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QUANTITATIVE AND PHARMACOKINETIC ANALYSIS OF NALOXONE IN PLASMA USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION AND SOLID-PHASE EXTRACTION

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SUMMARY

In this study we present a method for measuring naloxone in plasma after intravenous and oral administration of naloxone to humans, in order to study its pharmacokinetic profile. The method consists of a solid-phase extraction step followed by detection on a high-performance liquid chromatographic (HPLC) system equipped with an electrochemical dual-electrode detector. The extraction step employs cyanopropyl columns optimized for naloxone extraction to allow for elution of naloxone by the HPLC mobile phase; this eluate is then directly injected in the HPLC instrument. The HPLC system employs a radial compression phenyl column with a mobile phase containing 18% (v/v) acetonitrile and pentanesulfonic acid as ion-pairing agent; this system shows extraordinary high plate counts for naloxone. The detection limit is 3 ng (signal-to-noise ratio = 3) free naloxone per ml plasma. Following intravenous injection of 30 mg naloxone hydrochloride in two subjects, it was possible to determine the free naloxone concentration in the plasma for 8 h, more than four times the half-life of naloxone in plasma in humans.

INTRODUCTION

The presence of opiate receptors has been strongly suspected in the intestinal wall of man, because of their identification in the intestinal wall of some mammalian species [1] and the presence of endogenous opioids in the enteric nervous system. They have been implicated in the control of intestinal motility [1–4]. Therefore it has been hypothesized that naloxone, a specific opiate antagonist, would have some opioid blocking effect in the gut and reverse opioid induced hypomotility [1,5]. The plasma concentration of free, unmetabolized naloxone after oral administration is low (peak: 15 ng/ml of plasma as determined by radioimmunoassay after a 16-mg intake of naloxone hydrochloride) [6], requiring

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a sensitive and specific analytical technique to establish the pharmacokinetics of naloxone.

The use and abuse of the opiate alkaloids has led to a rapidly increasing interest in the quantitative analysis of these compounds and has produced a vast array of techniques for their separation and determination. High-performance liquid chromatography (HPLC) with ultraviolet (UV) absorption [7–9] and electrochemical detection (ED) [10–18], thin-layer chromatography (TLC) [19] and radioimmunoassay (RIA) [20,21] have all been employed in the separation and determination of the opiates. In this study ED was chosen for its higher sensitivity and selectivity, as compared to UV detection. In determination of the pharmacokinetics of naloxone, ED is capable of detecting free naloxone, while not detecting naloxone-3-glucuronide, the major degradation product of naloxone in man.

ED has been most widely reported using amperometric detection on a single glassy carbon electrode [10,11,13–18]. In this study, we used a detector with two porous graphite electrodes in series, allowing the first electrode to oxidize extraneous peaks, while not oxidizing the compounds of interest. The second electrode is used for measurement. Because of this screening capability, and because of its porous electrodes, this detection system has been shown to allow high sensitivity and selectivity for opiates extracted from plasma [12].

A solid-phase extraction is employed rather than liquid–liquid extraction, because of the former's higher precision and ease of use, thus introducing less variability [12] and suitability for large-scale sample processing. The extraction procedure was optimized to make a direct extraction into the mobile phase possible.

The analytical qualities of ED made its use the most promising in the present study in which we set out to measure the systemic bioavailability of naloxone after oral and intravenous administration in humans.

