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Column-switching solid-phase trace-enrichment high-performance liquid chromatographic method for measurement of buprenorphine and norbuprenorphine in human plasma and urine by electrochemical detection

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ABSTRACT

We describe a new high-performance liquid chromatographic method using electrochemical detection for the determination of buprenorphine and norbuprenorphine in plasma and urine. The minimum concentration for detection of buprenorphine and norbuprenorphine is 40 pg/ml. The intra-assay coefficient of variation (C.V.) in plasma and urine samples ranges from 6 to 17% depending on the drug concentration. At a plasma concentration of 500 pg/ml the inter-assay C.V. is 8% for buprenorphine and 9% for norbuprenorphine. The analysis duration is 16 min. After solid-phase extraction and evaporation a valve-switching system with two Rheodyne valves enables sample enrichment, optimal sample cleaning and rapid elution of long-retained substances.

INTRODUCTION

Buprenorphine is a relatively new analgesic with morphine-like action. It is chemically derived from thebaine and shows agonistic and antagonistic activity at the μ-opiate receptor [1]. Its analgesic potency exceeds that of morphine by 25- to 40-fold. Hence, therapeutic drug concentrations in the plasma are in the low-nanogram region and range between 50 and 300 pg/ml during the terminal phase of elimination. The only previously known buprenorphine metabolite, norbuprenorphine, is even more difficult to detect and only few data are available, mainly about its renal elimination and metabolism via the glucuronic acid pathway [2].

Previously published high-performance liquid

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chromatographic (HPLC) methods for the analysis of non-radioactively labelled buprenorphine from biological fluids have detection limits between 150 and 1000 pg/ml, are based on a pre-column derivatization, use complicated HPLC–mass spectrometric coupling and require time-consuming liquid–liquid extraction and re-extraction with highly toxic solvents for sample preparation or allow merely a screening analysis of high urinary concentrations [1,3–5]. A recently described HPLC method for the measurement of buprenorphine and norbuprenorphine in urine not only suffers from these disadvantages but is also hampered by co-eluting substances that interfere with buprenorphine measurements, at least at the detection limit [6]. On the other hand, the quality and reliability of radioimmunoassays and enzyme immunoassays depend on antibody specificity, and it appears that adequate binding is not always reliably achieved [7,8].

The current study was therefore initiated to develop a more reliable, sensitive and practicable HPLC method for the analysis of buprenorphine and norbuprenorphine in plasma and urine. Applying a solid-phase extraction (SPE) with C_{18} material, evaporation before HPLC analysis and a separate preparation and chromatographic procedure for buprenorphine and norbuprenorphine, a detection limit of 40 pg/ml and a concentration-dependent intra-assay variation of 6–17% could be achieved. On this basis, results of the pharmacokinetics of buprenorphine were evaluated in two patients each after subcutaneous and oral administration.

EXPERIMENTAL

Materials

Boehringer (Mannheim, Germany) generously supplied buprenorphine and norbuprenorphine. All salts and reagents were of analytical quality and were obtained from Merck (Darmstadt, Germany). All solvents (HPLC quality) were obtained from Baker (Gross-Gerau, Germany).

HPLC apparatus

The chromatographic system comprised two Jasco Model PU 880 analysis pumps (Chemdata, Gross-Zimmern, Germany), a pulsation reducer, an ESA Coulochem 5100 A electrochemical detector with an analytical measuring cell and a guard cell, Models 5010 and 5020, respectively (Chemdata), an injection valve from Rheodyne (Bischoff, Leonberg, Germany), Model 8125 equipped with a 2.5-ml sample loop, and two Rheodyne 7000 valves for column switching. Analysis, quantification and calibration were performed on a personal computer (PC) program (NINA) from Nuclear-Interface (Münster, Germany).

HPLC conditions

A LiChroCART 4 mm × 4 mm RP-18 cartridge from Merck containing 5-μm C_{18} material was used for the enrichment of buprenorphine and norbuprenorphine. This column and the in-line filter (0.5 μm porosity) of the enrichment system were changed after 50 injections of plasma or urine samples. A dry-filled 250 mm × 4 mm I.D. column with 40-μm silica material was placed between pump and injection valve to prevent alkalinic degradation of the enrichment column. The enrichment eluent consisted of a 0.05 *M* phosphate buffer adjusted to pH 8.5 with 1 *M* sodium hydroxide and 20% (v/v) acetonitrile for buprenorphine and 10% (v/v) acetonitrile for norbuprenorphine. The flow-rate in the enrichment system was 2 ml/min at room temperature. Owing to the optimal sample purity after SPE and enrichment, a 30 mm × 4 mm I.D., 5 μm particle size C_{18} cartridge from Macherey & Nagel (Düren, Germany) was sufficient for the chromatographic separation of buprenorphine or norbuprenorphine. The buffer for the elution contained 0.05 *M* sodium dihydrogenphosphate and 25% (v/v) acetonitrile for buprenorphine and the same salt concentration but 20% (v/v) acetonitrile for nobuprenorphine. Further conditions for the chromatographic separations were 0.8 ml/min flow-rate at room temperature.

