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Measurement of naloxone in plasma using high-performance liquid chromatography with electrochemical detection

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ABSTRACT

A new sample preparation technique, solid-phase extraction employing the ion-pair reagent octyl sodium sulfate (OSS), was developed for the selective isolation of nanogram quantities of naloxone from human plasma. Plasma samples containing naloxone, OSS, buffer, and naltrexone (internal standard) were applied to octyldecylsilane (C_{18}) cartridges, and the opiates were eluted with 100% methanol. An extraction efficiency of 90% was achieved. Extracts were then examined by reversed-phase high-performance liquid chromatography with electrochemical detection, with mobile phase composition adjusted to optimize separation and quantitation. An oxidizing potential of +0.93 V resulted in an assay sensitivity of 1.0 ng. Thus, plasma naloxone levels of *ca.* 20 ng/ml could be readily detected and quantified. The intra-assay coefficient of variation at plasma levels of 80 ng/ml was 7.2%. Plasma naloxone values obtained during a 5-h infusion of the drug (0.24 mg/min) in two normal human subjects were stable and reproducible. Thus, the method herein described is applicable to human studies employing naloxone infusion.

INTRODUCTION

Naloxone, 4,5-epoxy-3,14-dihydroxy-17-(2-propenyl)morphinan-6-one hydrochloride, is a non-specific opioid antagonist. It is widely used clinically for the treatment of opiate overdose. In addition, it has been shown to elevate blood pressure in patients with shock [1] and to have a mild diuretic effect in patients with liver cirrhosis [2]. Several methods to measure naloxone in biological specimens have been reported. Most utilize solvent extraction and isolation procedures with reversed-phase high-performance liquid chromatography (HPLC) equipped with either an ultraviolet [3–5] or an electrochemical [6–13] detector. Organic solvent extractions are time-consuming, difficult, and pose possible health and environmental risks if the proper safety precautions are not enforced. Ultraviolet detection methods are less sensitive than those employing electrochemical detection (ED) measurements [7,10]. Naloxone can be measured by radioimmunoassay [14]. Though sensitive, this method lacks specificity if the antiserum cross-reacts with the metabolites of naloxone. Gas chromatographic procedures have been described but they involve derivatization procedures in order to make the opiates volatile and resolvable [15,16].

We herein present an assay for naloxone which is rapid, reproducible, and sensitive. The method utilizes a C_{18} solid-phase extraction (SPE) column for isolating naloxone and the structurally related naltrexone, which is used as the internal standard. For quantitation, we used a reversed-phase HPLC–ED system.

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EXPERIMENTAL

Chemicals and equipment

HPLC-grade octyl sodium sulfate (OSS) was obtained from Kodak (Rochester, NY, USA). HPLC-grade methanol, HPLC-grade acetonitrile, boric acid and HCl were from Mallinckrodt (Paris, KY, USA). Ammonium dihydrogenphosphate and monochloroacetic acid (MCAA) were from Fisher (Itasca, IL, USA). The following were obtained from Sigma (St. Louis, MO, USA): NaOH, triethylamine (TEA), disodium ethylenediaminetetraacetic acid (Na_2EDTA) and naltrexone · HCl. Naloxone was from Merck–DuPont (Wilmington, DE, USA).

A working solution of 1 ng/μl naloxone was prepared by diluting a stock of 0.4 mg/ml naloxone in saline with the mobile phase solution. A working solution of 1 ng/μl naltrexone was made from a stock naltrexone solution of 0.1 mg/ml prepared in saline. Borate buffer (pH 9.0) was prepared as follows: (A) 17.5 ml of boric acid solution (1.237 g of boric acid, 90 ml of water and 10 ml of 1 *M* HCl); and (B) 83.5 ml of 0.1 M HCl.

The mobile phase consisted of 15% acetonitrile and buffer (0.1 *M* MCAA, 2.59 m*M* OSS and 2.39 m*M* EDTA, pH 4.5). The pH of the buffer was adjusted with NaOH. The mobile phase was filtered and degassed before it was placed in use. Double-distilled water was used throughout the procedure. Polypropylene tubes (75 mm × 12 mm) washed with 6.3 *M* nitric acid were used. Bonded-phase C_{18} SPE cartridges were employed (Sep-Pak, Waters, Millipore, Milford, MA, USA).