EXPERIMENTAL

Reagents

The mobile phase consisted of 5 m*M* sodium pentanesulfonic acid, monohydrate (HPLC grade, Fisher Scientific, Springfield, NJ, U.S.A.), 18% (v/v) acetonitrile, UV grade (American Burdick & Jackson Labs., Muskegon, MI, U.S.A.), 0.0045% (v/v) orthophosphoric acid–85% (w/v) (HPLC grade, Fisher Scientific) and 82% (v/v) water (distilled, deionized, by Millipore Milli-Q system, Waters Assoc., Bedford, MA, U.S.A.), pH 3.1 (ION85 ion analyzer, Radiometer, Copenhagen, Denmark). For the extraction, cyanopropyl columns, 100-mg/1.0-ml Bond-Elut® (Analytichem International, Harbor City, CA, U.S.A.) were used. Naloxone hydrochloride was donated by Key Pharmaceutical (Miami, FL, U.S.A.) and IVAX (Miami, FL, U.S.A.); 6.8 μl [^{3}H]naloxone [37.0 MBq/ml (1 mCi/ml) of ethanol, specific activity 1.54 TBq/mmol (41.5 Ci/mmol) obtained from New England Nuclear, Boston, MA, U.S.A.] was added to 10 ml water, to produce the internal standard solution with 38 000 dpm per 25 μl. A calibration solution was made up of 25 μl of internal standard solution plus 35 μl (1 ng/μl) of naloxone hydrochloride and 940 μl of mobile phase; 400 μl of this solution were injected

into the HPLC instrument. The area under the peak emerging at the retention time (t_R) of free naloxone (8.7 min) and dpm in the collected fraction were used to calculate the naloxone concentration in each sample (see *Procedure*).

Scintillation

Counting of the internal standard was accomplished by adding each of the collected fractions of 10 ml Hydrofluor® (National Diagnostics, Manville, NJ, U.S.A.) plus 2 ml glacial acetic acid (Fisher Scientific), followed by counting in a scintillation counter (Minaxi-Beta Tricarb 4000, Packard, Sterling, VA, U.S.A.).

HPLC system

The HPLC system consisted of a helium-sparged 2-l reservoir (eluent stabilization system, Waters Assoc.) feeding the pump (Model 6000A solvent delivery system, Waters Assoc.) with a pulse dampener in line. Between the injector (Model U6K universal chromatograph injector, Waters Assoc., loop volume 2 ml) and the column a graphite filter (0.2 μm, ESA Cat. No. 55-0448) was inserted to protect the column (Nova-Pak Phenyl Radial-Pak cartridge, 10 cm×8 mm, 4-μm spherical particles, Waters Assoc. Cat. No. 10658) from particles in the injected extract. Similarly the column was followed by the same type of filter to prevent silica fines, leaking of the column, from entering the detector cell. The detection system consisted of a Coulochem Model 5100A electrochemical detector equipped with a Model 5011 high-sensitivity analytical cell (ESA, Bedford, MA, U.S.A.). The chromatograms were plotted and measured by a Hewlett-Packard 3392A integrator (Hewlett-Packard, Avondale, PA, U.S.A.). The HPLC part of the procedure used the following conditions: temperature, ambient; flow rate, 2 ml/min; pressure, 114 bar. The detector conditions were: detector 1 potential, +0.35 V; detector 2 potential, +0.45 V; amplification, 50 (both detectors); response time (noise filter), 10 s.

Procedure

The 1-ml Bond Elut CN extraction columns (see *Reagents*) were activated by drawing through, under 0.3 bar of vacuum, 1 ml acetonitrile followed by 1 ml water. The plasma samples were centrifuged (conditions: 15 min, 1000 *g*) to remove particulate matter; 700 μl of plasma were mixed with 375 μl of water, 25 μl of 0.4 *M* pentanesulfonic acid and 25 μl of [^{3}H]naloxone (38000 dpm, as internal standard). This mixture was applied to the columns. A vacuum of 0.15 bar was applied, to draw the entire sample through. The columns were washed three times with 1 ml water and dried by drawing air through for 1 min. The naloxone was then eluted with 1 ml of HPLC mobile phase (see *Reagents*). A 400-μl volume of the eluate was injected into the HPLC system through a filter (Millex-GV$_4$, pore size 0.22 μm, diameter 4 mm, Millipore, Bedford, MA, U.S.A.), fitted with a blunt needle, using a 500-μl Hamilton syringe with a Luer lock fitting.

