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A QUANTITATIVE GAS–LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF PETHIDINE AND ITS METABOLITES, NORPETHIDINE AND PETHIDINE N-OXIDE IN HUMAN BIOLOGICAL FLUIDS

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SUMMARY

A sensitive and selective analytical method, to measure pethidine and its metabolites norpethidine and pethidine N-oxide simultaneously in human biological fluids is described. The procedure involves preliminary extraction of pethidine and norpethidine from the alkalinised fluid into diethyl ether, followed by concentration and analysis using a gas–liquid chromatograph with a flame ionisation detector. Two alternative gas–liquid chromatographic systems have been used successfully: one involves acylation of norpethidine prior to injection on the column, the other does not require derivative formation and was chosen for routine analysis. Benzphetamine hydrochloride is used as the internal standard for quantitation by the peak area ratio technique. The method will detect as little as 20 ng/ml of pethidine and norpethidine in a 5-ml sample and has been employed to measure pethidine, norpethidine and pethidine N-oxide in human urine, and pethidine and norpethidine in plasma and whole blood after a single intramuscular therapeutic dose.

INTRODUCTION

Pethidine was first introduced as a potent analgesic in 1939 by Eisleb and Schaumann[1]. After thirty-four years of clinical experience, pethidine is still one of the most widely used and most effective narcotic analgesics (Beckett and Casy[2], Murphree[3], Hellerbach *et al.*[4], Casy[5]). It is of particular value in obstetrics, to control post-operative pain, in the relief of severe attack of acute pain, and for chronic pains caused by malignant disease.

Methods reported previously for the determination of pethidine and its metabolites in biological fluids have been based on spectrophotometric measurements of coloured, lipid-soluble compounds formed by complexing with coloured organic acids (Brodie and Udenfriend[6], Brodie *et al.*[7], Way *et al.*[8,9], Burns *et al.*[10], Crawford and Rudofsky[11,12], Fochtman and Winek[13]). Though Asatoor *et al.*[14] claim that the methyl orange method is unaffected by naturally occurring urinary amines, Beckett *et al.*[15] have shown that drug metabolites and naturally occurring

amines react with methyl orange and are analysed with the drug. Separation of drug metabolites from the drug has been achieved by a counter-current distribution technique (Craig *et al.*[16], Plotnikoff *et al.*[17]) and by washing organic extracts with buffer before dye complexation (Asatoor *et al.*[14]). These methods are neither specific nor sensitive enough to be used in metabolic or pharmacokinetic studies.

Tracer techniques have been used in drug metabolism studies and ^{14}C-labelled pethidine has been used to estimate the total amount of drug and its metabolites in rat urine (Plotnikoff *et al.*[17]). However, these studies are expensive and of limited use in man.

Dal Cortivo *et al.*[18] developed a fluorometric technique to determine microgram amounts of pethidine from urine and plasma. The limits of detectability for the compound is around 300 ng/ml. Recovery of pethidine added to urine and plasma was about 65%. The method fails in the presence of fluorophores such as quinine, quinidine, and methapyrilene.

Methods based on gas–liquid chromatographic (GLC) techniques have been employed for quantitative determination of many drugs and their metabolites (Beckett *et al.*[19]), for example Beckett and Taylor[20] used a GLC method to compare blood concentrations of pethidine and pentazocine in mother and infant at the time of birth, but did not publish details of the method. Jenkins *et al.*[21] also described a GLC method for pethidine analysis and used it to study placental transfer in pregnant ewes. However, the chromatographs showed interfering peaks for 30 min after those of the pethidine and marker. There is no published GLC method which will assay both the parent drug, pethidine and its metabolites, norpethidine and the recently isolated pethidine N-oxide (Mitchard *et al.*[22]) in human biological fluids.

EXPERIMENTAL

Materials and apparatus

The following materials were used: AnalaR diethyl ether, freshly re-distilled; absolute alcohol, freshly re-distilled; ether-washed sodium hydroxide solutions (20 and 40 wt.%); 6 *N* hydrochloric acid; 30 wt.% titanium trichloride solution containing 24 wt.% hydrochloric acid (BDH, Poole, Great Britain); pethidine hydrochloride (Roche, Welwyn Garden City, Great Britain); norpethidine hydrochloride (Sterling-Winthrop, Rensselaer, N.Y., U.S.A.); benzphetamine hydrochloride (Upjohn, Kalamazoo, Mich., U.S.A.); furfurylmethylamphetamine hydrochloride and N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride (Aldrich, Milwaukee, Wisc., U.S.A.); chlorpheniramine hydrochloride B.P.; brompheniramine hydrochloride B.P.; methadone hydrochloride B.P.; pentazocine hydrochloride (Bayer, Great Britain); and trifluoroacetylimidazole (Pierce, Rockford, Ill., U.S.A.).

