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# Determination of naltrexone and its major metabolite, 6-β-naltrexol, in human plasma using liquid chromatography with electrochemical detection[1]

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**Abstract**

A sensitive and specific high-performance liquid chromatographic method with electrochemical detection was developed for the simultaneous determination of naltrexone and its major metabolite, 6-β-naltrexol, in human plasma. After alkalinizing 2 ml plasma samples with pH 9 sodium carbonate buffer, naltrexone and 6-β-naltrexol were extracted into dichloromethane and then back-extracted into 0.017 M phosphoric acid. A portion of the acid extract was chromatographed on a YMC phenyl column using a mobile phase of methanol–phosphoric acid (50 mM) (20:80, v/v) (pH* 3.2) at a flow-rate of 1.2 ml $min^{-1}$. Quantification was performed using an ESA Coulometric electrochemical detector. Acceptable intra-day and inter-day assay precision (RSD < 10%) and accuracy ( < 16%) for both compounds were observed over concentration ranges of 0.25–50.0 ng $ml^{-1}$ for naltrexone and 0.5–100 ng $ml^{-1}$ for 6-β-naltrexol. No degradation of either naltrexone or 6-β-naltrexol was observed in frozen human plasma stored at − 20°C over an 8 month period. The method is sufficiently sensitive and selective to quantify plasma concentrations of naltrexone and 6-β-naltrexol after oral doses of 50 mg of naltrexone to healthy subjects or alcoholic patients.

*Keywords:* Electrochemical detection; Human plasma; 6-β-naltrexol; Naltrexone; Quantitative determination

## 1. Introduction

Naltrexone, an opioid antagonist, has been marketed for over a decade for the clinical treatment of opiate dependence and has recently demonstrated effectiveness as adjunctive treatment for individuals with alcohol dependence undergoing psychosocial treatment programs [1–3]. Following oral administration of 50 or 100 mg oral doses, naltrexone undergoes rapid and nearly complete absorption (ca. 96%) with maximum plasma concentrations ranging from 9 to 44 ng $ml^{-1}$ within 1 h [4–6]. Naltrexone undergoes extensive hepatic metabolism primarily via reduction to a major human metabolite, 6-β-naltrexol. Like naltrexone, 6-β-naltrexol is believed to be a

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pure opioid antagonist and may contribute to the pharmacological blockage of opioid receptors [4]. For this reason, it is prudent to characterize the disposition of both naltrexone and 6-$\beta$-naltrexol. Naltrexone exhibits significant first-pass metabolism [5] which substantially limits the amount of intact naltrexone systemically available for quantification.

A variety of quantitative analytical methods, including gas chromatography (GC) [7–10], high-performance liquid chromatography with electrochemical detection (HPLC–EC) [5,11–13] and gas chromatography–mass spectometry (GC–MS) [14] have been published for the quantification of naltrexone from plasma, but only a few allow for the simultaneous detection of both naltrexone and 6-$\beta$-naltrexol at sensitivities of less than 1 ng ml$^{-1}$ required for clinical pharmacokinetic studies conducted at therapeutic doses (50 or 100 mg QD) [5,10,14]. Disadvantages to using the electron-capture GC [10] and the GC–MS [14] methods are attributed to the labor-intensive extraction steps required for these assays and while the electrochemical method [5] is simple and rapid, amperometric detection with a glassy carbon electrode hinders the reproducibility and robustness of the method. A recently published GC–MS–MS method [15] offers the advantage of a simpler liquid–liquid extraction procedure without compromising the 0.1 ng ml$^{-1}$ quantification limit of an earlier GC–MS method [14].

The method described in this paper has validated quantification limits of 0.25 and 0.50 ng ml$^{-1}$ for naltrexone and 6-$\beta$-naltrexol, respectively, when assaying 2 ml of human plasma, and provides a very economical alternative to mass spectrometric methods without sacrificing sensitivity or specificity. The method has been successfully applied in clinical trials evaluating the pharmacokinetics of naltrexone and 6-$\beta$-naltrexol in both healthy subjects and alcoholic patients.