In order to recover the internal standard, [^{3}H]naloxone, 3-ml fractions, corresponding to the naloxone peak 0.75 min before and after the peak, were collected from the outlet of the detector on a fraction collector (2112 RediRac, LKB,

Bromma, Sweden). Each fraction was then mixed with the scintillation cocktail and counted in the scintillation counter.

Each sample area score (A_{smp}) and each standard area score (A_{st}) was corrected by dividing by the dpm from the internal standard (dpm_{smp} and dpm_{st}, respectively). The internal standard, [^{3}H]naloxone, is only sensitive to loss of sample and not to change in detector sensitivity. In order to correct for this, samples were run in batches of eight samples plus two standards, starting and ending the analysis of each batch with a standard. The two standards were then used to adjust for change in electrode sensitivity by correcting the ratio (dpm_{st}/A_{st}) linearly between the first and the last standard. Thus

$$C_{smp,i}\ [ng/ml] = (35/V_{smp})\,(A_{smp}/dpm_{smp})\,r_i$$

where the corrected standard ratio of the ith sample is given by

$$r_i = (r_{last} - r_{first})\ (i/9) + r_{first}$$

$$r = (dpm_{st}/A_{st})$$

where i=the sample number i (1–8), first and last=the first and last standard in the batch and V_{smp}=volume of the plasma sample (in ml), in this study 0.7 ml. Linear regression analysis on the standard curve was performed using an IBM PC-AT and Symphony (Version 1.2) software (Lotus Development, Cambridge, MA, U.S.A.). The compartment analysis was done with the DRUGFUN procedure [22] running under the Prophet computer system (BBN, Cambridge, MA, U.S.A.).

RESULTS AND DISCUSSION

In this assay, the potential of detector 1 was set to +0.35 V instead of +0.25V as suggested by the hydrovoltammogram for naloxone (Fig. 1) in order to reduce

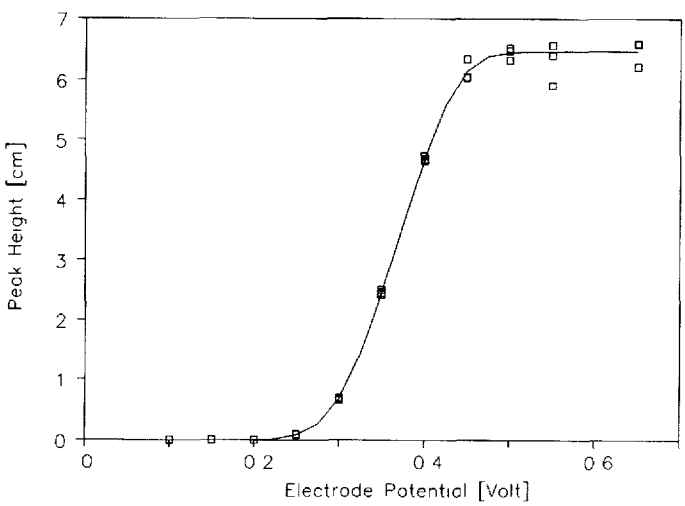

Fig. 1. Hydrodynamic voltammogram for naloxone. The detector response to injections of 20 ng of naloxone hydrochloride was recorded with detector 1 at +0.1 V and detector 2 varying from +0.1 V to +0.7 V. A cubic spline was performed on the means of three recordings at each potential.

