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Determination of buprenorphine by high-performance liquid chromatography with fluorescence detection: application to human and rabbit pharmacokinetic studies

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ABSTRACT

A rapid, sensitive, precise and accurate high-performance liquid chromatographic assay with fluorescence detection was developed for the determination of buprenorphine in human, rabbit, pig and dog plasma. It is comprised of only a one-step extraction procedure with hexane–isoamyl alcohol at pH 9.25 and reversed-phase chromatography on a μPorasil column. The recoveries of buprenorphine and nalbuphine (internal standard) were greater than 90%. Calibration graphs were linear over the concentration range 3–300 ng/ml with a coefficient of variation, both within-day and between-day, of less than 9% at any level. The limit of detection was 1.0 ng/ml of plasma based on a signal-to-noise ratio of 3. Eight other clinically used narcotics were investigated to check for potential interferences and their analytical conditions. The possible decomposed compounds of buprenorphine were also checked for the specificity of this assay. The method has been succesfully applied to the stability and pharmacokinetic studies of buprenorphine. Buprenorphine in plasma did not decompose significantly at −20°C for four weeks. Pharmacokinetic application in six rabbits and a surgical patient revealed that buprenorphine followed a linear three-compartment model with two distribution phases. The two distribution and elimination half-lives and the clearance of buprenorphine were 1.32, 24.8 and 230 min and 224 ml/min in human plasma, and 0.94, 12.5 and 232 min and 30 ml/min in rabbit plasma.

INTRODUCTION

Buprenorphine, N-cyclopropylmethyl-7α-[1(5)-hydroxy-1,2,2-trimethylpropyl]-6,14-*endo*-ethano-6,7,8,14-tetrahydronororipavine, is a relatively new morphine-like drug with partial agonist activity at the μ-opiate receptor and antagonist activity at the κ-opiate receptor [1]. As an analgesic agent, it is 25–30 times more potent than morphine and has been widely used in the treatment of acute and chronic pain [2–8]. It has also been used recently for the treatment of heroin addicts [3]. Its main advantages over morphine are a ceiling effect of respiratory

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depression, low tolerance liability and a lack of significant withdrawal symptoms [4]. Buprenorphine is available as an injection for intramuscular, intravenous, intrathecal or epidural administration, and as sublingual tablets. The usual recommended doses are 200–600 μg by intravenous or intramuscular injection every 6–8 h, 30–45 μg by intrathecal or 100–300 μg via an epidural route every 6–12 h, or 400 μg sublingually every 6–8 h [2–8].

A radioimmunoassay technique has been developed to determine the plasma level of parenterally administered buprenorphine in dogs and humans [9], but such techniques are sometimes non-selective and may suffer from interferences. A selected-ion monitoring method [10] was reported for the determination of buprenorphine and its metabolite with a low detection limit of 150 pg, but in included a crucial chemical degradation step and a multiple-step extraction procedure with only 30% recovery. A high-performance liquid chromatography (HPLC) assay [11] with UV detection (290 nm) was developed with a low detection limit of 2 ng on a column; however, the sample preparation was too time-consuming due to the three-step extraction. Another HPLC assay [12] with fluorescence detection (excitation 285 nm, emission 350 nm) has been used to determine plasma levels of buprenorphine using two extraction procedures and the lowest detection limit was 5 ng/ml in biological fluids. Martinez *et al.* [13] have described a model for the identification and determination of buprenorphine in plasma and urine by gas chromatography. Mullersman *et al.* [14] analysed buprenorphine in plasma and urine using electron-capture detection. Hackett *et al.* [15] have developed a screening method for buprenorphine in urine by thin-layer chromatography and HPLC with UV detection at 214 nm.

The purpose of the work reported here was to develop a simple, rapid, sensitive, precise and accurate HPLC method which is suitable for the pharmacokinetic study of buprenorphine. This method consists of a one-step extraction procedure with a low detection limit of 1.0 ng/ml buprenorphine in human, rabbit, pig and dog plasma. The stability of buprenorphine in frozen human plasma and pilot pharmacokinetic studies in a patient and rabbits were also investigated to test the suitability of this method for routine use.

