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High-performance liquid chromatography with electrochemical detection of buprenorphine and its major metabolite in urine

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ABSTRACT

A procedure was developed for the simultaneous determination of buprenorphine and its major metabolite, N-desalkylbuprenorphine, by reversed-phase high-performance liquid chromatography with electrochemical detection. The detection limit is about 100 pg/ml for the major metabolite and 250 pg/ml for buprenorphine.

INTRODUCTION

Buprenorphine (Temgesic) (Fig. 1) is a relatively new and powerful oripavine derivative, with both agonist and antagonist properties at the μ opiate receptor [1]. It is effective in the treatment of acute and chronic pain bij parenteral and sublingual administration [2]. The drug is about 30 times more potent than morphine [3] when administered intravenously or intramuscularly in doses of 5–10 μg/kg for post-operative pain in man.

Buprenorphine is metabolized by N-dealkylation and subsequent conjugation with glucuronic acid of both buprenorphine and the N-desalkyl metabolite. Buprenorphine has a long half-life of about 8 h [4]; the elimination of the N-desalkyl metabolite (Fig. 1) is even slower.

A low addiction potential has been reported [5] and suggestions for the use of buprenorphine in the management of opiate addicts have been made [6]. However, reports of misuse of the drug have been published [7–9]. Lethal overdose cases have never been described. A ban has been issued by the IOC Medical Commission on the use of buprenorphine together with other narcotic analgesics during Olympic Games [10]. The drug was rumoured to have been used in greyhound racing [11]. For screening urine samples, a rapid and sensitive method is needed.

Following intravenous, intramuscular [4] or sublingual administration of buprenorphine, urine concentrations of the drug are in the range of nanograms or even picograms per millilitre. Radioimmunoassay [13,14] and radioreceptorassay

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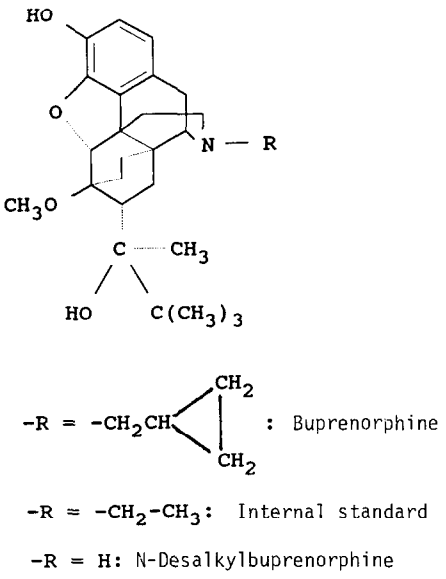

Fig. 1. Structures of buprenorphine, N-desalkylbuprenorphine and the ethyl derivative used as internal standard.

[15] have been used for the detection of buprenorphine. Although very sensitive, these techniques, as they are not specific owing to cross-reaction with metabolites and other structurally related drugs, may lead to the possibility of false-positive results. Several gas chromatographic methods have been described, all needing derivatization prior to analysis [16–18]. In a single subject, using chemical degradation of the buprenorphine molecule followed by treatment with pentafluoropropionyl anhydride, Blom *et al.* [19] were able to detect small amounts of unconjugated buprenorphine in both urine and plasma samples by gas chromatography–mass spectrometry with selected-ion monitoring.

Regarding the requirements for simplicity, specificity and sensitivity, high-performance liquid chromatography (HPLC) seems to be the technique of choice. HPLC methods published so far, however, are insufficiently sensitive [20,21] as they only allow concentrations higher than 10 ng/ml to be detected.