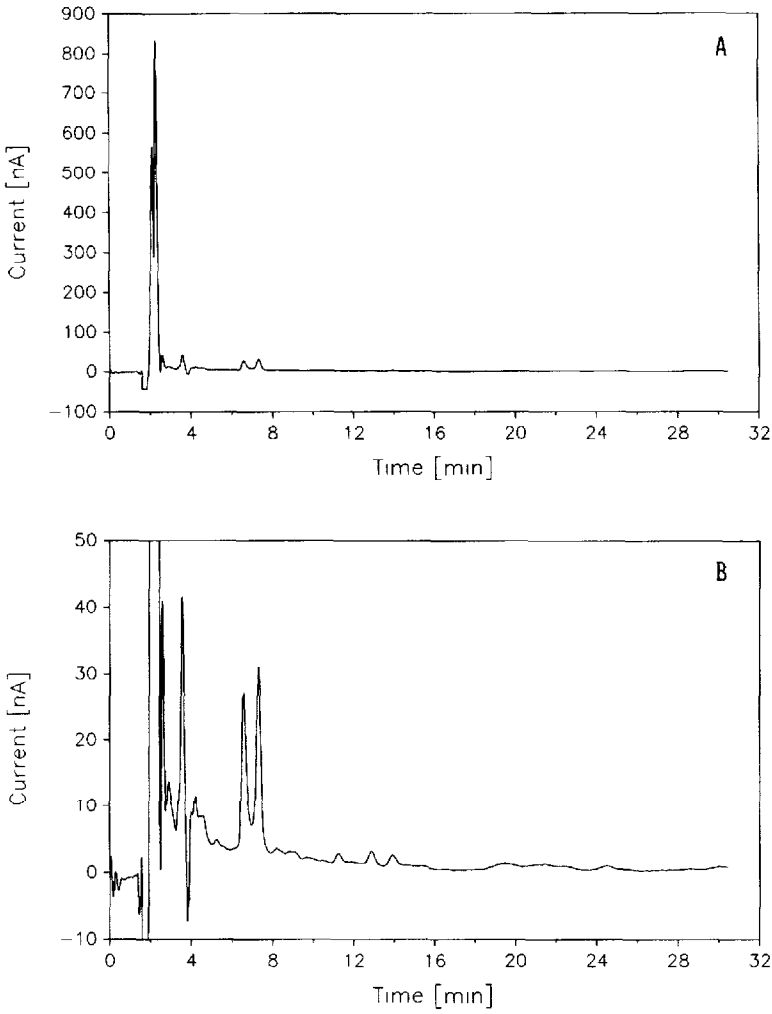

Fig. 2. Chromatogram from injection of extract of blood bank plasma: (A) Full chromatogram. (B) Magnified part of (A). The background noise is approximately equivalent to 0.5 ng naloxone.

an adjacent peak caused by inescapable sample contamination that interfered with the detection of naloxone.

Typical chromatograms of a plasma sample from standard outdated blood bank plasma and a sample of the same plasma spiked with naloxone are shown in Figs. 2 and 3, respectively. The sensitivity is not limited by the background noise generated by the pulsation of the flow from the pump, but rather by low levels of impurities as can be seen in Fig. 2B. The amplitude of the 'pump noise' was 0.1 nA, one order of magnitude below the 'impurity noise'. In order to obtain pump noise this low, it was essential to maintain the pump seals in good condition as well as keeping the level of electrochemically active pollution of the mobile phase low.

Even though we used exhaustive sample filtering and an extraction optimized for naloxone, the electrode eventually became contaminated, thus reducing its