EXPERIMENTAL

HPLC conditions

The HPLC system consisted of a pump (Spectroflow 400, Applied Biosystems, Ramsey, NJ USA), an automatic sampler (WISP 710B, Waters, Milford, MA, USA), a fluorescence detector (980 programmable fluorescence detector, Applied Biosystems) and an integrator (740 data module, Waters). A μPorasil column (300 mm × 2 mm I.D., 10 μm particle size, Waters) was used. For the column system, a pre-column (15 mm × 3.2 mm I.D., 7 μm particle size, Applied Biosystems, San Jose, CA, USA) was also used.

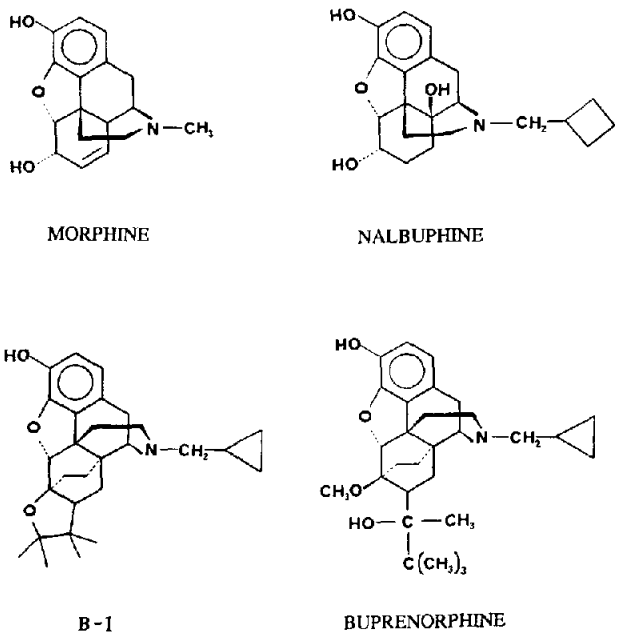

Fig. 1. Structures of buprenorphine, its acid-catalysed rearrangement product (B-1) and related morphinans.

Chemicals and reagents

Buprenorphine and nalbuphine (used as an internal standard, Fig. 1) were kindly supplied by the manufacturers (Macfarlan Smith, Edinburgh, UK and E. I. du Pont de Nemours, Wilmington, DE, USA). All chemicals were of analytical-reagent grade; all solvents were of HPLC grade. All aqueous solutions were prepared using Milli-Q water (Milli-RO 60, Millipore, Bedford, MA, USA).

Standard solutions

Buprenorphine. A stock solution of 300 μg/ml buprenorphine was prepared in acetonitrile and seven standard solutions of 6 μg/ml to 62.5 ng/ml in water were made by serial dilution. Aliquots of these standard solutions (50 μl in human, pigs or dogs and 25 μl in rabbits) were added to aliquots of blank plasma (0.95 ml in human, pigs or dogs, and 0.475 ml in rabbits) to give final concentrations of 3.13, 6.25, 12.5, 25, 50, 150 and 300 ng/ml.

Internal standard. A 1.0 mg/ml nalbuphine stock solution was prepared in acetonitrile and further diluted to give a working solution of 5 μg/ml in water. A 50-μl aliquot (250 ng) was added to each 1-ml aliquot of human, pig or dog plasma standard or specimen, and 25 μl (125 ng) to each 0.5 ml of rabbit plasma.

