Journal of Chromatography, 274 (1983) 219—230
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1589

SIMULTANEOUS DETERMINATION OF DISOPYRAMIDE AND ITS MONO-N-DEALKYL METABOLITE IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received August 10th, 1982; revised manuscript received November 23rd, 1982)

SUMMARY

A high-performance liquid chromatographic procedure is described for the determination of disopyramide and its mono-N-dealkyl metabolite which offers simplicity of extraction with excellent selectivity, sensitivity and reproducibility. The drug and metabolite, following basic diethyl ether extraction and back-extraction with acetic acid, are injected into a reversed-phase high-performance liquid chromatographic column and the absorbance of the eluate measured at 254 nm. Detectability limits of 0.05 μg/ml were obtained with both compounds, and studies of the reproducibility, precision, recovery, stability during storage and effect of time in separating plasma from erythrocytes are described. Applications of this high-performance liquid chromatographic procedure to plasma samples from patients on disopyramide therapy and to plasma and urine from a healthy dog administered single doses are reported.

INTRODUCTION

Disopyramide (I), [4-diisopropylamino-2-phenyl-2-(2-pyridyl) butyramide] (Fig. 1), is an antiarrhythmic agent which is active at the atrial as well as the ventricular level. It has pharmacological effects on the heart that are qualitatively similar to those of quinidine and procainamide. The major metabolite in man is the mono-N-dealkyl disopyramide (II, Fig. 1) [1] which is also found in dog. Species differences in the metabolism of I have also been described [2—4].

Many of the gas—liquid chromatographic (GLC) and high-performance liquid chromatographic (HPLC) procedures applied to analysis of I in biological fluids have been reviewed by Duchateau and Hollander [5]. Since that time, HPLC procedures by Ahokas et al. [6] and Flood et al. [7] have been described, as well as a new enzyme immunoassay by Johnston and Hamer [8]. However,

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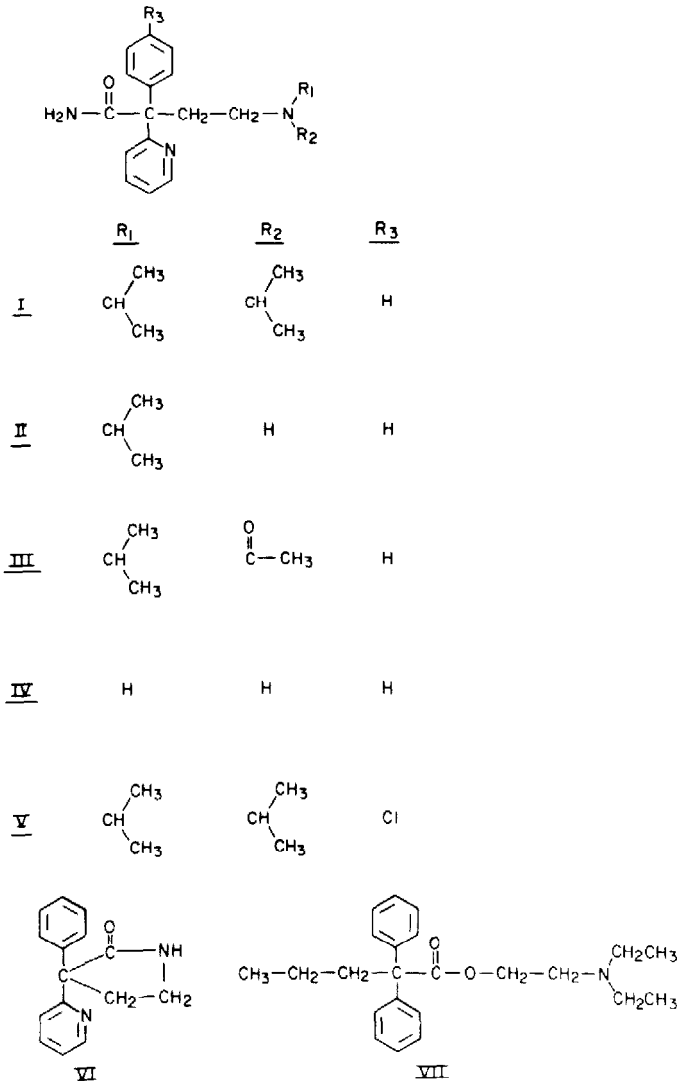