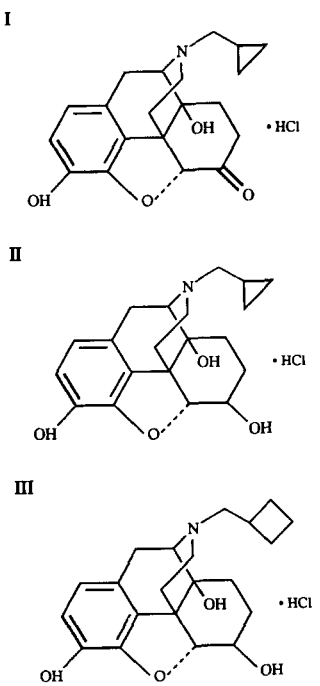


Fig. 1. Structures of naltrexone (**I**), 6-$\beta$-naltrexol (**II**) and the internal standard, nalbuphine (**III**).

## 2. Experimental

### 2.1. Chemicals and reagents

Naltrexone [17-(cyclopropylmethyl)-4,5-epoxy-3,14-dihydroxymorphinan-6-one], 6-$\beta$-naltrexol [17-(cyclopropylmethyl)-4,5-epoxymorphinan-3,6,14-triol] and the internal standard, nalbuphine [17-(cyclobutylmethyl)-4,5-epoxymorphinan-3,6,14-triol] (Fig. 1), were synthesized at The DuPont Merck Pharmaceutical Company (Wilmington, DE, USA). Unless stated otherwise, all solvents and reagents used were of HPLC grade. Methanol, ammonia solution and sodium hydroxide were purchased from J.T. Baker (Phillipsburg, NJ, USA), dichloromethane from Burdick and Jackson (Muskegon, MI, USA), 85% phosphoric acid from Fisher Scientific (Fair Lawn, NJ, USA) and sodium hydrogen carbonate from Aldrich Chemical (Milwaukee, WI, USA). Frozen heparinized control human plasma was supplied by Biological Specialty (Colmar, PA, USA).

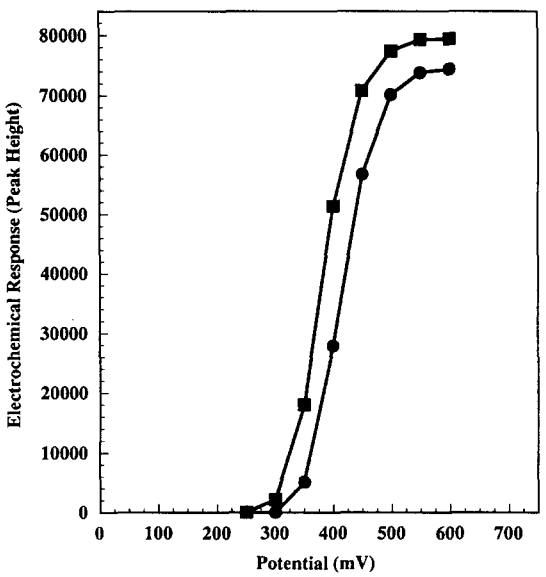


Fig. 2. Voltammograms for naltrexone (●) and 6-β-naltrexol (■).

### 2.2. Standard solutions

Primary standard solutions of naltrexone and 6-β-naltrexol were prepared in methanol at nominal concentrations of 10 μg $ml^{-1}$ (free base). Working standard solutions containing a mixture of naltrexone and 6-β-naltrexol were used for the preparation of plasma standards. These were prepared by diluting aliquots from each stock solution with methanol to yield nominal concentrations over a range of 0.005 to 1.0 μg $ml^{-1}$ for naltrexone and 0.01 to 2.0 μg $ml^{-1}$ for 6-β-naltrexol. All primary and working stock standard solutions were stored at 4°C and showed no degradation for at least 2 months. Plasma standard solutions were prepared daily by adding 100 μl aliquots of each working standard solution to 2.0 ml of control human plasma to yield final nominal plasma concentrations over the ranges 0.25–50 ng $ml^{-1}$ for naltrexone and 0.5–100 ng $ml^{-1}$ for 6-β-naltrexol.

