 6β-naltrexol were between 74.6 and 99.8%. The results for dog plasma were similar (naltrexone: 78.3–96.2% and 6β-naltrexol: 81.0–105.8%). There was no difference of recoveries at the different concentration levels as assessed by analysis of variance.

The response at the retention time of naltrexone and 6β-naltrexol in the chromatograms of blank matrix was less than 20% of the response of the calibration point with a concentration of 0.005 ng/mL in human plasma and less than 20% of the response of the calibration point with a concentration of 0.01 ng/mL in dog plasma. No significant interference was observed at the retention time of the internal standard for blank human EDTA plasma samples (Fig. 5A).

Carry-over was tested by alternately injecting the highest calibrator and non-enriched blank samples. No carry-over was detected.

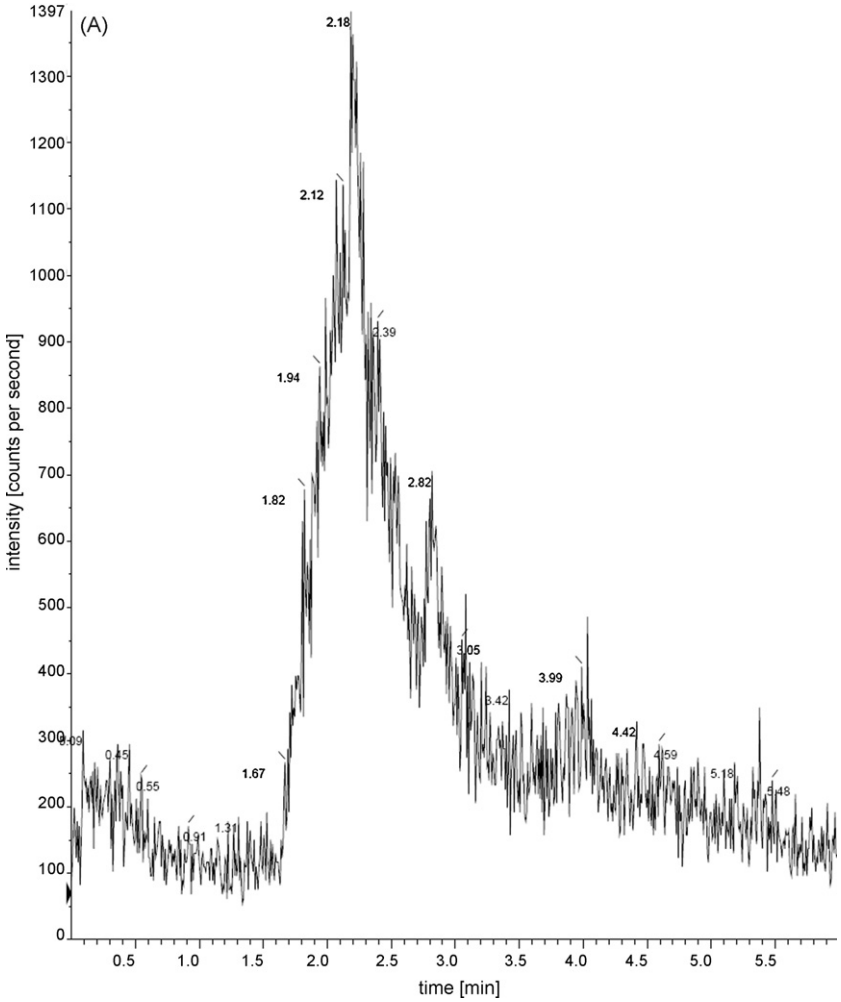

Fig. 5. Representative ion chromatogram after extraction of non-enriched (A) and blank human EDTA plasma enriched with naltrexone (B) or 6β-naltrexol (C) at the LLOQ. The LLOQs for naltrexone and 6β-naltrexol were 5 pg/mL. Please note that the Analyst software automatically adjusts the *y*-axis scale based on the highest signal and that, thus, the scales of the signal intensities in the three ion chromatograms are different. As mentioned in Section 2, the following ion transitions were monitored: naltrexone: $m/z = 342.3$ $[M+H]^+ \rightarrow 324.1$, and for 6β-naltrexol $m/z = 344.3$ $[M+H]^+ \rightarrow 326.1$. For the representative ion chromatogram of the blank sample shown in (A) the ion transition used for naltrexone detection was monitored.