The following apparatus were used: 10-ml-capacity centrifuge tube with well-fitted screw caps (Sovirel, Levallois-Perret, France); 15-ml stoppered evaporating tubes with finely tapered bases[23]; 10-μl Hamilton syringe (Hamilton, Reno, Nev., U.S.A.); mechanical tilt shaker; Hewlett-Packard Model 7610A high-efficiency gas chromatograph fitted with a flame ionization detector and Model 7650a electrometer linked to Model 3370b integrator and Model 7127A strip chart recorder (Hewlett-Packard, Avondale, Pa., U.S.A.); and U-shaped glass columns $\frac{1}{4}$ in. O.D.

Gas chromatography

Ethanolic solutions of the markers pethidine and norpethidine were injected on to the following chromatographic systems at various oven temperatures:

(1) Chromosorb G AW-DMCS (60–80 mesh) coated with 3% (w/w) OV-17, 4 ft. × ¼ in. O.D. glass.

(2) Chromosorb G AW-DMCS (60–80 mesh) coated with 2% (w/w) Carbowax 6000 and 5% (w/w) potassium hydroxide, 3 ft. × ¼ in. O.D. glass.

(3) Chromosorb G AW-DMCS (80–100 mesh) coated with 2.5% (w/w) SE-30, 4 ft. × ¼ in. O.D. glass.

(4) Gas-Chrom Q AW-DMCS (80–100 mesh) coated with 3% (w/w) Poly I 110, 4 ft. × ¼ in. O.D. glass.

(5) Chromosorb G AW-DMCS (85–100 mesh) coated with 10% (w/w) Apiezon L and 10% (w/w) potassium hydroxide, 4 ft. × ¼ in. O.D. glass.

(6) Chromosorb W AW-DMCS (80–100 mesh) coated with 8% Carbowax 20M and 2% (w/w) potassium hydroxide, 4 ft. × ¼ in. O.D. glass.

Retention times, resolution and symmetry of the peaks for these systems were then established.

All columns were conditioned at 20° below that of the maximum recommended temperature of the relevant stationary phases for 24 h. Each column was then silanized twice *in situ* with 10-μl aliquots of hexamethyldisilazane before use.

Compounds which were investigated as possible internal markers were: furfurylmethylamphetamine hydrochloride, benzphetamine hydrochloride, N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride, methadone hydrochloride, chlorpheniramine hydrochloride, and brompheniramine hydrochloride.

General procedure for assaying pethidine and norpethidine

A sample of 5.0 ml plasma or urine or 2.5 ml blood lysed with 2.5 ml of ice-cold water, in a 10-ml glass centrifuge tube, was made alkaline (pH 10–12) with 0.5 ml of 20% sodium hydroxide and 1 ml of the marker solution (2 μg/ml for plasma and whole blood and 20 μg/ml for urine) was added. The alkaline solution was extracted with diethyl ether (4 × 3.0 ml) using a mechanical tilt-shaker (for 5 min) centrifuged (at 3000 *g* for 5–10 minutes) to break emulsion, and the ether extracts were transferred carefully into a 15-ml evaporating tube with a tapered base[23]. The bulked ether extracts were concentrated by evaporation at 45° on a water-bath to about 20 μl. Ethanol (10 μl) was added to the concentrate. An aliquot (2 μl) of the final concentrate was injected on to the GC column with a 10-μl Hamilton syringe. The concentration of pethidine and norpethidine present in a sample was determined from the ratio of the integrated peak areas of pethidine and norpethidine to the marker.

Procedure for assaying pethidine N-oxide in urine

A 5-ml urine sample was assayed for total pethidine in the same manner as described in the *General procedure*. Another 5-ml urine sample from the same specimen was assayed for pethidine N-oxide by the method described for methadone N-oxide (Beckett *et al.*[24]). The difference between the first and second assay values would give the amount of pethidine N-oxide present in the sample.