Sample preparation

A Visiprep SPE vacuum manifold system manufactured by Supelco was used for sample prepa-

ration and enrichment. Buprenorphine or norbuprenorphine was collected from plasma, serum or urine onto C_{18}, 40 μm particle size Supelclean LC-18 SPE 1-ml tubes, which were activated with 4 ml of methanol and 4 ml of water. After addition of up to 5 ml of sample, tubes were subsequently alkalinized using 400 μl of a 0.05 *M* borate buffer previously adjusted to pH 9.1 with 1 *M* sodium hydroxide. Matrix components were eluted with 600 μl of a 0.05 *M* sodium dihydrogenphosphate buffer with 20% (v/v) acetonitrile. Owing to its lower lipophilicity, this preparatory step was omitted for the analysis of norbuprenorphine. The valves of the SPE system were cleaned by flushing with 5 ml of methanol after removal of the extraction tubes. Subsequently the tubes were flushed to dryness with nitrogen over 5 min. The extraction of buprenorphine or norbuprenorphine from the tubes was undertaken using slow elution with 3 ml of chloroform into 5-ml glass vials. After evaporation to dryness under a soft stream of nitrogen, the residue was reconstituted in 0.5 ml of 0.05 *M* sodium dihydrogenphosphate, 50% (v/v) acetonitrile, pH 2.5 adjusted with phosphoric acid, by shaking the samples at 70°C for 20 min. This procedure was extremely important for resolving buprenorphine or norbuprenorphine quantitatively. After resolving, the samples were diluted with 3 ml of water and 0.5 ml of the above-mentioned borate buffer. A 2-ml volume of the diluted samples was twice injected subsequently into the HPLC enrichment system for one chromatographic analysis.

Quantification of buprenorphine and norbuprenorphine

Buprenorphine and norbuprenorphine were quantified using the external standard method. For this, the peak areas from 10 ng, 5 ng, 2 ng, 1 ng, 750 pg, 500 pg and 200 pg of buprenorphine or norbuprenorphine were plotted against the concentrations. The calibration data were fitted to a linear, unweighted, forced-through-zero model computed by the calibration function of the PC chromatography program (NINA). The resultant linear regression curves were used to calculate the drug concentrations in the samples.

Valve switching

Fig. 1 shows the valve-switching procedure with two Rheodyne valves. Enrichment of buprenorphine or norbuprenorphine was performed by valve A. Under the above-mentioned conditions the substances bind to the C_{18} material at the inlet of the enrichment column. Maximal elution of matrix components requires rinsing 2 min after the first 2-ml injection of the prepared sample at a flow-rate of 2 ml/min and a subsequent 4-min rinsing after the last injection. The substances were eluted from eluent B after cessation of the flow in valve A. This marks the start of the chromatogram and means time (t) = 0. Valve B is switched after 8 min and works as backflush valve for eluting long-retained substances retrogradely from the analytical cartridge. At the same time valve A is turned to the position for enrichment of the next sample. After another 8 min strongly retained substances were eluted retrogradely from the analytical column and the enrichment of the next sample is completed.

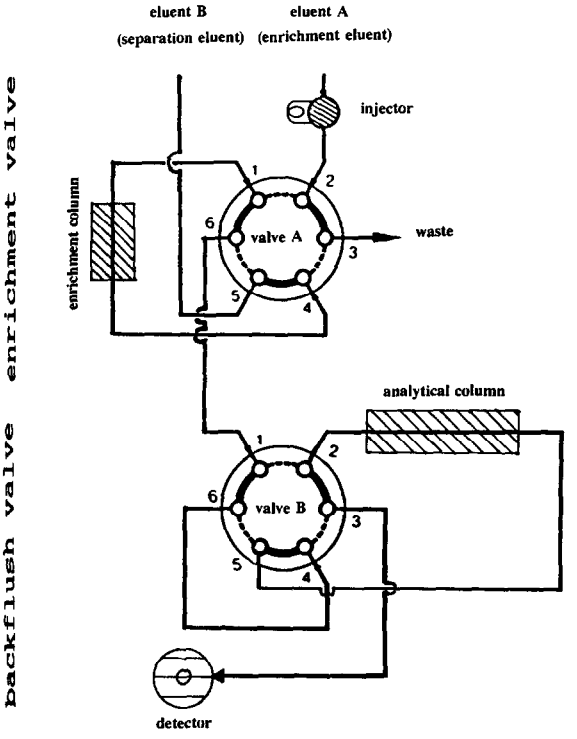

Fig. 1. Valve system diagram with two Rheodyne 7000 valves for sample enrichment and column backflushing.