Sample preparation

To 1 ml of human, pig or dog and 0.5 ml of rabbit plasma placed in a 10-ml capacity glass culture tube, fitted with a PTFE-lined screw cap, 50 or 25 μl of internal standard solution and 1 or 0.5 ml of 0.5 *M* sodium carbonate buffer (pH 9.25) were added. The samples were extracted with 3 or 1.5 ml of a mixture of hexane–isoamyl alcohol (9:1, v/v) by mixing for 30 min on a rotary shaker. After centrifugation at 1880 g for 20 min, the glass tubes were put into a freezer (−20°C) for 1 h. After the lower layer (plasma) was frozen, the organic layer was poured into another 10-ml glass tube and evaporated to dryness under a stream of filtered dry air. The samples were reconstituted by 250 μl of mobile phase. Aliquots of 200 μl were injected into the HPLC system.

Chromatography

The assay for buprenorphine was performed using a mobile phase of 5 m*M* sodium acetate buffer (pH 3.75)–acetonitrile (20:80, v/v) and a fluorescence detector (excitation 210 nm, emission 345 nm, slit width 5 nm). A flow-rate of 1.0 ml/min at 25°C was used and yielded a back-pressure of about 100 bar.

Calibration graphs

Calibration graphs were obtained by the assay of extracts of blank plasma samples spiked with buprenorphine to cover the concentration range 3–300 ng/ml and the internal standard. Quantitation was obtained by the measurement of drug concentrations against the peak-height ratio of buprenorphine/nalbuphine. The concentrations of unknown samples were determined by using the linear regression line (unweighted) of the concentration of the calibration standard *versus* peak-height ratios.

Repeatability, precision and accuracy

The repeatability of the method was estimated by comparing the linear regression slopes, intercepts and correlation coefficients of the standard graphs from human plasma. Precision and accuracy were determined by processing spiked human samples at seven concentrations (3–300 ng/ml) with respect to a calibration graph run each day. The precision of the method was expressed as the within-day and between-day coefficient of variation (%) and the accuracy was shown as the mean deviation of all concentrations from the theoretical value.

Selectivity and resolution

To determine the selectivity and resolution of these HPLC systems, the method reported by Cone *et al.* [16] was followed, in which buprenorphine was treated under extreme acidic (pH 1, with hydrochloric acid) and alkaline (pH 12, with sodium hydroxide) conditions in an autoclave (112°C and 75.8 kN/m^2 for 30 min). The final products were injected into the HPLC system.

Stability and recovery

To determine the stability of buprenorphine in frozen plasma, aliquots of seven standard buprenorphine solutions (50 μl) were added to a batch of blank human plasma (0.95 ml) and immediately frozen to $-20°C$. The buprenorphine concentration was determined at 0, 2, 3 and 4 weeks and the buprenorphine calibration graph was constructed for each experiment.

The extraction recovery of buprenorphine and the internal standard in human plasma was determined at all levels of the calibration graph by comparing the peak heights obtained by the direct injection of standard aqueous solutions to those obtained after the whole extraction procedure.

Pharmacokinetic studies

Animals. Following the guidelines of the American Association for the Accreditation of Laboratory Animal Care, six two-month-old male New Zealand white rabbits weighing between 1.9 and 2.1 kg were used. After placing the animals in a restraining box, their ears were shaved with a razor blade. Following the sucessful cannulation of the auricular artery and vein in the opposite ear, 7 ml of blood were collected form the artery at zero time and 1 ml at 2, 3, 5, 7, 12, 20, 30, 45, 60, 120, 180 and 240 min after the intraveneous injection of buprenorphine (0.3 mg/kg).

Humans. This analytical method has also been used in a preliminary pharmacokinetic study of intraveneous buprenorphine in a 45-year-old female patient after thyroid goiter resection for pain relief. In the post-anaesthetic room, 15 ml of blood were collected from a forearm vein into a heparinized tube at zero time and 3 ml at 1, 3, 5, 7, 9, 12, 17, 22, 30, 40, 120 and 220 min after the intravenous administration of buprenorphine (6 μg/kg).

