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Development and validation of a liquid chromatography–mass spectrometry method for the quantitation of naltrexone and 6β-naltrexol in guinea pig plasma

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Abstract

A quantitative liquid chromatographic–electrospray ionization mass spectrometry method for the determination of naltrexone and 6β-naltrexol in guinea pig plasma has been developed and validated using naloxone as an internal standard. A single step precipitation-extraction technique was carried out to extract the plasma samples using acetonitrile:ethyl acetate (1:1, v/v). The chromatographic separation was performed on a C_{18} column using a mobile phase consisting of 35:65 (v/v) acetonitrile:2 mM ammonium acetate with 0.01 mM ammonium citrate at a flow rate of 0.25 mL/min. The analyte was detected after positive electrospray ionization using selected ion monitoring (SIM) mode. The mean recoveries for naltrexone, naltrexol, and naloxone were 91.7, 89.3, and 99.0%, respectively. The lower limit of quantification (LLOQ) for naltrexone and 6β-naltrexol was 1.25 ng/mL, and the limit of detection (LOD) was 0.75 ng/mL. The method was applied to a pharmacokinetic study in order to assess the drug disposition of naltrexone in guinea pigs.
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Keywords: Naltrexone; 6β-Naltrexol; Pharmacokinetics

1. Introduction

Naltrexone, an opioid antagonist, commonly used for the treatment of narcotic addiction [1], has recently been prescribed as an adjunct in the treatment of alcohol dependence [2,3]. Naltrexone undergoes extensive hepatic metabolism primarily via reduction to its major metabolite in humans, 6β-naltrexol. 6β-Naltrexol is believed to be a major contributor to the pharmacologic effect of naltrexone [1]. For this reason, it is worthwhile to characterize the disposition of both naltrexone and 6β-naltrexol. A sensitive and simple analytical method is necessary for the pharmacokinetic analysis of naltrexone and its metabolite, 6β-naltrexol, in plasma samples from small animal models like guinea pigs, where the volume of plasma is very low (<200 μL). A variety of quantitative analytical methods, including thin layer chromatography (TLC) [4], gas chromatography (GC) [5–7], high-pressure liquid chromatography (HPLC) with electrochemical detection (ECD) [8–11], and GC–MS (mass spectrometry) [12] have been reported for the quantification of naltrexone and 6β-naltrexol in plasma. The method based on TLC may not be selective and sensitive for routine analysis of the drugs in plasma. HPLC with ECD detection hinders the reproducibility and robustness of the method, because the cell can be easily contaminated, especially in the analysis of plasma samples. Disadvantages to using the GC and the GC–MS methods are attributed to the elaborate sample preparation and various derivatization techniques required for these assays. Two methods have been reported on the simultaneous analysis of naltrexone and 6β-naltrexol by GC–MS/MS [13,14] in biological specimens with a sensitivity of at least

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1 ng/mL. However, both methods required a derivatization technique and larger volumes (1 mL) of sample. Mason et al. reported a LC–MS/MS method [15] for quantification of naltrexone and 6β-naltrexol in human plasma with a sensitivity of 0.25 ng/mL. However, no information is available on the sample preparation and analytical conditions. In this manuscript, a relatively simple, selective, and sensitive LC–MS method for the determination of naltrexone and 6β-naltrexol in guinea pig plasma using a single-step precipitation-extraction method is described.

2. Experimental

2.1. Materials and chemicals

Naltrexone was obtained from Mallinckrodt Inc. (St. Louis, MO) and 6β-naltrexol was obtained from the National Institute on Drug Abuse (NIDA Drug Supply, Research Triangle Park, NC). The internal standard, naloxone, was obtained from Sigma (St. Louis, MO). Ammonium acetate, ethyl acetate, and acetonitrile (HPLC grade) were obtained from Fisher Scientific (Fairlawn, NJ). Ammonium citrate was obtained from Alfa Aesar (Ward Hill, MA). Water was purified by a Barnstead nanopure Diamond™ Ultrapure water system (Barnstead International, Dubuque, Iowa).

2.2. Calibration standards and quality control samples

Standards and quality control samples (QCs) were made from stock solutions (1 mg/mL, naltrexone and naltrexol in acetonitrile). Working calibration standards at concentrations of 1.25–500 ng/mL in plasma were prepared fresh daily. Five levels of QC samples, 5, 50, 100, 200 and 400 ng/mL, were prepared in plasma for the determination of inter-day accuracy and precision. A stock solution of naloxone (1 mg/mL) was prepared in acetonitrile, from which a 500 ng/mL internal standard (IS) working solution was prepared in acetonitrile as well.

