Journal of Chromatography, 565 (1991) 247–254
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5758

Determination of pethidine and its major metabolites in human urine by gas chromatography

K. CHAN*

Department of Pharmacology, The Chinese University of Hong Kong, Shatin, N.T. (Hong Kong)

and

O. W. LAU and Y. C. WONG

Department of Chemistry, The Chinese University of Hong Kong, Shatin, N.T. (Hong Kong)

(First received October 1st, 1990; revised manuscript received November 26th, 1990)

ABSTRACT

Procedures based on gas chromatography were established to determine pethidine and its major metabolites in human urine. The chromatographic system consisted of a glass column packed with 3% (w/w) SP2250 on Chromosorb W (80–100 mesh) linked to a nitrogen–phosphorus detector. Diethyl ether was used as the extraction solvent. Pethidinic and norpethidinic acids, and their conjugated metabolites (after β-glucuronidase treatment) were determined after conversion into pethidine and norpethidine by acid-catalysed esterification. The retention times of pethidine, norpethidine and chlorpheniramine (internal standard) were 3.3, 4.5 and 7.5 min, respectively. The amount of unchanged drugs and metabolites excreted varied considerably among the subjects. The mean 24-h urinary recoveries in eight patients of pethidine, norpethidine, pethidinic acid, norpethidinic acid, and the glucuronides of pethidinic and norpethidinic acids were 6.62 ± 5.05, 4.33 ± 1.19, 18.9 ± 6.29, 9.10 ± 4.26, 15.1 ± 3.02 and 7.57 ± 2.28%, respectively. This indicates that the major metyabolic pathways of pethidine in the eight patients were hydrolysis followed by conjugation. Over 60% of the dose was accounted for in 24 h after intramuscular administration of 1 mg/kg pethidine.

INTRODUCTION

Pethidine (P) is extensively metabolized in the liver, mainly by demethylation, hydrolysis and conjugation. Major metabolites include norphetidine (N), pethidinic and norpethidinic acids (PA and NA) and conjugates of pethidinic and norpethidinic acids (PC and NC). There are well established methods for the analysis of P and N, such as colourimetry [1,2] ion-selective electrodes [3], gas chromatography (GC) [4–6] and high-performance liquid chromatography (HPLC) [7] and the analysis of PA and NA [8,9], but information is scare on the measurement of P together with all its major metabolites in biological materials. In attempt to achieve this, we initially attempted the simultaneous HPLC assay of *p*-aminobenzoic acid and its conjugates [10] in urine samples. We found that this method was not applicable because, using UV detection at low wavelength (*ca.*

0378-4347/91/$03.50

205 nm), the analytical peaks of PA, NA, PC and NC were subject to the interferences from endogenous urinary substances. These peaks were not resolved even when an ion-pair reagent (heptanesulphonic acid) was added.

We now report a reliable GC method to determine pethidine and, its major metabolites in human urine, from patients who were given a single intramuscular dose of pethidine. The method was based on a modification of our recently developed GC assay for monitoring the plasma levels of P and N [11].

EXPERIMENTAL

Materials and apparatus

Pethidine hydrochloride was obtained from May and Baker (Dagenham, U.K.), norpethidine hydrochloride from Sterling Winthrop Research Institute (New York, NY, U.S.A.), chlorpheniramine hydrochloride from Astra Pharmaceutical Production (Södertälje, Sweden) and β-glucuronidase (from *Helix pomatia*) from Sigma (St. Louis, MO, U.S.A.). Pethidinic and norpethidinic acids were synthesized by alkaline hydrolysis of their respective esters, and their purities were checked by thin-layer chromatography [12]. Absolute ethanol, acetic acid, diethyl ether, methanol and sodium acetate (all of analytical grade) were purchased from Merck (Darmstadt, Germany). The following glassware was used: 15-ml centrifuge tubes with well fitting screw caps containing PTFE linings; 15-ml Quick-fit glass tubes with tapered base of 50 μl. All glassware was cleaned and silanized with 3% hexamethyldisilizane (HMDS) in chloroform before use. Stock solutions containing 1 mg/ml P, N, PA and NA were prepared in methanol and stored at −20°C before use. The freeze drier (Savant Instruments, New York, NY, U.S.A.) consisted of a speed vacuum concentrator (SYC-100H), a refrigerated condensation trap (RT-100A) and a rotary vacuum pump (VP 100).