Both rabbit and human plasma were collected by centrifugation and immediately frozen to $-20°C$ until assay. Plasma concentrations were fitted to a three-compartment open model using the computer program PCNONLIN [17]; a weighing function of the reciprocal of concentration was used. Initial parameter estimations were required for non-linear regression and were obtained using the linear regression computer program CSTRIP [18]. Pharmacokinetic parameters such as half-lives, clearance, apparent central compartment volume of distribution and area under the plasma concentration–time graph were calculated for the rabbits and the patient by standard formulae [19].

RESULTS AND DISCUSSION

Chromatography

As the chromatograms of extracts from humans, rabbits, pigs and dogs are similar, only the typical chromatograms of extracts from the human patients are shown (Fig. 2). These are extracts of drug-free plasma, spiked samples with buprenorphine and the internal standard, nalbuphine. No interfering peaks were

TABLE I

RETENTION TIMES, CAPACITY FACTORS, COLUMN SELECTIVITY AND LOW DETECTION LIMITS OF VARIOUS DRUGS ON A SILICA GEL COLUMN

Conditions: flow-rate = 1.0 ml/min; Waters 441 UV detector (214 nm); ABI 980 fluorescence detector (210/345 nm); temperature = 25°C.

Drug	Acetonitrile 5 m*M* sodium acetate buffer (pH 3.75)[a]										
	75:25			80:20			85:15			80:20 (low detection limit, ng/ml)	
	RT	*k'*	*A*	RT	*k'*	*A*	RT	*k'*	*A*	UV (214 nm)	F (210/345 nm)
Buprenorphine	8.08	2.11	1.00	8.83	2.40	1.00	10.9	3.19	1.00	1.3	1.0
Nalbuphine	8.53	2.28	1.08	11.7	3.50	1.45	16.1	5.19	1.63	1.5	10
Butorphanol	7.06	1.72	0.82	9.31	2.58	1.08	12.8	3.82	1.23	6.3	12
Morphine	10.8	3.15	1.49	16.5	5.35	2.23	24.9	8.58	2.69	3.8	7.5
Ethylmorphine	11.0	3.23	1.53	14.9	4.73	1.97	21.5	7.27	2.28	3.6	4.8
Codeine	10.7	3.12	1.48	16.0	5.15	2.15	23.8	8.15	2.55	2.9	4.6
Nalorphine	8.79	2.38	1.13	11.6	3.46	1.44	14.4	4.54	1.42	3.1	5.6
Fentanyl	7.12	1.74	0.82	9.39	2.61	1.09	12.6	3.85	1.21	5.5	–
Pethidine	8.22	2.16	1.02	11.2	3.31	1.38	15.6	5.00	1.57	8.8	–
Tramadol	8.68	2.34	1.11	11.0	3.23	1.35	14.9	4.73	1.48	6.3	–

[a] RT = retention time (min); *k'* = capacity factor; *A* = column selectivity compared with buprenorphine.

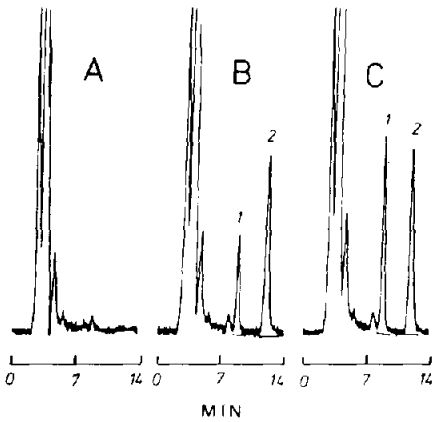

Fig. 2. Chromatograms of extracts from (A) blank plasma, (B) plasma spiked with buprenorphine (25 ng/ml) and (C) sample from a patient (buprenorphine 36 ng/ml). Peaks: 1 = buprenorphine; 2 = internal standard (nalbuphine 250 ng/ml).

detected in the blank plasma nor in the samples from the patients or animals. Certain drugs which are commonly used clinically during general anaesthesia and the post-operative period were selected to check for their potential interference in the assay of buprenorphine. These included thiopentone, succinylcholine, pancuronium, diazepam, atropine and neostigmine. After injection directly into the HPLC system, no interfering peaks were found during the analysis.