Fig. 1. Chemical structures of disopyramide (I), mono-N-dealkyl disopyramide (II), acetylated mono-N-dealkyl disopyramide (III), di-N-dealkyl disopyramide (IV), *p*-chloro-disopyramide or SC 13068 (V), 3-phenyl-3-(2-pyridyl)-2-pyrrolidone or SC 32046 (VI) and β-diethylaminoethyl-2,2-diphenylpentanoate or SKF-525A (VII).

most of these methods do not allow simultaneous estimation of I and II. Methods which describe this capability include thin-layer chromatography (TLC) [9—11], GLC [12—14] and HPLC [15—17], but these either include cumbersome extraction procedures or do not attain the required sensitivity.

This paper describes a new HPLC procedure, involving a simple extraction, with system conditions to provide increased selectivity, sensitivity and reproducibility for estimation of I and II as well as some minor biotransformation products. Further, some comparison with GLC is given together with applications to patient and animal studies.

EXPERIMENTAL

HPLC

An HPLC system was assembled from an Altex Model 110A pump (Beckman Instruments, Toronto, Canada), a Model U6K injector (Waters Scientific, Mississauga, Canada), a Waters 202 UV detector operated at 254 nm and an Altex Ultrasphere ODS 5-μm stainless-steel column 150 mm × 4.6 mm I.D. and was employed for all analyses. The mobile phase was pumped at a flow-rate of 2.0 ml/min at room temperature producing a back pressure of 138 bars. A Brownlee Labs. (T.M.A. Scientific Supply, Mississauga, Canada) RP-8 column, 250 mm × 4.6 mm I.D., 10 μm, operated at 2.0 ml/min (back pressure of 55 bars) was used as an alternative.

The chromatographic mobile phase consisted of aqueous 0.05 *M* acetic acid—aqueous 0.05 *M* ammonium formate—distilled water—acetonitrile (9:13.5:22.5:55) and was prepared daily.

Gas—liquid chromatography with alkali flame ionization detector and with mass spectrometry

A gas chromatograph (Model 5730A; Hewlett-Packard, Avondale, PA, U.S.A.) equipped with an alkali flame ionization detector (AFID) was used. The 1.83 m × 2 mm I.D. coiled-glass column was packed with 5% OV-17 on Gas-Chrom Q, 100—120 mesh (Chromatographic Specialities, Brockville, Canada). The injection port and detector temperatures were maintained at 300°C and the column at 250°C. The detector bead current was adjusted to obtain maximum sensitivity. The carrier gas, helium, was maintained at a flow-rate of 30 ml/min and the air and hydrogen flow-rates were adjusted for optimum sensitivity.

A Hewlett Packard 5985 GLC—mass spectrometry (MS)—data system, operated in the electron impact mode with 70 eV as the ionization beam energy, was used for GLC—MS analysis with source maintained at 200°C. The chromatographic column was coiled glass, 1.22 m × 2 mm I.D., packed with 3% OV-17 on Gas-Chrom Q, 100—120 mesh. The injection port and detector temperatures were 275°C. The column oven temperature was maintained at 240°C for 1 min after the injection, then temperature programmed at 5°C/min to a final temperature of 280°C which was held for 5 min. Helium was used as carrier gas with a flow-rate of 30 ml/min.

Standards

Disopyramide (SC-7031), mono-N-dealkyl disopyramide (SC-24566), pyrrolidone metabolite (SC-32046) and *p*-chlorodisopyramide (SC-13068) were kindly supplied by Searle Pharmaceuticals (Oakville, Canada) and throughout this report will be identified as I, II, VI and V, respectively (Fig. 1).