Calibration graphs

Standard solutions of pethidine and norpethidine were prepared by dissolving

both compounds together in distilled water to make up two standard solutions of 1 μg/ml and 10 μg/ml. These were diluted to give two series of solutions covering the concentration ranges of 20 ng–1 μg/ml and 1 μg–10 μg/ml, which were then analysed and the peak area ratios plotted against the corresponding concentrations. The first range was used for assaying pethidine and norpethidine in plasma and blood and the second range for the drugs in urine.

Recovery, selectivity, reproducibility and storage

Recovery. Solutions of pethidine and norpethidine (equivalent to 600 ng/ml extract) in plasma, urine, and lysed whole blood (50% blood + 50% distilled water) were prepared by dissolving the drug in these fluids with the aid of gentle shaking. Aliquots (8 × 5.0 ml) were assayed on System 6 described earlier (8% Carbowax 20M + 2% KOH). An ethereal solution of pethidine and norpethidine (600 ng/ml of each) and marker (400 ng/ml) was prepared by dissolving in a little absolute alcohol (1 ml) and made up to volume with diethyl ether, and the solution was kept at 4° before use. This ethereal solution (5 ml) was concentrated as described in the *General procedure.* The results were related to the 100% value obtained from a standard ethereal solution.

Selectivity. Samples of urine, plasma and lysed whole blood from patients on a variety of drugs were analysed to find out if they produced peaks after chromatography which interfered with those of pethidine, norpethidine, and benzphetamine.

Reproducibility. Seven replicate samples of pethidine and norpethidine in urine (600 ng/ml) were assayed by the *General procedure* and the peak area ratios of the drugs to the marker were calculated.

Storage. Samples of plasma, lysed whole blood and urine were analysed immediately and after storage at —20° for seven days.

RESULTS AND DISCUSSION

Choice of GLC system

The performance of the six GLC systems investigated are summarised in Table I. System 5 (10% Apiezon L + 10% KOH) and System 6 (8% Carbowax 20M + 2% KOH) were satisfactory for analysis of pethidine and norpethidine (Tables II and III and Figs. 1 and 2). System 5 required prior acylation of norpethidine by the addition of trifluoroacetylimidazole (5 μl) to the ethanolic concentrate before injection on to the analysing column (Fig. 1). System 6 did not require derivative formation and was, therefore, chosen for routine analysis. Compounds such as methadone, pentazocine, chlorpheniramine and brompheniramine were considered unsuitable as internal markers because their retention times in all systems investigated were between 15 to 20 min longer than that of pethidine. N-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine was chosen as internal marker for analysis in System 5 and benzphetamine for System 6. Their respective retention times, symmetry factors, and resolution are shown in Tables II and III.

Recovery, selectivity, reproducibility and storage

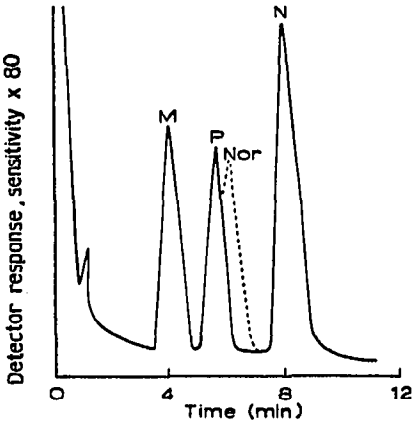
8% Carbowax 20M + 2% KOH was chosen for routine analysis.

TABLE I

A SUMMARY OF GLC SYSTEMS INVESTIGATED

Temperatures: both injection temperature and detector temperature were 50° above the oven temperature. Gas flow-rates: nitrogen carrier, 40 ml/min; hydrogen, 33 ml/min; and air, 400 ml/min.

No.	*System*	*Oven temperature (°C)*	*Retention time (min)*		*Remarks*
			Pethidine	*Norpethidine*	
1	3% OV-17, 4 ft.	180	5.0	5.8	Both peaks are tailing and too close.
2	2% Carbowax + 5% KOH, 3 ft.	165	6.8	12.8	Norpethidine peak too broad and tailing.
3	2.5% SE-30, 4 ft.	150	3.0	4.0	Too close and tailing.
4	3% Poly I 110, 3 ft.	165	7.0	—	Good pethidine peak, norpethidine not resolved.
5	10% Apiezon L + 10% KOH, 4 ft.	210	5.2	6.0	Good peaks, too close, required acylation.
6	8% Carbowax 20M + 2% KOH, 4 ft.	195	6.0	10.2	Good peaks, suitable for routine analysis.



