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Determination of plasma nefopam by liquid chromatography and electrochemical detection

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SUMMARY

A liquid chromatographic method for the determination of plasma nefopam is presented. A combination of liquid- and solid-phase extraction and electrochemical detection gave clean extracts and, hence, a low limit of detection. Calibration curves were linear over at least two orders of magnitude (1–100 ng/ml) making the method suitable for pharmacokinetic studies.

INTRODUCTION

Nefopam, a non-narcotic analgesic, is a benzoxazocine compound structurally related to the anti-cholinergic drug orphenadrine (Fig. 1). In their 1980

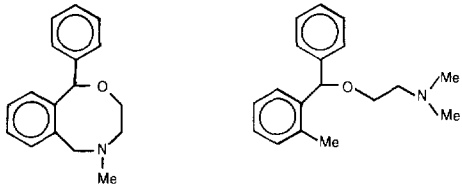

Fig. 1. Structures of nefopam and orphenadrine The N-methyl group in nefopam is replaced by ethyl in the internal standard.

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review of the pharmacological properties, Heel et al. [1] noted that there was only limited information available on the pharmacokinetic properties of nefopam and that this was primarily in the form of unpublished reports. Little has changed in the intervening years; for example, although nefopam can be administered orally, there are no data on its bioavailability. Gas chromatographic methods with flame ionisation detection for plasma nefopam require relatively large volumes of plasma, 5 ml for a sensitivity of 5 ng/ml [2] and 2 ml for a sensitivity of 10 ng/ml [3]. Using nitrogen-selective detection, Chang et al. [4] detected 5 ng/ml in a 1-ml plasma sample. Plasma and milk concentrations have been reported [5] using reversed-phase liquid chromatography (LC) with ultraviolet (UV) detection at 215 nm [6]. For a 2-ml sample the detection limit (80% confidence) was 1.6 ng/ml [5]. We report a new LC method with electrochemical detection (ED), which is capable of assaying 1 ng in a 1-ml plasma sample, with an intra-assay coefficient of variation of 10.6%.

EXPERIMENTAL

Materials

Nefopam hydrochloride and N-desmethylnefopam were gifts from Riker Labs. (Loughborough, U.K.). Orphenadrine hydrochloride was purchased from Sigma (Poole, U.K.). Nefopam N-oxides were obtained by oxidizing nefopam base with 3-chloroperbenzoic acid [7] in ethyl acetate. N-Ethyl and N-propyl homologues of nefopam were prepared by reacting desmethylnefopam with the appropriate iodoalkane and separating the required products by thin-layer chromatography (TLC; SiO_2; chloroform–ethanol–18 *M* ammonium hydroxide, 80:10:1, v/v). [N-Methyl-^{3}H]nefopam was prepared by reacting an excess of desmethylnefopam with [^{3}H]methyl iodide (3.15 TBq/mmol; Amersham International, Amersham, U.K.). The product was isolated by TLC as described above. The purity was determined as >97% by TLC.

HPLC-grade acetonitrile was purchased from Fisons Scientific Apparatus (Loughborough, U.K.). Hipersolv-grade cyclohexane was from BDH (Poole, U.K.). Bond Elut columns (100 mg sorbent, 1 ml volume) were from Jones Chromatography (Hengoed, U.K.).

Plasma samples were from healthy male volunteers (41–60 years old) who had received an intramuscular injection of nefopam hydrochloride (20 mg). Blood samples (10 ml) were drawn into heparinised tubes, plasma was separated by centrifugation and stored at −20°C until assay. The protocol was approved by the Tower Hamlet District Ethics Committee. Blood bank plasma for the preparation of standard solutions was provided by the National Blood Transfusion Service (Brentwood, U.K.).