Stock solutions (100 μg/ml) of I, II and V were prepared by dissolving appropriate amounts of the base in 0.01 *N* aqueous hydrochloric acid. Working plasma solutions containing I (0.5—4.0 μg/ml) and II (0.125—3.0 μg/ml) were prepared fresh daily by appropriate dilution of stock solutions with blank plasma. Working solutions of the internal standard V (4 μg/ml) were obtained by diluting the stock solution with distilled water.

Chemicals and reagents

Diethyl ether (Mallinckrodt, Montréal, Canada) was glass-distilled prior to use. HPLC-grade acetonitrile, chloroform and methanol (Fisher Scientific, Ottawa, Canada), distilled in glass ethyl acetate (Burdick and Jackson Labs., Muskegon, MI, U.S.A.), Analar grade amyl acetate and acetic anhydride (BDH, Toronto, Canada) and all other analytical-grade chemicals were purchased from commercial sources. Compound SKF 525A [β-diethylaminoethyl-2,2-diphenylpentanoate] (Smith, Kline & French Labs., Philadelphia, PA, U.S.A.) was used as an internal standard for the GLC procedure and identified as VII (Fig. 1).

Patients

All plasma samples were obtained from patients admitted to the cardiac unit of a local hospital and treated with disopyramide (Rythmodan capsule).

Dog

Separate doses of disopyramide phosphate capsules (Norpace) equivalent to 150 mg (1 capsule) and 750 mg (5 capsules) base respectively were given orally to a healthy male Labrador dog (33.1 kg) with 28 days between doses. The animal was placed in a metabolism cage and total urine was collected for the 24 h prior to ingestion and for 24-h periods up to 72 h. The urine was divided in two: one portion being stored at 4°C and the other at −15°C prior to extraction. Blood samples (10 ml) were taken from the saphenous vein into heparinized tubes at appropriate time intervals up to 72 h. The tubes were centrifuged, plasma was separated and stored at 4°C till analysis. For both doses, the dog was fasted overnight, no food was given till the 7-h sample post dose, but water was permitted ad libitum after drug administration.

Extraction procedure

HPLC analysis. To a plasma sample (1 ml) was added 0.5 ml of an aqueous solution of the internal standard V (4 μg/ml). The contents were mixed (Vortex Genie, Fisher Scientific) for 15 sec before adding 0.1 ml of concentrated ammonium hydroxide and 6 ml diethyl ether. The drug and the metabolite, along with V, were extracted into the ethereal phase by shaking on a rotary mixer (Roto-Rack, Fisher Scientific) for 15 min followed by centrifugation. A portion of the organic layer (5.0 ml) was transferred to a conical tube containing 0.2 ml of an 0.1 *M* aqueous acetic acid solution. The contents were mixed and centrifuged for 5 min each. Aliquots, 25—100 μl, of the aqueous layer were analysed by HPLC.

Urine was similarly processed; 9-ml aliquots of urine being basified with 1.5 ml concentrated ammonium hydroxide and extracted with 9 ml diethyl ether on the rotary mixer. The ethereal layer, separated by centrifugation, was then evaporated to dryness under a light stream of nitrogen at 55°C, the residue dissolved in 300 μl acetonitrile and appropriate aliquots injected onto the chromatograph.

GLC—AFID and GLC—MS analysis. The extraction procedure for plasma analysis was essentially that of Johnston and McHaffie [18]. The urine samples were extracted as described previously for HPLC. Acetylation of plasma or urine extracts was accomplished by dissolving the dry residue from the ethereal

extract with 3 ml of chloroform. To this solution, 25 μl of acetic anhydride were added before mixing on a Vortex mixer. This organic solution was evaporated in a dry bath at 55°C under light stream of nitrogen, the residue dissolved with 300 μl of ethyl acetate or methanol and aliquots (1–5 μl) injected into the GLC or GLC—MS system.