Low detection limit, column retention time, capacity factors and column selectivity of various narcotics

The programmable fluorescence detector used in this method consisted of two wavelengths (excitation and emission) working in series. The screen mode of operation was selected to improve the detector selectivity and sensitivity. The low detection limit, retention times, capacity factors and column selectivity of buprenorphine and the other narcotic analgesics listed in Table I, which are widely used clinically were investigated to check for their potential interference and their respective analytical conditions.

In different column tests, the μPorasil column exhibited a great selectivity. The retention times, capacity factors, column selectivities and low detection limits of buprenorphine and other morphine analogues were determined by elution with various ratios of acetonitrile–buffer (Table I). Using the mobile phase acetonitrile–buffer (80:20), buprenorphine and the internal standard had retention times of 8.8 and 11.7 min, respectively. The low detection limits of buprenorphine, defined as a signal-to-noise ratio greater than 3, were 12, 3.1, 1.3 and 1.0 ng/ml of plasma at 285/345, 230/345, 214/345 and 210/345 nm (excitation/emission), respectively. The detection limits of some other narcotic analgesics are also shown in Table I.

Selectivity and resolution

After autoclaving buprenorphine under extreme acidic and alkaline conditions, only the acid-catalysed rearrangement products of buprenorphine was found by fluorescence detection. The chemical structure of the acid-catalysed rearrangement product of buprenorphine is shown in Fig. 1. When injected directly into the HPLC system, buprenorphine and its acid-catalysed rearrangement product were found to have capacity factors of 1.8 and 1.4, respectively, and a selectivity factor of 0.778.

Repeatability, precision and accuracy

Over a period of 41 days, the calibration graphs ($n = 12$) were linear in the concentration range 3–300 ng/ml with correlation coefficients of 0.999 ± 0.001 (mean ± S.D.) and with a minimum intercept of 0.0172 ± 0.042 (mean ± S.D.). The slopes averaged 0.0255 ml/μg with a coefficient of variation of 11.02%. Precision and accuracy studies in plasma showed an acceptable coefficient of variation (< 10%) and high accuracy for both within-day ($n = 6$) and between-day ($n = 11$) studies, as shown in Table II.

Stability and recovery

The stability of buprenorphine in spiked human plasma was determined after freezing for four weeks at −20°C. The results are given in Table III and indicate

TABLE II

PRECISION AND ACCURACY OF BUPRENORPHINE DETERMINED BY THE HPLC METHOD

Known concentration (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	Coefficient of variation (%)	Accuracy (% mean deviation)
Within-day (n = 6)			
3.13	3.22 ± 0.27	8.3	2.9
6.25	6.62 ± 0.39	5.9	5.9
12.5	13.2 ± 0.9	6	6
25.0	25.1 ± 1.6	6.5	0.3
50.0	50.5 ± 3.1	6.1	1.0
150	146 ± 4	3	−3
300	302 ± 3	1	1
Between-day (n = 11)			
3.13	3.19 ± 0.27	8.4	2.0
6.25	6.46 ± 0.39	6.1	3.4
12.5	12.9 ± 0.9	7	3
25.0	25.0 ± 1.7	6.8	−0.2
50.0	50.4 ± 3.0	5.9	0.7
150	147 ± 4	3	−2
300	301 ± 3	1	0