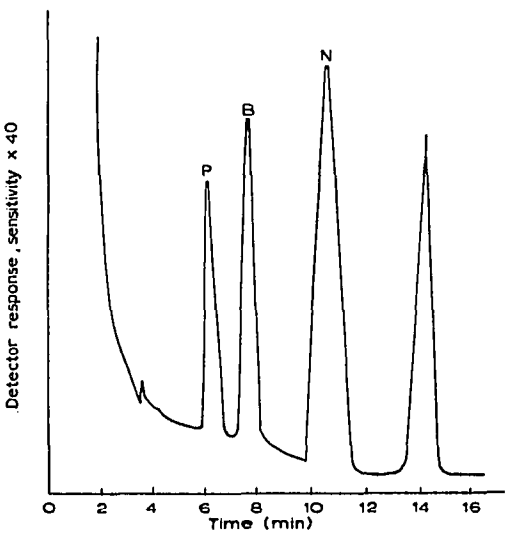


Fig. 1. Chromatograms of pethidine (P), norpethidine (Nor), N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (M), and N-trifluoroacetyl-norpethidine (N). System 5: 10% Apiezon L + 10% KOH.

Fig. 2. Chromatograms of pethidine (P), benzphetamine (B), and norpethidine (N). System 6: 8% Carbowax 20M + 2% KOH.

TABLE II

PERFORMANCE OF SYSTEM "10% APIEZON L + 10% KOH", 4 ft.

Temperatures: injection 260°, oven 210°, and detector 260°. Gas flow-rates: nitrogen carrier, 40 ml/min; hydrogen, 33 ml/min; and air, 400 ml/min.

Drug	*Retention time (min)*	*Symmetry factor (limit 0.95 to 1.05)*	*Resolution between marker (>1.0)*
Pethidine	5.2	1.02	1.2
Norpethidine	6.0	1.01	
N-Trifluoroacetyl-norpethidine	7.2	0.98	3.0
N-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (marker)	3.8	1.00	

TABLE III

PERFORMANCE OF SYSTEM "8% CARBOWAX 20M + 2% KOH", 4 ft.

Temperatures: injection 250°, oven 195°, and detector 250°. Gas flow-rates: nitrogen carrier, 40 ml/min; hydrogen, 33 ml/min; and air, 400 ml/min.

Drug	*Retention time (min)*	*Symmetry factor (limit 0.95 to 1.05)*	*Resolution between marker (>1.0)*
Pethidine	6.0	1.00	1.2
Norpethidine	10.2	0.98	3.0
Benzphetamine	7.3	1.05	

Recovery. The relative recoveries of pethidine from urine, plasma and lysed whole blood were 96.8 ± 2.5%, 95.5 ± 3.8% and 93.9 ± 4.9%, respectively, and the corresponding values for norpethidine were 95.5 ± 3.0%, 96.4 ± 3.2%, and 91.0 ± 4.5%.

Selectivity. During the course of these studies it was established that substances in samples obtained from patients on a variety of drugs, particularly morphine, diamorphine, pentazocine and methadone, did not interfere in the analysis of pethidine and norpethidine.

Reproducibility. The repeated assays of the same urine samples containing both pethidine and norpethidine indicated that the reproducibility of the peak area ratio of pethidine to marker was 100 ± 1.21% and that of norpethidine was 100 ± 3.87%.

When peak height ratios were compared, the corresponding values were 100 ± 3.96% and 100 ± 6.1%. Calibration graphs (Fig. 3) for pethidine and norpethidine were linear over the ranges 20 ng–1 μg/ml for blood and 1 μg–10 μg/ml for urine. The graphs were found to be reproducible when repeated six times during the studies.

Storage. Samples of urine, plasma and lysed whole blood whether fresh or stored at −20° for seven days did not give peaks which would interfere with the measurement of peaks corresponding to pethidine, norpethidine, and the marker in the chromatogram (Figs. 1 and 2). There was no appreciable loss of the drugs from the samples after storing at −20° for seven days.