Electrochemical detection

Fig. 2 depicts the hydrodynamic voltammogram of buprenorphine set to a voltage range of 180–780 mV (signal/voltage). The most sensitive detection was achieved when the measuring electrode was at a voltage of 480 mV, indicated by the curve of signal-to-noise ratio *versus* voltage. Because higher oxidation potentials produce higher noise drift, the signal-to-noise ratio against voltage was used to determine the optimal voltage. As the relationship between signal and voltage under defined conditions is specific for one substance, the presence of buprenorphine in patient samples could be proved by the ratio of signal/voltage at 480/680 mV, 480/580 mV and 580/680 mV. The guard cell (placed between pump B and the enrichment column) was set at 500 mV and detector 1 of the analytical cell at 160 mV. At this voltage up to 10 ng of buprenorphine or norbuprenorphine failed to provoke a signal on cell 1 so that the actual detector cell 2 lost none of its possible response.

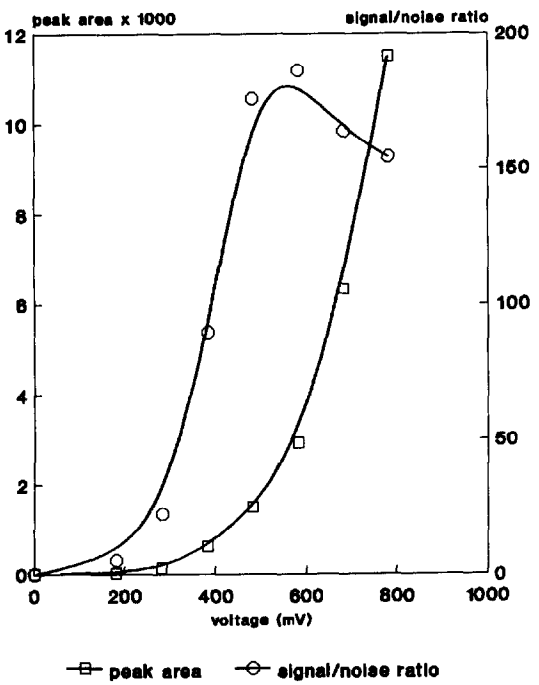

Fig. 2. The left ordinate shows the hydrodynamic voltammogram of 5 ng of buprenorphine. Current–voltage curve determined by application of 180, 280, 380, 480, 580, 680 and 780 mV oxidation potential. The right ordinate depicts the signal-to-noise ratio at the different potentials for buprenorphine. The optimal potential was found to be between 480 and 580 mV.

Pharmacokinetic calculation

The pharmacokinetics of buprenorphine was evaluated in two patients receiving 5 μg/kg buprenorphine following a surgical intervention. The plasma kinetics was analysed over the following 24 h. Calculation of the pharmacokinetic parameters from the measured data was carried out using the "Topfit" pharmacokinetic computer program [9,10].

In two additional patients, receiving 1 mg buprenorphine per day by sublingual application, plasma and urine concentrations of buprenorphine and norbuprenorphine were analysed under pharmacokinetic "steady-state" conditions at day 5.

RESULTS

Separation parameters

Figs. 3a and 4a show a standard chromatogram with 2 ng of buprenorphine and norbuprenorphine, from which capacity factor, plates/m, and tailing factor were calculated. The following values for buprenorphine and norbuprenorphine were obtained. Buprenorphine: capacity factor = 16.1, plates/m = 31225, peak tailing = 1.68; norbuprenorphine: capacity factor = 17.7, plates/m = 13100, peak tailing = 1.79.