Liquid chromatography

The chromatographic system consisted of an Altex 110 pump, a Kontron MSI 660T autosampler fitted with a 50-μl injection loop and either an Environmental Science Assoc. Coulochem Model 5100A electrochemical detector (Severn Analytical, Shefford, U.K.) or a Hitachi 110-10 spectrophotometer fitted with an Altex 155-00 flow cell. The analytical wavelength was 267 nm. An additional membrane pulse dampener (Severn) was placed in series between the pump and injection valve. The silica column, Spherisorb S5W (150 mm $\times$ 4.6 mm I.D.) was slurry-packed in methanol or purchased ready-packed (Phase Separations, Queensferry, U.K.).

Eluents were based on mixtures of acetonitrile and 0.1 *M* ammonium nitrate buffer which were filtered to 0.2 μm before use. For plasma extracts, pH 9.1 buffer (7 volumes) was mixed with 93 volumes of acetonitrile. The flow-rate was 1 ml/min. The 5010 analytical cell electrodes were operated in the 'screen mode' with the first electrode at +0.5 V and the second at +0.8 V relative to the palladium reference electrodes.

pH-controlled solvent extraction

The cyclohexane–buffer partition characteristics of nefopam, desmethylnefopam and N-ethylnornefopam were determined using standard techniques [8]. Samples were assayed by high-performance liquid chromatography (HPLC) with UV detection, using orphenadrine as internal standard. The change in apparent partition coefficient, APC, as a function of pH was fitted iteratively and solved for pK_a and true partition coefficient (TPC) [8].

Ionisation constants

Ionisation constants for nefopam and desmethylnefopam were determined spectrophotometrically [9] using a Kontron Uvikon 810. The analytical wavelengths were 267 and 273 nm, respectively, for nefopam and the desmethyl compound. The pH values of Britton–Robinson buffers were determined using a Beckmann 500 pH meter, standardized with pH 4.0 and pH 7.0 buffer solutions and checked with a solution of 0.05 *M* disodium tetraborate (pH 9.23 at 20°C) [9].

Plasma extractions

Plasma samples (1 ml) and internal standard solution (N-ethylnornefopam, 25 ng in 25 μl water) were pipetted into 10-ml screw-capped glass tubes. Sodium hydroxide solution (0.1 *M*, 1 ml) and cyclohexane (5 ml) were added and the capped tubes rolled on a Denley, Spiramix, for 15 min. After centrifugation (1000 *g*, 15 min) to separate the layers, the organic phases (4 ml) were transferred to prepared diol columns.

Diol columns were prepared by sequentially washing with methanol (2 $\times$ 1 ml), water (2 $\times$ 1 ml), 0.1 *M* hydrochloric acid (1 ml), water (1 ml), methanol

(1 ml) and cyclohexane (1 ml) under vacuum. After the cyclohexane extracts had been drawn through the prepared columns, they were washed with cyclohexane (1 ml), acetonitrile (1 ml) and finally HPLC eluent (0.1 ml). The columns were centrifuged at 500 *g* for 1 min to remove excess solvent. The compounds were eluted by centrifugation as above, with 500 μl of HPLC solvent, the eluents being collected in 10-ml screw-capped glass tubes. The contents of the tubes were mixed on a vortex mixer to ensure homogeneity of the contents, and 200 μl were transferred to 0.25-ml polyethylene microcentrifuge tubes. The tubes were sealed with polypropylene autosampler caps (Chromacol, London, U.K.). After centrifugation for 1 min at 10 000 *g* the tubes were placed in the autosampler.

Standard solutions were prepared in blood bank plasma at 100, 50, 25, 10, 5, 2, 1 and 0 ng/ml and taken through the extraction procedure. Calibration curves were prepared as peak-height ratio of nefopam to internal standard against known concentration. Estimates of precision were obtained from replicate assays of standard solutions.

Recovery experiments

Stock nefopam and [^{3}H]nefopam solutions were diluted with blood bank plasma to give solutions containing 100, 10 and 1 ng/ml drug and 520 Bq/ml radioactivity. Replicate samples ($n=6$) were taken through a modified plasma extraction procedure in which the nefopam was eluted from the Bond Elut columns with 0.5 ml eluent, directly into mini-scintillation vials, and 4 ml scintillation cocktail (NE 260, Nuclear Enterprises, Reading, U.K.) were added. Quench corrections were made using the external standard method.