Calibration curves

Peak height ratios were calculated by dividing the height of the peak due to the drug or the metabolite by the height of the peak due to the internal standard. Calibration curves were assembled from the results obtained by analysing spiked control plasma by plotting the peak height ratios against the concentration of I or II. Calibration curves were run with every set of unknowns.

RSULTS AND DISCUSSION

HPLC analysis

Under the conditions described in the Experimental section, analysed on a Ultrasphere ODS column, the compounds I, II, and V gave sharp and symmetrical peaks with retention times of 4.2, 3.1 and 5.4 min, respectively. The total analysis time for one injected sample was approximately 8 min. Fig. 2 shows chromatograms obtained by processing a 1-ml sample of blank plasma

TABLE I

HPLC CALIBRATION CURVE OF DISOPYRAMIDE AND MONO-N-DEALKYL DISOPYRAMIDE EXTRACTED FROM PLASMA

Concentration (μg/ml)	*n*	Mean ratio	R.S.D. (%)
A: Disopyramide			
0.50	24	0.43	5.7
1.00	24	0.84	3.2
2.00	16	1.70	3.1
3.00	14	2.50	1.5
4.00	6	3.27	1.3
		Mean	3.0
Slope = 0.822, intercept = 0.027 and coefficient of determination r^2 = 0.998			
B: Mono-N-dealkylated disopyramide			
0.125	3	0.13	7.7
0.25	14	0.28	3.6
0.50	20	0.64	4.7
1.00	14	1.26	3.2
1.50	7	1.86	4.8
2.00	4	2.54	3.9
3.00	2	3.88	—
		Mean	4.8
Slope = 1.285, intercept = —0.024 and coefficient of determination r^2 = 0.996			

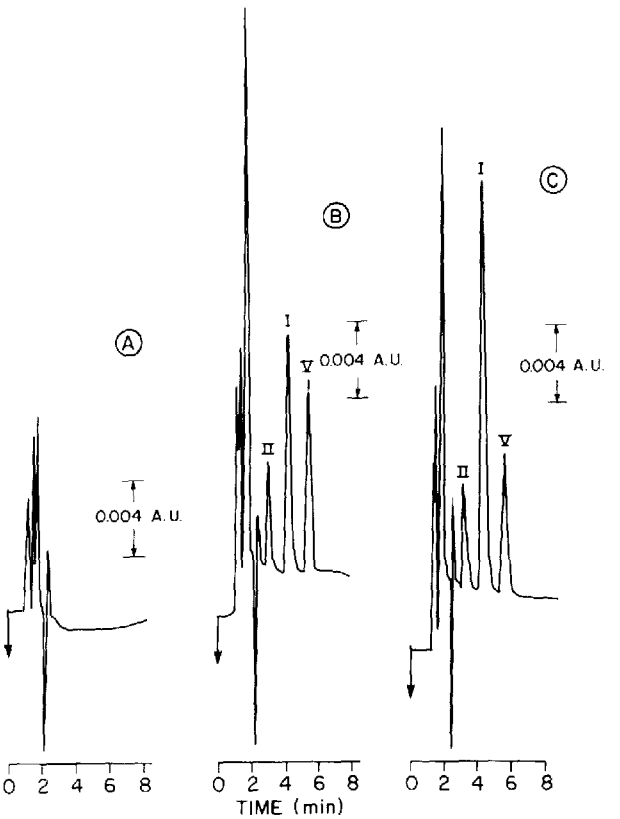

Fig. 2. HPLC chromatograms from human plasma. A, blank plasma; B, plasma spiked with 0.50 μg of mono-N-dealkyl disopyramide and 1.00 μg of disopyramide; C, plasma from a patient estimated to contain 0.78 μg of the mono-N-dealkyl and 2.58 μg of disopyramide. Peaks: I = disopyramide; II = mono-N-dealkyl disopyramide; V = *p*-chlorodisopyramide, the internal standard.

(A), plasma containing added drug and metabolite (B) and plasma from a patient treated with disopyramide (C). The peaks observed in blank plasma did not interfere with the peaks due to I, II and V.

