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Note

Simple high-performance thin-layer chromatography method for the determination of disopyramide and its mono-N-dealkylated metabolite in serum

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Disopyramide is an antiarrhythmic agent used in the treatment of ventricular and supraventricular arrhythmias, whose plasma therapeutic range is 1.5—8 μg/ml [1]. Of an administered dose, 46- 60% is excreted unchanged, predominantly in urine. A further 15—20% is metabolized to mono-N-dealkylated disopyramide (MND), which then also undergoes renal elimination [2]. After a single dose of disopyramide (3 mg/kg), serum concentrations of MND are usually very low (< 0.4 μg/ml) [3], but after long-term disopyramide therapy, MND concentrations amount to approximately 1 μg/ml [4]. A large increase in the ratio of MND to disopyramide concentrations has been observed in chronic renal insufficiency or in the case of simultaneous therapy with phenytoin, known as an enzyme inducer [4].

In animal experiments, MND is only slightly less active than the parent compound against supraventricular arrhythmia but is inactive against ventricular arrhythmia [5]. In addition, MND might exert a positive inotropic effect on isolated cardiac muscle [6].

Several methods for the determination of disopyramide in serum, plasma or urine have been described, which are based mainly on gas—liquid chromatography (GLC) [1, 3, 7—12] or on high-performance liquid chromatography (HPLC) [13—16]. In GLC, quantitation of MND requires derivatization of the molecule, because it usually breaks down and is eluted as three poorly separated peaks under GLC conditions suitable for disopyramide [1—3]. Simultaneous GLC determination of disopyramide and its metabolite requires a complex and tedious sample preparation; therefore, most GLC methods are not suited to measure MND concentrations [7—12]. HPLC quantitation does not require any derivatization of MND, so that sample preparation is simpler and more rapid than for GLC [13—16]. Usually, GLC and HPLC techniques require

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a relatively large serum sample (500–1000 μl). Fluorimetric assay of disopyramide is more rapid and the equipment simpler than that of GLC or HPLC, but it does not distinguish between the parent drug and the metabolite [17, 18]. Conventional thin-layer chromatography (TLC) has also been used for the determination of disopyramide and MND, but scraping of the plates and other steps make the method tedious and unsuitable for routine analysis [18]. Recently, a conventional TLC method using in situ quantitation of disopyramide and MND by fluorescence spectrophotometry has been described [19].

The method of high-performance thin-layer chromatography (HPTLC) described in this paper allows a simple, rapid, reliable and relatively inexpensive assay of disopyramide and its mono-N-dealkylated metabolite in serum, from a small sample (100 μl).

EXPERIMENTAL

Materials

Disopyramide, as disopyramide phosphate (Norpace® or SC 13957), mono-N-dealkylated disopyramide (SC 24566) and *p*-chlorodisopyramide (SC 13068) were supplied as pure compounds by Searle (Lausanne, Switzerland).

All reagents were of analytical grade and purchased from Merck (Darmstadt, G.F.R.).

All the glassware was washed with hot dichromate–sulfuric acid solution prior to use.

Equipment

Precoated HPTLC plates silica gel 60 F_{254}, 10 × 10 cm were obtained from Merck.

Linomat III, an automatic sample applicator for HPTLC plates, type 27804, HPTLC linear-developing chamber, type 28510, TLC/HPTLC Scanner, standard version, type 76500 were all from Camag (Muttenz, Switzerland).

A potentiometric recorder was connected to the 100-mV and the remote control scanner outputs.

Sample preparation

Extraction. Serum (100 μl) was placed into a 10-ml conical glass tube. Then, 100 μl of the internal standard solution (6 μg/ml of *p*-chlorodisopyramide in 0.1 *M* hydrochloric acid), 200 μl of saturated aqueous sodium carbonate solution and 1000 μl of chloroform were added. The tube was shaken vigorously for 15 sec, centrifuged for 15 min at 2000 *g*, then the organic layer was transferred into a second conical tube and centrifugation repeated. The organic phases were collected and evaporated to dryness under a stream of nitrogen.

Sample application. The dry extract was dissolved in 50 μl of chloroform and 30 μl of this solution were applied to the HPTLC plate by the Linomat as narrow, 5 mm long bands. The selected application speed was 0.25 μl/sec and the plate table speed 15 mm/sec. Usually, eighteen samples were applied to opposite sides of one plate by successive 5-mm displacement of the plate table.

Chromatography

The HPTLC plate was placed in the developing chamber, the silica layer being directed upwards; 2 ml of mobile phase (a 2% solution of concentrated ammonia solution in ethanol) were placed in each lane and development started immediately. The developing chamber was protected by a plastic hood (part of the equipment). After 25 min, both solvent fronts met and the plate was removed from the chamber and dried under a gentle stream of compressed air.

Quantitation

The HPTLC plates were inserted into the scanner for the UV absorption measurements of the bands in each track (λ = 254 nm, micro slit 3 mm). The scanning speed of the plate tracks was 0.5 mm/sec. Serum levels of disopyramide and MND were deduced from the peak height ratio to internal standard.

Calibration

Calibration graphs were obtained by adding known amounts of disopyramide and MND to blank human serum samples, which were then handled the same as patient sera. The regression lines were computed by the G02CAF program from the FORTRAN NAGLIB computer program library [20].

Reproducibility

The intra-assay reproducibility in serum was determined at three concentrations: 1.0, 4.0 and 8.0 μg/ml for disopyramide and 0.43, 1.7 and 3.4 μg/ml for MND, respectively. Eight different samples were prepared at each concentration.

