Journal of Chromatography, 106 (1975) 151–158

CHROM. 7979

DETERMINATION OF DISOPYRAMIDE AND ITS MONO-N-DEALKYLATED METABOLITE IN BLOOD SERUM AND URINE

T. C. HUTSELL and S. J. STACHELSKI

Department of Analytical Resources and Methods, Searle Laboratories, P.O. Box 5110, Chicago, Ill. 60680 (U.S.A.)

(First received July 29th, 1974; revised manuscript received September 23rd, 1974)

SUMMARY

A rapid, precise and accurate assay for disopyramide and mono-N-dealkylated disopyramide concentrations in blood serum and urine is described. The method involves extraction of the drugs from a basic aqueous medium into chloroform, derivatization of the metabolite, purification of the extract and gas chromatographic analysis using an OV-17 liquid phase and flame ionization detection. Unique characteristics of the procedure, direct derivatization in the organic phase and the use of Florisil to separate the drugs from interfering materials, should be applicable to the analysis of other basic drugs in biological specimens.

INTRODUCTION

The use of disopyramide phosphate* (Fig. 1) as an antiarrhythmic agent has been described[1,2]. Pharmacokinetic and biotransformation studies reveal that, in man, disopyramide (DIS) has a plasma half-life of 7.0 h, is excreted largely unchanged in the urine and is metabolized primarily to mono-N-dealkylated disopyramide[3,4] (MND; Fig. 1). Quantitation of DIS in blood plasma or serum and urine in previous studies was performed using a method[3] based upon the fluorescence exhibited by DIS in strong-acid solution. However, this procedure is non-specific because DIS and MND have identical fluorescence spectra. In addition, the fluorescence technique is subject to interferences, which often lead to variable blanks, and lacks the sensitivity required to determine DIS and MND concentrations in blood serum. The methodology described herein allows accurate and precise quantitation of DIS as well as MND using a new specimen purification procedure and derivative-preparation pro-

* Compounds used in this study are as follows: disopyramide phosphate (4-diisopropylamino-2-phenyl-2-(2-pyridyl) butyramide phosphate; also known as SC-7031 phosphate, SC-13957 and Norpace®), mono-N-dealkylated disopyramide (4-isopropylamino-2-phenyl-2-(2-pyridyl)butyramide; also known as SC-24566), *p*-chlorodisopyramide (4-diisopropylamino-2-(*p*-chlorophenyl)-2-(2-pyridyl) butyramide; also known as SC-13068). All compounds were obtained from Searle Laboratories, Chicago, Ill., U.S.A.

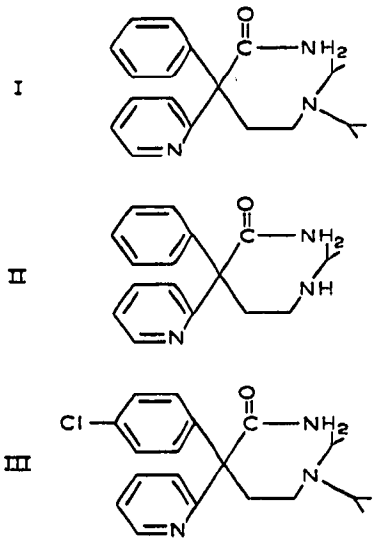

Fig. 1. Structural formulas for disopyramide (I), its mono-N-dealkylated metabolite (II) and the internal standard (III) used in the assay procedure.

cedure that may be applicable to a wide range of basic drug determinations in biological specimens.

EXPERIMENTAL

Materials

Reagents. Florisil (60–100 mesh, Floridin Company, Pittsburgh, Pa., U.S.A.), as received, was hydrated to 7% (w/v) with deionized water and was stored in a tightly closed polyethylene bottle. All other reagents were analytical-reagent grade and were used as supplied except for chloroform and methanol, which were distilled before use. Nanograde® (registered trademark of Mallinckrodt, St. Louis, Mo., U.S.A.) chloroform and methanol may be used without distillation. Potassium hydroxide, acetic anhydride, methanol and chloroform were obtained from Mallinckrodt; sodium hydroxide and ammonium hydroxide were purchased from J. T. Baker (Phillipsburg, N.J., U.S.A.). As an internal standard, *p*-chlorodisopyramide (Fig. 1) was used.