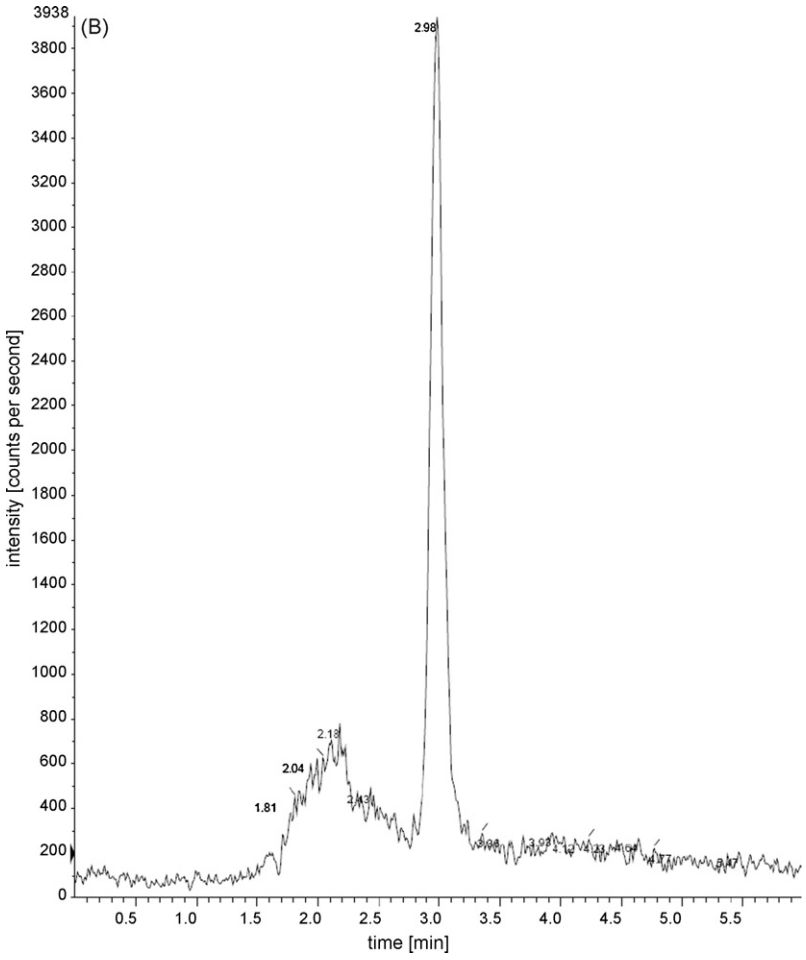

Fig. 5. (*Continued*)

In human EDTA plasma, the lower limit of quantitation for naltrexone and 6β-naltrexol was 5 pg/mL (Fig. 5B and C), and the assay was linear from 0.005 to 100 ng/mL ($y = 0.9412x + 0.8394$, $r = 0.9996$, $n = 6$) for naltrexone and ($y = 0.9964x + 1.2534$, $r = 0.9997$, $n = 6$) for 6β-naltrexol. The lower limits of quantitation for naltrexone and 6β-naltrexol in dog plasma were 10 pg/mL, and calibration curves were linear from 0.01 to 100 ng/mL ($y = 0.9967x + 1.0311$, $r = 0.9995$, $n = 6$) for naltrexone and ($y = 0.9967x + 0.911$, $r = 0.9994$) for 6β-naltrexol ($n = 6$).