EXPERIMENTAL

Materials and reagents

The following analytical-reagent grade materials were used: sodium dihydrogenphosphate monohydrate, disodium hydrogenphosphate dihydrate, tetrabutylammonium sulphate (E. Merck, Darmstadt, Germany) and sodium 1-heptanesulphonate (Janssen Chimica, Beerse, Belgium). Toluene and acetonitrile were of HPLC grade. All extraction tubes were sonicated with warm nitric acid, thor-

oughly rinsed with doubly distilled water and siliconized using a water-soluble siliconizing fluid (Aquasil) (Pierce, Rockford, IL, U.S.A.). SPE columns were obtained from Analytichem International (Harbor City, CA, U.S.A.) (Bond Elut; 3 ml) and from E. Merck (Extrelut-3; 15 ml)

β-Glucuronidase–arylsulphatase from *Helix pomatia* (EC 3.2.1.31 and EC 3.1.6.1) (Boehringer, Mannheim, Germany) was used for deglucuronidation. Buffer solution (pH 3.0) was prepared by mixing 0.02 mol of sodium dihydrogenphosphate monohydrate, 10 mmol of sodium 1-heptanesulphonate and 0.01% tetrabutylammonium sulphate. The buffer solution used for extraction was prepared by mixing 0.02 mol of disodium hydrogenphosphate dihydrate and 0.02 mol of sodium dihydrogenphosphate monohydrate (97:3).

Buprenorphine and N-desalkylbuprenorphine were synthesized in the laboratory according to Kleemann and Engel [22]. The synthesis of the internal standard, N-ethyl-7-[1-(5)-hydroxy-1,2,2-trimethylpropyl]-6,14-*endo*-ethano-6,7,8,14-tetrahydronororipavine, was performed following a similar reaction scheme but using ethyl bromide instead of methylcyclopropyl bromide in the last reaction step. The synthesized compounds were characterized by mass, nuclear magnetic resonance and infrared spectrometry.

Preparation of standard solutions

Stock solutions were prepared by dissolving buprenorphine (10.0 mg), N-desalkylbuprenorphine (10.0 mg) and internal standard (10.0 mg) in 100 ml of acetonitrile. These solutions were kept in a freezer (− 18°C). Working solutions consisted of 0.1 ng/μl in the mobile phase.

Apparatus

A Model Eldec 201 amperometric detector (Chromatofield/Instrulab, Chateauneuf-les-Martigues, France) was used. This instrument was coupled to a Merck-Hitachi Model L-6200 intelligent pump equipped with a 20-μl injector (Rheodyne, Berkeley, CA. U.S.A.). The chromatograms were recorded with a Merck-Hitachi Model D-2500 chromato-integrator.

The extractions were carried out with a rotary mixer. The extraction tubes were centrifuged with a Heraeus Sepatech Labofuge A.

Chromatographic conditions

HPLC was carried out on a LiChrosorb CN (5 μm) column (25 cm x 0.4 cm I.D.) (E. Merck). The optimum mobile phase for separating both buprenorphine and its major metabolite was found to be acetonitrile–phosphate buffer (pH 3.0) (13:87). The mobile phase was filtered through a 0.2-μm filter and completely degassed with helium before use. Chromatography was carried out at a flow-rate of 1.0 ml/min at ambient temperature. Electrochemical detection was performed at a potential of 0.75 V and a sensitivity of 1 nA.

Extraction procedure

Urine (2 ml) was first centrifuged for 10 min at 572 *g*. A 100-μl volume of internal standard solution, corresponding to 10 ng of internal standard, together with 1 ml of phosphate buffer (pH 8.5), was added to the supernatant. The samples were extracted with 5 ml of toluene on a rotary mixer for 30 min. After centrifugation for 10 min at 1880 *g* the organic layer was transferred into another tube and washed with 1 ml of phosphate buffer (pH 8.5). The organic layer was separated again by centrifugation and was subsequently re-extracted for 30 min into 1 ml of hydrochloric acid (0.1 mol/l). The separated organic layer was discarded. A 1-ml volume of sodium hydroxide (0.1 mol/l) and 1 ml of phosphate buffer (pH 8.5) were added to the remaining aqueous layer and the latter was extracted with 5 ml of toluene on a rotary mixer for 30 min. The organic layer was separated by centrifugation for 10 min at 1880 *g*, transferred into siliconized tubes and evaporated to dryness under a stream of nitrogen at 60°C. The residue was reconstituted in 60 μl of acetonitrile–phosphate buffer (pH 3.0) (13:87) and 20-μl aliquots were injected into the chromatograph.