The typical calibration curves obtained were linear with negligible intercepts over the range studied: 0.50—4.00 and 0.125—3.00 μg/ml for I and II, respectively. The accuracy and precision of the HPLC procedure are demonstrated in Table I. The overall mean relative standard deviations for I and II were 3.0 and 4.8% over their respective ranges.

The overall mean recoveries (± S.D.) of I, II and V from plasma were 72.8 ± 2.9%, 55.8 ± 1.5% and 73.8 ± 3.1%, respectively. The percentages recovered from different plasma concentrations for the drug and its metabolite, and the internal standard are demonstrated in Table II. There was no marked change in recovery over the concentration ranges investigated.

Results of the application of the present method to the determination of plasma I and II concentrations in samples from patients treated with disopyramide are shown in Table III. Those from dog experiments are

TABLE II

RECOVERY OF DISOPYRAMIDE, MONO-N-DEALKYL DISOPYRAMIDE AND *p*-CHLORODISOPYRAMIDE FROM PLASMA (HPLC EXTRACTION)

Compound	Amount added to 1 ml of plasma (μg)	*n*	Mean percentage recovered	R.S.D. (%)
Disopyramide	0.50	7	73.0	6.1
	1.00	10	76.7	4.7
	2.00	5	71.5	3.6
	3.00	6	69.8	5.1
	mean	—	72.8	4.9
Mono-N-dealkyl disopyramide	0.25	7	55.1	5.2
	0.50	6	56.2	5.0
	1.00	6	57.6	5.1
	1.50	5	54.2	5.2
	Mean	—	55.8	5.1
p-Chlorodisopyramide	2.00	25	73.8	4.2

TABLE III

PATIENT PLASMA LEVELS OF DISOPYRAMIDE AND MONO-N-DEALKYL DISOPYRAMIDE BY HPLC

Patient	Collection time (h)			Plasma concentration (μg/ml)	
				I	II
1	08:00			2.15	0.69
	11:00			1.79	0.75
2	08:00			2.36	0.84
3	13:00			1.35	0.64
4	unknown			0.95	0.54
5	22:00			2.55	0.72
6	11:00			2.99	0.78
7	09.30			2.24	3.74
8	11:00			2.62	0.99
9	11:00			2.85	0.83
10	11:00			2.90	1.02
11	11:00			2.00	1.88
12	11:00			1.84	0.37
13	19:00			1.72	1.56
14	11:00			1.83	0.31
15	08:00	dosed	0 h	no sample	
	10:00		2 h	3.33	0.94
	13:00		5 h	3.01	0.87
	15:00		7 h	2.85	0.80
	19:00		11 h	1.70	0.66
16	08:00			3.56	1.35
17	11:00			7.87	1.58
18	11:00			3.77	0.95

illustrated in Fig. 3. Each quoted estimation is the mean from replicate analysis.

The HPLC conditions used for plasma level determinations are also applicable to urine analysis and chromatograms are shown in Fig. 4. The urine extraction was judged to be adequate because no endogenous peaks eluted at the retention times of I, II and VI (Fig. 4A). This procedure readily allows quantitation of the drug and the mono-N-dealkyl (II) and di-N-dealkyl (VI) metabolites.