2.3. Extraction procedure

All samples, QCs, and standards with a sample volume of 0.1 mL spiked with 20 μL of IS working solution were extracted with 1 mL of acetonitrile:ethyl acetate (1:1, v/v). The mixture was vortexed for 30 s and centrifuged at 10,000 × *g* for 20 min. The supernatant was pipetted into a 3 mL glass test tube and evaporated at 37 °C under nitrogen. The residue was reconstituted with 100 μL of acetonitrile and sonicated for 15 min. The samples were transferred into autosampler vials containing low volume inserts and 20 μL was injected onto the HPLC column.

2.4. LC–MS conditions

Chromatography was performed on a Waters Symmetry® C_{18} (2.1 mm × 150 mm, 5 μm) column at 35 °C with a mobile phase consisting of acetonitrile:ammonium acetate (2 mM) containing 0.01 mM of ammonium citrate (35:65, v/v) set at a flow rate of 0.25 mL/min. A Waters Symmetry® C_{18} (2.1 mm × 10 mm, 3.5 μm) guard column was also used.

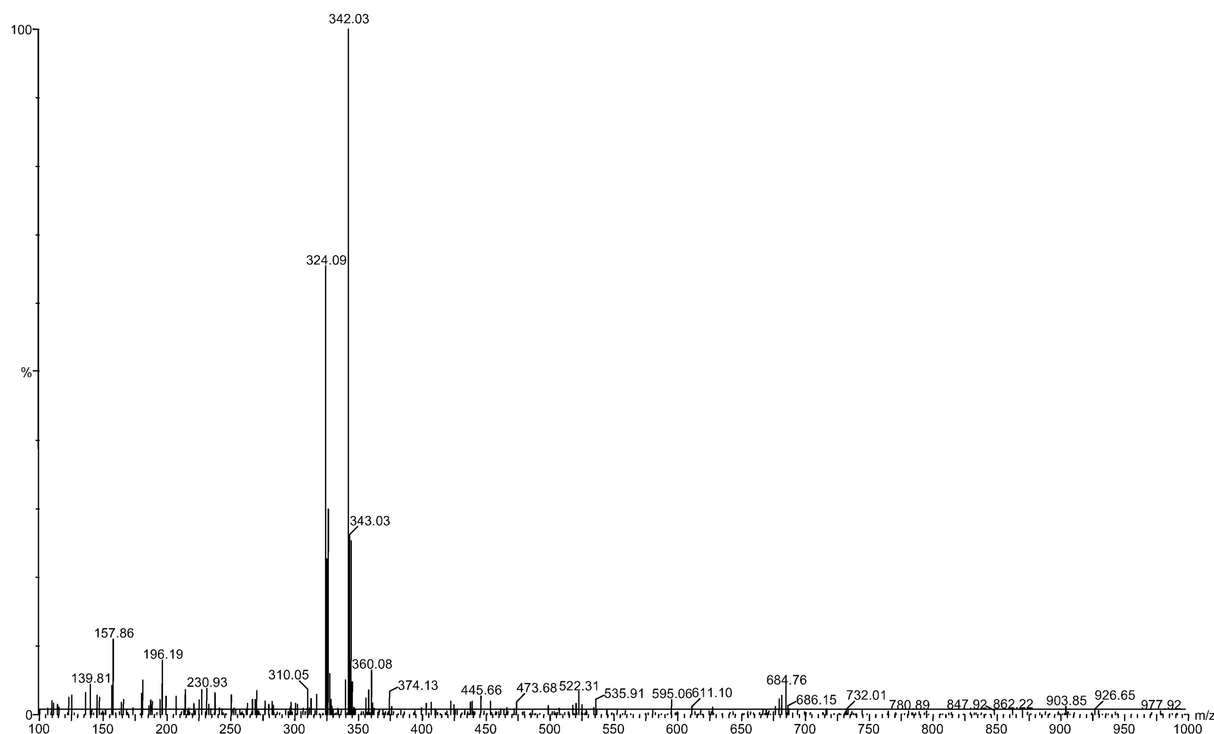

Fig. 1. Full scan mass spectrum of naltrexone (*m*/*z* 324).