Assay accuracy and precision were determined by using four different concentrations of naltrexone and 6β-naltrexol in human plasma (0.03, 0.3, 3, and 30 ng/mL). The results for intra-day precision and accuracy are listed in Table 1. The results for intra-day precision and accuracy for EDTA dog plasma were in the same range. At 0.03 ng/mL, inter-day variability was 10.1% and accuracy was 103.7% for naltrexone. At the same concentration of 6β-naltrexol, inter-day variability was 10.2% and accuracy 105.6%. At the other concentrations tested (0.3, 3, and 30 ng/mL), inter-day variability was ≤6.5 and accuracy was between 105.4 and 106.2% for naltrexone. For 6β-naltrexol at these concentrations, inter-day variability was ≤5.7% and inter-day accuracies depending on the concentrations tested were between 104.5 and 106.8%.

Validity of sample dilution for the measurement of naltrexone was established up to 1:64. However, dilution of samples with blank EDTA plasma for analysis of 6β-naltrexol was only valid up to a dilution 1:16. Dilution 1:32 resulted in measurement of concentrations that were only 70.9% of the nominal and thus outside of the acceptance limits of 85–115% of the nominal values.

Naltrexone and 6β-naltrexol were stable in non-extracted human EDTA plasma over at least three freeze–thaw cycles. Interestingly, after protein precipitation, samples showed significant instabilities already during the first freeze–thaw cycle, indicating that extracted samples cannot be stored long-term. In human and dog plasma, naltrexone and 6β-naltrexol were stable at room temperature for at least 12 h (benchtop stability) before extraction. On the other hand, extracted samples were instable when stored at −20 °C, +4 °C, 25 °C and 37 °C for 48 h. Extracted samples were stable in the autosampler at +4 °C for 24 h, but not for 48 h. The results of the stability studies are shown in detail in the Supplementary materials.

The assay has proven to be robust and reliable during the analyses of thousands of samples from several pharmacokinetics studies. A representative example of the results after intra-muscular injection of a naltrexone depot formulation is shown in Fig. 6.

4. Discussion

In the past, a major problem with the development of naltrexone sustained release formulations has been that there is poor correlation between systemic exposure and naltrexone's ability to efficaciously block opioid receptors. Even at plasma concentrations that were not measurable, naltrexone efficiently antagonized opioid challenges [13–15]. One of the likely reasons is simply that the analytical methods used in these studies were lacking the necessary sensitivity. In addition to a LLOQ in human plasma of 5 pg/mL, our