Gas chromatography

The gas chromatograph was a Varian Model 6000 equipped with a nitrogen–phosphorus detector. A coiled glass column (2.4 m × 2 mm I.D., 6 mm O.D., Supelco, Bellefonte, PA, U.S.A.) was packed with 3% (w/w) SP2250 on Chromosorb W, 80–100 mesh. The column was silanized with two 50-μl volumes of 3% HMDS and conditioned at 300°C overnight before use. The temperature of the column was at 230°C and that of the detector and injector was 260°C. The signal was recorded and displayed by at a flat-bed recorder (Linseis, Model L6512). The gas flow-rates were: nitrogen (carrier gas), 30 ml/min; air, 175 ml/min; hydrogen, 4.5 ml/min.

Analytical procedures for P and N (assay I)

A flow diagram of the assay procedures is shown in Fig. 1. To a 0.25-ml urine sample, 0.75 ml of acetate buffer (pH 5.4) was added in a 15-ml centrifuge tube. The tube was incubated at 37°C in a water-bath for 18 h, and then 20 μl of 5 *M*

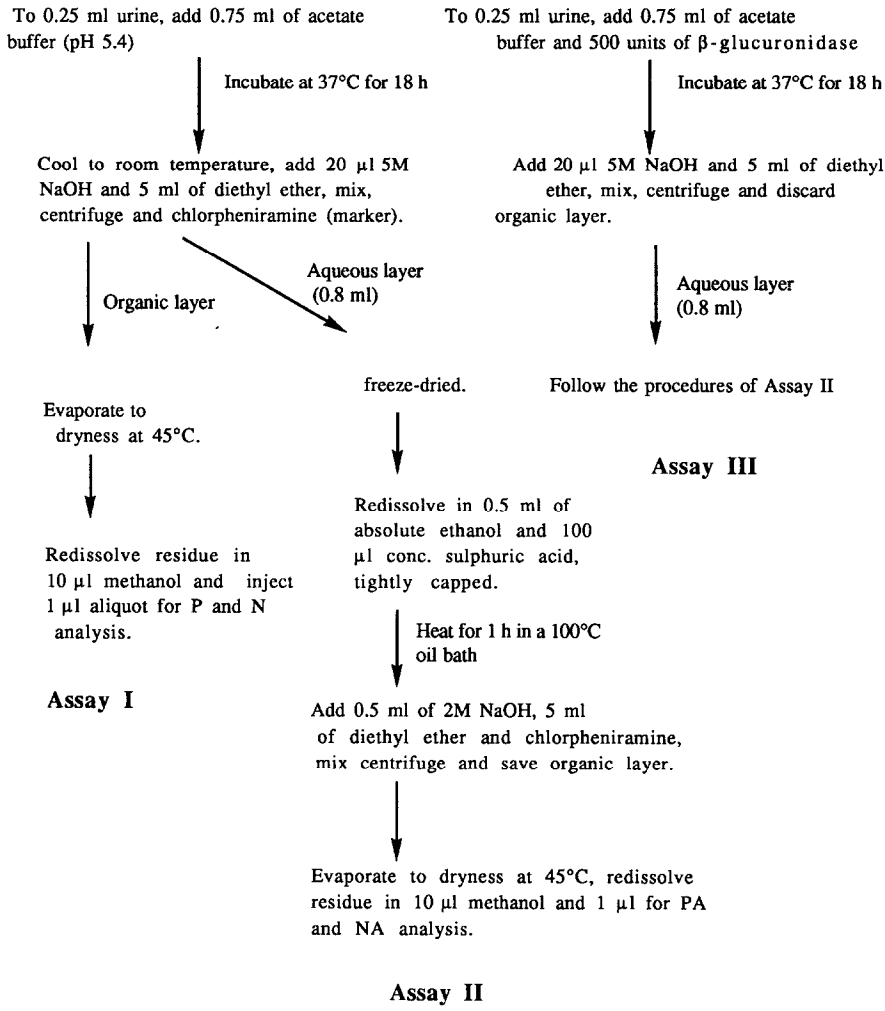

Fig. 1. Flow diagram of the analytical procedures for the assay of P and its major metabolites in urine samples.

sodium hydroxide solution, 5 µl of the internal standard chlorpheniramine hydrochloride (0.4 mg/ml), and 5 ml of freshly distilled diethyl ether were added to the mixture. The tube was mixed for 5 min on an automatic shaker and centrifuged to break the emulsion. The ether layer was transferred to a 15-ml evaporating tube and evaporated under a gentle stream of nitrogen at 45°C. The residue was redissolved in 10 µl of methanol, and 1-µl aliquots were analysed.

Analytical procedures for PA and NA (assay II)

The aqueous phases (0.8 ml) from the above procedure were evaporated to dryness by freeze drying using the Savant freeze-dryer. The residue was dissolved

in 0.5 ml of absolute ethanol and transferred to a 15-ml centrifuge tube containing 100 μl of concentrated sulphuric acid. The tube was tightly capped and heated at 100°C in an oil-bath for 1 h. After cooling to room temperature, 0.5 ml of 2 *M* sodium hydroxide was added, followed by 5 μl of chlorpheniramine hydrochoride (0.4 mg/ml) and 5 ml of diethyl ether. The subsequent procedure was identical with that of assay I.