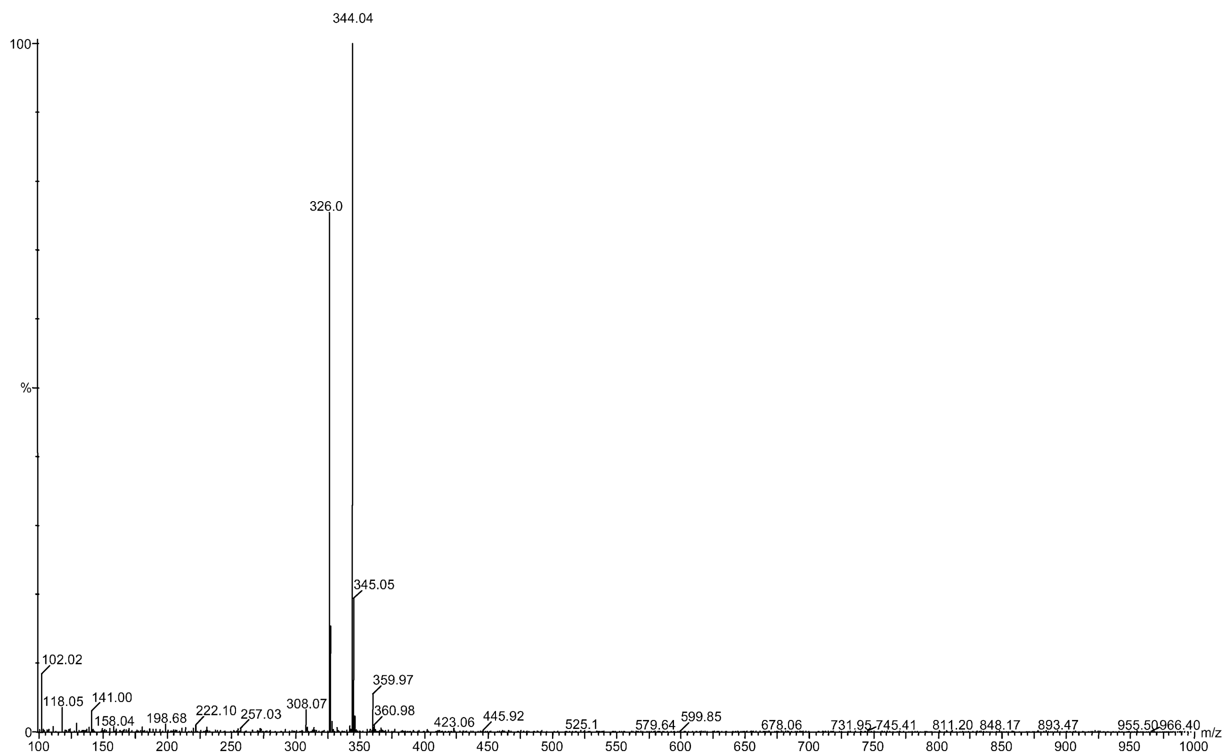

Fig. 2. Full scan mass spectrum of 6β-naltrexol (*m/z* 344).

The LC–MS system consisted of a Waters Alliance 2690 HPLC pump (Waters, Milford, MA, USA), a Waters Alliance 2690 autosampler, and a Micromass ZQ detector (Waters, Milford, MA, USA) using electrospray ionization (ESI) for ion production. Selected ion monitoring (SIM) was performed in positive mode for naltrexone, *m/z* 324 [342 ≫ 324] (dwell time 0.30 s) (Fig. 1), naltrexol, *m/z* 344 $[M+\mathrm{H}]^+$ (Fig. 2), and naloxone, *m/z* 310 [364 ≫ 310] (dwell time