TABLE III

STABILITY OF BUPRENORPHINE IN FROZEN PLASMA

Initial concentration (ng/ml)	Concentration found (mean ± S.D., n = 3) (ng/ml)		
	14 days	21 days	28 days
3.13	3.16 ± 0.28	3.17 ± 0.15	3.12 ± 1.34
6.25	6.51 ± 0.52	6.26 ± 0.25	5.79 ± 0.30
12.5	13.6 ± 0.5	12.2 ± 1.5	11.2 ± 0.2
25.0	26.0 ± 0.4	25.1 ± 1.9	24.4 ± 0.5
50.0	50.1 ± 4.2	51.4 ± 1.3	49.1 ± 3.3
150	143 ± 4	148 ± 10	156 ± 7
300	303 ± 2	301 ± 21	297 ± 21

that no significant degradation occurred over a four-week period. Garrett and Chandran [12] predicted that buprenorphine in buffer is stable between pH 2 and 12 at 25°C for at least 12.5 years. The internal standard (nalbuphine) was also stable in frozen plasma for at least eighteen weeks [20]. The absolute recoveries of buprenorphine and the internal standard were greater than 90% and independent of the buprenorphine concentration (Table IV). Compared to other extracted organic solvents or mixtures such as dichloromethane, benzene, diethyl ether, chloroform or hexane–isopropanol, the mixture hexane–isoamyl alcohol gave a greater recovery and chromatograms with less background noise for both human and animal samples. The extraction procedure, involving only one step, is fairly rapid and the freezing method also simplified the procedure of solvent transfer.

TABLE IV

ABSOLUTE RECOVERIES OF BUPRENORPHINE AND NALBUPHINE (INTERNAL STANDARD) FROM SPIKED PLASMA SAMPLES (n = 6)

Drug	Concentration (ng/ml)	Recovery (mean ± S.D.) (%)	Coefficient of variation (%)
Buprenorphine	3.13	99.1 ± 7.8	7.9
	6.25	96.4 ± 4.4	4.5
	12.5	97.8 ± 9.5	9.7
	25.0	98.2 ± 2.5	2.5
	50.0	95.3 ± 1.5	1.6
	150	96.7 ± 1.8	1.9
	300	97.3 ± 4.0	4.1
Mean value	–	97.2 ± 4.5	4.6
Nalbuphine	250	92.1 ± 5.5	6.0

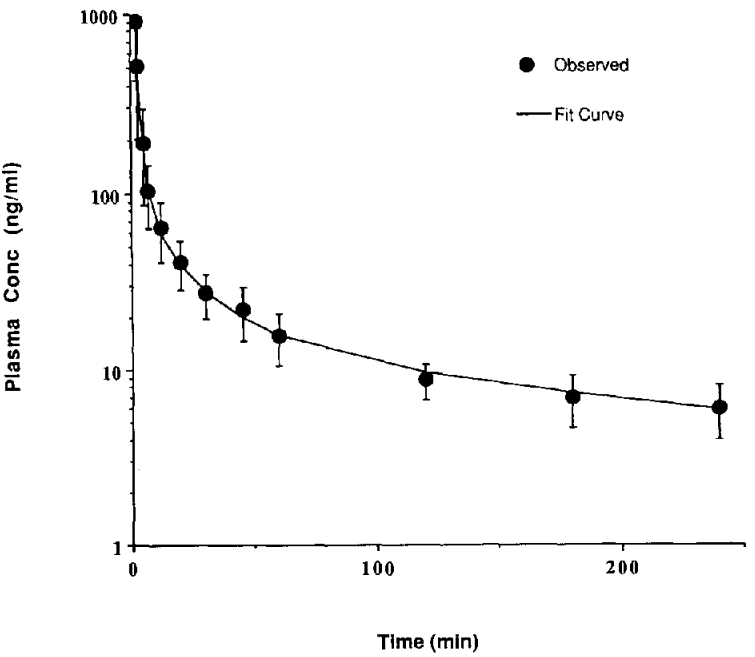

Fig. 3. Plasma concentration–time profile of buprenorphine (mean ± S.D.) in six rabbits receiving intravenous buprenorphine (300 μg/kg).