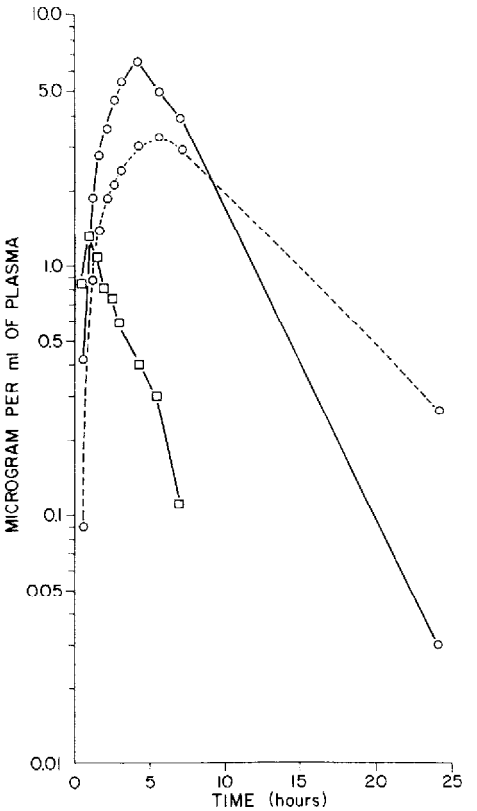

Fig. 3. Plasma concentration profiles obtained from the same dog following ingestion of 150 mg Norpace capsule and five 150-mg Norpace capsules. □——□, disopyramide (150 mg); ○——○, disopyramide (750 mg); ○— — —○, mono-N-dealkyl disopyramide (750 mg).

The RP-8 column sorbent was selective for the analysis of I, II, V and VI and gave sharp and symmetrical peaks eluting at 4.1, 3.1, 5.3 and 2.1 min, respectively under the conditions described in the Experimental section. It appears to compare favorably with the Ultrasphere ODS column in terms of sensitivity and stability.

Although the GLC–AFID procedure was adequate for the determination of I in plasma (0.15—2.00 μg/ml), problems were encountered with the determination of II (Fig. 5). Derivatization of II with acetic anhydride was required prior to GLC—AFID analysis because otherwise the metabolite did not elute as a single peak. Although this additional step did not affect the determination

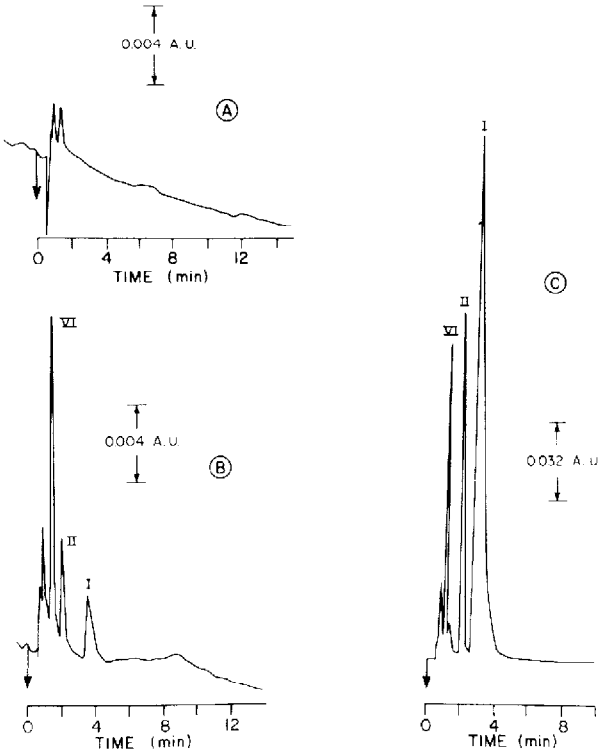

Fig. 4. Typical HPLC chromatograms of dog urine after oral dose of Norpace capsule(s). A, blank control urine; B, 24—48 h urine collection, 150 mg; C, 0—24 h urine collection 750 mg. Peaks: I = disopyramide, II = mono-N-dealkyl disopyramide and VI = cyclic di-N-dealkyl disopyramide (SC 32046).

of I, it did not prove possible to obtain reproducible calibration curves for II and the significant negative intercepts noted may indicate instability. This problem was not overcome despite modification of various conditions such as injector and detector temperatures, column phases, solvents, temperature and time for reaction. At higher concentrations, i.e. greater than 4 μg of II per ml plasma, this problem would possibly be less important. Because of these difficulties, the simultaneous determination of I and II by GLC—AFID was abandoned in favor of the HPLC method.