In a separate experiment, 2 ml of urine, diluted with 1.0 ml of citrate–phosphate buffer (pH 5.5) (0.01 mol/l), were incubated overnight at 38°C with 200 μl of β-glucuronidase–arylsulphatase containing 20 000 Fishman Units of β-glucuronidase and 10 000 Units of arylsulphatase. The mixture was then extracted as described above.

Calibration graphs were constructed by spiking drug-free urine samples with known amounts of buprenorpine, N-desalkylbuprenorphine (0.1, 0.25, 0.5, 1.0, 2.0, 5.0 and 10 ng/ml) and 100 μl of the internal standard solution (0.1 ng/μl). The samples were extracted as described above.

RESULTS AND DISCUSSION

Extraction procedure

Several techniques were tried for extracting buprenorphine and N-desalkylbuprenorphine simultaneously from human urine. Solid-phase extraction (SPE) was examined using C_2, C_{18}, CN and Extrelut-3 columns. Alternatively, liquid–liquid extraction procedures were studied. Chloroform, diethyl ether, hexane, toluene, chloroform–isopropanol (90:10), hexane-ethyl acetate (90:10), toluene–hexane (90:10) and toluene–hexane (90:20) were tried as extraction solvents. Toluene combined with an acidic clean-up procedure was found to be the most suitable solution for obtaining extracts free from interfering impurities. This procedure was further used in this study.

Choice of detection potential

The best detection potential was established by constructing a hydrodynamic voltammogram (Fig. 2A) and plotting the detector response (area under the curve; AUC) *versus* the applied potential (V). The resulting potential of the work-

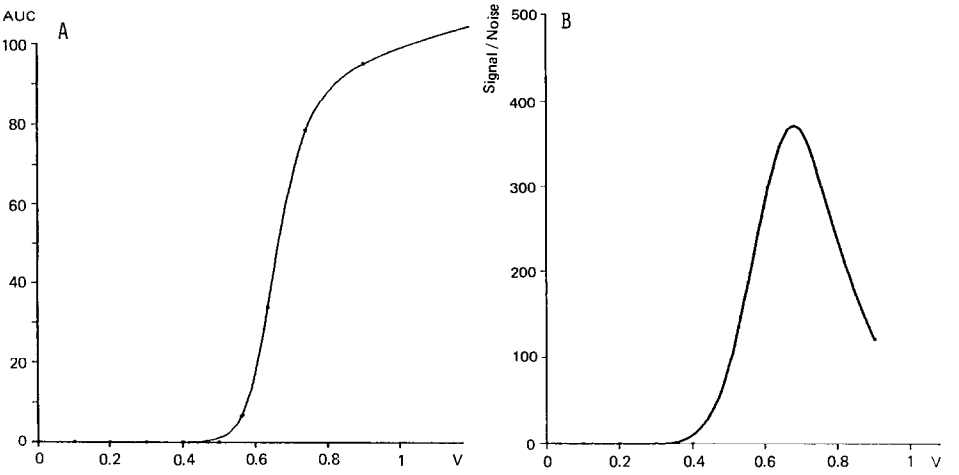

Fig. 2. (A) Current–voltage curve determined by application of different oxidation potentials. (B) Determination of optimum potential by measuring the signal-to-noise ratio.

ing electrode was obtained from the maximum of the signal-to-noise ratio *versus* the applied potential (Fig. 2B). Any subsequent increase in potential decreased the detection efficiency, owing to an increased background current.