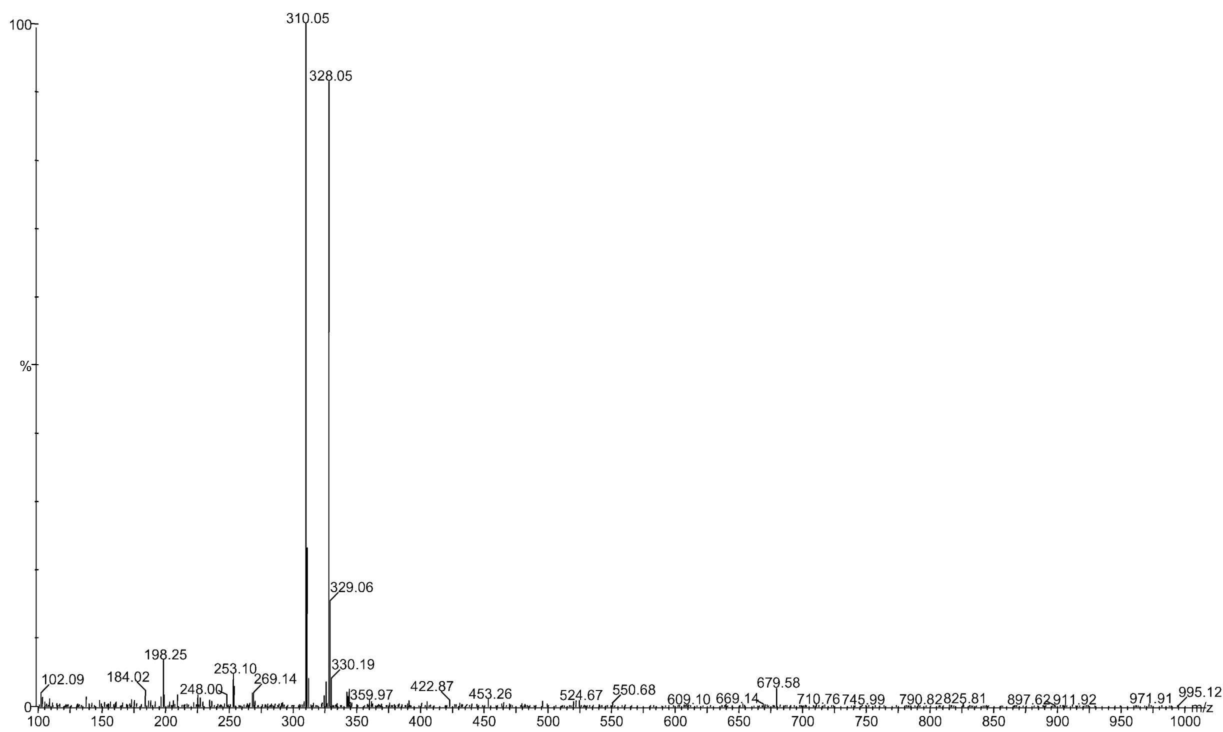

Fig. 3. Full scan mass spectrum of naloxone (*m/z* 310).

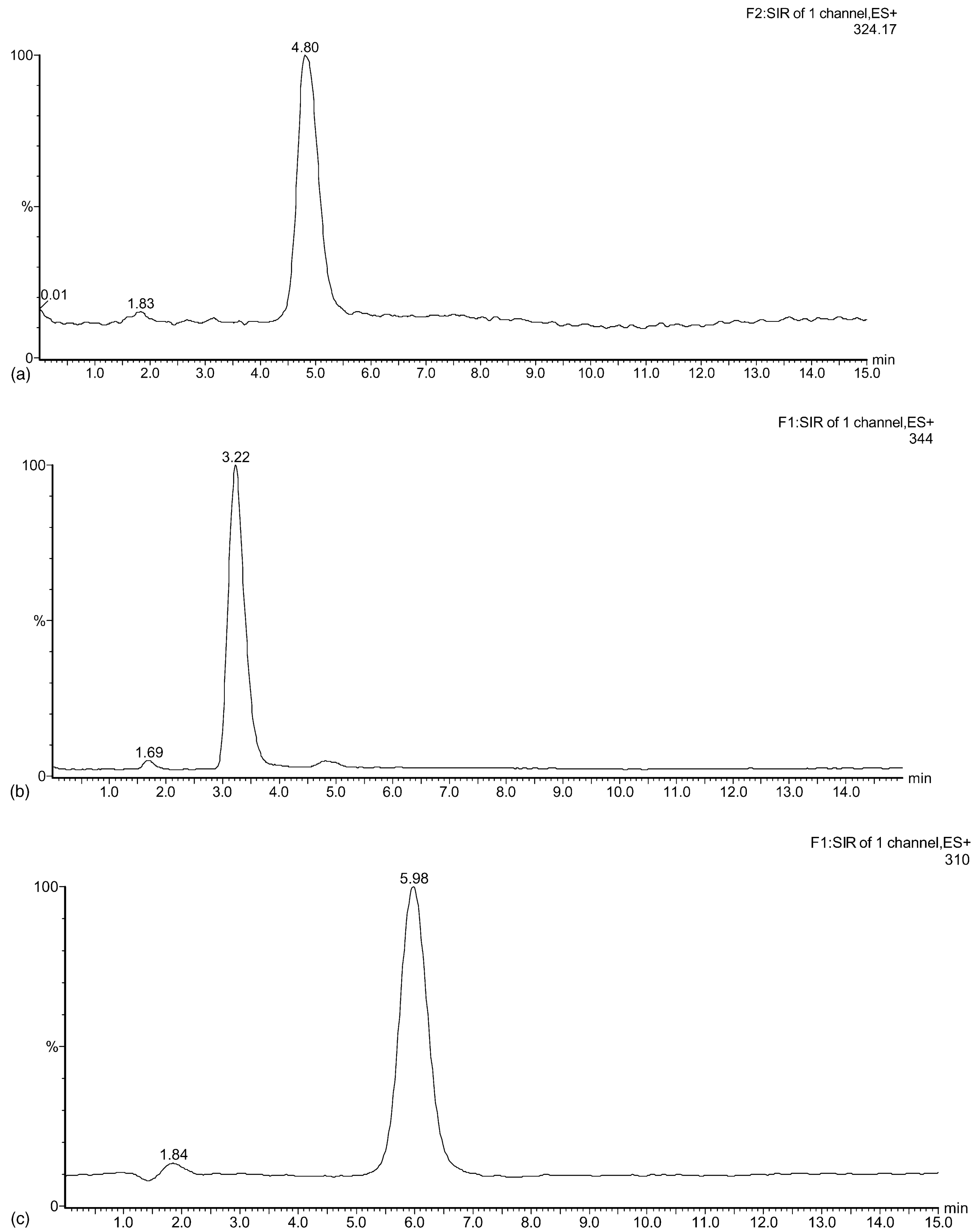

Fig. 4. Typical HPLC/MS ion chromatograms spiked with 25 ng/mL of naltrexone, 6β-naltrexol and 100 ng/mL of naloxone in guinea pig plasma (a) naltrexone (4.80 min); (b) 6β-naltrexol (3.22 min); (c) naloxone (5.98 min).