### 2.3. Instrumentation and chromatographic conditions

The chromatographic system consisted of a Spectra-Physics SP8810 isocratic pump (San Jose, CA, USA), a Waters 712 autosampler (Waters Associates, Milford, MA, USA) and a Coulochem II 5200 electrochemical detector (ESA, Bedford, MA, USA). A Model 5020 guard cell (ESA), operated at a cell potential of + 0.7 V, was used to oxidize impurities from the mobile phase to reduce system noise and background current prior to sample introduction. An ESA Model 5011 high-sensitivity analytical cell with dual porous graphite working electrodes was operated at two cell potentials. The first electrode was set at + 0.30 V and was used as a screen to eliminate oxidation of endogenous components of the plasma extract at the second electrode, operated at + 0.65 V, where analyte detection occurred. The mobile phase was methanol–phosphoric acid (50 mM) (20:80, v/v) and was adjusted to pH* 3.2 using ammonia solution. The flow-rate was 1.2 ml $min^{-1}$ with recirculation of the mobile phase, which assisted in decreasing background current as many impurities were irreversibly oxidized. Analyte separation was achieved using a 100 × 4.6 mm i.d. (3 μm) YMC phenyl column (YMC, Wilmington, NC, USA). Data acquisition and integration were performed using Waters Ease Chromatography Software, (v. 3.0).

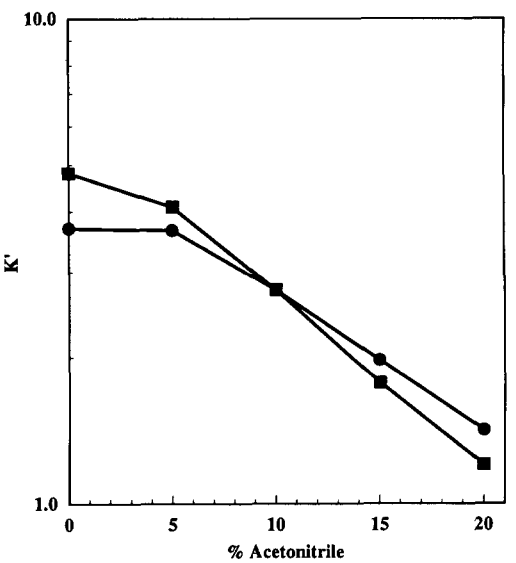


Fig. 3. Effect of mixtures of acetonitrile and methanol on chromatographic selectivity (as measured by the capacity factor, $k'$) of naltrexone (●) and 6-β-naltrexol (■). Total organic solvent was maintained at 20% of mobile phase.