Chromatography

The above conditions allow a good separation of buprenorphine, its major metabolite and the internal standard. The addition of tetrabutylammonium sulphate to the mobile phase was necessary to improve the peak shape, by saturating the unbound silanol groups of the stationary phase. Earlier experiments were conducted, using a synthesized homologue with a propyl side-chain as an internal standard instead of the ethyl derivative. This compound, however, was incompletely separated from the buprenorphine peak. The actual retention times for buprenorphine, N-desalkylbuprenorphine and internal standard are 7.0, 9.0 and 12.6 min, respectively.

Detection limit

The detection limit for the major metabolite was about 150 pg/ml and for buprenorphine 250 pg/ml. Reliable concentrations, however, could only be calculated for concentrations above 250 pg/ml N-desalkylbuprenorphine and 500 pg/ml buprenorphine.

Linear calibration graphs were obtained for urine by plotting the area under the curve ratio of both buprenorphine and N-desalkylbuprenorphine to the internal standard *versus* buprenorphine and N-desalkylbuprenorphine concentration, respectively. The graphs were linear up to a concentration of 100 ng/ml, with a correlation coefficient $r > 0.99$.

TABLE I

REPRODUCIBILITY OF BUPRENORPHINE (B) AND N-DESALKYLBUPRENORPHINE (NB) ASSAY IN URINE SAMPLES ($n=6$)

Concentration (ng/ml)			R.S.D. (%)	
Added	Found		B	NB
	B	NB		
0.5	0.43	0.49	8.1	5.2
5.0	4.4	4.8	6.3	4.5
20.0	18.0	19.2	5.8	4.1

Reproducibility

The precision and accuracy of the assay (within-day analysis) for buprenorphine and N-desalkylbuprenorphine were evaluted over the concentration range 0.5–20 ng/ml in urine. The results are shown in Table I. The relative standard deviation (R.S.D.) varied from 4.1 to 8.1% over the concentration range 0.5–20 ng/ml in urine and the measured concentrations of buprenorphine and N-desalkylbuprenorphine ranged from 86 to 98% of the added amount in the spiked urine samples. In order to ensure an acceptable between-day reproducibility, it is essential to clean the working electrode every day.

Recovery

Recoveries were studied by comparing the area under the curve for both buprenorphine and N-desalkylbuprenorphine after extraction from urine and the area under the curve obtained after injection of a standard solution at the same concentration. The recovery depends on the concentration range as shown in Table II. The R.S.D.'s varied from 2 to 11% for urine samples containing 1–50 ng/ml of both buprenorphine and N-desalkylbuprenorphine.

TABLE II

RECOVERY OF BUPRENORPHINE AND N-DESALKYLBUPRENORPHINE IN URINE

Concentration (ng/ml)	Buprenorphine		Desalkylbuprenorphine	
	Mean ($n=8$) recovery (%)	R.S.D. (%)	Mean ($n=8$) recovery (%)	R.S.D. (%)
1	64	11	68	10
2	68	6	72	6
10	73	5	77	4
50	89	3	95	2.5