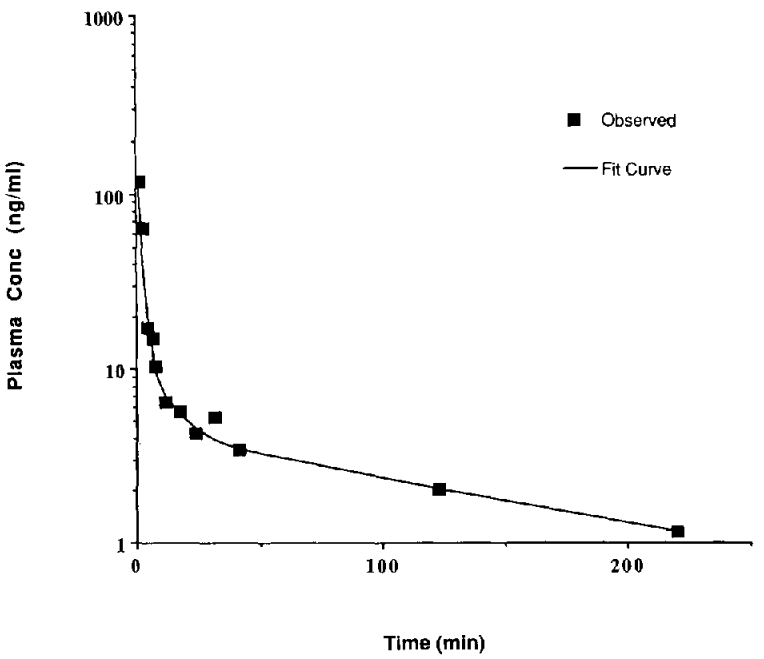

Fig. 4. Plasma concentration time profile of buprenorphine in a surgical patient receiving intravenous buprenorphine (6 μg/kg).

TABLE V

PHARMACOKINETIC PARAMETERS OF BUPRENORPHINE IN A SURGICAL PATIENT AND SIX RABBITS AFTER INTRAVENOUS BUPRENORPHINE INJECTION

Parameter[a]	Unit	Human	Rabbit
Dose	μg/kg	6.00	300
A	ng/ml	193	4090 ± 2320
B	ng/ml	5.19	126 ± 77
C	ng/ml	2.36	15.0 ± 4.1
α	1/min	0.524	0.759 ± 0.271
β	1/min	0.028	0.079 ± 0.049
γ	1/min	0.003	0.004 ± 0.002
$T_{1/2}(\alpha)$	min	1.32	0.94 ± 0.25
$T_{1/2}(\beta)$	min	24.8	12.5 ± 7.4
$T_{1/2}(\gamma)$	min	231	232 ± 150
AUC_0^∞	ng min/ml	1339	11 470 ± 5350
Cl	ml/min	224	59.3 ± 19.4
Body weight	kg	50.0	2.0 ± 0.2

[a] Equation: plasma concentration (C_p) = $Ae^{-\alpha t} + Be^{-\beta t} + Ce^{-\gamma t}$; A, B, C = intercepts; α, β, γ are the first-order rate constants for the central, tissue, and deep tissue compartments: $T_{1/2}$ = half-life of the first-order rate constant; AUC_0^∞ = area under the time–concentration graph to time infinity; Cl = total plasma clearance.

This allows the analysis of at least 80 samples per day using the automated HPLC system.

Pharmacokinetic studies

The HPLC method has been used intensively in this laboratory. The observed and fitted plasma concentration–time curve for the intravenous injection of buprenorphine into six rabbits and an adult human patient after thyroid goiter resection are shown in Figs. 3 and 4. The data were successfully fitted to a three-compartment model with two distribution and one elimination phase. The plasma level of intravenous buprenorphine declined very rapidly in the patient and rabbits, as expected for this very lipophilic drug. Slow elimination phases were found with similar elimination half-lives of 231 and 233 min in the patient and rabbits, respectively (Table V).

CONCLUSIONS

This method is sufficiently simple, rapid, sensitive, specific and accurate for the determination of buprenorphine in large amounts of human and animal biological fluids. It has been extensively used in these laboratories for pharmacokinetic studies of the drug in human patients and animals.

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