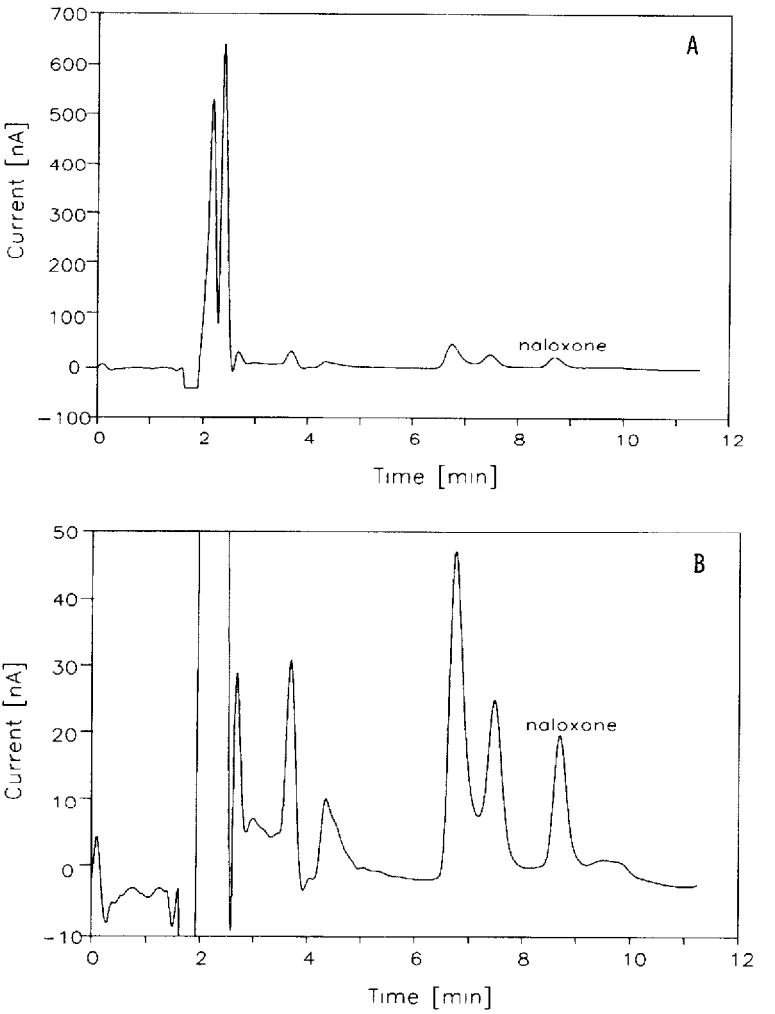

Fig. 3. Chromatogram from injection of extract of blood bank plasma (same plasma as in figure 2) spiked with 10 ng naloxone. (A) Full chromatogram. (B) Magnified part of (A).

sensitivity. This problem was easily dealt with by flushing the electrode with 6 *M* nitric acid, which usually completely restores the electrode performance.

Fig. 4 represents the elution pattern of [^{3}H]naloxone extracted from plasma on the cyano columns. The samples, which were run in triplicate, showed a very low coefficient of variation. Thus, although the recovery was somewhat low (45%), very little variability was observed and improved selectivity of the extraction was gained using this type of column.

The standard curve was prepared with naloxone-spiked plasma in the range 3–500 ng/ml. Even in this wide range, the assay showed a high degree of linearity on a line forced through (0,0) (slope = $2.52 \cdot 10^5$ area score per ng naloxone hydrochloride, $r^2 = 0.995$). Also the intra- and inter-assay coefficients of variation (C.V.) were low (Table I).