RESULTS AND DISCUSSION

Chromatographic conditions

The LC separation was developed using unmodified silica columns and alkaline eluents as we had successfully used these conditions for other basic, centrally acting drugs [10–12]. Preliminary investigations of methanol–acetonitrile mixtures showed that the best results, with regard to peak shape and sensitivity, were obtained when methanol was omitted from the eluent. The conditions were optimised to separate nefopam, desmethylnefopam and ethylnornefopam. This was achieved with a mixture of ammonium nitrate buffer (0.1 *M*, pH 9.1) and acetonitrile (7:93, v/v). Under these conditions all three compounds were adequately resolved (Fig. 2). The k' values of the compounds increased as the pH of the eluent increased but the rate of increase of k' for the secondary amine was greater than the rates for the tertiary amine compounds. Consequently, at lower pH values the desmethyl compound and the ethyl homologue were not resolved and at higher pH values the desmethyl compound and nefopam co-eluted. The propyl homologue, being the most li-

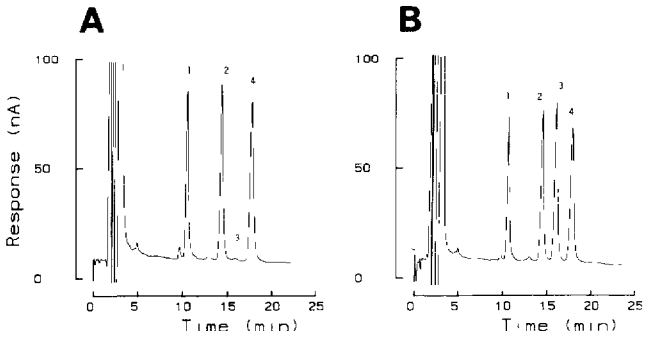

Fig. 2 Chromatogram of reference materials. (A) 100 ng each, (B) 100 ng each except desmethylnefopam (7 μg). Peaks: 1 = N-propylnornefopam; 2 = N-ethylnornefopam; 3 = desmethylnefopam; 4 = nefopam.

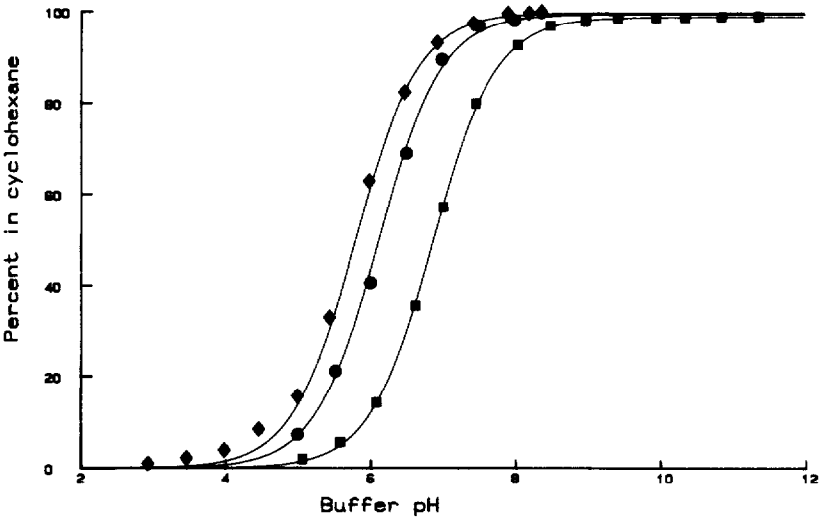

Fig. 3. pH-controlled extraction of nefopam (●), desmethylnefopam (♦) and N-ethylnornefopam (■). The solid lines represent the fitted curves.

pophilic, had the lowest k' values. Additional selectivity was imparted by the differences in electroactivity. At a working potential of 0.8 V the response to desmethylnefopam was approximately 1–2% of that for the parent compound (Fig. 2).