Stability of I and II in plasma

A stability study was undertaken over a 31-day period to determine the effect of storage on the concentration of I and II. A control human blank plasma was spiked with I and II giving a final concentration of 1.8 and 1.0 μg/ml, respectively with aliquots being stored at —20°C and at 4°C until analysis time. There was no difference in the estimated levels for either I or II under either conditions of storage over the 31 days as found from triplicate HPLC analysis at various time intervals (mean I, 1.75 μg/ml ± 5%, mean II, 0.98 μg/ml ± 6%) indicating that the substances are stable in plasma for at least 1 month under those conditions.

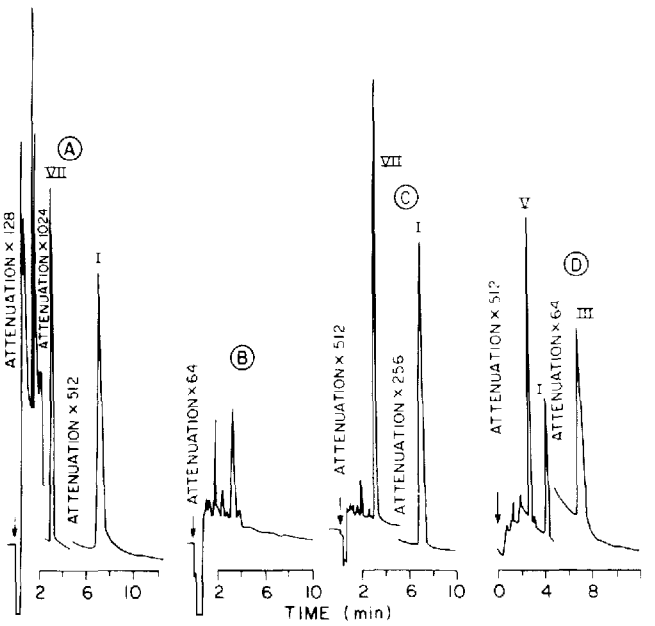

Fig. 5. Typical GLC—AFID chromatograms of I and VII when injected as a methanolic standard solution containing 20 and 15 μg/ml of I and VII respectively (A), blank plasma (B), spiked plasma at 1 μg/ml of I to which 1 μg of VII (internal standard) was added (C), of I, II acetylated (III) and V acetylated obtained at column temperature of 270° C from a 1-ml methanolic standard solution containing 3 μg I, 1.5 μg II and 2 μg V after derivatization (D). Peaks: I = disopyramide; III = monoacetylated derivative of mono-N-dealkyl disopyramide; V = *p*-chlorodisopyramide (internal standard); VII = SKF 525A (internal standard).

Effect of blood collection method

Blood was withdrawn from healthy subjects using heparinized evacuated glass tubes (Vacutainer, green rubber stopper) avoiding contact with the stopper. The samples were divided into two aliquots and spiked with I and II to obtain concentrations of 2 μg I and 1 μg II per ml and 1.5 μg I and II per ml. Equal volumes of each spiked blood sample were transferred to a green rubber stopper Vacutainer and to a polypropylene tube respectively. The tubes were stoppered and placed on a Roto-Rack for 2 min at 5 rpm, centrifuged, then plasma was removed and analysed in duplicate by HPLC. Ratios of the concentrations found from the Vacutainer tube over the polypropylene tube were 0.96 and 1.02 for disopyramide at 2.00 and 1.50 μg/ml levels and 1.01 for II at the 1.5 μg/ml concentration values which indicated no substantial difference.

A similar experiment was carried out during the dog study (750 mg) with blood collected by glass syringe and by Vacutainer on three separate occasions. Again there were no substantial differences in the values from the two collection procedures as determined by HPLC.

The above experiments showed that these materials used for blood collection did not affect the plasma drug or metabolite concentrations.

