Journal of Chromatography, 132 (1977) 503–510

CHROM. 9620

HIGH-PRESSURE LIQUID CHROMATOGRAPHIC ANALYSIS OF DRUGS IN BIOLOGICAL FLUIDS

III. ANALYSIS OF DISOPYRAMIDE AND ITS MONO-N-DEALKYLATED METABOLITE IN PLASMA AND URINE*

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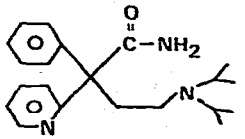
(Received July 27th, 1976)

SUMMARY

A high-pressure liquid chromatographic analysis for disopyramide (I) and its mono-N-dealkylated metabolite (II) in plasma and urine is described. The analysis, in which I and II together with an internal standard are chromatographed as ion pairs with heptanesulphonic acid, employs a simple and rapid method of sample preparation. The method is more sensitive, reproducible, and rapid than previously reported gas chromatographic methods.

INTRODUCTION

Disopyramide phosphate (I) is a new antiarrhythmic agent which is undergoing clinical evaluation. Many of the studies with this drug have been summarized in the proceedings of a recent symposium[3]. Previous methods used to estimate disopyramide in biological fluids have included a nonspecific fluorescence method which also measures the mono-N-dealkylated metabolite II[4], and two gas chromatographic (GC) methods[5,6]. One of the GC methods measures both I and II but has a complex sample preparation, including multiple extractions and evaporations, a derivatization, and separate chromatography for the analysis of I and II[5]. The other method, although more straightforward, measures only I and has not been described for the analysis



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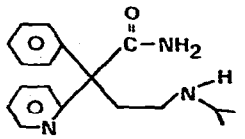

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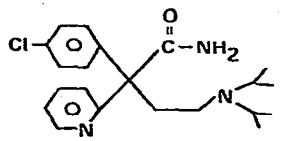

III

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* For Parts I and II of this series, see refs. 1 and 2.
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of urine samples[6]. Both of the above methods also have non-zero intercepts of their calibration curves, which limits their application at low concentrations.

The technique described in this paper allows simultaneous analysis of both I and II from plasma or urine. It is more sensitive and reproducible, as well as being simpler and more rapid than previously reported methods. The non-linearity of response at low concentrations, observed with GC methods, is also avoided.

EXPERIMENTAL

Materials

Disopyramide (I) [4-diisopropylamino-2-phenyl-2-(2-pyridyl) butyramide phosphate], its mono-N-dealkylated metabolite (II) [4-isopropylamino-2-phenyl-2-(2-pyridyl) butyramide] and the internal standard (III) [4-diisopropylamino-2-*p*-chlorophenyl-2-(2-pyridyl) butyramide] were kindly supplied by Searle Labs. (Chicago, Ill., U.S.A.). Stock solutions of I, II, and II in 0.01 *M* H_3PO_4 were stored at 4° for approximately 4 months, with no detectable decomposition. The methanol used for the chromatography was "distilled in glass" grade and was purchased from Burdick and Jackson Labs. (Muskegon, Mich., U.S.A.). The 0.005 *M* solution of heptanesulphonic acid, adjusted to about pH 3.5, was prepared from a commercial reagent purchased from Waters Assoc. (Milford, Mass., U.S.A.).

Sample preparation

A schematic outline of this procedure is given in Fig. 1. Between 0.1 and 1.0 ml of plasma or urine containing an estimated 0.05–4.0 μg of I and/or II was added to a 15-ml capacity glass tube fitted with a PTFE-lined screw-capped top, together with 200 μl of 2 *N* NaOH and 200 μl of an aqueous solution containing 2 μg of the internal standard (III). Water was added to those tubes containing less than 1 ml of plasma or urine, so that each tube contained the same volume of aqueous phase. Diethyl ether (3 ml) was than added to the tube, and the aqueous phase was extracted using gentle hand tilting for 5 min. After centrifugation to separate the two phases, the aqueous phase was frozen using an acetone–dry ice bath, and the diethyl