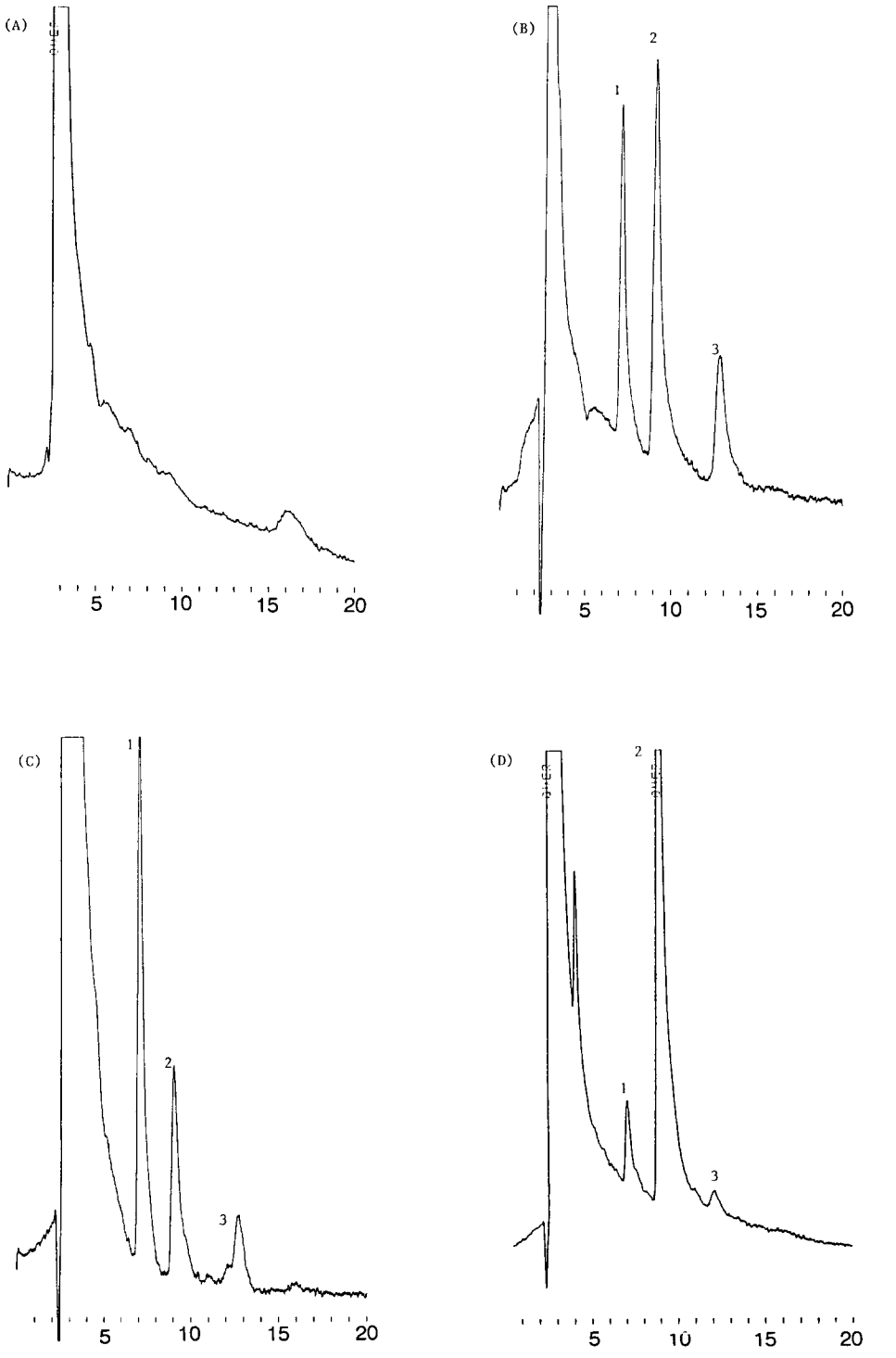

Fig. 3.

