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QUANTITATIVE THIN-LAYER CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF PROCAINAMIDE AND ITS MAJOR METABOLITE IN PLASMA

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SUMMARY

A sensitive and accurate spectrodensitometric method was developed for the determination of procainamide and its major metabolite, N-acetylprocainamide, in plasma. The method involves extraction into organic solvent at alkaline pH, separation by thin-layer chromatography and direct measurement of the absorbance of the compounds on the plate at 275 nm. Quantities as low as 10 ng could be measured and a linear relationship was obtained between peak areas and amounts of the compounds in the spots from 10 to 200 ng. The recovery of both drugs from plasma was from 95.4 to 104.8%. The method is sensitive and specific, and procainamide was well separated from N-acetylprocainamide at all investigated concentrations. The method is recommended for clinical assays and pharmacokinetics studies.

INTRODUCTION

Procainamide (PA) was studied in the dog and Rhesus monkey and 50—67% was found to be excreted unchanged in dogs and 22—49% in monkeys after intravenous administration. The main metabolite was identified as N-acetylprocainamide (NAPA) in the monkeys but not in the dogs [1]. When administered orally to patients and normal subjects, 19.2—48.8% of the drug was excreted unchanged in patients and 57.1—79.5% in normal subjects and in both cases the major metabolite was identified as NAPA [2]. Further examination of the metabolic pattern of PA showed a substantial amount of NAPA in the plasma samples of patients receiving PA and also in some experimental animals [3]. When NAPA was administered intraperitoneally to mice, it prevented coarse ventricular fibrillation, caused by deep chloroform anesthesia, and resultant hypoxia. NAPA also reduced aconitine-induced arrhythmia to atrial flutter or atrial tachycardia in dogs. NAPA was not deacetylated during these tests in animals and was assumed to be a better therapeutic agent than PA because it was less toxic. The metabolism of PA was studied in cardiac patients receiving

PA and the acetylated drug accounted for 16—63% of the administered drug and it was considered to be the chief metabolite in man (plasma level between 1 and 15 μg/ml, at times higher than that of PA) [4]. The metabolite showed weaker effect than PA on the maximal electrical driving velocity of isolated atrial strips from guinea pigs. The pharmacokinetics of the N-acetylated metabolite of PA was studied in man and the assumption, raised by previous authors, about NAPA antiarrhythmic efficacy in man [3] was strengthened, although the question of the NAPA effect in cardiac patients was left unsolved [5]. The acetylation of PA was studied in healthy volunteers and was found to be subject to the same genetic polymorphism as that of isoniazid and some sulfonamides (slow/fast acetylators) [6]. Since these results showed that NAPA may be similar in therapeutic effect to the parent drug and since the plasma concentration of NAPA may be as high as or even higher than that of the original drug in patients, it becomes important to have a reliable method to measure both compounds from one sample of biological fluid.

The first techniques used for PA determination in biological material have been spectrophotometric and fluorimetric [7, 8]. Both methods seem sensitive enough for routine measurement of PA in biological fluids but they do not provide for determination of NAPA. A gas—liquid chromatographic method was developed for PA determination in plasma [9] which gave good results at concentrations of 2 to 20 μg/ml. Another specific gas chromatographic method was divised for the determination of PA and NAPA in plasma and urine using 4-amino-N-(2-piperidinoethyl)benzamide as internal standard [10]. An attempt was made to compare the fluorimetric method [8] with the gas chromatography determination of PA [9, 10] and it was concluded that, if NAPA also has significant antiarrhythmic activity in man, the different NAPA/PA plasma ratios may require concomitant determination of both compounds to ensure adequate therapy and dose regimen adjustments [11]. The gas chromatographic methods enabled one to measure both PA and NAPA in one sample of the biological fluid, but they lack sensitivity having a lower limit of reliable reading at 2 μg/ml plasma. Measurements of lower levels are frequently needed. The spectrophotometric and fluorimetric methods require separation of PA and NAPA prior to measurement, making this procedure a tedious one.

This paper describes a thin-layer chromatographic method based on the separation of PA and its major N-acetylated metabolite on silica gel plates followed by direct determination of their absorbances. The method of quantitation on a chromatoplate has the advantage of greater precision and sensitivity because the compounds are concentrated over a small surface area. It may be used to measure very small amounts of PA and NAPA (10 ng) and therefore the determination can be made with small volumes of plasma. The procedure is fast, requiring 1.5—2 h from receipt of the samples to the output of final results for a group of samples.

PA is completely resolved from NAPA and interfering biological contaminants and both compounds appear as distinct spots on the chromatoplates. The spots are scanned at a fixed wavelength and the absorbance is recorded, while peak areas are integrated automatically.

The described method was applied to plasma samples from patients after oral doses of 250 mg of PA and typical results are presented.