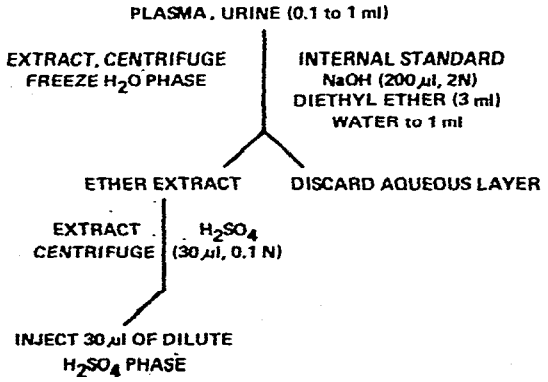

Fig. 1. Schematic outline of the sample preparation used for the analysis of I and II in plasma or urine.

ether poured into a second 15-ml tube having an elongated cone at its base of approximately 50-μl capacity, which contained 30 μl of 0.1 N H_2SO_4. The diethyl ether phase was extracted using a Vortex mixer for 1 min, and the organic and aqueous phases separated by centrifugation. All or part of the dilute sulphuric acid, sampled through the diethyl ether with a 50-μl syringe, was injected into the high-pressure liquid chromatograph.

A Varian Model 8500 gradient elution high-pressure liquid chromatograph was used with a 25 cm × 2 mm I.D. octadecylsilane reversed-phase column designed for ion-paired chromatography (Varian, Palo Alto, Calif., U.S.A.). One pump of the dual-pump chromatograph contained a 0.005 M solution of heptanesulphonic acid in methanol, while the other pump contained the same concentration of this reagent in water. The flow-rates of the pumps were adjusted to produce a total flow-rate of 60 ml/h with a solvent composition of 53% methanol. Minor adjustments in the ratio of water to methanol (1–2%) were required from time to time to produce standard retention times. The compounds of interest were detected using a Varian 254-nm fixed-wavelength UV absorption detector. Pressure was maintained in the detector to prevent the formation of bubbles by means of a 0.5-μm-porosity stainless-steel frit placed on the efferent side of the detector. The detector flow cell, and the connections leading to and from it, were thermally insulated with sponge rubber tubing, in order to minimize baseline drift resulting from refractive index changes due to thermal fluxes. The chromatographic peaks were visualized by connecting the output of the detector to a Varian A25 dual-pen recorder. In order to increase the working range of the system, one pen of the recorder was set at a 1-mV span, while the other was varied between 2 and 10 mV, depending on the expected size of the signal.

Calibration and accuracy

The method was calibrated by adding known amounts, from 0.05–4.0 μg, of I and II to plasma or urine, which were then taken through the analytical procedure. The peak height ratio of I and II to the internal standard (III) was plotted *versus* the amount of I and II added, in order to construct the calibration curves. For each particular batch of unknown samples, the method was calibrated and an estimate of accuracy was obtained by running standards of 0.1, 0.5, 1.0, 2.0, and 4.0 μg of I and II. The peak height ratio of each standard was then divided by the amount of standard added, to give the normalized peak height ratio. The average normalized peak height ratio was used to calculate the amount of I and II in the unknown samples, and the coefficient of variation was used to estimate accuracy. Similar working curves were prepared for urine, diluted 1:10 with water, in the range of 0.1–4.0 μg, equivalent to 1–40 μg/ml of original urine.

The reproducibility of the method at a given concentration was investigated by the analysis of replicates (ten samples) at concentrations of 0.5 and 2.0 μg of I and II.

The effect of sample size on the method was determined by adding 2 μg of I and II to samples containing 0.1–1.0 ml of plasma or urine, which were then assayed as previously described but without the addition of water to produce a standard volume of aqueous phase during the initial extraction. The efficiency of the extraction procedure was investigated by comparing the peak height of extracted I and II with an equal amount of I and II injected directly into the chromatograph.