Analytical procedures for total PA and NA (assay III)

To a 0.25-ml urine sample, 075 ml of acetate buffer (pH 5.4) and 500 U of β-glucuronidase were added. The tube was incubated at 37°C for 18 h, and the mixtures was centrifuged after the addition of 20 μl of 5 *M* sodium hydroxide solution and 5 ml of diethyl ether. The aqueous layer (0.8 ml) was saved, and the subsequent procedure was identical with that of assay II. The amounts of PC and NC were measured from the difference between assays II and III.

Quantitation

Calibration graphs were constructed by spiking P, N, PA and NA into drug-free urine samples. Diluted stock standard solutions were prepared to cover the concentration range 0.4–6.4 μg/ml for P and N and 0.8–12.8 μg/ml for PA and NA. Between-day standards of concentration 3.2 μg/ml were determined to obtain the between-batch variation of the assays. The calibration graph measurements were repeated six times. The recoveries of P and N after esterification of PA and NA at 3.2 μg/ml in the urine samples were determined.

RESULTS AND DISCUSSION

Enzyme incubation period and choice of extraction solvent

From the information obtained from previous papers, we noticed that the reaction time for enzymic hydrolysis of conjugated metabolites at 37°C was usually long, *e.g.* 10 h for conjugated N-hydroxynorpethidine [13], 18 h for conjugated 2-hydroxyimipramine [14], and 24 h for morphine-6-glucuronide [15]. Therefore we decided to use a relatively long incubation time (18 h) so that the enzymic process would be complete.

During the development stages of the previous work [11], various commonly used solvents were investigated for the extraction of P and N from plasma. The percentage recovery was determined for each drug by comparing the peak heights of extracted plasma with the peak heights of methanolic standards at 0.1 μg/ml (external standard quantitation). It was shown that diethyl ether gave higher recoveries (>90%) but dirtier chromatograms. The interfering peaks in the chromatograms were due to endogenous materials in the plasma [11], and these substances were not extracted in urine samples, therefore diethyl ether was chosen as the extraction solvent in this assay.