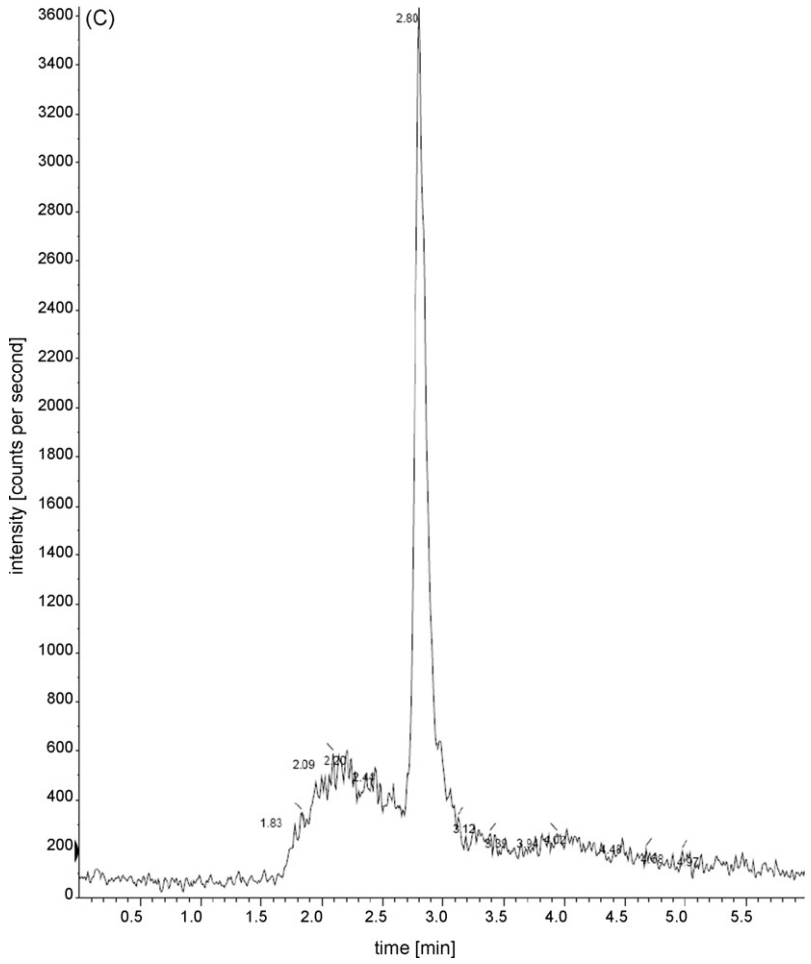

Fig. 5. (*Continued*)

Table 1
Intra- and inter-day precisions of the LC/LC-MS/MS analysis of naltrexone (A) and 6β-naltrexol (B) in human plasma

Concentration [ng/mL]	Precision R.S.D.%	Accuracy % of nominal
(A) Naltrexone		
Intra-day		
0.03	9.1	110.7
0.3	2.7	105.1
3.0	2.4	104.9
30	2.1	105.3
Inter-day		
0.03	10.1	103.7
0.3	5.5	105.4
3.0	6.5	105.7
30	4.6	106.2
(B) 6β-Naltrexol		
Intra-day		
0.03	5.2	106.3
0.3	4.2	103.7
3.0	3.0	105.7
30	4.8	104.3
Inter-day		
0.03	10.2	105.6
0.3	5.7	106.8
3.0	4.7	104.5
30	5.4	104.6

Intra-day precision and accuracy: $n = 6$/concentration; inter-day precision and accuracy: $n = 6$/concentration/day measured over 5 days. Inter-day precision is reported as relative standard deviations in % (R.S.D.%, as estimated by analysis of variance).

assay involves automatic sample online extraction and allows for the simultaneous quantification of the major naltrexone metabolite 6β-naltrexone.

The reasons for the high sensitivity included the online extraction/concentration step on the extraction column, the use of an APCI interface reducing the effect of ion suppression and the use of a high-end Sciex API5000 tandem quadrupole mass spectrometer. During the early stages of method development an electrospray ionization interface was used but it was quickly realized that ion suppression constituted a major problem. The online extraction procedure uses a very high solvent flow of 5 mL/min and extraction of an individual sample takes only 1 min. Systematic online extraction "break through" experiments showed that there was little migration of the analytes on the extraction column. After extraction, analytes were backflushed as a condensed band from the extraction column. This "volume-less" injection strategy resulted in sharper peaks that allowed for a more reliable identification of the beginning and end of peaks by the integration software and required less frequent manual re-integration of samples with analyte concentrations close to the lower limit of quantitation. It also allowed for the use of larger sample volumes without a negative impact on chromatographic separation or peak shape. In our experience online extraction procedures have not only the advantage of being significantly less labor-intense, they also result in better reproducibility, are less prone to random errors and allow for a complete automated documentation of each individual sample using the Analyst software. One of the critical steps during the extraction