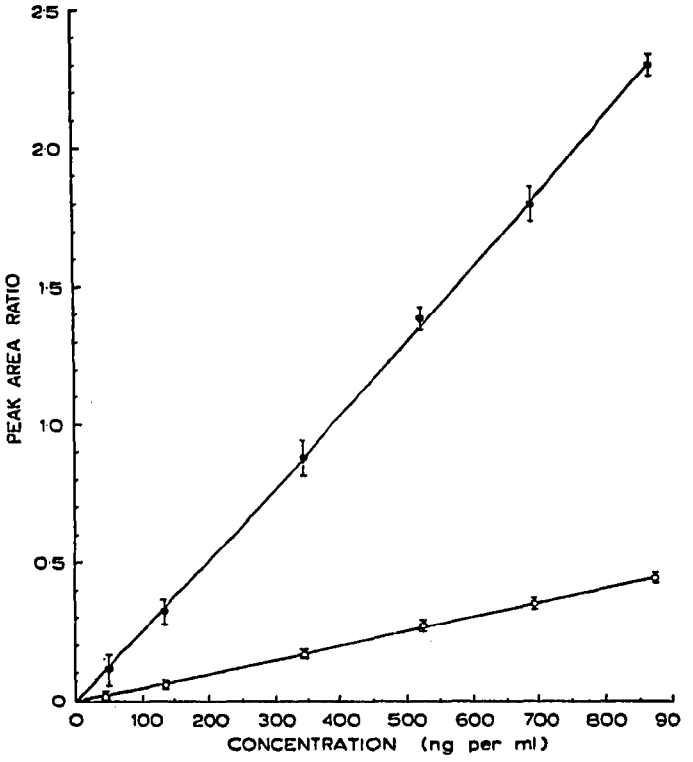


Fig. 3. A typical calibration curve for pethidine (●—●) and norpethidine (○—○).

Application

The procedure has been used to measure pethidine and norpethidine concentrations in human plasma, whole blood, and urine, and pethidine N-oxide in urine after a single intramuscular dose of pethidine (1.5 mg/kg). The blood profile of a male

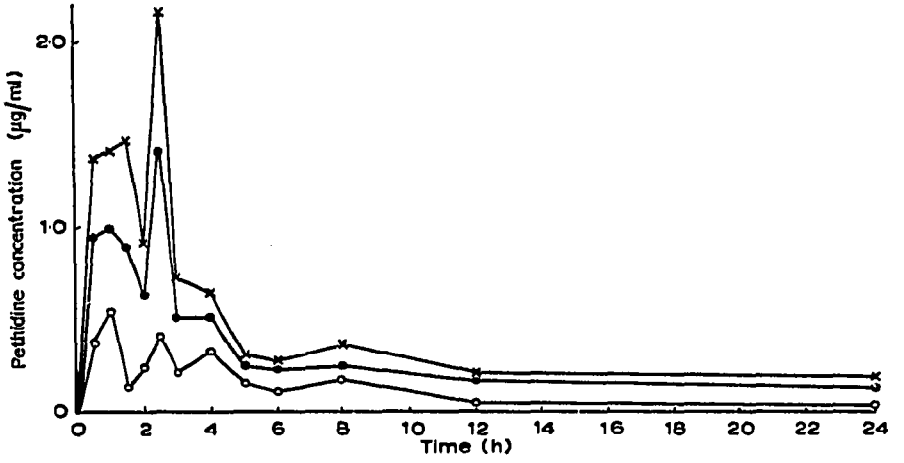


Fig. 4. Profiles of plasma (×—×), whole blood (●—●), and erythrocyte (○—○) concentrations with time after an intramuscular dose of pethidine (100 mg).

hospitalised subject (age 59 years, weight 70 kg) obtained in a preliminary study is shown in Fig. 4. The peak concentration at 2 h 30 min showed that a blood pethidine concentration of 1.41 μg/ml coincided with a plasma concentration of 2.16 μg/ml and erythrocyte concentration of 0.41 μg/ml. The corresponding values for blood norpethidine, plasma norpethidine and erythrocyte norpethidine were 0.656 μg/ml, 1.05 μg/ml, and 0.13 μg/ml, respectively. When the urine was acid (pH 5.0), 34.8% of the total dose was recovered after 48 h as pethidine, while only 0.55 % of the total dose was recovered as norpethidine. A separate study showed that pethidine N-oxide was only present in urine between the third and twelfth hour after intramuscular administration of pethidine and only about 0.03 % of the total dose was recovered in this form.

A more detailed report of this work is in preparation.

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