0.30 s) (Fig. 3). Capillary voltage was 4.5 kV and cone voltage was 30 V. The source block and desolvation temperatures were 120 and 250 °C, respectively. Nitrogen was used as a nebulization and drying gas at flow rates of 50 and 450 L/h, respectively. The retention times for naltrexone, naltrexol and naloxone were 4.81 ± 0.15, 3.20 ± 0.11, and 5.84 ± 0.20 min (Fig. 4), respectively. Calibration graphs were constructed using a linear regression of the ratio of the drug peak-area to internal standard versus nominal drug concentrations.

2.5. Validation

The method was validated for accuracy, precision, selectivity, calibration curve range, and reproducibility over a concentration range of 1.25–500 ng/mL using five calibration standards, each containing the two analytes of interest, and three replicates of QC samples at each concentration level in three separate runs.

The matrix effect (co-eluting, undetected endogenous matrix compounds that may influence the analyte ionization) was investigated by extracting "blank" normal plasma and reconstituting with acetonitrile containing a known amount of the analytes, analyzing the reconstituted extracts, and then comparing the peak areas of the analytes with that of analytes in acetonitrile. The extraction recoveries of naltrexone, naltrexol, and naloxone were calculated by comparing the peak areas of extracted plasma standards to the peak areas of post-extraction plasma blanks spiked at corresponding concentrations. The extraction recoveries of naltrexone and 6β-naltrexol in QC samples were also performed to prove consistency across the complete dynamic range.

2.6. Stability studies

The stabilities of naltrexone, 6β-naltrexol, and the IS were investigated in the stock solutions and in the final extracts. The stabilities of the analytes and IS in the stock solution were determined at room temperature and at 4 °C. The concentration of IS in the QC samples was 100 ng/mL. Freshly prepared QC samples were stored for 48 h at room temperature, and 1 week at 4 °C. For each of the storage conditions, three replicates were analyzed at five concentration levels. The analyte and IS samples were processed immediately at each individual time point and compared with that of freshly prepared solutions. The post-preparative stabilities of the analytes and IS in the final extracts were studied at three concentrations at autosampler temperature (12 °C) for 48 h. The drug concentrations in the final extract QC samples were compared at 0 and 48 h. The analytes and IS were considered to be stable in the final extract (post-preparative) when 85–115% of the initial concentration was found. The stability limit in the stock solutions was set at 95–105% of the initial concentrations [16].

2.7. Pharmacokinetic analysis

The pharmacokinetic analysis of naltrexone plasma concentration versus time profiles after intravenous bolus administration was carried out by fitting the data to a three compartment model (WinNonlin Professional, version 4.0, Pharsight Corporation, Mountain View, California) with the following exponential expression:

$$C(t) = A\mathrm{e}^{-\alpha t} + B\mathrm{e}^{-\beta t} + C\mathrm{e}^{-\gamma t} \quad (1)$$

where $C(t)$ is the plasma concentration of drug at time 't'; A, B and C are preexponential constants; fast distribution rate constant, α; slow distribution rate constant, β; terminal elimination rate constant, γ; and t is time. The pharmacokinetic parameters, such as terminal elimination half-life, $t_{1/2(\gamma)}$; distribution half lives, $t_{1/2(\alpha)}$ and $t_{1/2(\beta)}$; steady-state volume of distribution, V_{ss}; area under the curve from 0 to infinity, $AUC_{0-\infty}$; and total body clearance (Cl_{tot}) were estimated using the software. The peak plasma concentration (C_{max}) after the IV bolus dose of naltrexone was used to calculate the initial volume of distribution by the following equation:

$$V = \frac{\text{dose}}{C_{max}} \quad (2)$$

3. Results and discussion

The initial development step for the LC–MS method consisted of a mobile phase of 2mM ammonium acetate: acetonitrile (35:65) at a flow rate of 0.25 mL/min, but tailing was observed with naltrexone and 6β-naltrexol. In order to improve the peak shapes, a concentration of 0.01 mM ammonium citrate was added. Typical ion chromatograms obtained with blank guinea pig plasma spiked with 25 ng/mL naltrexone, naltrexol and IS working solution are shown in Fig. 4. The representative chromatograms of processed blank plasma are shown in Fig. 5. The total run time for each sample was about 15 min. Naltrexone, 6β-naltrexol, and naloxone peaks were well resolved and free of interference from endogenous compounds in the plasma. Only three additional peaks were observed at 1.69–1.95 min, and these were well separated from the drug peaks. Standard curves prepared for naltrexone and 6β-naltrexol in plasma were linear over a range of 1.25–500 ng/mL. The mean ($n = 3$) calibration curves for naltrexone and 6β-naltrexol were $y = 0.0262x - 0.0749$, $R^2 = 0.999$ and $y = 0.0177x + 0.0069$, $R^2 = 0.999$, respectively, where y and x are the peak area ratio of analyte to internal standard and concentration (ng/mL) of analyte, respectively.