Recovery of the esterification of PA and NA

The mean ± S.D. conversions of PA and NA into the corresponding esters at 3.2 μg/ml were 65.4 ± 3.7 and 63.7 ± 2.6%, respectively. These comparatively low recoveries were mainly due to incomplete reaction (equilibrium occurred during acid-catalysed esterfication), degradation or possible adhesion of the molecules to the screw-cap linings during the course of the reaction. Although the recovery might be increased if the reaction were performed under reflux, the major advantage of using screw-capped tubes is that a large number of samples can be determined at a time.

The percentage conversion of PC and NC into PA and NA, respectively, during acid-catalysed hydrolysis was also accessed. This was achieved by saving the aqueous layers (of those samples not treated with β-glucuronidase after hydrolysis and extraction with ether, *i.e.* assay II) and neutralization with appropriate volume of 5 *M* sodium hydroxide. The aqueous mixtures were adjusted with buffer to pH 5.4 and incubated, and then subjected to assay III. These results (amounts of PC and NC) were compared with the values obtained from the difference between assays II and III. It was found that a proportion of PC and NC were hydrolysed to their respective acids, but the amount did not usually exceed 7% ($n = 8$). In spite of this low percentage conversion, the present method still provides a good estimate of the amount of these compounds in patients' urine samples.

Performance of the GC procedure

The peaks of P, N and chlorpheniramine (internal standard) were well resolved with good symmetry (Fig. 2), and the retention times were 3.3, 4.5 and 7.5

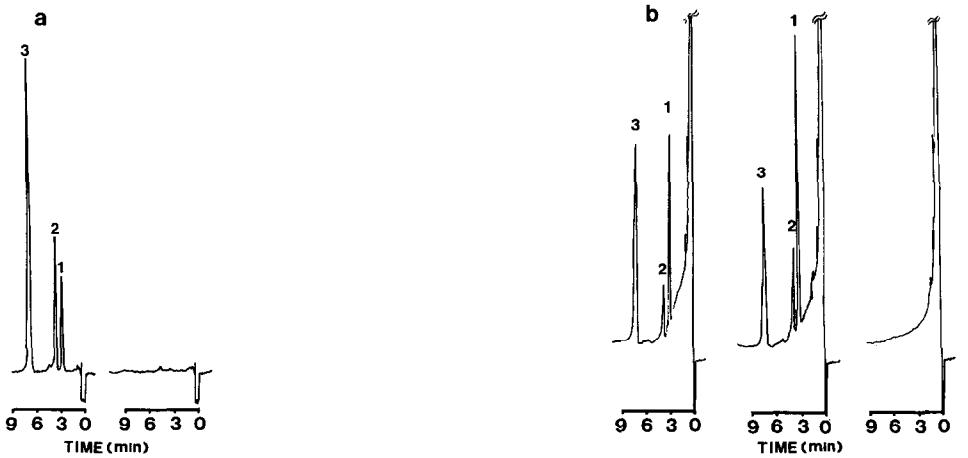

Fig. 2 (a) Chromatograms showing (left) peaks of P (1) at 0.71 μg/ml, N (2) at 2.8 μg/ml and the internal standard, chlorpheniramine (3) at 8.0 μg/ml in a patient's urine sample and (right) blank urine. (b) Chromatograms showing (left) a patient's urine sample after acid-catalysed esterification, (middle) the same patient's sample after both acid-catalysed esterification and β-glucuronidase hydrolysis (note the increase in height of peaks 1 and 2) and (right) blank urine. Peak numbers as in (a).