Specificity

In order to assess the specificity of the HPLC assay plasma and urine samples were collected from ten healthy volunteers and ten patients before therapy with buprenorphine. Under the conditions described no co-eluting peak with buprenorphine or norbuprenorphine was found in any specimen (Figs. 5b and d and 6b and d). After adding 400 pg/ml buprenorphine or norbuprenorphine to the samples prior to processing, a new peak with the retention time of buprenorphine or norbuprenorphine could be detected (Figs. 3b, 4b, 7a and 8a). In six patient plasma samples 30 min after subcutaneous injection of buprenorphine a corresponding peak with an

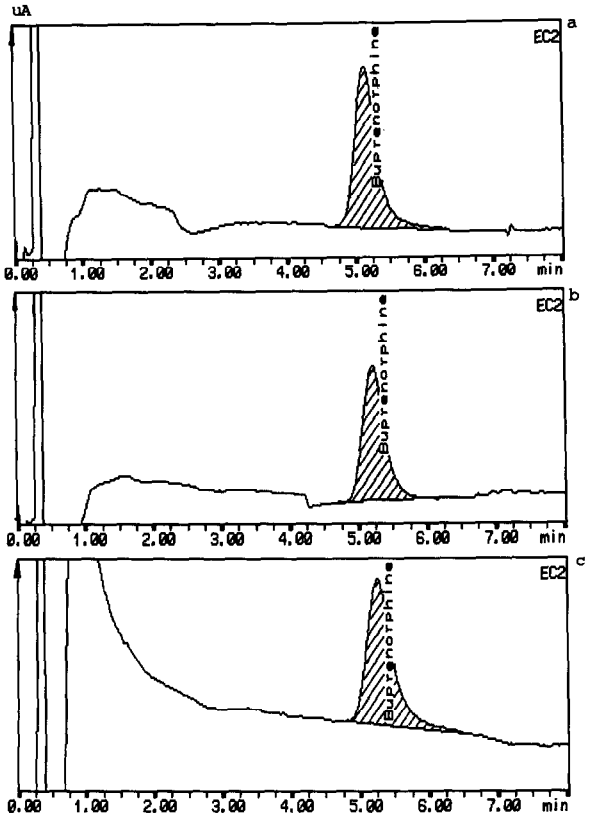

Fig. 3. HPLC separation of (a) a standard mixture of 2 ng of buprenorphine, (b) plasma (5 ml) spiked with 400 pg/ml buprenorphine and (c) 1 ml of patient plasma 30 min after 5 μg/kg subcutaneous buprenorphine administration. The ordinate of the chromatograms has a range of 25 μA.

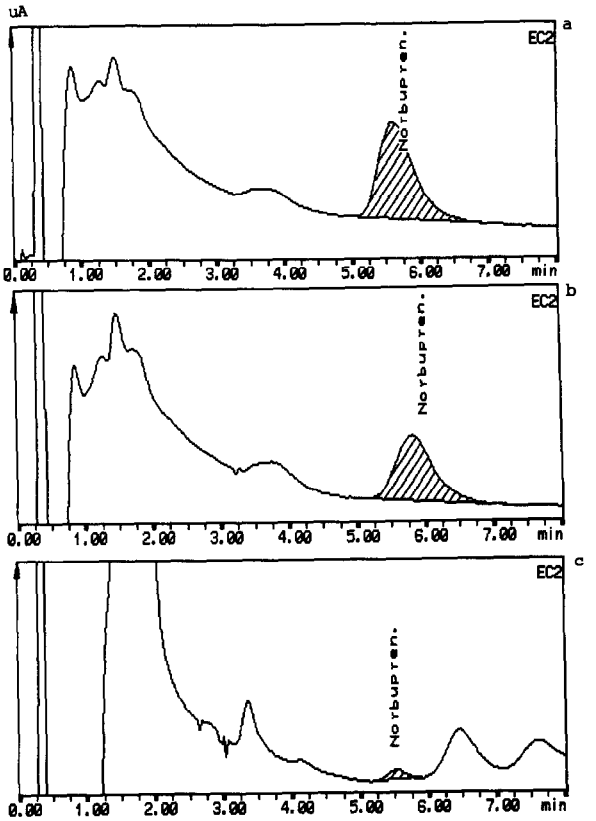

Fig. 4. HPLC separation of (a) a standard mixture of 2 ng of norbuprenorphine, (b) plasma (5 ml) spiked with 400 pg/ml norbuprenorphine and (c) 5 ml of patient plasma five days after sublingual administration of 1 mg of buprenorphine. The ordinate of the chromatograms has a range of 50 μA.