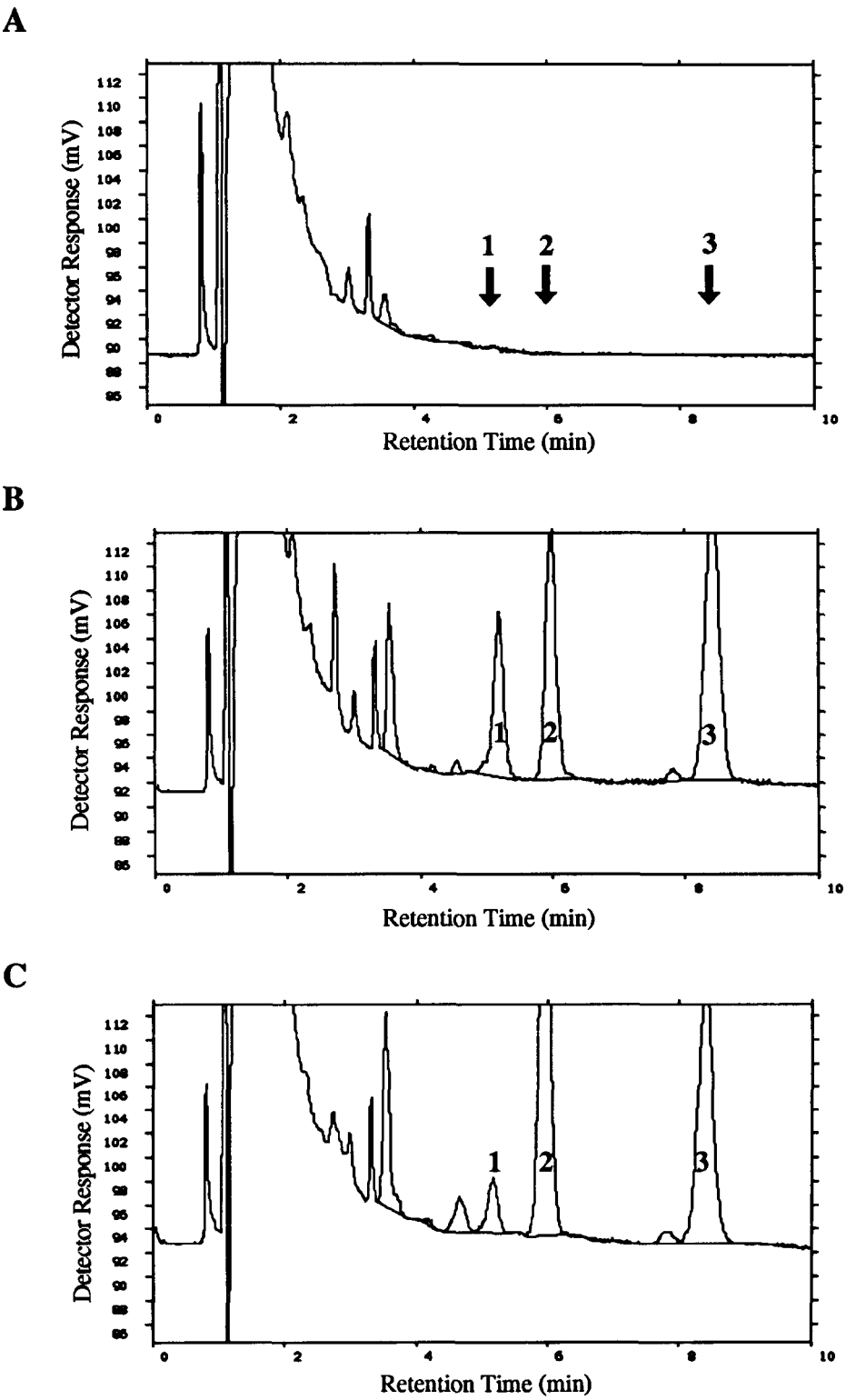


Fig. 4. Representative chromatograms (YMC phenyl column) of naltrexone, 6-$\beta$-naltrexol and internal standard (IS, nalbuphine) in human plasma (denoted 1, 2 and 3, respectively). (A) Control plasma; (B) control plasma spiked with naltrexone, 6-$\beta$-naltrexol and IS at concentrations of 5, 10 and 10 ng ml$^{-1}$, respectively; and (C) post-dose plasma sample from an alcoholic patient 8 h after oral dosing with 50 mg of naltrexone·HCl; the concentrations of naltrexone and 6-$\beta$-naltrexol are equivalent to 1.76 and 19.7 ng ml$^{-1}$, respectively.

Table 1
Intra-day assay precision and accuracy for naltrexone in human plasma

| Concentration added (ng ml$^{-1}$) | Concentration found (ng ml$^{-1}$) | Difference (%) | Mean concentration found ± SD (ng ml$^{-1}$) | Mean difference (%)[a] | RSD (%)[b] |
|---|---|---|---|---|---|
| 0.25 | 0.25 | 0.0 | 0.26 ± 0.02 | 7.2 | 7.7 |
| | 0.28 | 12.0 | | | |
| | 0.23 | −8.0 | | | |
| | 0.25 | 0.0 | | | |
| | 0.29 | 16.0 | | | |
| 0.50 | 0.51 | 2.0 | 0.49 ± 0.03 | 2.8 | 6.1 |
| | 0.45 | −10.0 | | | |
| | 0.51 | 2.0 | | | |
| | 0.50 | 0.0 | | | |
| | 0.50 | 0.0 | | | |
| 5.00 | 5.19 | 3.8 | 5.09 ± 0.07 | 1.8 | 1.4 |
| | 5.12 | 2.4 | | | |
| | 5.01 | 0.2 | | | |
| | 5.04 | 0.8 | | | |
| | 5.08 | 1.6 | | | |
| 15.0 | 15.3 | 2.0 | 15.1 ± 0.2 | 1.1 | 1.3 |
| | 14.9 | −0.7 | | | |
| | 15.0 | 0.0 | | | |
| | 15.2 | 1.3 | | | |
| | 15.2 | 1.3 | | | |
| 50.0 | 51.4 | 2.8 | 51.6 ± 0.5 | 3.2 | 1.0 |
| | 52.0 | 4.0 | | | |
| | 51.0 | 2.0 | | | |
| | 51.5 | 3.0 | | | |
| | 52.1 | 4.2 | | | |

[a] Mean percentage difference calculated without regard to sign.
[b] Relative standard deviation for concentration.