Effect of the pre-analysis storage time of blood samples

Blood from a healthy human subject was spiked with I and II to give a final

concentration of 2.0 and 1.0 μg/ml, respectively. The blood was divided into three aliquots and the tubes kept at room temperature. One tube was centrifuged immediately (0 h) and plasma was analysed in duplicate by HPLC. The second aliquot was centrifuged and the plasma analysed after standing 1.5 h at room temperature. The percentages found compared to the 0-h sample were 100.4% for I and 89.4% for II. After standing 2.45 h, the third aliquot was centrifuged and analysis of plasma yielded 101.9% and 62.8% of the 0-h value for I and II, respectively. This experiment demonstrated that while the time of standing before centrifugation and transfer of plasma did not affect the disopyramide plasma level, the mono-N-dealkyl disopyramide plasma concentration decreased significantly, i.e. 37.2% in 2.45 h.

In contrast to the above result, Daniel and Subramanian [19] using GLC—FID reported a loss of 30% for disopyramide in 1 h when blood (spiked with 5.5 μg/ml) was stored at ambient temperature. Johnston and McHaffie [18], however, again with GLC—FID, could not confirm that latter observation in plasma from a volunteer treated with 400 mg of I.

Knowing that I and II are stable in plasma, as demonstrated previously, the experiment completed with spiked blood indicated the importance of minimizing the time between blood sampling and centrifugation, particularly when the mono-N-dealkyl metabolite is being estimated.

Dog studies

The plasma concentrations of I found following the 150 and 750-mg doses of disopyramide to the dog are illustrated in Fig. 3. With the lower dose, the drug was not detectable after 7 h nor was metabolite II detectable at any time, while with the higher dose II was also followed over 24 h. The elimination half-life was about 1.8—2.5 h for I and 5.0 h for II, values in agreement with Karim et al. [4]. The dog was unaffected by the low dose but was noticeably docile and vomited about 7 h after the larger dose. The peak for I was also delayed with the higher dose (from 1 to 4 h).

Typical HPLC chromatograms obtained from urine extracts are shown in Fig. 4. Collection of fractions from the liquid chromatograph followed by mass fragmentography indicated that VI was the cyclic stable product formed after di-N-dealkylation as described by Karim et al. [2]. GLC—MS analysis of the acetylated urine extracts also confirmed the presence of I, II and VI with retention times of 3.6, 7.4 and 2.1 min, respectively from comparison with the mass spectra obtained from authentic samples. In the HPLC examination of the urine extracts (Fig. 4) the amount of VI increased with time relative to I and II and persisted even with the low dose to 48 h. With the higher dose only small amounts of I and II were detectable in the 48—72 h collection, while VI again persisted.

Patients

Results of the application of the HPLC method to patient plasma samples are given in Table III. Values for I ranged widely from 0.95 to 7.87 μg/ml and II for 0.31 to 3.74 μg/ml with means of 2.65 and 1.04 μg/ml, respectively. These ranges although wide are in agreement with values in various studies in the literature [12, 20].

CONCLUSION

The described HPLC procedure, for the simultaneous determination of disopyramide and mono-N-dealkyl disopyramide in plasma, is rapid, sensitive, specific and easily reproducible. It has adequate sensitivity for plasma level determination with detectability limit as low as 0.05 μg/ml of either I or II. Storage of plasma at 4°C and −20°C for a period as long as 31 days did not affect the amount of I and II recovered. However, blood should be centrifuged immediately after collection because loss of II but not I occurs on standing at room temperature. The different tubes used for blood collection had no apparent influence on the plasma levels of I and II. This HPLC method is adequate for plasma level determinations of disopyramide and mono-N-dealkyl disopyramide after single or therapeutic doses in pharmacokinetic studies.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. W.M. McLean and Mr. M.G. Tierney from Ottawa General Hospital and to Dr. D.S. Beanlands from Ottawa Civic Hospital for providing patient samples for plasma level determination. The authors express their appreciation to Mr. J.C. Ethier, Bureau of Drug Research, Health Protection Branch, Ottawa, for obtaining MS data, to Dr. S. Sved, Bureau of Drug Research for his contribution to helpful discussions, to Messrs. J. Evans and L. Martin for dog studies and to Mr. A. Trottier for drafting.

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