pH partitioning

The effect of buffer pH on the extraction of the compounds can be seen in Fig. 3. The figure shows the experimental points and the modelled curves [8] for percentage extraction from aqueous to organic solution. The estimated pK_a

values (± S.D.) for nefopam, desmethylnefopam and the ethyl homologue were 8.4 ± 0.09, 8.8 ± 0.03 and 8.6 ± 0.04, respectively. As expected the ethyl homologue was the most lipophilic having a cyclohexane–water partition coefficient of 660. The TPC values for nefopam and its desmethyl metabolite were 197 and 88, respectively. Once TPC and pK_a are known the extraction curve for any volume ratio can be derived.

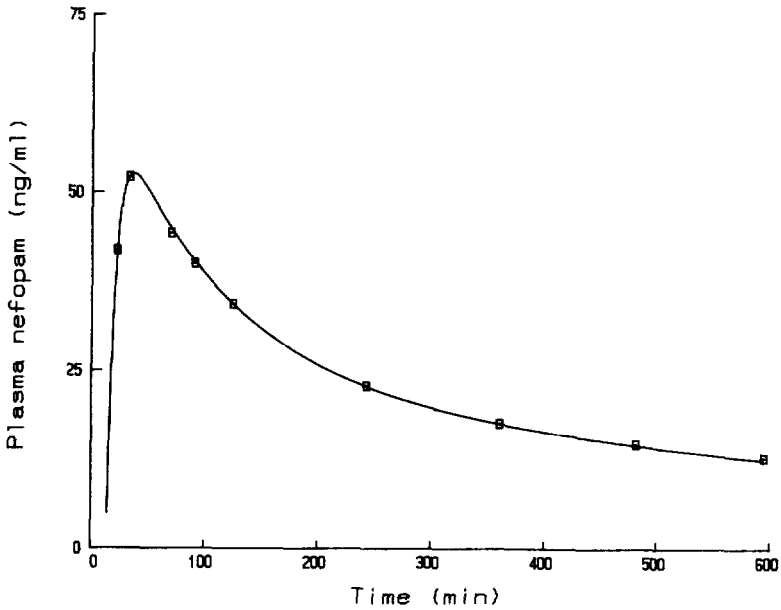

Fig. 4. Plasma concentration–time curve following a 20-mg intramuscular dose in subject 1 Solid line was fitted to a two-compartment model with first-order input using Grafit [17].

TABLE I

INTRA- AND INTER-ASSAY COEFFICIENTS OF VARIATION OBTAINED FROM REPLICATE ASSAYS OF KNOWN SOLUTIONS

Concentration (ng/ml)	Intra-assay			Inter-assay		
	n	Found (ng/ml)	C.V. (%)	*n*	Found (ng/ml)	C V. (%)
1.0	8	1.06	10.6	6	0.95	10.6
2 0	8	2.50	22.5	6	2.01	5.2
2.0	7	2.32	10.7	–	–	–
5.0	7	5.1	1.4	7	4.8	5.8
25.0	7	25.0	2.0	7	25 8	2.4
100.0	7	101 9	1.41	7	99 8	0.5

Ionisation constants

The ionisation constant of nefopam determined by pH partitioning was at variance with literature values of 9.2 [13] and 9.36 [5]. Using spectrophotometry we obtained a pK'_a value of 8.24 ± 0.05 ($\pm$ scatter, $n=7$) which is in keeping with our partition results and the value of 8.18 obtained by Ehrsson and Eksborg [2] using potentiometric titration. The spectrophotometric pK'_a of the desmethyl compound was 8.9 ± 0.2, in reasonable agreement with the value obtained by partitioning.