EXPERIMENTAL

Materials and methods

PA (250-mg Pronestyl capsules) was obtained from Squibb & Sons, Princeton, N.J., U.S.A. and NAPA was synthesized from PA by the method of Dreyfuss et al. [1]. All other chemicals were of analytical grade. Solvents used were tested by thin-layer chromatography to assure that no traces of UV-absorbing substances were present. Thin-layer plates were 20 × 20 cm silica gel 60 on glass (E. Merck, Darmstadt, G.F.R.), which were activated at 100° for 20 min and cooled in a desiccator prior to use.

Stock solution of PA and NAPA contained 1 mg/ml in methanol. Standard solutions were prepared by dilution of the stock solution to yield concentrations of 100 μg/ml of PA and NAPA.

Application of standard solutions and extracts was achieved with a mechanical spotter which enables application of 10 spots simultaneously and automatically, leaving blank channels between spots, and yielding small spots of uniform diameter. No additional drying time was needed for the spots since gentle heat was applied to the plate during spotting.

Excellent movement and separation of PA and NAPA was achieved with a developing solvent consisting of benzene—ammonium hydroxide 28%—dioxane (10:15:80).

The plates were air-dried after development and scanned at moderate speed on a chromatogram analyzer at 275 nm recording the absorption curve and integrating the peaks simultaneously with an automatic integrator.

Apparatus

For automatic spotting of plates the Multi-Spotter AIS (Analytical Instrument Specialties) supplied with temperature and speed control was used. Ultraviolet scanning was done with a UV—VIS-2 Chromatograph Scanner (Farrand) coupled with a strip chart recorder Model 100 (Farrand), and a CDS 101 Chromatography Data System (Varian).

Extraction and development procedures

To 0.5—1.0 ml of plasma in a 20-ml glass centrifuge tube was added 0.5 ml of 0.5 *N* sodium hydroxide and the mixture was mechanically shaken for 10 min with 3—4 ml of dichloromethane as extracting solvent. The tube was then centrifuged at 1300 *g* and the organic layer was transferred to a second tube. The aqueous layer was re-extracted in the same manner with 3 ml of dichloromethane and the combined organic layers were evaporated to dryness at 40° under a stream of nitrogen. The solid residue was dissolved in 100 μl of ethyl acetate. Three spots of 1 μl each of the ethyl acetate solution were applied to a thin-layer plate along with a series of extracts from plasma spiked with measured amounts of PA and NAPA, covering the concentration range of 0.10 to 20 μg/ml and standards of PA and NAPA in methanol. The plate was developed in the solvent described above in a saturated tank allowing the solvent to migrate about 15 cm (time: about 25 min).

A calibration curve of integrated peak areas (square millimeters) against concentration (microgram per milliliter of plasma) of PA and NAPA obtained from

the respective standards was constructed and it was used for determination of the drugs in plasma specimens of patients by interpolation.

Recovery

The recovery of the added PA and NAPA, separately and when together, was determined by comparison of the absorbance peak areas obtained from spiked plasma with those of the standards scanned on the same plate.

Standard curves relating areas to drug amounts were calculated using the method of least squares.

RESULTS AND DISCUSSION

Typical recordings obtained by scanning are illustrated in Fig. 1. Calibration curves relating concentration of PA and NAPA to peak areas are shown in Fig. 2. The curves were consistently linear for spots containing 10 to 200 ng.

Recovery from spiked plasma samples was calculated by comparison with the methanol standards which had been spotted directly on the plates, and those data are included in Table I. It is seen that recovery is essentially complete, but it is still recommended that both methanol standards and extracts from spiked plasma samples be run concurrently with samples containing unknown quantities of PA and NAPA so that failure to extract completely might be detected.

Table II presents data obtained by this method on two patients at 2 and 4 h after administration of 250 mg of PA. In these cases, 1-ml samples of plasma were used, although smaller samples would be equally suitable if the extraction residue is redissolved in a smaller volume of dissolving solvent or a larger volume is applied to the plate.

The UV scanner used can be used also to measure fluorescence, and since both PA and NAPA are fluorescent, scanning in the fluorescent mode was com-

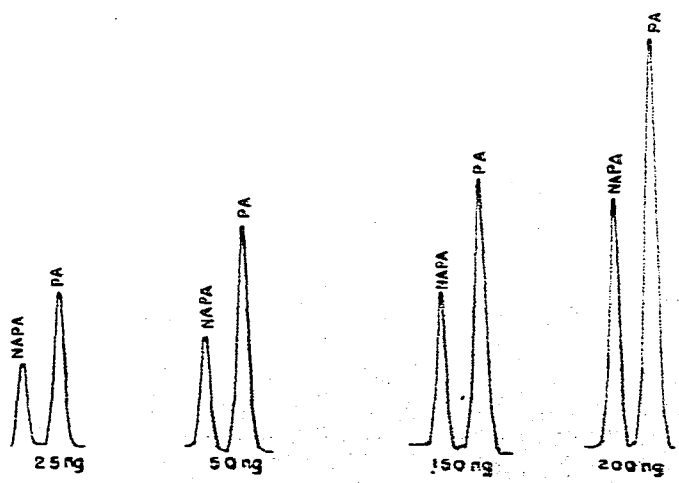

Fig. 1. Peaks observed when scanning the absorbance of procainamide and N-acetylprocainamide standards on silica gel 60.