A Perkin Elmer (Norwalk, CT, USA) Series 410 LC pump in conjunction with a Bioanalytical Systems (West Lafayette, IN, USA) LC-4A amperometric electrochemical detector with a glassy carbon electrode was used in this study. A Coulochem 5100A electrochemical detector (ESA, Bedford, MA, USA) with a standard analytical cell was also used for some of the samples. The column used was a 150 mm × 4.6 mm reversed-phase C_{18} 5-μm column (Econosphere, Alltech/Applied Science, State College, PA, USA). The mobile phase was continuously recirculated through the system at a flow-rate of 1 ml/min. The chart speed was set at 5 mm/min. The potential was set at +0.79 to +0.93 V, with best results obtained at +0.89 V (see Results and discussion).

Sample collection

Healthy human subjects were given a 5.0-mg intravenous bolus of naloxone followed by a constant infusion of the drug at a rate of 0.06 mg/min for 5 h. On a separate day, subjects were given a 20-mg bolus followed by a 0.24 mg/min infusion for 5 h. A 10-ml volume of heparinized blood was collected at baseline and every hour during the infusion period. Blood specimens were centrifuged for 20 min at 1500 *g* at room temperature, and the plasma poured off into polypropylene tubes and stored at −70°C until needed.

Extraction and chromatography of the opiates

Samples were prepared by mixing the following: 250 μl of plasma, 750 μl of water, 1000 μl of borate buffer, 200 μl of 0.3 m*M* OSS, and 80 ng of naltrexone as an internal standard. For recovery studies of naloxone, 0–100 ng of naloxone were added to drug-free plasma obtained from healthy subjects prior to naloxone infusion. The SPE columns were conditioned by first washing with 2 ml of methanol and then 2 ml of 0.109 m*M* OSS using a pressure of 7 kPa. The mixture containing the samples was then added and allowed to adsorb to the SPE cartridges at a very reduced pressure (approximately 0.007 MPa). The loaded columns were next washed four times with 2.5 ml of distilled water each time and followed by 2 × 2.5 ml of methanol–water (50:50, v/v) at a pressure of 7 kPa. The opiates were eluted with 2 ml of 100% methanol without applied pressure. The eluates were evaporated under nitrogen at 65°C and the dried extracts were resuspended in 100 μl of mobile phase solution. A volume of 20 μl or less was injected into loop of the HPLC system.

In general, extracts were injected into the HPLC system for measurement of naloxone within 24 h of the extraction. However, we found that extracts could be stored for several weeks at −70°C without appreciable loss of naloxone.

TABLE I

EFFECT OF pH, TEA AND NaOH ON SENSITIVITY AND RETENTION TIMES

The mobile phase consisted of 14% acetonitrile, 86% 0.1 *M* MCAA, 2.39 m*M* EDTA and 1.29 m*M* OSS.

pH	Naloxone		Naltrexone	
	Retention time (min)	Peak height (cm)	Retention time (min)	Peak height (cm)
3.2	6.0	1.9	8.6	0.9
4.5 with TEA	3.4	3.4	4.8	1.8
4.5 with NaOH	7.0	5.5	10.4	2.8

Storage of extracts in excess of two months resulted in a 50-100% reduction in measurable naloxone. Unextracted plasma samples stored at −70°C were stable for at least six months.

RESULTS AND DISCUSSION

Recovery of naloxone and naltrexone

Reported recoveries of naloxone using liquid-phase extraction procedures are 67–88% [3,4,9,11]. In most of these methods, a larger volume of sample was used compared to the present method [3,4,6,9,11–13,17]. An SPE procedure using a CN column has been described [10]. However, these investigators reported only a 45% recovery for naloxone.

We were able to achieve substantially better recoveries for both naloxone and naltrexone using the C_{18} SPE method described. Addition of the ion-pair reagent OSS to the sample markedly affected opiate recovery. In the absence of OSS, recovery was only 65%. With 0.027 *M* OSS (*i.e.*, after addition of 200 μl of 0.3 *M* OSS), the recovery of both naloxone and naltrexone was approximately 90% at levels of 40–200 ng/ml for each.