Equipment. Gas chromatography was performed using a Packard (Downers Grove, Ill., U.S.A.) gas chromatography system (Model 878 electrometer–voltage supply, Model 888 flame ionization detector, Model 804 coiled-column air oven, Model 873 temperature controller, Model 824 dual flow controls and Honeywell Electronik 194 linear recorder) containing a 0.61 m × 2 mm I.D. glass U-shaped column packed with 2.6% OV-17 (Ohio Valley Specialty Chemical, Marietta, Ohio, U.S.A.) on 80–100 mesh Chromosorb W-HP (Supelco, Bellefonte, Pa., U.S.A.). Gas chromatographic parameters are shown in Table I.

Methods

Preparation of standards. Two stock standard solutions each of DIS, MND

TABLE I

GAS CHROMATOGRAPHIC PARAMETERS FOR THE ASSAY OF DIS AND MND IN BIOLOGICAL SPECIMENS

Parameter	*Assay system*	
	DIS	*MND*
Injector temperature (°C)	230	250
Column temperature (°C)	210	230
Detector temperature (°C)	260	260
Gas flow-rates (ml/min)		
Carrier (helium)	60	60
Air	350	350
Hydrogen	20	20
Relative retention times		
Disopyramide	1.00	0.30
Internal standard	1.90	0.57
MND-acetate	3.50	1.00
Absolute retention times (min)		
Disopyramide	6.25	2.50
Internal standard	11.88	4.69
MND-acetate	21.88	8.43
Electrometer sensitivity (A/mV)	$8–16 \cdot 10^{-12}$	$8–16 \cdot 10^{-12}$

and the internal standard were prepared. A primary stock standard (1 mg/ml) was prepared by dissolving 50 mg of pure compound in 5 ml of 1 *M* hydrochloric acid and diluting to 50 ml with deionized water. A secondary stock standard (10 μg/ml) was made by diluting 1 ml of the first stock solution to 100 ml with 0.1 *M* hydrochloric acid. These stock solutions were stored at room temperature and remained stable throughout the course of this work (4 months). Working standards of DIS and MND were stored at room temperature and were prepared once weekly by diluting appropriate aliquots of the respective secondary stock solution with deionized water to provide drug concentrations of 0.2, 0.4, 1.0, 3.0 and 5.0 μg/ml. A working internal standard solution was prepared by diluting aliquots of the secondary stock internal standard with 0.1 *M* hydrochloric acid to provide concentrations of 1.2 and 3.7 μg/ml.

Assay procedure. Because MND is present in a lower concentration in biological fluids than DIS, separate preparations of serum or urine are recommended for optimal quantitation of each of the analytes.

Depending upon expected concentration of drug, transfer up to 3.0 ml serum or up to 4.0 ml of urine to a clean 25 mm × 150 mm PTFE-lined screw-capped culture tube (Scientific Products, McGaw Park, Ill., U.S.A.). Standards are prepared in duplicate by adding 1 ml of human serum or 0.1 ml of human urine known to be free of DIS and MND to 1-ml aliquots of each working standard. Tubes containing human serum or urine known to be free of DIS and MND were analyzed along with standards and samples to serve as a check for contaminants in reagents and glassware.

Add 1 ml appropriate internal standard solution (3.7 μg/ml for assay of DIS, 1.2 μg/ml for assay of MND) and 0.5 ml of 60% (w/v) potassium hydroxide; dilute with water if necessary to a total volume of 4.5 ml for serum or 6.5 ml for urine. Add 20 ml of chloroform and shake on a mechanical shaker (A. H. Thomas Company,