Possible interference by other basic cardiovascular drugs, likely to be administered to patients concurrently with disopyramide, was investigated by chromatographing samples of these drugs under the same conditions as those used for disopyramide.

RESULTS AND DISCUSSION

Under the chromatographic conditions described in Experimental, the retention times of I, II, and III were 4.4, 2.5, and 7.8 min, respectively. Fig. 2A shows the chromatogram of the extract of 1 ml of human plasma containing 1.0 μg of I (peak b) and 1.0 μg of II (peak a), together with 2.0 μg of the internal standard (III) (peak c). Fig. 2B shows the chromatogram of the extract of the same volume of a control plasma from this subject, and Fig. 2C the chromatogram of an extract of 1 in 10 dilution of control urine. Chromatograms of control urine samples contained peaks which interered with peaks I and II. In most urine samples this interference corresponded to peaks equivalent to approximately 0.1 μg of I and II. For this reason, calibration curves in urine were constructed using diluted urine (see Experimental) in the concentration range equivalent to 1–40 μg, where this degree of interference did not seriously detract from the accuracy of the method. In no case were peaks observed in the chromatograms of control plasma or diluted control urine which

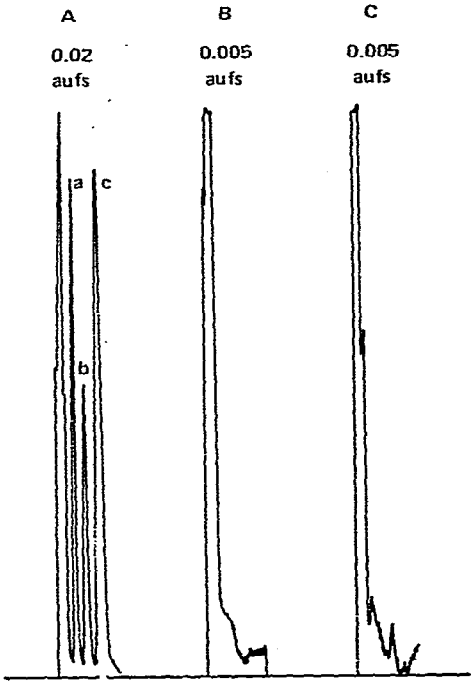

Fig. 2. (A) Chromatogram of an extract of 1 ml of plasma containing 1 μg of I (peak b), 1 μg of II (peak a), and 2 μg of III (peak c). (B) Chromatogram of an extract of control plasma. (C) Chromatogram of an extract of control urine. The detector settings used in B and C are at four times the sensitivity used for A.

interfered with those of I, II, and III, other than the small peaks seen in Fig. 2C. The chromatograms in Figs. 2B and C were obtained with the detector set at four times the sensitivity of that used in Fig. 2A.

A typical calibration curve from plasma is shown in Fig. 3. The curves for both I and II are linear in the range of 0.05–4.0 μg and pass through the origin. The coefficient of variation for the normalized peak height ratio (see Experimental) was 3.5% for I and 4.3% for II. The calibration curves for urine are also linear in the range of 1–40 μg and pass through the origin, the coefficients of variation of the normalized peak height ratio for I and II being 3.9% and 4.8%, respectively. This estimate of accuracy may be less than that which actually occurred during the routine application of the method, where sample volumes were chosen to give peak height ratios as close as possible to one, in order to increase accuracy. Analysis of repetitive samples at concentrations of 0.5 and 2.0 μg/ml had coefficients of variation, respectively, of 3.9% and 2.7% for I and 2.7% and 1.2% for II.