Optimization of the mobile phase composition

In order to arrive at a suitable solvent system for the analytical column, we investigated the effects of pH, basic solutions for adjusting the pH, ion-pair concentration, and methanol–acetonitrile organic modifiers. When the pH of the mobile phase was increased from 3.2 to 4.5 using either TEA or NaOH, an increase in sensitivity (peak height) for naloxone was noted; this effect of pH on sensitivity was greatest when NaOH was used as the alkalinizing agent (Table I). However, divergent effects on retention time were noted with the two pH modifiers. As observed by others [18], use of NaOH resulted in a somewhat longer retention of the opiates on the column.

The ion-pair reagent OSS probably exerts its effects by adsorbing onto the column and undergoing an ion-exchange type of reaction with available counter-ions. Utilizing this agent, we were able to increase the retention time for the opiates and avoid co-elution of interfering peaks (Table II). The retention time was also affected

TABLE II

EFFECT OF OSS AND ACETONITRILE ON RETENTION TIMES

The mobile phase consisted of 15 or 20% acetonitrile, 85 or 80% 0.1 *M* MCAA, 2.39 m*M* EDTA and 1.29, 2.59 or 3.45 m*M* OSS (pH 4.5 with NaOH).

Acetonitrile (%)	OSS (m*M*)	Retention time (min)	
		Naloxone	Naltrexone
15	1.29	5.2	7.4
15	2.59	6.2	8.6
15	3.45	6.8	9.8
20	1.29	4.2	5.8
20	3.45	4.6	6.2

by the concentration of acetonitrile employed, with higher concentrations leading to decreased retention times (Table II).

Evaluation of oxidizing potentials

As noted previously by others [7,9,11], the use of a high applied potential to oxidize the opiates resulted in higher sensitivity, yet also increased baseline instability and noise and necessitated more frequent cleaning of the electrode. The effect of different applied potentials on opiate sensitivity is given in Table III, with each point representing the mean of four injections. We found that an applied voltage of +0.89 V gave adequate sensitivity with an acceptable signal-to-noise ratio. At this voltage, a noticeable loss in sensitivity occurred after about 25–30 injections. O'Connor *et al.* [9] reported similar responses for voltages in the +0.75 to +1.05 V range.

Electrochemically, naloxone and naltrexone have the same *o*-dialkoxy functional groups, with the difference occurring at C-17, where naloxone has a 2-propanyl group and naltrexone has a tertiary amine contained in a cyclopropyl group. The greater peak heights seen with naloxone in our studies are probably explained by the more electroactive tertiary amine of naloxone. Garrett *et al.* [17] have reported similar findings.

TABLE III

EFFECT OF VOLTAGE ON PEAK HEIGHT

The mobile phase consisted of 20% methanol, 13% acetonitrile, 67% 0.05 *M* phosphate buffer pH 4.5, 2.39 m*M* EDTA and 1.29 m*M* OSS.

Voltage (V)	Peak height (cm)		Naltrexone/naloxone peak-height ratio
	Naloxone	Naltrexone	
0.79	3.7	3.4	0.92
0.81	6.1	4.8	0.79
0.83	8.6	5.3	0.62
0.85	10.0	6.9	0.69
0.87	11.7	8.0	0.68
0.89	13.7	9.0	0.66
0.91	19.0	11.8	0.62
0.93	21.4	13.4	0.63

Assay chromatography and sensitivity

Using the chromatographic conditions that are listed in Table III at a flow-rate of 0.8 ml/min, separation and quantitation of naloxone were achieved. The retention times were 6.1 and 8.0 min for naloxone and naltrexone, respectively (Fig. 1).

With a clean oxidizing electrode, 1.0 ng of naloxone injected into the column gave a signal-to-noise ratio of 10:1. Thus, assuming 90% recovery of opiates during extraction and a 20-μl injection volume, our assay could be used to measure plasma naloxone concentrations of *ca.* 20 ng/ml. By increasing the amount of plasma placed on the SPE column or by increasing the quantity of extract injected, sensitivity can be increased further if necessary.