Philadelphia, Pa., U.S.A.) for 30 min. Centrifuge (average 500 *g*, 5 min); remove and discard the upper aqueous layer. If a serum sample becomes emulsified, it may be necessary to centrifuge the sample at a higher *g* force and/or for a longer period of time. This may be especially true for aliquots of serum $>$ 1 ml or for samples high in lipid content. Decant chloroform into a second tube containing 25 μl of acetic anhydride and mix. Add 100 $\pm$ 10 mg hydrated Florisil with a small scoop; cap tightly and shake 10 min on the mechanical shaker. Centrifuge as above, decant and discard the chloroform. Wash the Florisil with an additional portion of approximately 10 ml of chloroform. Centrifuge and discard this chloroform wash as before. Elute the drugs from the Florisil with two 5-ml portions of methanol containing 1 % (v/v) ammonium hydroxide. Evaporate the combined basic-methanol eluates in a water bath at 40° under a stream of nitrogen. Transfer the dried residue to a 1-dram glass vial with two 1-ml portions of basic-methanol; evaporate to dryness under nitrogen. Immediately prior to gas chromatography, add 25 μl of chloroform to dissolve the residue; mix and inject 1–4 μl into the gas chromatographic column.

Calculations. On linear graph paper, a standard curve is prepared by plotting the ratio of peak height of drug standard to peak height of internal standard *vs.* known amount (μg) of drug standard. Unknown sample concentrations are obtained using the experimentally determined ratio of peak height (sample) to peak height (internal standard) and reading the drug concentration from the graph constructed using known standard solutions. Final unknown sample concentration (μg/ml) is obtained by dividing the graph-derived amount of sample (μg) by the volume of sample (ml) used in the assay.

RESULTS AND DISCUSSION

DIS and the acetate derivative of MND are easily separated and quantified by gas–liquid chromatography (Fig. 2). Although both the DIS and the MND-acetate can be quantitated from a single chromatographic injection, this was considered impractical for routine analysis of large numbers of specimens because of the longer retention time and the lower concentration of MND-acetate relative to that of DIS. Thus, a set of chromatographic conditions was employed which provided optimal quantitation for each compound (Table I). Underivatized MND eluted from the gas chromatographic column as three poorly separated peaks under the experimental conditions employed, whereas the acetate derivative of MND provided a single peak with a retention time short enough to be used in the routine analysis of large numbers of specimens. Acetate derivatives are usually prepared by adding pyridine and acetic anhydride to the residue remaining after evaporation of the organic extract. However, addition of acetic anhydride directly to the chloroform extract as in the current procedure yielded acetate derivatives that were chromatographically indistinguishable from those prepared using pyridine and acetic anhydride reagents. This derivatization technique obviates the necessity for extracting the acetate derivatives or, if the acetylation reaction mixture is injected directly into the gas chromatographic column, it circumvents the resulting broad solvent peak that can interfere with analyte peaks.

Chloroform extracts from serum and urine contained large amounts of substances that produced peaks on the gas chromatogram with retention times similar to those of DIS and MND-acetate. Separation of the analytes from these interfering

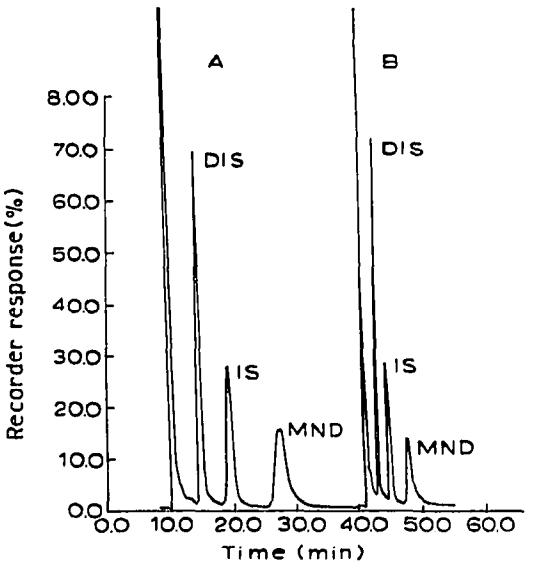

Fig. 2. Gas–liquid chromatograms for DIS (A) and MND-acetate (B), each with internal standard (IS). Chromatographic conditions as shown in Table I except for electrometer sensitivity, which was $128 \cdot 10^{-12}$ A/mV. Each peak represents approximately 1.0 μg compound.

substances was achieved by the addition of 7% hydrated Florisil directly to the chloroform extract. The interfering materials remain in the chloroform solution that is aspirated and discarded; the drugs are adsorbed on the Florisil and are easily eluted with basic methanol. Using this technique, up to 3 ml of serum or 4 ml of urine can be processed in the assay procedure with no interfering peaks visible on the chromatogram. This Florisil separation technique should prove generally applicable to the analysis of other basic drugs in biological specimens.