### 2.4. Sample preparation

A constant volume of plasma (2.0 ml) was extracted for all standard, quality control and unknown clinical samples by diluting low-volume or anticipated high-concentration unknown samples with blank human plasma. To this 2.0 ml plasma sample, 200 μl of aqueous internal standard (nalbuphine, 100 ng ml$^{-1}$) were added, followed by the addition of 1.0 ml of 1 M sodium carbonate buffer (pH 9.0). After brief vortex mixing ( < 10 s), the sample was extracted with 10 ml of dichloromethane by mechanical rotation (Rugged Rotator, Kraft Apparatus, Mineola, NY, USA) for 30 min. After centrifugation for 10 min at 1240*g*, the aqueous layer was aspirated to waste and the remaining organic layer was transferred to a 15 ml conical tube containing 200 μl of 0.017 M phosphoric acid. The conical tube was mixed for 4 min on a multi-tube vortex mixer (SMI, Emeryville, CA, USA) at high speed. After centrifugation for 10 min at 1240*g*, the acid layer was transferred to a glass autosampler insert/vial and a 150 μl injection was made on to the HPLC column.

### 2.5. Validation procedures

Plasma standards ranging from 0.25 to 50 ng ml$^{-1}$ and 0.5 to 100 ng ml$^{-1}$ for naltrexone and 6-β-naltrexol, respectively, were prepared daily to create independent calibration curves for validation or subject samples. Calibration curves were constructed by a power curve fit of the peak-

Table 2
Intra-day assay precision and accuracy for 6-$\beta$-naltrexol in human plasma

| Concentration added (ng ml$^{-1}$) | Concentration found (ng ml$^{-1}$) | Difference (%) | Mean concentration found ± SD (ng ml$^{-1}$) | Mean difference (%)[a] | RSD (%)[b] |
|---|---|---|---|---|---|
| 0.50 | 0.45 | −10.0 | 0.46 ± 0.03 | 8.8 | 6.5 |
| | 0.43 | −14.0 | | | |
| | 0.42 | −16.0 | | | |
| | 0.48 | −4.0 | | | |
| | 0.50 | 0.0 | | | |
| 1.00 | 1.00 | 0.0 | 1.00 ± 0.03 | 1.8 | 3.0 |
| | 0.96 | −4.0 | | | |
| | 1.00 | 0.0 | | | |
| | 1.00 | 0.0 | | | |
| | 1.05 | 5.0 | | | |
| 10.0 | 9.65 | −3.5 | 9.78 ± 0.08 | 2.2 | 0.8 |
| | 9.79 | −2.1 | | | |
| | 9.86 | −1.4 | | | |
| | 9.83 | −1.7 | | | |
| | 9.76 | −2.4 | | | |
| 50.0 | 50.4 | 0.8 | 50.0 ± 0.7 | 1.0 | 1.4 |
| | 48.9 | −2.2 | | | |
| | 50.0 | 0.0 | | | |
| | 49.8 | −0.4 | | | |
| | 50.7 | 1.4 | | | |
| 100 | 101 | 1.0 | 102 ± 1 | 1.6 | 1.0 |
| | 102 | 2.0 | | | |
| | 101 | 1.0 | | | |
| | 101 | 1.0 | | | |
| | 103 | 3.0 | | | |

[a] Mean percentage difference calculated without regard to sign.
[b] Relative standard deviation for concentration.