Solid-phase extraction

Although plasma samples can be applied directly to prepared solid-phase extraction columns, we preferred to effect a preliminary separation using liquid–liquid extraction. This had the advantage of producing cleaner samples and avoided problems caused by protein binding [14]. Nefopam was retained on several acid-treated Bond Elut phases including C_{18}, C_8, C_2 and CN. It was assumed that the retention mechanism was adsorption on residual silanols and it seemed logical to investigate the diol phase, which proved to be the most satisfactory phase. The smallest column size (100 mg sorbent) was adequate and provided a sharp elution pattern so that the compounds could be concentrated in HPLC eluent without the need for evaporation. The elution profiles of nefopam and its ethyl homologue were very similar.

Using radiolabelled nefopam, the combination of liquid- and solid-phase extractions gave overall recoveries (mean $\pm$ S.D.) of 93.9 ± 1.7, 95.3 ± 1.6 and $94.4 \pm 2.4\%$ at 100, 10 and 1 ng/ml of plasma, respectively. Adsorption onto glassware, as reported by other workers [6], was not a problem and there was no need to silanize the apparatus. The absolute recovery of ethylnornefopam was not determined but the peak-height ratio nefopam to internal standard of a standard solution did not change significantly when it was taken through the extraction method.

Amine oxides

Nefopam N-oxides were prepared to check that they would not interfere in the assay. As nefopam is chiral [15] and the tertiary amine is asymmetric, oxidation produced diastereoisomeric N-oxides in approximately equal amounts. Under the conditions used for plasma assays, the N-oxides chromatographed as a broad, partially resolved peak with a retention time approximately eleven times that of nefopam. Although the extraction of N-oxide into cyclohexane was expected to be negligible, a possible interference could be caused by reversion of amine oxide under the alkaline conditions of the liquid–liquid extraction [16]. When high concentrations (1 μg/ml) of the N-oxides were taken through the method, no reduction to parent compound could be detected.