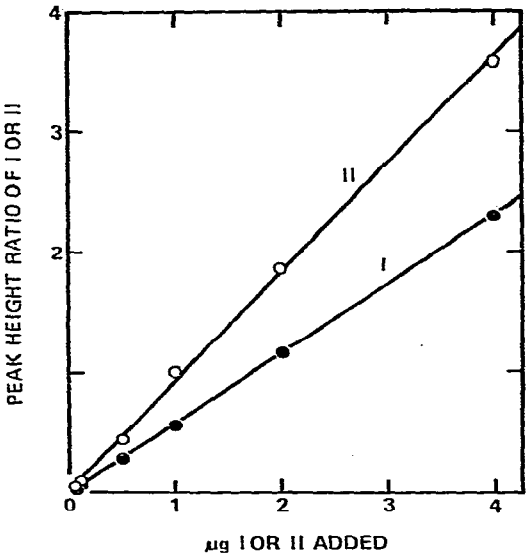

Fig. 3. Typical calibration curves for I and II. The vertical scale shows peak height ratios of I or II over the amount of III added.

During the course of these studies, it was found that increasing the size of the plasma of urine samples in the range of 0.1–1.0 ml produced a decrease in the peak height ratio of both I and II. This presumably reflects the greater lipid/water partition coefficient of the internal standard (III) compared to I and II. In order to prevent this phenomenon, water was added to those tubes containing less than 1 ml of biological fluid, so that a standard volume of aqueous phase was present in the initial diethyl ether extraction. Under these conditions, the peak height ratio of I and II had a coefficient of variation of 1.8% and 4.4%, respectively, in the range of 0.1–1.0 ml of plasma or urine, with no apparent trend in the data. Using the conditions described for the extraction, 97 ± 3% of I and 63 ± 2% of II present in biological samples were injected into the chromatograph (see Experimental).

The working range of the analysis for I and II is 0.05–4.0 μg from plasma and 1–40 μg from urine. The minimal detectable concentrations, with a signal-to-noise ratio of 5:1, of I and II correspond to 0.025 and 0.01 μg.

Under the chromatographic conditions used, procainamide, N-acetylprocainamide, lidocaine, quinidine, and propranolol had retention times of 1.9, 2.1, 2.9, 5.8, and 6.4 min, respectively. Of these commonly employed antiarrhythmic drugs, only lidocaine caused significant interference with the peaks corresponding to I, II, or III. In this case the peak due to lidocaine was sufficiently resolved from II to enable the presence of the two drugs to be recognized, but accurate quantitation of II in the presence of lidocaine is not feasible.

Reversed-phase chromatography has, in recent years, proved to be one of the most versatile alternatives for separation of compounds using high-pressure liquid chromatography (HPLC). However, the application of this technique to the chromatography of strongly basic compounds, using conventional solvents, has been limited by the stability characteristics of reversed-phase columns. In order to obtain symmetrical peaks and reasonably efficient separations with reversed-phase columns, it has proved necessary to suppress the ionization of acidic or basic compounds. While this has not been a problem with most organic acids, the chromatographic solvents required to suppress the ionization of strongly basic compounds (pH 9 and above) attack the exposed areas of the silica in such columns, resulting in rapid column deterioration.

The use of a large organic anion such as heptanesulphonic acid, and the chromatography of the bases of interest as ion pairs, results in symmetrical peaks and reasonably efficient separations under conditions (pH 3–4) which result in column stability. The ion-paired technique also offers advantages for the analysis of compounds in biological fluids, in terms of the simplicity of the sample preparation. Because the solvent injected onto the column is an aqueous solution, a simple two-stage procedure can be used to extract selectively and concentrate basic compounds at the expense of acidic and neutral compounds (see Fig. 1). This technique avoids the evaporation of organic solvents, which is time-consuming and is more likely to result in the concentration of interfering compounds than is the present technique.

A summary of the characteristics of the method reported here, compared to those of previously published chromatographic methods for the analysis of I and II, is given in Table I. A major advantage of the present method is its simplicity and rapidity. The complex extraction, evaporation, and derivatization procedures described in other methods are replaced by a simple two-stage technique which requires approximately 15 min. Both I and II are determined in a single procedure, whereas other methods required separate chromatographic conditions[5] or measured only I[6]. The time required for chromatographing the samples, as measured by the retention time of the last peak, is also shorter in the present method than for those described by other workers. It should be noted that, in order to determine both I and II by the previously described GC method, two samples must be chromatographed[5] (see Table I).