height ratio (PHR) of either naltrexone or 6-$\beta$-naltrexol to the internal standard against the corresponding known amount of the respective analyte. The power curve regression equation was PHR = $a$(amount)$^b$, where $a$ and $b$ are the regression coefficients. The intra- and inter-day precision (relative standard deviation, RSD) of the method was assessed by assaying plasma quality control samples at five concentrations (0.25, 0.50, 5.0, 15 and 50 ng ml$^{-1}$ for naltrexone and 0.50, 1.0, 10, 50, and 100 ng ml$^{-1}$ for 6-$\beta$-naltrexol) on days 1 and 2 ($n = 1$ per concentration) and day 3 ($n = 5$ per concentration). The accuracy of the assay was evaluated as the absolute value of the percentage difference between the assayed concentration and spiked concentration for the replicate ($n = 5$) quality control samples assayed on day 3. The specificity of the assay was determined by assaying blank control plasma from several different human donors to ensure that no endogenous chromatographic interferences were observed. Extraction efficiency was determined by comparing the peak heights of extracted plasma standards with those of corresponding unextracted standards. The stability of naltrexone and 6-$\beta$-naltrexol in human plasma stored at −20°C was evaluated. Plasma samples were spiked at concentrations of 1.5 and 85.0 ng ml$^{-1}$ for naltrexone and 3.0 and 85.0 ng ml$^{-1}$ for 6-$\beta$-naltrexol and assayed in duplicate immediately and at various times after storage. In addition, the stability of naltrexone and 6-$\beta$-naltrexol extracted from human plasma into 0.017 M phosphoric acid (HPLC autosampler stability) was determined.

### 2.6. Application to clinical trials

This assay method was used to quantify naltrexone and 6-β-naltrexol plasma levels in both healthy subjects (after a single 100 mg oral dose of naltrexone) and in alcoholic patients (after a 50 mg oral dose of naltrexone). Venous blood samples were collected at various time points for up to 48 h following drug administration. Plasma samples, obtained following centrifugation of the blood, remained frozen ( − 20°C) until assayed.

## 3. Results

### 3.1. Detector and chromatography optimization

Optimal cell potentials were determined for naltrexone and 6-β-naltrexol by constructing a hydrodynamic voltammogram with a standard mixture containing both analytes (Fig. 2). A potential of + 0.65 V for the second detector electrode was ultimately selected since this resulted in an optimal response for both naltrexone and 6-β-naltrexol. Chromatographic selectivity using a mixture of acetonitrile and methanol was also examined and is depicted in Fig. 3. It was desirable to have naltrexone elute prior to 6-β-naltrexol since lower plasma concentrations of parent drug relative to its metabolite are almost always attained. A 50 mM ammonium phosphate buffer comprised of varying proportions of acetonitrile and methanol (total organic content maintained at 20%) was evaluated. It was found that a mixture of 20% methanol, with no acetonitrile, resulted in the desired column selectivity and provided good separation between naltrexone and 6-β-naltrexol. In addition, the apparent mobile phase pH (3.2) was investigated and found to be a critical factor in providing adequate separation of naltrexone from a small, prior-eluting endogenous plasma substance.

Table 3
Inter-day assay precision for naltrexone and 6-β-naltrexol in human plasma

| Compound | Concentration added (ng ml$^{-1}$) | Mean concentration found (ng ml$^{-1}$)[a] | RSD (%) |
|---|---|---|---|
| Naltrexone | | | |
| | 0.25 | 0.27 | 7.4 |
| | 0.50 | 0.49 | 6.1 |
| | 5.00 | 4.94 | 4.7 |
| | 15.0 | 15.3 | 0.5 |
| | 50.0 | 51.2 | 1.5 |
| 6-β-Naltrexol | | | |
| | 0.50 | 0.47 | 4.3 |
| | 1.00 | 0.97 | 8.2 |
| | 10.0 | 9.89 | 2.4 |
| | 50.0 | 50.5 | 0.7 |
| | 100 | 98.9 | 2.8 |

[a] $n = 3$.

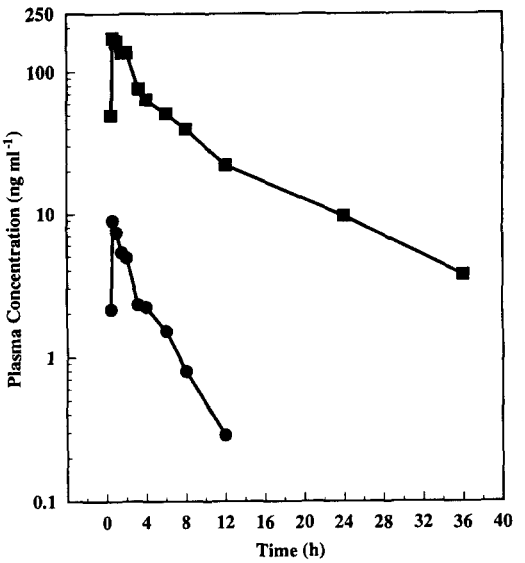


Fig. 5. Time course of naltrexone (●) and 6-β-naltrexol (■) in plasma after oral administration of 50 mg of naltrexone to an alcoholic patient.