The mean absolute recoveries of naltrexone, 6β-naltrexol, and naloxone (IS) determined in triplicate in the concentration range of 1.25–500 ng/mL were 91.7% (%CV 4.6), 89.3% (%CV 7.2), and 99.0% (%CV 5.4), respectively. The absolute recoveries of naltrexone and 6β-naltrexol in the QC samples are listed in Table 1. The absolute recovery values for QC samples were in between 82.2 and 95.5% for naltrexone

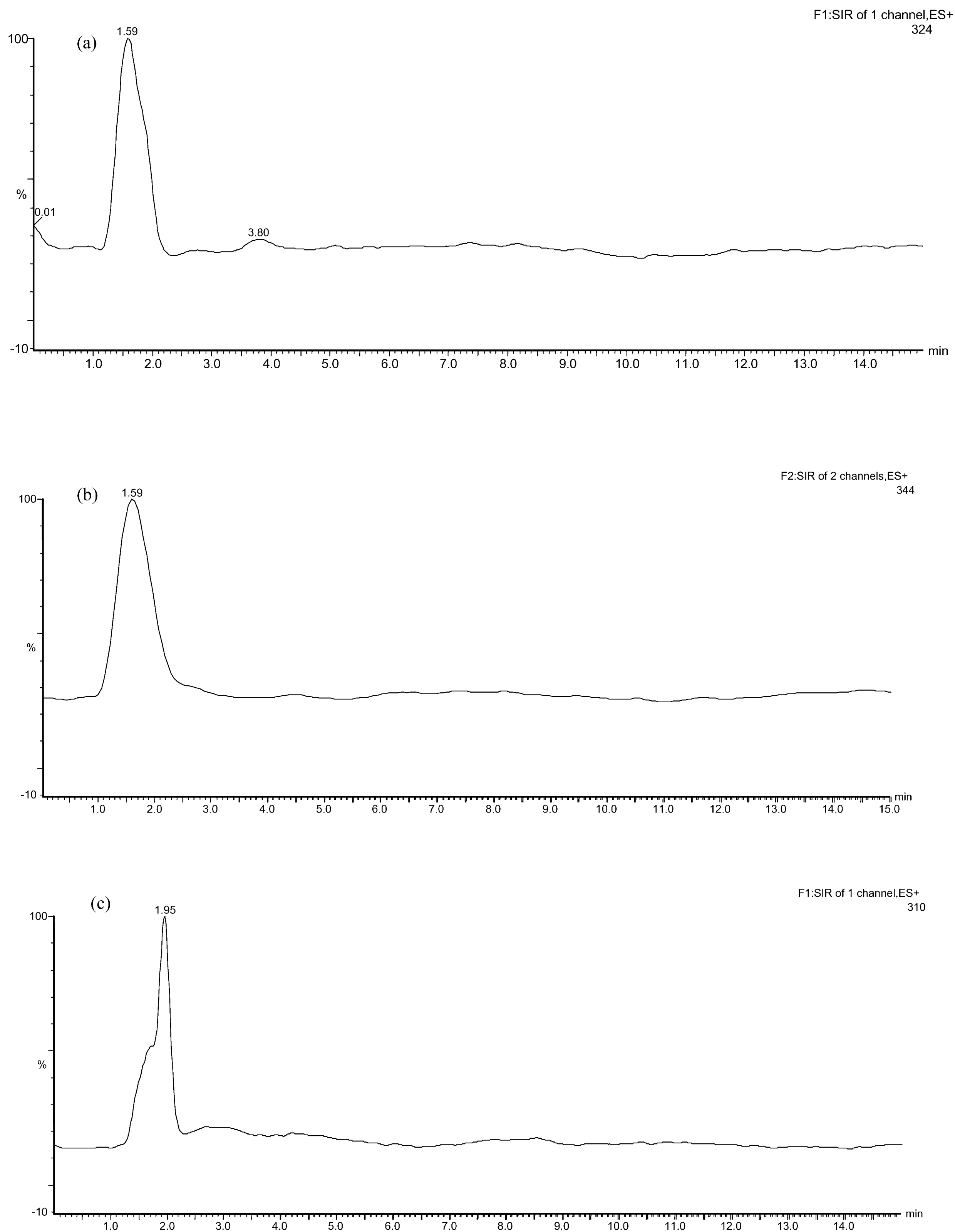

Fig. 5. The representative HPLC/MS ion chromatograms of processed blank guinea pig plasma (a) naltrexone; (b) 6β-naltrexol; (c) naloxone.