The sensitivity and reproducibility of the method reported here is better than, or comparable to, those previously reported[5,6]. The lower range of the plasma concentration curve is 1/10th of that reported using GC methods. The problem of nonlinearity of response at low concentrations of I and II, encountered with both of the

TABLE I

COMPARISON OF ANALYTICAL CHARACTERISTICS OF CHROMATOGRAPHIC METHODS FOR DISOPYRAMIDE (I) AND ITS MONO-N-DEALKYLATED METABOLITE (II) IN BIOLOGICAL FLUIDS

	Ref. 5		*Ref. 6**	*This paper*	
	I	*II*	*I*	*I*	*II*
Chromatographic method used	GC; separate analysis recommended for I and II		GC	Simultaneous ion-paired HPLC for I and II	
Sample preparation	Multiple extractions and evaporations	Multiple extractions, evaporations, and derivatization	Multiple extractions and evaporations	Double extraction (see Fig. 1)	
Plasma					
Working range, μg	0.5–5.0	0.5–5.0	0.5–10	0.05–4.0	0.05–4.0
Coefficient of variation at given concentrations ( ), μg	10.6 (0.3), 9.3 (1.18)	—	2.7 (10)	3.0 (0.5), 2.7 (2.0)	2.7 (0.5), 1.2 (2.0)
Urine					
Working range, μg	5–50**	5–50**	—	1–40**	1–40**
Coefficient of variation at given concentration ( ), μg	12.7 (1.5), 6.2 (7.5)	—	—	3.0 (average for range)	2.7 (average for range)
Linear response at low concentrations	no	no	no	yes	yes
Retention time of last peak, min	11.9	8.3	≃7	6.8	6.8

* Analysis reported for I only.

** Based on 1 ml of undiluted urine.

GC methods, was not experienced with the present HPLC procedure. Lack of linearity is presumably due to loss of I and II on the GC column, resulting from adsorption or thermal degradation, and may reflect the polar nature of these compounds. HPLC offers an attractive alternative to GC in such situations.

The reproducibility of the present method is approximately three times greater than one of the previously published methods for I[5] and comparable to a second[6] as measured by the coefficient of variation (see Table I). Direct comparisons of this sort are difficult, however, as the estimates of reproducibility were made at different concentrations for all three methods. No reproducibility data have previously been reported for II.

In summary, it is felt that the method described in this paper offers significant advantages in terms of rapidity, simplicity, sensitivity, and reproducibility over previously published methods for the determination of disopyramide and its mono-N-dealkylated metabolite in biological fluids.

ACKNOWLEDGEMENTS

We wish to thank Ms. Mary Parrish for excellent help in the preparation of

this manuscript. We also wish to thank Dr. T. C. Hutsell for the samples of disopyramide and its homologues used in the course of this study. This work was supported in part by NIH Program Project Grant No. 1-P01-HL-15833.

REFERENCES

1 T. D. Bjornsson, T. F. Blaschke and P. J. Meffin, *J. Pharm. Sci.*, in press.
2 P. J. Meffin, S. R. Harapat and D. C. Harrison, *J. Pharm. Sci.*, in press.
3 Norpace Seminar, *Angiology*, 26, Suppl., Part 2 (1975).
4 R. E. Ranney, R. R. Dean, A. Karim and F. M. Radzialowski, *Arch. Int. Pharmacodyn. Ther.*, 191 (1971) 162.
5 T. C. Hutsell and S. J. Stachelski, *J. Chromatogr.*, 106 (1975) 151.
6 A. M. J. A. Duchateau, F. W. H. M. Merkus and F. Schobben, *J. Chromatogr.*, 109 (1975) 432.