The chromatographic system used was a 'π-electron interaction' system (mo-

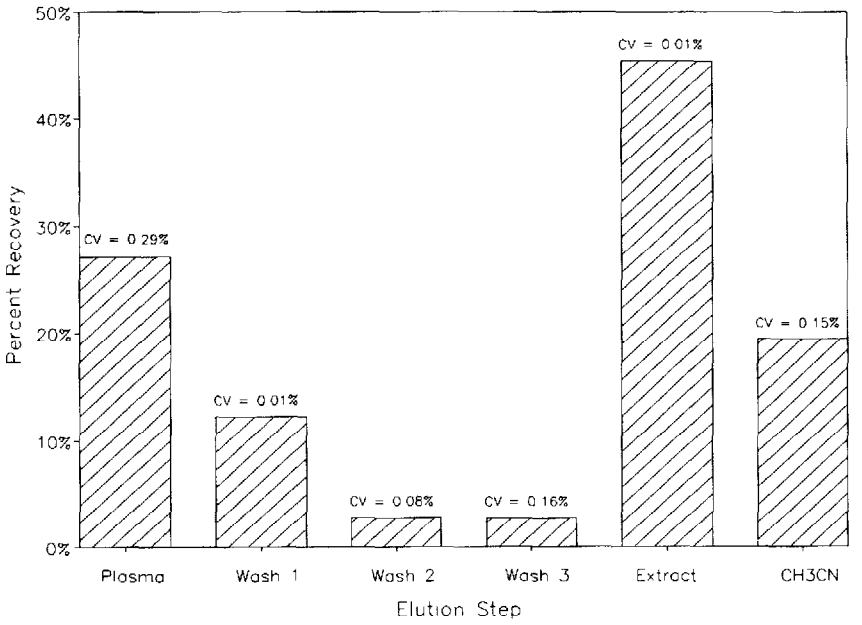

Fig. 4. Distribution of 10 ng [^{3}H]naloxone hydrochloride during extraction of plasma on cyano solid-phase columns. x-Axis represents the stage in the extraction: plasma: the application of the plasma sample; wash 1-3: the 3× wash with 1 ml water; extract: the extraction with 1 ml mobile phase; CH_3CN: extraction of the remaining counts with 100% acetonitrile. y-Axis represents percentage of counts out of total counts recovered in each fraction. The coefficient of variation (C.V.) ($n=3$) is indicated on top of each bar.

TABLE I

CHARACTERISTIC PARAMETERS OF THE NALOXONE ASSAY

Sensitivity: sample concentration when naloxone signal-to-background noise = 3. r^2: the correlation coefficient of the linear fitted standard curve. Intra-assay C.V.: on each of three different days three samples each containing 50 ng/ml naloxone hydrochloride were analyzed; the C.V. of the three samples for each of the three days was calculated, the average of these C.V.s was taken and is reported as intra-assay C.V. Inter-assay C.V.: the C.V. calculated collectively of all the nine samples. Accuracy: the deviation of the average of the nine samples from the expected value of 50 ng/ml.

Parameter	Value
Sensitivity (ng/ml)	3
r^2	0.995
Intra-assay C V. (%)	2.10
Inter-assay C.V. (%)	2.48
Accuracy (%)	0.02

bile phase: acetonitrile, stationary phase: phenyl function) since initial experiments with 'σ-electron interaction' systems (mobile phase: methanol; stationary phase: C_{18} function) gave disappointing results (plate count at half height $N_{\frac{1}{2}h}$ = 1500) in terms of separation, because of low column efficiency for naloxone, as alkaloids analyzed by σ-systems have done for other chromatographers [23].

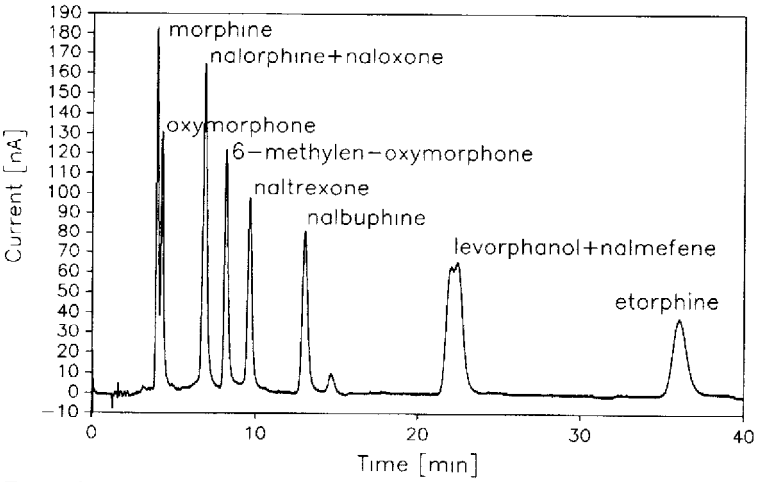

Fig. 5. Chromatogram of a mixture of 10 ng of each of ten different opiates (etorphine 20 ng).