average concentration of 2.9 ng/ml could be identified with the same retention time as buprenorphine and a signal/voltage ratio (see above) as in the standard (Fig. 3c). The values for the ratios were: 480/680 mV, samples = 0.238 ± 0.006, standard = 0.240 ± 0.004; 480/580 mV, samples = 0.516 ± 0.004, standard = 0.514 ± 0.005; 580/680 mV, samples = 0.461 ± 0.005, standard = 0.459 ± 0.003. In the plasma and urine samples of the patients with sublingual buprenorphine application 250 pg/ml and 1.8 ng/ml buprenorphine were found on average (Fig. 7b). In addition, norbuprenorphine was detectable in the plasma and urine samples of these patients, with an average concentration of 60 pg/ml and 19 ng/ml, respectively (Figs. 4c and 8b). While blank plasma samples before sublingual buprenorphine application show a chromatogram similar to blanks from healthy volunteers without detectable peaks except the injection front, norbuprenorphine and three new unidentified peaks were found under pharmacokinetic "steady-state" conditions after five days of continuous buprenorphine administration. Presumably this may indicate the further metabolism of buprenorphine and norbuprenorphine to the glucuronic acid species and a potential metabolite of buprenorphine with C-7 side-chain rearrangement as described by Cone *et al.* [11].

Linearity and recovery

The linearity of buprenorphine and norbupre-

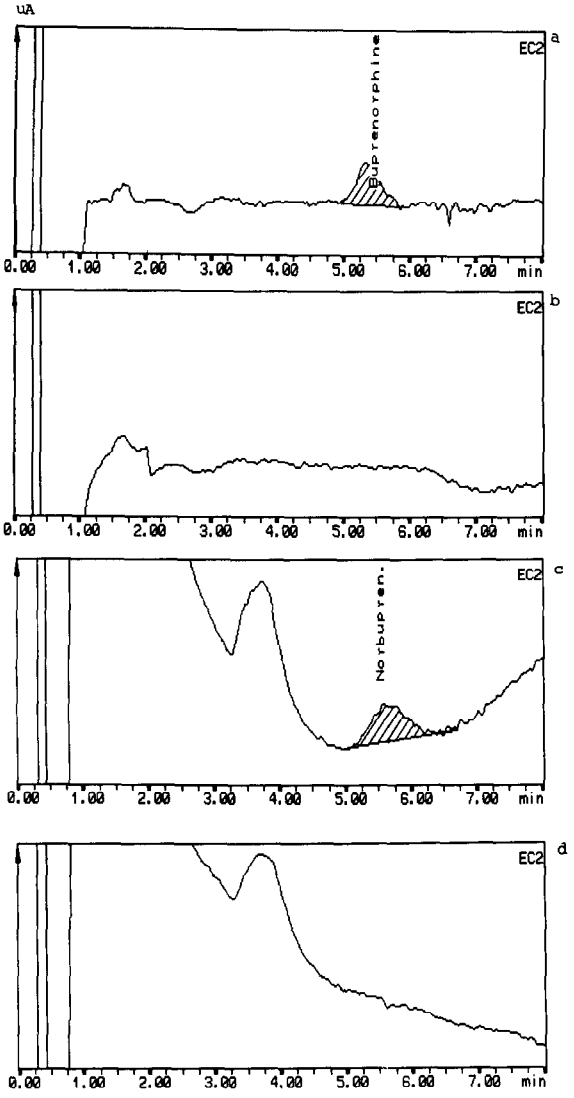

Fig. 5. HPLC separation of (a) 5 ml of plasma spiked with 40 pg/ml buprenorphine, (b) 5 ml of blank plasma, (c) 5 ml of plasma spiked with 40 pg/ml norbuprenorphine and (d) 5 ml of blank plasma. The ordinate of the chromatograms has a range of 8 μA.