Table 1
Recovery data for QC samples of naltrexone and 6β-naltrexol ($n = 3$)

Naltrexone			6β-Naltrexol		
Concentration (ng/mL)	Recovery (%)	%CV	Concentration (ng/mL)	Recovery (%)	%CV
5	82.2	6.5	5	89.3	5.4
50	87.2	5.3	50	91.3	3.6
100	88.2	3.2	100	92.4	3.1
200	94.3	4.6	200	98.6	4.9
400	95.5	3.0	400	98.3	4.3

Table 2
Intra-day and inter-day quality control results of naltrexone

Intra-day variation				Inter-day variation			
Concentration (ng/mL)[a]	Mean concentration found (ng/mL)	%CV[b]	%Accuracy	Concentration (ng/mL)	Mean concentration found (ng/mL)	%CV[b]	%Accuracy
5	5.0	5.8	100.2	5	4.98	4.9	99.6
50	49.95	3.9	99.9	50	48.6	1.9	97.2
100	98.1	4.8	98.1	100	101.2	4.0	101.2
200	195.9	2.5	98.0	200	199.7	6.0	99.8
400	400.5	3.3	100.1	400	401.1	4.7	100.3

[a] $n = 3$.
[b] %CV: coefficient of variation.

and between 89.3 and 98.6% for 6β-naltrexol. No significant matrix effect was observed for the analytes in the plasma samples. The peak areas of analytes in the reconstituted QC samples had a coefficient of variation of 6%, indicating that the extracts were "clean" with no co-eluting compounds influencing the ionization of the analytes.

The LLOQ, defined as that concentration of naltrexone and naltrexol which can still be determined with acceptable [16] precision (%CV < 10) and accuracy, was found to be 1.25 ng/mL and the LOD for naltrexone and 6β-naltrexol was 0.75 ng/mL. Results of the intra-day and inter-day validation assays presented in Tables 2 and 3 indicated that the accuracy of the assay was >95% and the CV did not exceed 7%. Naltrexone, 6β-naltrexol and the IS were stable (Table 4) in the stock solution at room temperature and at 4 °C for the time periods studied. The post-preparative stability studies (Table 5) indicated that the stabilities of naltrexone, 6β-naltrexol and the IS were guaranteed for at least 48 h at 12 °C. Due to the high selectivity of MS detection; no interfering peaks were found when blank plasma extracts were analyzed. The ionization response monitored by injecting a system performance verification standard at the beginning and at the end of each batch indicated that the system response remained stable.

The described method was applied to a pharmacokinetic study of an intravenous dose of naltrexone in guinea pigs. All animal studies were approved by the University of Kentucky IACUC. Representative plasma profiles of observed and predicted concentrations of naltrexone, and observed concentrations of 6β-naltrexol after an intravenous bolus dose of naltrexone in guinea pigs (3 mg/kg) are shown in Fig. 6. It can be seen from the plasma profiles of naltrexone and 6β-naltrexol that drug could still be detected even after 20 h. The plasma profile of naltrexone in the guinea pig followed a three compartmental model. The observed plasma concentration of naltrexone was in good agreement (correlation = 0.978) with the predicted plasma concentration, and the pharmacokinetic parameters of naltrexone are shown in Table 6. The maximum plasma concentration of naltrexone obtained after intravenous administration of 3 mg/kg in the guinea pigs was 1039.5 ± 612.3 ng/mL, and it sharply declined to 9.2 ± 3.5 ng/mL after 2 h. The maximum plasma concentration of the naltrexol metabolite was 60.7 ± 18.2 ng/mL with a T_{max}

Table 3
Intra-day and inter-day quality control results of naltrexol

Intra-day variation				Inter-day variation			
Concentration (ng/mL)[a]	Mean concentration found (ng/mL)	%CV[b]	%Accuracy	Concentration (ng/mL)	Mean concentration found (ng/mL)	%CV[b]	%Accuracy
5	4.9	6.2	97.8	5	5.2	2.3	103.2
50	50.2	2.6	100.5	50	49.1	2.1	98.2
100	102.4	5.3	102.4	100	98.7	4.2	98.7
200	196.3	2.0	98.2	200	198.5	3.7	99.3
400	399.5	5.6	99.9	400	402.7	4.7	100.7

[a] $n = 3$.
[b] %CV: coefficient of variation.