### 3.2. Chromatography and assay validation

Representative chromatograms of control plasma, control plasma spiked with naltrexone, 6-β-naltrexol and the internal standard, and an 8 h post-dose plasma sample from an alcoholic patient after an oral dose of 50 mg of naltrexone are shown in Fig. 4. Analysis of control plasma from several different donors indicated the absence of interfering endogenous compounds, confirming adequate assay specificity. There was sufficient separation of the analytes and internal

standard, with retention times of 5.0, 6.0, and 8.4 min for naltrexone, 6-$\beta$-naltrexol and the internal standard, respectively.

The lower limits of quantification for naltrexone and 6-$\beta$-naltrexol were 0.25 and 0.50 ng $ml^{-1}$, respectively. The intra-day reproducibility and accuracy of the assay, determined by the analysis of five replicate plasma spikes at five different plasma concentrations, are shown in Tables 1 and 2. The RSD ranged from 1.0 to 7.7% for naltrexone and from 0.8 to 6.5% for 6-$\beta$-naltrexol. Individual percentage differences ranged from 0.0 to $\pm$16.0% for both naltrexone and 6-$\beta$-naltrexol, with a mean of $\pm$1.1 to 7.2% for naltrexone and $\pm$1.0 to 8.8% for 6-$\beta$-naltrexol. The inter-day reproducibility, determined over three separate assay days, is shown in Table 3. The RSD ranged from 0.5 to 7.4% for naltrexone and from 0.7 to 8.2% for 6-$\beta$-naltrexol. A good relationship ($r^2 > 0.999$) between PHR and the extracted amount of plasma standard was observed for both naltrexone and 6-$\beta$-naltrexol.

The overall extraction recovery averaged 95 $\pm$ 13.6% for naltrexone and 74 $\pm$ 5.2% for 6-$\beta$-naltrexol and these recoveries were independent of concentration. No degradation of naltrexone or 6-$\beta$-naltrexol was observed in spiked human plasma following storage at $-20$°C for at least 8 months. Additionally, the stability of both compounds extracted from human plasma into 0.017 M phosphoric acid was demonstrated for up to 40 h, indicating that extracted samples could remain on the HPLC autosampler for this time period.

## 4. Discussion

The present method provided the necessary sensitivity for the simultaneous quantification of naltrexone and 6-$\beta$-naltrexol in human plasma following low clinical doses of oral naltrexone. Fig. 5 shows representative plasma concentration–time profiles of naltrexone and 6-$\beta$-naltrexol in an alcoholic patient following a single 50 mg oral dose of naltrexone. In this patient, naltrexone and 6-$\beta$-naltrexol both peaked at approximately the same time (40 min) and at concentrations of 9.0 and 171 ng $ml^{-1}$, respectively; however, the naltrexone concentration declined faster than that of its metabolite. These results in a single patient are similar to other pharmacokinetic results in healthy subjects [5,16] and patients [16], where naltrexone and 6-$\beta$-naltrexol plasma concentrations typically decline in a biphasic fashion with terminal disposition half-lives of ca. 4 and 13 h, respectively.

Prior to the development of the present analytical method, naltrexone in plasma was measured using a published method for nalbuphine [17] which was readily amenable to naltrexone [5]. Recent and sporadic specificity problems attributed to the extraction solvent (toluene) and/or HPLC column manufacturing changes, in addition to day-to-day instability of the amperometric detector electrode (glassy carbon), prompted major modifications to the extraction procedure and chromatographic conditions. Most significantly, the use of a porous graphite electrode minimized previous analytical problems associated with the instability of the glassy carbon electrode. The present method was used continuously for over a 3 month period with no associated instrument problems, although periodic (ca. weekly) rinsing of the HPLC system and column has been performed with reimplementation of mobile phase flow immediately following system rinsing.

In summary, a simple, sensitive and robust HPLC method with coulometric detection was developed for the determination of naltrexone and 6-$\beta$-naltrexol in human plasma. This method offers an economical alternative to GC–MS and still provides sufficient sensitivity to study the clinical pharmacokinetics of naltrexone and 6-$\beta$-naltrexol.

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