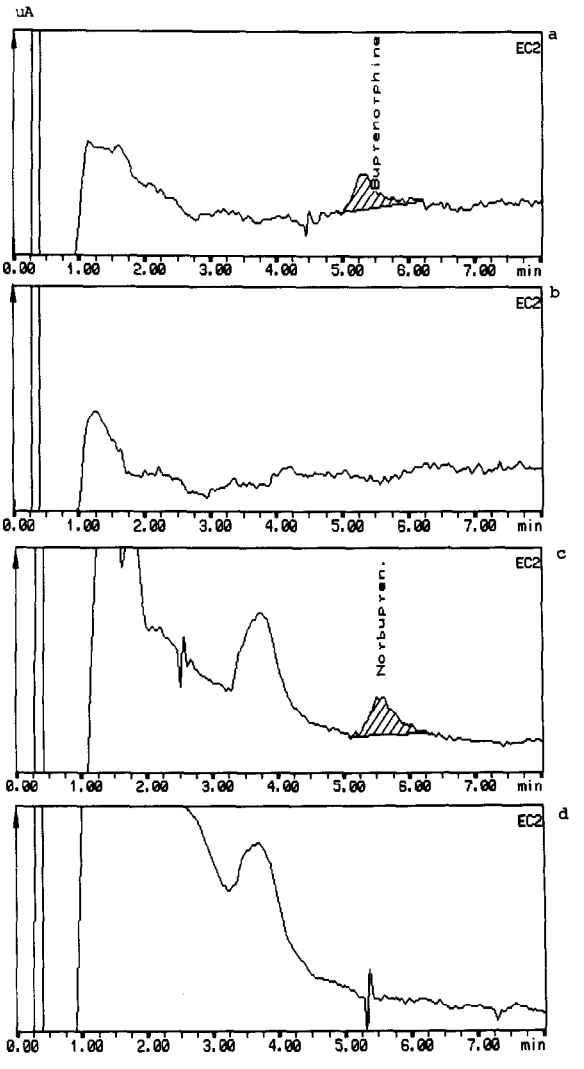

Fig. 6. HPLC separation of (a) 5 ml of urine spiked with 40 pg/ml buprenorphine, (b) 5 ml of blank urine, (c) 5 ml of urine spiked with 40 pg/ml norbuprenorphine and (d) 5 ml of blank urine. The ordinate of the chromatograms has a range of 8 μA.

norphine assays was assessed for concentrations between 40 pg and 2 ng per ml of plasma from 5-ml plasma samples. The regression equations for the peak area *versus* drug concentration were calculated for 10 ng, 5 ng, 2 ng, 1 ng, 750 pg, 500 pg and 200 pg of buprenorphine and norbuprenorphine in the aqueous standard and in plasma samples. The correlation coefficients for the standards and plasma samples were 0.991 and 0.989, respectively, for buprenorphine and for norbuprenorphine the values were 0.978 and 0.972. The peak areas of plasma concentrations plotted against the aqueous standard concentrations were used to define the regression curve, which indicated the average recovery over all concentrations by the slope of the curve. The recovery for buprenorphine was found to be 82% and that of norbuprenorphine 76% in plasma samples.

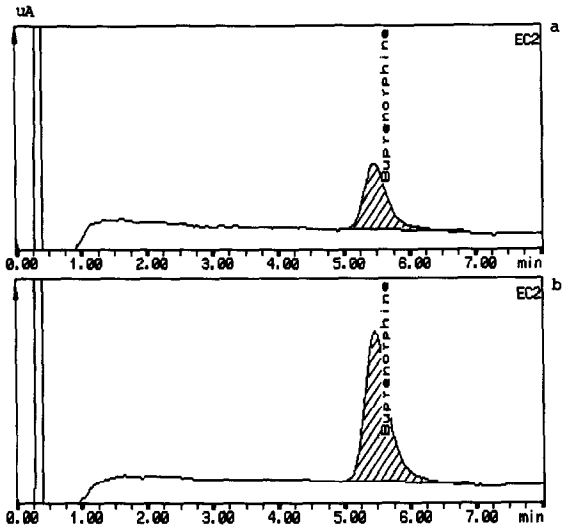

Fig. 7. HPLC separation of (a) 5 ml of urine spiked with 400 pg/ml buprenorphine and (b) 2 ml of patient urine five days after daily sublingual administration of 1 mg of buprenorphine. The ordinate of the chromatograms has a range of 50 μA.

Intra-assay variation and recovery

Ten plasma samples of 2 ng/ml, 500 pg/ml, 200 pg/ml and 40 pg/ml as well as ten urine samples with the same concentrations were prepared and analysed for the intra-assay variation

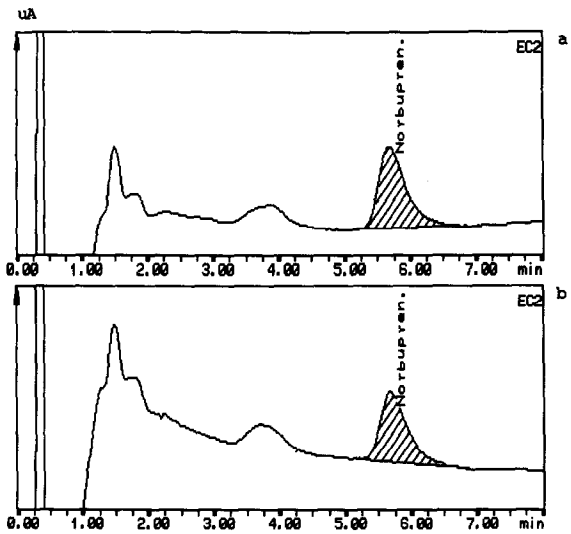

Fig. 8. HPLC separation of (a) 5 ml of urine spiked with 400 pg/ml norbuprenorphine and (b) 100 μl of patient urine five days after daily sublingual administration of 1 mg of buprenorphine. The ordinate of the chromatograms has a range of 50 μA.