TABLE I

CALIBRATION AND PRECISION OF P AND N ASSAY (n = 6)

Concentration (μg/ml)	Peak-height ratio (mean ± S.D.) (C.V., %)	
	Pethidine	Norpethidine
0.4	0.165 ± 0.00849 (5.15)	0.0673 ± 0.00408 (6.06)
0.8	0.347 ± 0.0178 (5.13)	0.142 ± 0.00738 (5.20)
1.6	0.624 ± 0.0274 (4.39)	0.267 ± 0.0112 (4.19)
3.2	1.28 ± 0.0448 (3.50)	0.541 ± 0.0211 (3.90)
4.8	1.96 ± 0.0820 (4.18)	0.813 ± 0.0273 (3.36)
6.4	2.62 ± 0.107 (4.08)	1.12 ± 0.0295 (2.61)
Batch standard at 3.2 mg/ml (n = 10)	1.29 ± 0.0519 (4.02)	0.537 ± 0.0226 (4.21)
Calibration graph	$y = 0.409x - 0.00513$ (r = 0.9998)	$y = 0.175x - 0.00786$ (r = 0.9994)

min, respectively. After enzyme treatment and esterification in acidic medium, the peak heights of P and N increased (Fig. 2b), indicating that PC and NC were converted into the corresponding acids. Data showing the performance of the GC procedure are summarized in Tables I and II. It was found that the calibration graphs were linear over the range 0.4–6.4 μg/ml for P and N (r = 0.9998 and 0.9994, respectively) and 0.8–12.8 μg/ml for PA and NA (r = 0.9994 and 0.9988, respectively). The between-day coefficients of variation at 3.2 μg/ml were 4.02,

TABLE II

CALIBRATION AND PRECISION OF PA AND NA ASSAY (n = 6)

Concentration (μg/ml)	Peak-height ratio (mean ± S.D.) (C.V., %)	
	Pethidinic acid	Norpethidinic acid
0.8	0.186 ± 0.00926 (4.98)	0.0795 ± 0.00548 (6.89)
1.6	0.317 ± 0.0873 (2.78)	0.164 ± 0.00100 (6.10)
3.2	0.802 ± 0.0413 (5.15)	0.339 ± 0.0203 (5.99)
4.8	1.25 ± 0.0745 (5.96)	0.462 ± 0.0196 (4.24)
6.4	1.78 ± 0.0667 (5.21)	0.627 ± 0.0245 (3.91)
12.8	3.52 ± 0.141 (4.01)	1.35 ± 0.0326 (2.41)
Batch standard at 3.2 mg/ml (n = 10)	0.807 ± 0.0412 (5.11)	0.327 ± 0.0207 (6.32)
Calibration graph	$y = 0.283x - 0.0860$ (r = 0.9994)	$y = 0.105x - 0.0151$ (r = 0.9988)

4.21, 5.11 and 6.32%, respectively. The corresponding lowest detection limits of P and N were 0.01 μg/ml and of PA and NA were 0.02 μg/ml. These values were sufficiently sensitive in the quantitation of their urinary concentrations within 24 h after administration.

Application of the assay

Eight Chinese patients who underwent either hernia repair or excision exostosis were given a single dose (1 mg/kg of the total body weight) of pethidine intramuscularly. Bulked urine samples were collected in plastic containers until 24 h after an intramuscular dose, the pH values were measured, and the samples were immediately stored at −20°C before analysis. Samples were assayed in duplicate. The amounts of P and its metabolites were found to vary considerably among the eight subjects (Table III). The excretion of P, N, PA, NA, PC and NA was 0.97–15, 2.0–6.0, 12.9–30.1, 4.18–17.2, 11.4–20.4 and 4.7–10.6%, respectively, over 24 h after dosing. The percentages of unchanged drug and the N-demethylated metabolite excreted were lower than those of the others metabolites. This showed that hydrolysis and subsequent conjugation of the acid were the dominant routes of P metabolism in the patients. The mean dose recovery (61.6 ± 5.14%) was lower than 100% because proportion of these compounds was excreted after 24 h, and because other metabolites, such as pethidine N-oxide, 4-hydroxypethidine and N-hydroxynorpethidine, were not included in this study. None of the patients suffered from urinary acidosis. The pH values (5.15–6.46) of the urine samples were found to be within the normal range. Since pethidine is weakly basic (pK_a = 8.63), it is likely that patients with lower pH urine excreted P more readily, and these observations were correlated with the previous findings