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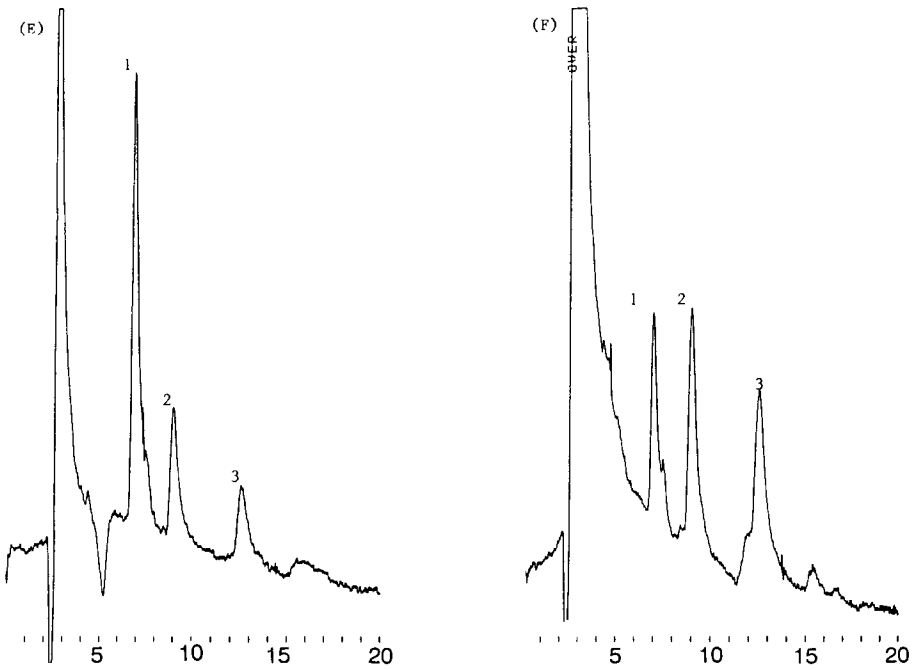

Fig. 3. (A) Chromatogram of blank urine. (B) Chromatogram of a standard solution containing 5 ng of N-desalkylbuprenorphine (1), 5 ng of internal standard (2) and 5 ng of buprenorphine (3). (C) Urine spiked with 2 ng/ml buprenorphine, 10 ng/ml N-desalkylbuprenorphine and 5 ng/ml internal standard. (D) Urine extract after a single therapeutic dose (intramuscular) of 0.3 mg of buprenorphine (10 h after injection). (E) Unknown urine sample containing 10 ng/ml each of N-desalkylbuprenorphine and buprenorphine. (F) Unknown urine sample containing 20 ng/ml N-desalkylbuprenorphine and 8 ng/ml buprenorphine.

As about 50% of buprenorphine and its major metabolite are excreted in the form of glucuronides, deglucuronidation with β-glucuronidase–arylsulphatase, prior to extraction, should theoretically result in an increased concentration. A limited number of experiments, however, showed that the extracts obtained from deglucuronized urine samples were no longer suitable for analysis with electrochemical detection owing to the presence of many co-extracted materials.

When using SPE for the extraction of urine samples with either Bond Elut columns (C_2, C_{18} or CN) or Extrelut-3 columns, the recoveries as determined with high concentrations ($>$ 50 ng/ml) were generally satisfactory ($>$90%). The major problem with SPE was the difficulty in obtaining sufficiently pure extracts. Indeed, when applying the sensitivity of 1 nA necessary for the detection of concentrations of buprenorphine and its metabolite down to the level of 250 pg/ml in urine, these compounds were hidden by co-extracted impurities. Liquid–liquid extractions as described in this paper yielded pure extracts.

Sample analysis

Chromatograms of some actual samples are shown in Fig. 3. They include the result for a urine sample collected 10 h after intramuscular injection of a single therapeutic dose of 0.3 mg of buprenorphine to a volunteer. The unconjugated buprenorphine concentration is calculated to be *ca.* 500 pg/ml and that of the N-desalkyl metabolite *ca.* 2 ng/ml (Fig. 3D). Fig. 3 also includes the chromatograms for two unknown samples. In Fig. 3E, a high buprenorphine concentration is seen with about an equal concentration of the major metabolite; in Fig. 3F the latter predominates.

CONCLUSION

A sensitive and selective method has been developed for the determination of buprenorphine in urine samples. This method allows the detection of both unconjugated buprenorphine and N-desalkylbuprenorphine, even after a single therapeutic dose, using a relatively simple pretreatment of the biological samples. It constitutes a sensitive routine procedure for the confirmation of this drug in pre-screened urine samples by radioimmunoassay for doping control. The applicability of this method is being studied on plasma and urine samples from patients and persons suspected of drug misuse.

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