of buprenorphine and norbuprenorphine. For every analysis 5 ml of plasma or urine were prepared. Table I shows the standard deviations and average recoveries. The average values for the recoveries over all concentrations calculated from the intra-assay variation were 79 and 75% for buprenorphine and norbuprenorphine, respectively, and confirm the calculated value using linear regression analysis of spiked plasma samples to aqueous standards.

Inter-assay variation and stability of buprenorphine and norbuprenorphine

The inter-assay variance was evaluated using 10 ml of plasma containing 5 ng of buprenorphine or norbuprenorphine. A 1-ml aliquot of this sample was prepared and analysed on a daily basis on ten consecutive days. The average value of these analyses calculated by the external standard method using linear regression of plasma samples (see above) showed a concentration of 0.48 ng/ml for buprenorphine with a standard deviation of ± 0.036 ng/ml and 0.51 ng/ml for norbuprenorphine with a standard deviation of ± 0.046 ng/ml. A plasma sample of buprenorphine and norbuprenorphine with a concentration of 5 ng/ml was left at room temperature for two weeks and no measurable degradation was found. These data confirm previous reports by Garrett and Chandran [12].

Detection limit of buprenorphine and norbuprenorphine

The detection limit of buprenorphine and norbuprenorphine was defined as the signal with one fifth of the magnitude of the average background noise. On this basis the detection limits for buprenorphine and norbuprenorphine were found to be 200 pg. By preparing 5 ml of plasma or urine the resulting sensitivity of buprenorphine and norbuprenorphine is 40 pg/ml. Figs. 5a and c and 6a and c depict the chromatograms of the detection limits of both substances and the corresponding blank analysis of the same amount of 5 ml of prepared plasma or urine (Figs. 5b and d and 6b and d). Attenuation and detector gain were exactly the same for spiked and blank plasma or urine.

TABLE I

INTRA-ASSAY VARIANCE OF BUPRENORPHINE AND NORBUPRENORPHINE ANALYSED FROM PLASMA AND URINE

Buprenorphine (pg/ml)	Standard deviation (pg/ml)	Average recovery (%)	Norbuprenorphine (pg/ml)	Standard deviation (pg/ml)	Average recovery (%)
Plasma (n = 10)					
2000	±97.5	78	2000	±143.2	76
500	±34.2	83	500	±26.9	73
200	±19.7	80	200	±21.3	74
40	±4.9	77	40	±4.4	70
Urine (n = 10)					
2000	±117.4	79	2000	±105.3	78
500	±50.3	75	500	±37.4	73
200	±18.3	81	200	±23.0	75
40	±4.2	77	40	±5.5	81

Pharmacokinetics of buprenorphine

Fig. 9 shows the concentration–time curves for buprenorphine obtained from two patients after subcutaneous application of 5 μg/kg body weight buprenorphine. Table II depicts the corresponding pharmacokinetic parameters of plasma concentrations. Peak plasma concentration and AUC (area under the curve) values are similar to the results of other groups, while terminal elimination half-life values are substantially higher than previously described, most probably because of the higher sensitivity of the described analytical procedure [13].

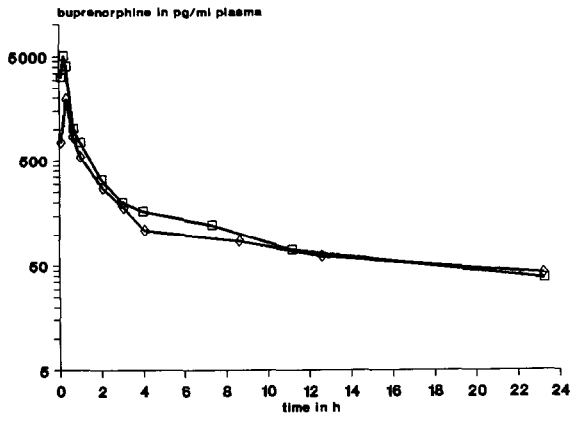

Fig. 9. Concentration–time curve of buprenorphine from two patients after subcutaneous administration of 5 μg buprenorphine per kg body weight.