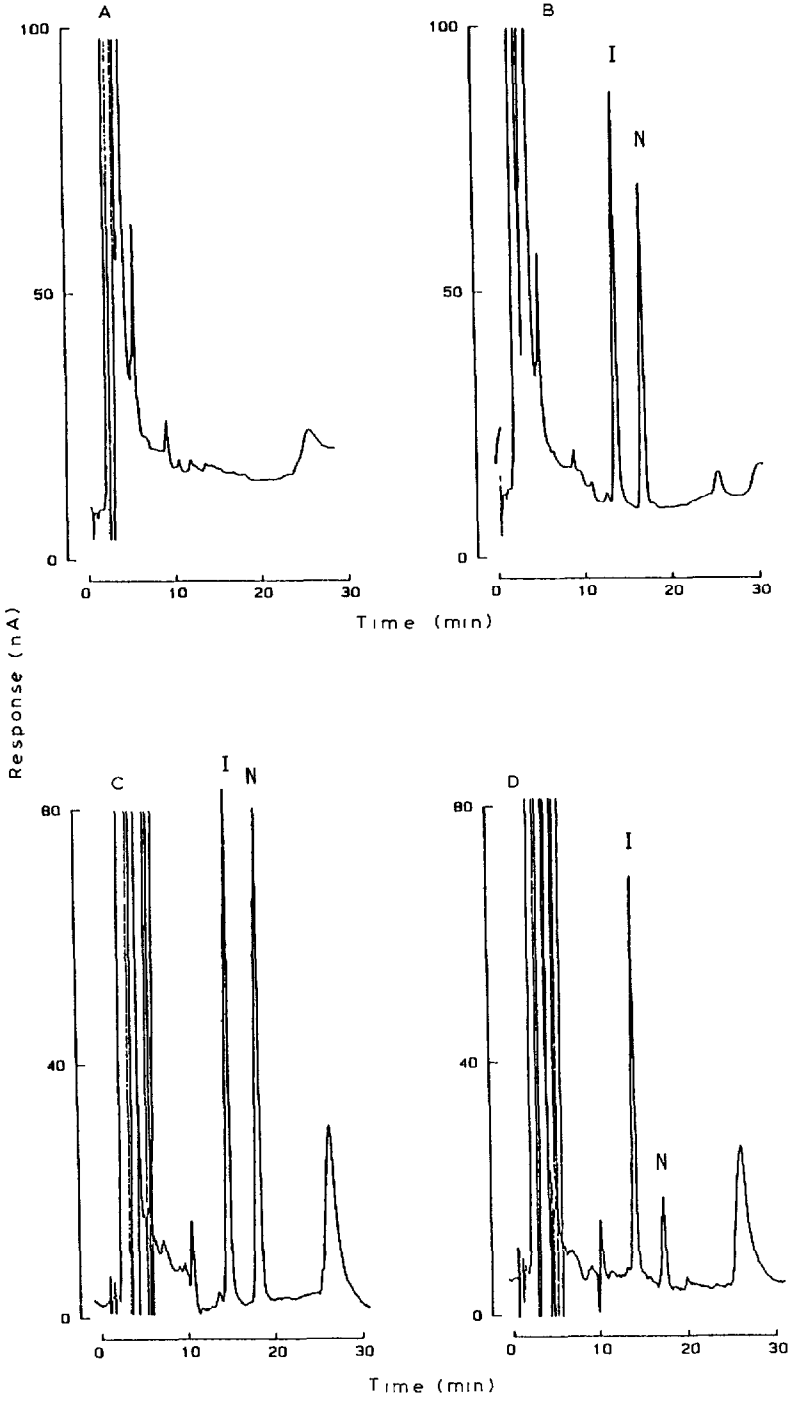

Fig 5 Chromatograms of plasma extracts. (A) Drug-free plasma, (B) 20 ng/ml standard, (C subject 3 plasma at 5 min; (D) subject 3 plasma at 13 h Peaks I = internal standard, N = nefopam

Precision, accuracy and sensitivity

An indication of the precision of the method can be obtained from the data of Table I. The intra-assay coefficients of variation (C.V.) for samples containing between 5 and 100 ng/ml were 2% or less. The accuracy in this concentration range was good, relative errors (bias) being about 2%. For the 2 ng/ml sample the CV increased to 22.5%, with a relative error of 25% for a mean of eight samples. One sample gave a particularly high value and when this was omitted from the calculation the C.V. fell to 10.7% and the bias to 16%. The reason for this is not known. The intra-assay precision at 1 ng/ml was 10.6%.

The calibration curves were linear over the range 1–100 ng/ml, coefficients of regression being >0.9997. The minimum detectable quantity of nefopam was 50 pg injected. This quantity gave a signal-to-noise ratio of 3.5–4. This is in keeping with the fact that a 1 ng/ml standard plasma solution gave a signal-to-noise ratio of 7. Although it should be possible to detect as little as 0.5 ng/ml with a signal-to-noise ratio of ≈ 3, we have only tested the accuracy and precision of the method to 1 ng/ml and set the limit of quantification (LOQ) at this value. An LOQ of 1 ng/ml should be sufficient for most pharmacokinetic studies.

Plasma concentrations in volunteers

A plasma concentration–time curve, following a single intra-muscular injection of 20 mg nefopam hydrochloride in subject 1 is presented in Fig. 4. The concentration rose rapidly, a peak value of 52 ng/ml being measured in the 30-min sample. The concentrations fell to 13 ng/ml at 10 h (the last-time sample for this subject). Although the number and distribution of data points were such that iterative curve fitting was not possible, using a graphic program (Grafit [17]) three exponential phases were identified. The two declining phases had half-times of 69 and 570 min. After a lag-time of 13 min the plasma concentrations rose rapidly with a half-time of 4.8 min. Assuming 100% bioavailability of the intramuscular dose, the clearance was 0.8 l/min. Other subjects showed similar patterns. The lag-time in subject 3 was less as can be seen from the chromatogram of the 5-min sample (Fig. 5) and the half-time of the terminal phase was less, the concentration falling to 3 ng/ml by 13 h.

CONCLUSIONS

LC–ED offers a simple but sensitive assay procedure for plasma nefopam. With a limit of quantification of 1 ng/ml, it is eminently suitable for pharmacokinetic studies.

ACKNOWLEDGEMENTS

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