Analysis of aqueous solutions to which DIS and MND were added indicated that a strongly basic medium was necessary for optimal extraction of both compounds into chloroform (Fig. 3). In addition to chloroform, several other organic solvents

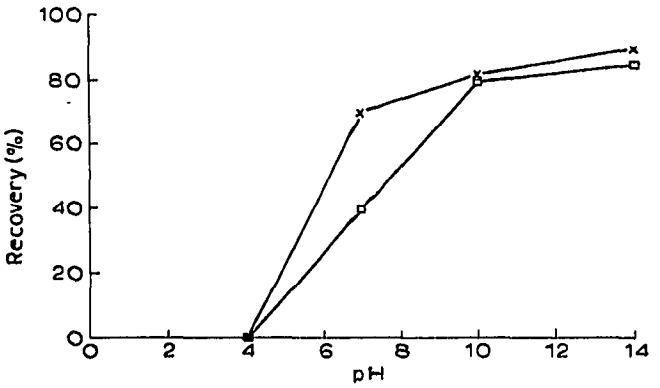

Fig. 3. Effect of pH of aqueous medium on recovery of DIS (×—×) and MND (□—□).

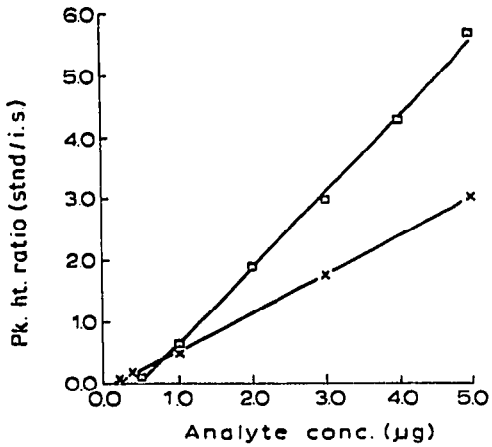

Fig. 4. Typical standard curves prepared by analyzing 1 ml of serum or 0.1 ml of urine to which known amounts of DIS (×—×) or MND (□—□), respectively, were added.

were evaluated in the extraction procedure. Chloroform was the least polar solvent that provided acceptable recoveries; therefore, it was used throughout these studies. Adding known quantities of DIS and MND to serum or urine and analyzing for each compound gave approximately 80% recoveries when compared with unextracted standards and essentially quantitative recoveries (> 95%) when compared with standards that were carried through the assay procedure. Although only one standard curve for each compound is shown (Fig. 4), standard curves for one or both compounds in serum, urine or chloroform solution were essentially identical; standard curves were linear over the concentration range tested (0.5–5.0 μg/ml). These results indicate that the internal standard chosen for this study, *p*-chlorodisopyramide, provides a very reliable mechanism to compensate for any procedural errors that may occur in carrying out the assay.

Since single injections into the gas–liquid chromatograph of 10 ng disopyramide or 20 ng MND-acetate can be quantitated using the procedure described herein, the effective limits of detection (corrected for sample volume and dilution during analysis) relative to serum or urine are 85 ng DIS/ml and 170 ng MND-acetate/ml or 65 ng DIS/ml and 125 ng MND-acetate/ml, respectively. These detection limits are well

TABLE II

PRECISION OF THE ANALYSIS OF DIS

Aliquots of pooled serum and urine specimens were analyzed (one each per day per technician) using the described method. Results of four technicians were used.