Jhangiani and Bello [7] and Suthheimer et al. [24] obtained an $N_{\frac{1}{2}h}$ of 1200 for morphine and naloxone, respectively, while both employing a system of a C_{18} column with a methanol-based mobile phase. The present technique achieved a half-height plate count of 6000, demonstrating the superior performance of radial compression phenyl columns for use with alkaloid opioids and probably other basic aromatic amines as well (Fig. 5). The high efficiency of the phenyl column may be ascribed both to the acetonitrile–phenyl chemistry and to the radial compression packing of the column. The radial compression technique has been claimed to achieve perfect sphere packing, a configuration which counteracts bandspreading of peaks caused by a type of flow turbulence known as 'eddy diffusion' [25,26].

Even though the HPLC separation can be extended to a variety of opiate agonists and antagonists as demonstrated in Fig. 5, a more hydrophobic extraction column would probably be necessary for some of the other opiates. A number of opiates were extracted using various columns from the Analytichem® development kit (see Experimental) and it was found that compounds like morphine and naltrexone would need more hydrophobic columns such as C_2 or phenyl to be retained.

Application

The analytical procedure was applied in a pharmacokinetic study of naloxone in humans. In two experiments in healthy volunteer subjects, a bolus injection of 30 mg naloxone hydrochloride was administered intravenously (i.v.) and blood drawn at regular intervals for 24 h. The plasma was analyzed according to the above procedure and the free naloxone concentration plotted against time (Fig. 6). Neither curve declined steadily, but rather showed several areas of plateau. We have observed similar plateauing with another basic amine drug, methadone [27,28] and we have hypothesized [27,28] that when a patient is allowed to eat during the study, the enterohepatic recycling causes a surge of bile acids through