DISCUSSION

In spite of its broad clinical application, little information is available about the pharmacokinetics of buprenorphine, a new thebaine derivative with high analgesic potential. In addition, the few published reports on this issue suffer from incomplete data, short evaluation times and complicated, time-consuming and relatively insensitive analytical procedures [13]. These limitations arise from the low therapeutic concentrations of

TABLE II

PHARMACOKINETIC PARAMETERS OF TWO PATIENTS AFTER SUBCUTANEOUS ADMINISTRATION OF 5 μg/kg BUPRENORPHINE

Patient No.	Terminal half-life (h)	AUC concentration (pg h/ml plasma)	Peak concentration (pg/ml)
1	14.25	4245	4865
2	19.36	4438	2357

buprenorphine in biological samples and inadequate assays for its detection.

The current paper describes a new sensitive HPLC method using a valve-switching solid-phase trace-enrichment procedure. This assay allows a detection limit of as low as 40 pg/ml buprenorphine or norbuprenorphine with a concentration-dependent inter-assay variation of 9–17%. Similar data have been achieved by a few other investigators but only by using labour-intensive and expensive techniques such as gas chromatography–mass spectrometry [2,14]. Other HPLC methods of similar complexity are not as sensitive [3–6,10,11,15]. Enzyme immunoassays and radioimmunoassays are more complicated (derivatization) and suffer from the usual problems of selectivity [7,16]. The method described here is easily reproducible, rapid and relatively inexpensive. Simple alteration of the proportion of acetonitrile in the enrichment and analytical eluent allows the analysis of both buprenorphine and norbuprenorphine in separate steps requiring approximately 10 min for inter-assay adjustment.

Preliminary data indicate that this procedure can be applied for broad clinical use and for the monitoring of drug levels during therapeutic interventions. This knowledge might be useful in optimizing buprenorphine therapy and to gain further insights into the metabolism of this agent.

REFERENCES

1 C. Rossano, L. F. De Luca, V. Firetto, F. Fossi, C. L. Castiglioni and P. Carminati, *Clin. Ther.*, 5 (1982) 61.
2 Y. Blom, U. Bondersson and E. Anggard, *J. Chromatogr.*, 338 (1985) 89.
3 G. Mullersman, S. Toufflin and H. Derendorf, *J. Pharm. Biomed. Anal.*, 5 (1987) 303.
4 J. Noda and T. Kojima, *Hiroshima J. Med. Sci.*, 34 (1985) 261.
5 S. T. Ho, J. J. Wang and W. Ho, *J. Chromatogr.*, 570 (1991) 339.
6 L. Debrabandere, M. Van Boven and P. Daenens, *J. Chromatogr.*, 564 (1991) 557.
7 A. J. Bartlet, J. G. Lloyd-Jones, M. J. Rance, I. R. Flockhart, G. J. Po, M. R. D. Bennett and R. A. Moore, *Eur. J. Pharmacol.*, 18 (1980) 339.
8 G. K. L. Tiong and J. E. Olley, *Naunyn-Schmiedeberg's Arch. Pharmacol.*, 338 (1988) 202.
9 G. Heinzel, R. Hammer, M. Wolf, F. W. Koss and G. Bozler, *Arzneim.-Forsch.*, 27 (1977) 904.
10 G. Heinzel, G. Bozler and J. M. van Rossum (Editors), *Pharmacokinetics During Drug Development: Data Analysis and Evaluation Techniques*, Gustav Fischer Verlag, Stuttgart, New York, 1982, p. 207.
11 E. J. Cone, C. W. Gorodetzky, D. Yousefnejad, W. F. Buchwald and R. E. Johnson, *Drug Metab. Dispos.*, 12 (1984) 577.
12 R. Garrett and V. R. Chandran, *J. Pharm. Sci.*, 74 (1985) 515.
13 R. E. S. Bullingham, H. J. McQuay and R. A. Moore, *Clin. Pharmacokin.*, 8 (1983) 332.
14 M. Ohtani, F. Shibuya, H. Kotaki, K. Uchino, Y. Saitoh, F. Nakagawa and K. Nihitateno, *J. Chromatogr.*, 487 (1989) 469.
15 L. P. Hackett, L. J. Dusci, K. F. Ilett, S. S. W. Seow and A. J. Quigley, *J. Chromatogr.*, 374 (1986) 400.
16 J. W. Villinger, R. A. Boas and K. M. Taylor, *Life Sci.*, 29 (1981) 229.