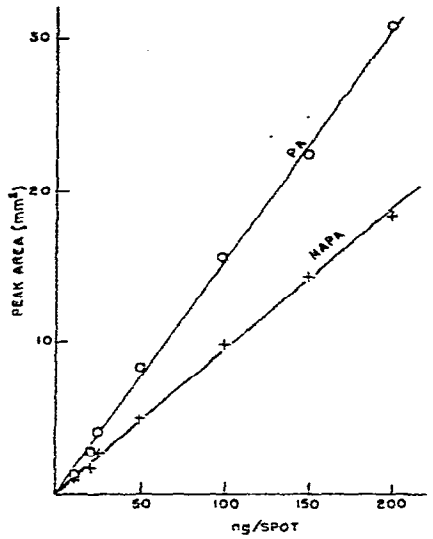

Fig. 2. Procainamide and N-acetylprocainamide standard curves in methanol.

TABLE I

RECOVERY OF PA AND NAPA FROM SPIKED HUMAN PLASMA SAMPLES

Amounts added to plasma (ng/ml)		Number of samples	Recovered (ng/ml)		Percent recovery ± coeff. variation	
PA	NAPA		PA	NAPA	PA	NAPA
25	–	6	25.1	–	100.2 ± 2.49	–
50	–	6	48.9	–	97.9 ± 3.07	–
100	–	6	98.2	–	98.2 ± 0.55	–
200	–	6	191.6	–	95.8 ± 1.68	–
–	25	6	–	22.7	–	90.6 ± 4.78
–	50	6	–	47.7	–	95.4 ± 2.40
–	100	6	–	95.9	–	96.0 ± 1.18
–	200	6	–	201.5	–	100.8 ± 1.48
25	25	3	25.6	26.2	102.5 ± 3.20	104.8 ± 5.82
50	50	3	50.2	49.4	100.4 ± 1.27	98.8 ± 3.20
100	100	3	97.1	100.1	97.1 ± 1.63	100.1 ± 0.79
200	200	3	194.4	200.0	97.2 ± 2.70	100.0 ± 0.72

pared with UV absorption. The sensitivity was found to be no greater and this method was abandoned.

This method was also compared with a published gas chromatographic method [9] on both spiked plasma and patient samples. Results are presented in Table III. It can be seen that the two methods give comparable results, though the gas–liquid chromatographic procedure is not sensitive enough and therefore is not useful below a plasma concentration of 1–2 μg/ml. The lower

TABLE II

PLASMA CONCENTRATION OF PA AND NAPA AT 2 AND 4 h AFTER AN ORAL DOSE OF 250 mg OF PA

Patient	Time after dose (h)	PA concentration (μg/ml)	NAPA concentration (μg/ml)
E.G.	2	4.8	3.0
	4	3.0	2.0
L.R.	2	6.0	3.8
	4	3.8	3.0

TABLE III

COMPARISON OF THE DETERMINATION OF PLASMA CONCENTRATIONS OF PA AND NAPA USING QUANTITATIVE THIN-LAYER AND GAS CHROMATOGRAPHY

Quantities PA and NAPA added (μg/ml)	TLC assay (μg/ml)		GLC assay (μg/ml)	
	PA	NAPA	PA	NAPA
0.5	0.45	0.51	not detected	
0.5	0.52	0.51	not detected	
1.0	1.10	0.95	not detected	
1.0	1.08	1.04	not detected	
2.0	1.92	1.95	1.84	1.90
2.0	1.98	2.03	1.98	1.90
5.0	5.08	4.89	5.00	4.91
5.0	5.0	5.04	4.92	4.95
10.0	9.90	10.05	9.89	9.90
10.0	10.04	9.95	10.06	9.96

limit of sensitivity of this thin-layer chromatography procedure is around 40 times lower than is required for the therapeutic PA plasma concentration levels.

The use of an automatic plate spotter and the thin-layer spectrophotometer equipped with a recorder and integrator makes possible the processing of up to 10 samples on a single plate. Thus, a considerable savings of time in handling a large number of samples is achievable.

This new procedure for the quantitative determination of PA and NAPA in plasma shows that thin-layer chromatography can offer an accurate, fast, sensitive and specific method for quantitative determinations.

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