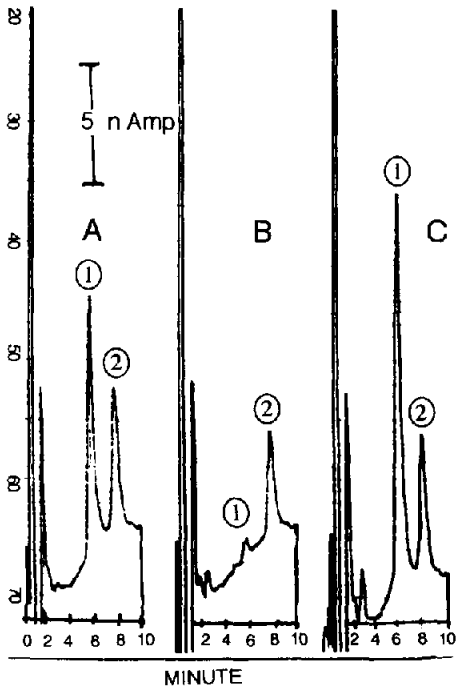

Fig. 1. Typical chromatograms of (A) extracted standards containing naloxone and naltrexone (20 ng each), (B) subject's plasma prior to naloxone infusion and (C) subject's plasma 2 h after naloxone infusion. Naltrexone (internal standard) was added to the subject's plasma prior to extraction. Peaks: 1 = naloxone; 2 = naltrexone.

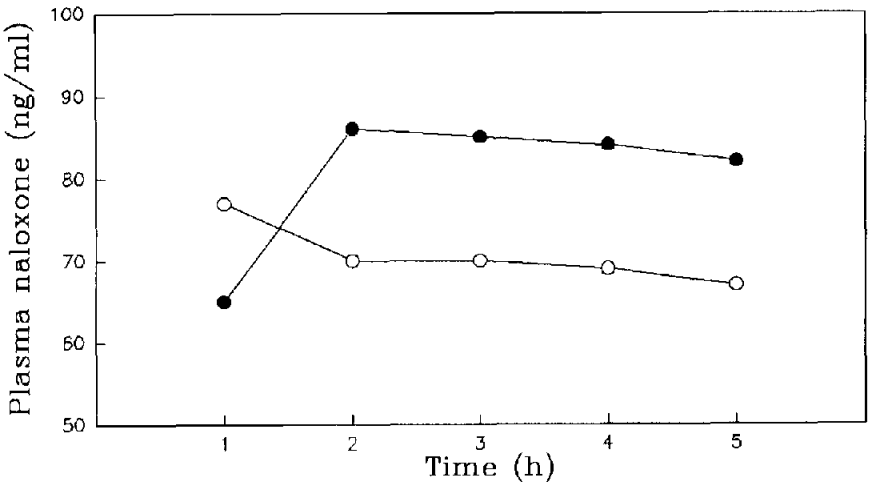

Fig. 2. Plasma naloxone values of two subjects given a 20-mg bolus of naloxone at time 0 followed by a 5-h infusion at a rate of 0.24 mg/min.

Precision

Using this assay method, the intra-assay precision was as follows (n = 7 each): mid-level control, 79 ± 5.0 ng/ml (C.V. 6.3%); high-level control, 161 ± 6.9 ng/ml (C.V. 4.3%). The inter-assay averages (n = 8 each) were: low-level control, 42 ± 3.2 ng/ml (C.V. 7.6%); mid-level control, 81 ± 7.0 (C.V. 8.6%).

Biochemical evaluation

In two healthy subjects given intravenous naloxone (20-mg bolus followed by a 0.24 mg/min infusion), plasma naloxone levels 5 h after the start of the infusion were 69 and 84 ng/ml, respectively. Steady-state plasma levels of naloxone during the last 4 h of infusion are shown in Fig. 2.

CONCLUSION

Naloxone is a widely used drug, and a method to accurately measure its concentration in plasma is of interest. The assay we have described is relatively simple and allows for a more routine clinical application. The retention times for the opiates using the chromatographic system described are conveniently short, yet allow for good separation and quantitation of naloxone. The structurally related naltrexone is used as the internal standard. The detection limit of the method is 1.0 ng/ml naloxone (signal-to-noise ratio of 10:1). Both opiates gave identical recoveries with the SPE procedure.

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