Specimen	*Known value (μg/ml)*	*No. of replicates*	*Assayed value (μg/ml)*	*Standard deviation (μg/ml)*	*Relative standard deviation (%)*
Serum	0.30	44	0.29	0.03	10.6
Serum	1.18	42	1.14	0.11	9.3
Urine	1.50	26	1.42	0.19	12.7
Urine	7.50	26	7.51	0.46	6.2

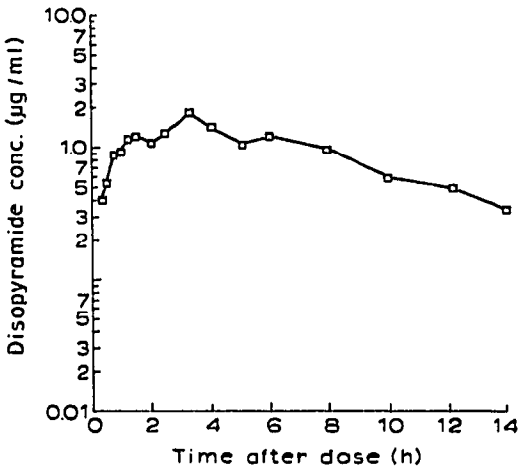

Fig. 5. Time course of serum DIS concentration in a patient given a single oral dose of DIS (3 mg/kg body weight).

below the expected drug concentrations in biological specimens from patients given therapeutic doses of disopyramide[3].

Precision of the method was estimated by adding known quantities of DIS to large volumes of serum and urine. Aliquots of the large serum and urine samples were stored frozen; one aliquot each of serum and urine was analyzed each day along with routine specimens for at least one month. Even though four different technicians were performing these analyses, the relative standard deviation was approximately 10% (Table II).

Several drugs that represented potential interferences were analyzed using the described method: fursemide, pentaerythrityl tetranitrate, isosorbide dinitrate, reserpine, dipyridamole, quinidine, papaverine and morphine. None of the tested compounds interfered with the assay.

TABLE III

CONCENTRATIONS OF DIS AND MND IN URINE FROM A PATIENT TREATED WITH A SINGLE DOSE (3 mg/kg) OF DISOPYRAMIDE PHOSPHATE

Specimen collection period (h)	*Urine volume voided (ml)*	*DIS*		*MND*	
		Concentration (µg/ml)	*Total (mg)*	*Concentration (µg/ml)*	*Total (mg)*
−10–0	333	0	0	0	0
0–2	155	101	16	26.7	4.1
2–4	67	305	20	101	6.8
4–6	114	130	15	53.1	6.1
6–8	385	25.6	9.9	15.2	5.9
8–10	655	20.8	13.6	6.9	4.5
10–12	548	17.1	9.4	9.1	5.0
12–18	330	31.0	10.2	22.4	7.4
18–24	160	18.9	3.0	23.9	3.8

The developed method was used routinely to analyze serum and urine specimens from patients given single therapeutic doses (3 mg/kg) of DIS (Fig. 5 and Table III). Serum concentrations of MND were very low (< 0.4 μg/ml); only about 15% of the analyzed serum samples contained detectable quantities of MND.

The only problem encountered in the routine analytical work was the inconsistent appearance of chromatographic peaks with retention times similar to those of the analytes, apparently produced by glassware contaminants. All glassware used in the procedure was soaked overnight in dichromate–sulfuric acid cleaning solution and rinsed several times with deionized water to remove these contaminants; no extraneous chromatographic peaks were observed when glassware cleaned in this manner was used in the assay procedure. The assay has been used in the authors' laboratory for the analysis of over 1000 biological specimens.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge stimulating discussions with Dr. Aziz Karim and the technical assistance of Mr. William Sherman during the course of these studies.

REFERENCES

1 C. M. Mokler and C. C. van Arman, *J. Pharmacol. Exp. Ther.*, 136 (1962) 114.
2 M. J. Katz, C. E. Meyer, A. El-eth and S. J. Slodki, *Curr. Ther. Res.*, 5 (1963) 343.
3 R. E. Ranney, R. R. Dean, A. Karim and F. M. Radzialowski, *Arch. Int. Pharmacodyn. Ther.*, 191 (1971) 1.
4 A. Karim, R. E. Ranney and S. J. Kraychy, *J. Pharm. Sci.*, 61 (1972) 888.