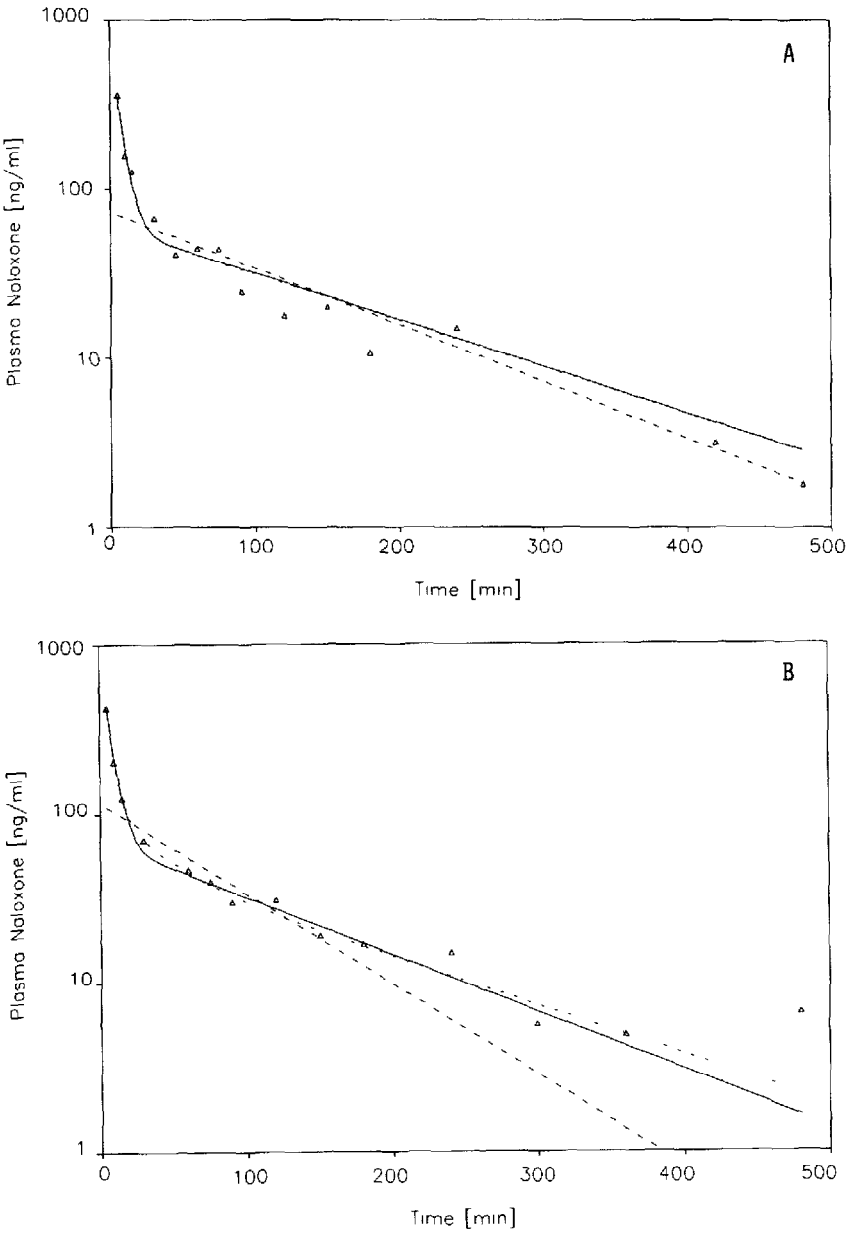

Fig. 6. Intravenous bolus injection study in two patients (A and B) each receiving 30 mg of naloxone hydrochloride. The time course of naloxone concentration in plasma is followed for 8 h (triangles). Compartmental analysis of the data using one-, two- and three-compartment models are shown by the dashed, solid and dotted lines, respectively. The model parameters are reported in Table II.

the liver. The bile acids may, by a detergent effect, release tissue-bond naloxone back into circulation.

As can be seen from Fig. 6, this method allows free naloxone concentration changes to be studied for 8 h, a time span equivalent to more than four half-lives of naloxone (see Table II). Therefore this method is sufficiently sensitive and

TABLE II

CLEARANCE AND TERMINAL HALF-LIFE IN TWO PATIENTS EACH GIVEN A BOLUS INJECTION OF 30 mg NALOXONE HYDROCHLORIDE INTRAVENOUSLY

Clearances were calculated either from the model (Model) or from the area under the curve (AUC).

Compartment model	Clearance (l/min)		Terminal half-life (min)
	Model	AUC	
Patient A			
1	3.17	2.59	90.6
2	2.22	2.21	110.1
3	2.23	2.21	110.9
Patient B			
1	3.17	2.31	55 9
2	2.16	1.99	88.2
3	2.06	1.91	104.3

specific to obtain the pharmacokinetic profile of naloxone after an i.v. dose. In Fig. 6 compartmental analyses were performed on the i.v. data. One-, two- and three-compartment models were tested with the data (see Table II) [22]. As it appears from Fig. 6, the three-compartment model does not add any significant improvement of fit. This is in agreement with pharmacokinetic practice where two-compartment models are generally used. The results are within the range found by Aitkenhead et al. [10] using a two-compartment model.

In a single oral study a bolus dose of 30 mg naloxone hydrochloride was given and blood collected at regular intervals for 24 h. The procedure turned out to be less suited for oral studies at the present dose level, since only the peak level of naloxone was detectable ($C_{plasma} = 3.6$ ng/ml at a peak time of 70 min).

CONCLUSION

The method described permits sensitive and selective determination of free naloxone in plasma, while eliminating cross-reaction with and false identification of other naloxone metabolites, especially the major metabolite in humans, naloxone 3-glucuronide. The method is well suited for i.v. studies of the pharmacokinetics of naloxone in humans, although, at present, it does not have the sensitivity required for oral studies, in which the same dose as in the i.v. study (30 mg naloxone hydrochloride) is administered. The use of solid-phase extraction with the HPLC mobile phase and dual-electrode coulometric detection makes the method rapid, accurate and precise.

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