TABLE III

RECOVERY OF P AND ITS METABOLITES OVER 24 h IN PATIENTS' URINE

Patient	pH	Recovery (%)						
		P	N	PA	NA	PC	NC	Total
1	5.98	5.71	3.82	18.4	9.32	14.7	5.66	57.6
2	5.15	15.0	4.46	13.6	8.02	17.1	7.65	65.8
3	6.26	0.968	3.79	14.6	17.2	20.4	9.26	66.2
4	5.39	13.3	2.04	12.9	8.09	11.4	10.6	58.3
5	6.46	5.68	4.39	19.6	13.2	17.5	8.11	68.5
6	5.77	3.72	5.37	26.4	4.18	12.8	4.65	57.1
7	5.63	6.64	6.00	15.5	7.69	14.2	4.88	54.9
8	6.07	1.94	4.77	30.1	5.06	12.6	9.72	64.2
Mean		6.62	4.33	18.9	9.10	15.1	7.57	61.6
S.D.		5.05	1.19	6.29	4.26	3.02	2.28	5.14

[16]. It was also speculated that Oriental subjects were better demethylators whereas Caucasians produced more hydrolysed metabolites [17,18]. This investigation could be extended if more urine samples from different races were studied by using the described method.

REFERENCES

1 L. Przyborowski, P. Zawisza and A. Wojtczak, *Farm. Pol.*, 44 (1988) 466.
2 H. Farsam and M. R. Nadjari-Moghaddam, *J. Pharm. Biomed. Anal.*, 2 (1984) 543.
3 M. Anastasid, K. C. Theodore and P. D. Eleftherios, *Analyst*, 110 (1985) 1091.
4 P. G. Quinn, B. R. Kuhnert, C. J. Kaine and C. D. Syracuse, *Biomed. Environ. Mass. Spectrom.*, 13 (1986) 133.
5 L. M. Babiak, W. F. Cherry, S. Fayz and K. S. Pang, *Drug Metab. Dispos.*, 12 (1984) 698.
6 P. Hartvig and C. Fagerlund, *J. Chromatogr.*, 274 (1983) 355.
7 R. C. Meatherall, D. R. P. Guay and J. L. Chalmers, *J. Chromatogr.*, 338 (1985) 141.
8 S. Y. Yeh, H. A. Krebs and A. Changchit, *J. Pharm. Sci.*, 70 (1981) 867.
9 I. W. Wainer and J. E. Stambaugh, *J. Pharm. Sci.*, 67 (1978) 116.
10 K. Chan, J. O. Miner and D. J. Birkett, *J. Chromatogr.*, 426 (1989) 103.
11 Y. C. Wong, O. W. Lau, K. Chan, C. Aun, D. M. Lowe and I. T. Houghton, *J. Biopharm. Sci.*, 1 (1990) 267.
12 K. Chan, *J. Pharm. Pharmacol.*, 31 (1979) 672.
13 W. G. Stillwell, C. S. Myran and J. T. Stewart, *Res. Commun. Pathol. Pharmacol.*, 14 (1976) 605.
14 T. A. Sutfin, C. L. DeVane and W. J. Jusko, *Psychopharmacology*, 82 (1984) 310.
15 J. Svensson, A. Rane, J. Sawe and F. Sjoqvist, *J. Chromatogr.*, 230 (1982) 427.
16 K. Chan, J. Tse, F. Jennings and M. L'E. Orme, *Methods Find. Exp. Clin. Pharmacol.*, 9 (1987) 49.
17 K. Chan, J. Tse, F. Jennings and M. L'E. Orme, *Methods Find. Exp. Clin. Pharmacol.*, 9 (1987) 243.
18 K. Chan, J. Tse, F. Jennings and M. L'E. Orme, *Methods Find. Exp. Clin. Pharmacol.*, 12 (1990) 61.