Table 4
Stability of naltrexone, 6β-naltrexol and IS in stock solutions ($n = 3$)

Drug	Storage condition	Concentration (ng/mL)	Mean Concentration (ng/mL)	Mean Concentration recovered (ng/mL)	%Deviation	%CV
Naltrexone	At 25 °C for 48 h	5	5.21	5.35	2.69	3.2
		50	51.25	50.50	−1.46	1.33
		100	100.52	99.58	−0.94	1.80
		200	200.21	198.36	−0.92	5.65
		400	398.01	400.19	0.55	1.36
	At 4 °C for 1 week	5	5.15	4.96	−3.69	2.00
		50	49.00	50.02	2.08	2.55
		100	100.89	99.88	−1.00	5.66
		200	200.14	199.63	−0.25	2.01
		400	397.21	400.41	0.81	3.32
6β-Naltrexol	At 25 °C for 48 h	5	4.85	5.15	6.19	2.5
		50	50.00	49.05	−1.90	1.02
		100	100.35	99.25	−1.10	4.09
		200	199.25	200.25	0.50	4.65
		400	400.65	398.33	−0.58	4.01
	At 4 °C for 1 week	5	4.98	4.93	−1.0	3.4
		50	51.66	48.62	−5.88	4.22
		100	99.02	98.55	−0.47	4.01
		200	201.62	197.32	−2.13	1.5
		400	401.25	398.14	−0.78	1.89
IS	At 25 °C for 48 h	100	100.25	97.1	−3.14	3.85
	At 4 °C for 1 week	100	101.63	100.2	−1.41	1.02

Table 5
Post-preparative stability of naltrexone, 6β-naltrexol and IS at 12 °C for 48 h ($n = 3$)

Drug	Concentration (ng/mL)	Mean concentration (ng/mL)	Mean concentration recovered (ng/mL)	%Deviation	%CV
Naltrexone	5	4.98	4.86	−2.41	0.99
	50	51.21	50.90	−0.61	1.65
	100	99.20	101.15	1.97	3.69
	200	199.01	200.21	0.60	2.5
	400	400.25	399.85	−0.10	1.69
6β-Naltrexol	5	5.21	5.35	2.69	1.8
	50	49.52	51.56	4.12	2.01
	100	99.60	99.00	−0.60	2.85
	200	198.65	197.54	−0.56	5.63
	400	399.60	400.54	0.24	6.01
IS	100	100.1	97.10	−3.00	5.30

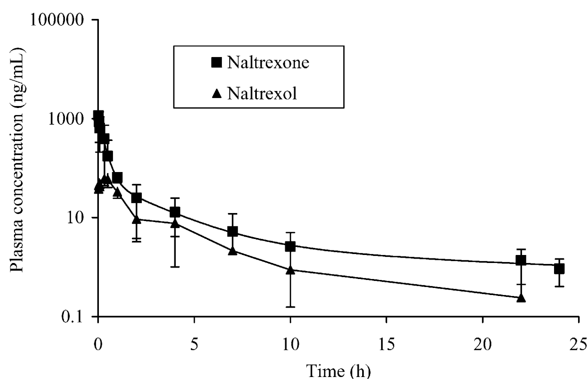

Fig. 6. Mean (±S.D.) plasma profiles of naltrexone and 6β-naltrexol after intravenous administration of naltrexone (3 mg/kg) in guinea pigs ($n = 3$).

Table 6
Pharmacokinetic parameters of naltrexone after intravenous administration (3 mg/kg) in guinea pigs ($n = 3$)

Parameter	Mean ± S.D.
C_{max} (ng/mL)	1039.5 ± 612.3
AUC (ng/mL)h	430.7 ± 105.8
AUMC (ng/mL)h^2	1095.2 ± 132.3
Cl (L/h)	7.14 ± 1.68
V_{ss} (L/kg)	15.78 ± 3.44
α (1/h)	3.75 ± 0.77
β (1/h)	0.45 ± 0.03
γ (1/h)	0.07 ± 0.02
$t_{1/2(\alpha)}$ (h)	0.19 ± 0.04
$t_{1/2(\beta)}$ (h)	1.54 ± 0.09
$t_{1/2(\gamma)}$ (h)	9.81 ± 2.43
Initial V_d (L/kg)	3.49 ± 1.53
MRT (h)	8.53 ± 0.71

of 15 min (Fig. 6). The mean terminal elimination half-life ($t_{1/2(\gamma)}$), steady-state apparent volume of distribution (V_{ss}), and total clearance (Cl) of naltrexone were 9.81 h, 15.78 L/kg and 7.14 L/h, respectively.

4. Conclusion

A LC–MS method for the estimation of naltrexone and 6β-naltrexol, its metabolite, in guinea pig plasma was successfully developed and validated. The method is sensitive and simple with an LLOQ of 1.25 ng/mL for naltrexone and 6β-naltrexol using a 0.1 mL aliquot of sample. It has been shown in a pharmacokinetic study with guinea pigs that naltrexone and 6β-naltrexol could be quantitated after a 3 mg/kg dose of naltrexone. Thus, the method is appropriate for monitoring naltrexone and its metabolite, 6β-naltrexol, in pharmacokinetic studies.

Acknowledgments

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