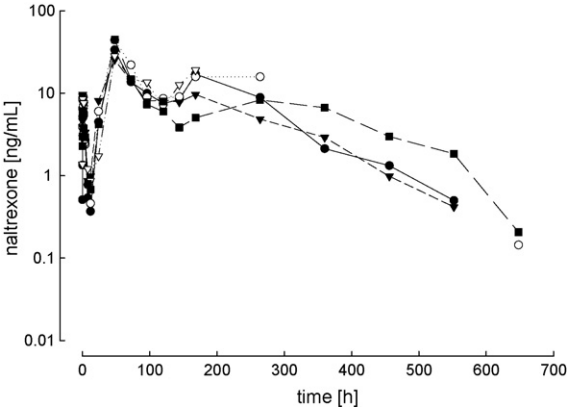

Fig. 6. Pharmacokinetics of naltrexone after intra-muscular injection of a depot formulation in dogs. Individual concentration–time profiles of five dogs are presented in this semi-log plot. The protocol was approved by an internal animal use and care committee and all animals received humane care following national and international guidelines. Two hundred milligrams naltrexone in the form of the sustained release Vivitrol formulation (Alkermes, Cambridge, MA) was administered as an intra-muscular bolus injection (2.9 mL volume). Blood samples were collected from the jugular vein for determination of the plasma concentrations of naltrexone and its major metabolite. The test animals were not fasted prior to blood collection. Blood samples (approximately 4 mL each) were collected and transferred into appropriately sized collection tubes containing K_3EDTA (15% solution). Plasma was separated and stored at −70 °C before analysis. Key pharmacokinetic parameters as estimated using a non-compartmental model (WinNonlin 5.0 professional, Pharsight. Mountain View, CA) were: time-to-maximum concentration 48 h, maximum concentration (median, range): 33.5 (25.4–44.8) ng/mL, area-under-the-time–concentration curve during the observation period: 4087 (2116–4838) ng/mL h. The pharmacokinetic parameters for 6β-naltrexol were: time-to-maximum concentration 48 h, maximum concentration (median, range): 0.95 (0.8–1.6) ng/mL, area-under-the-time–concentration curve during the observation period: 69.2 (58.6–85.6) ng/mL h.

is protein precipitation. $ZnSO_4$ results in efficient protein precipitation without causing trapping of protein-bound compounds. During the several thousand analyses we have run with this assay, there has never been a problem with protein precipitation on the online extraction column. The protein pellets in the 96-well plates are sufficiently stable during the time in the autosampler.

The analytes were stable on the column under the conditions used. A temperature of 65 °C resulted in sharper peaks and more reliable peak baseline recognition during automated integration especially at low concentrations.

It must be noted that the ion transitions monitored for naltrexone and 6β-naltrexol were −18 amu indicating the loss of water. Since this is not a very specific fragmentation reaction, it is not an obvious first choice. However, only these ion transitions gave the necessary sensitivity and the validation results showed that a lack of specificity was not a problem.

In general, deuterated internal standards are regarded as ideal. Deuterated naltrexone was not available to us. However, sinomenine is structurally similar, commercially easily available and, as demonstrated by the results of our validation, was a suitable choice.

Our assay was validated for human and dog EDTA plasma as matrix. Previous studies had shown that the protein precipitation step is vulnerable against matrix degradation resulting in poor absolute recovery [17]. EDTA plasma showed better stability in combination with our extraction procedure than other anticoagulants. This is also the reason why samples need to be stored in a −80 °C freezer for long-term storage. Comparison of human and dog plasma showed that there were no differences regarding linearity, accuracy and precision. The only minor difference was that the assay for naltrexone and 6β-naltrexol in dog plasma was slightly less sensitive than for human plasma with a LLOQ of 10 pg/mL.

An important finding during the validation was the instability of samples after protein precipitation during the freeze–thaw process. Thus long-term storage of samples after protein precipitation in a freezer is not an option. The best stability was achieved at +4 °C (at least 24 h). The use of a temperature controlled autosampler proved critical to ensure with-in batch stability.

As of today, the assay has successfully been used to measure several thousand plasma samples from pharmacokinetics studies. The online extraction procedure proved to be very robust and not a single sample has been lost. The extraction column is changed after every 500 sample injections and the analytical column after every 1500 injections. No loss of performance was noted during these periods of use and the column life spans are probably even longer.

Acknowledgment

This study was supported in part by the United States National Institutes of Health, grant P30 DK048520 Mass Spectrometry Core.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2008.08.021.

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