Interferences

The R_F values of two typical basic compounds susceptible to interfere with the drug assay, i.e. amitriptyline and imipramine, were compared with those of disopyramide, MND and internal standard under the same extraction and chromatographic conditions.

Patients sera

Determination of serum concentrations of disopyramide and MND was performed in three patients on various preparations and dosages of disopyramide.

RESULTS AND DISCUSSION

Under the chromatographic conditions described, MND, disopyramide and the internal standard gave symmetrical, well defined peaks, the R_F values of which were 0.26, 0.46 and 0.52, respectively (Fig. 1A).

The calibration graphs were linear in the range of 0.5—10.0 μg/ml for disopyramide and 0.25—5.0 μg/ml for MND (Fig. 2). Equations of the computed regression lines were $Y = -0.005 + 0.173\ X$ and $Y = -0.011 + 0.084\ X$ for disopyramide and MND, respectively. The correlation coefficients were 1.000 and 0.998, respectively.

In preliminary assays, the silica layer was facing downwards and a counterplate was used so as to realize the conventional sandwich configuration of the

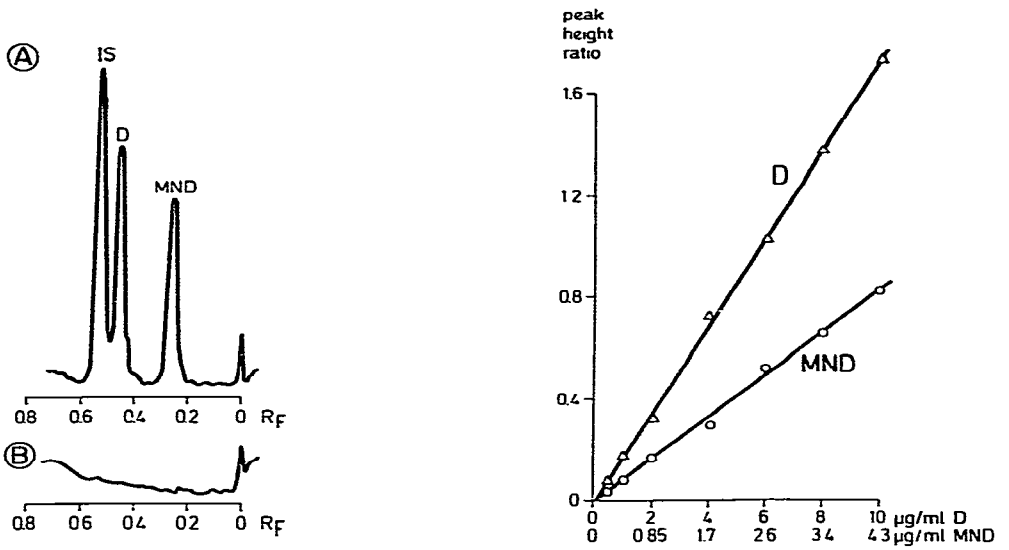

Fig. 1. (A) Typical HPTLC chromatogram from a serum sample containing mono-N-dealkylated disopyramide (MND) 1.7 µg/ml, disopyramide (D) 4.0 µg/ml and *p*-Cl-disopyramide, internal standard (IS) 6.0 µg/ml. (B) Blank serum (same extraction and chromatographic conditions as in A).

Fig. 2. Calibration graph for the determination of disopyramide (D) and its mono-N-dealkylated metabolite (MND) in serum. The peak height ratios of D or MND to internal standard are plotted against the serum concentrations of D or MND, respectively.

developing chamber. The development of the HPTLC plates yielded poor results, due to an incurvation of the chromatographic bands. The scanning of such bands revealed peak broadening and overlapping. Experimental conditions were modified in such a manner as to obtain straight bands perpendicular to the mobile phase flow. These experimental conditions were realized by changing the conventional operation of the developing chamber, that is by developing the HPTLC plate with the silica layer directed upwards. Under these conditions only, the peaks were clear-cut, reproducible and without overlapping.

From the data shown in Table I it may be seen that intra-assay coefficients of variation were in the range 2.4—7.8% for disopyramide and 7.8—17.7% for MND; this reproducibility is comparable with that of the GLC [1, 3, 7—12], HPLC [13—16] and TLC [19] methods. The coefficient of variation for MND is relatively large at the low serum concentrations of the metabolite. At high serum concentrations of MND, a situation that has been observed in some patients [4] and may be of clinical significance, the coefficient of variation is reasonably good.

No serum components, even if extracted simultaneously with disopyramide, interfered with the assay (Fig. 1B). The R_F values of amitriptyline and imipramine, two basic drugs that were co-extracted with disopyramide, were close to unity.

In the three patients whose relevant clinical data are listed in Table II, significant levels of disopyramide were measured in each case. The relatively low

TABLE I

INTRA-ASSAY COEFFICIENTS OF VARIATION OF HPTLC DETERMINATION OF DISOPYRAMIDE AND ITS MONO-N-DEALKYLATED METABOLITE (MND) IN SERUM

Eight samples were prepared at each concentration.

Compound	Serum concentration (μg/ml)		Coefficient of variation (%)
	Expected	Assayed	
Disopyramide	1.0	0.86	7.8
	4.0	4.2	2.4
	8.0	7.9	4.0
MND	0.43	0.33	17.7
	1.7	1.9	13.1
	3.4	3.4	7.8

TABLE II

CLINICAL CHARACTERISTICS AND SERUM CONCENTRATION OF CREATININE, DISOPYRAMIDE AND ITS MAIN METABOLITE IN PATIENTS ON THERAPY

	Subject		
	U.P.	L.B.	O.M.
Age (years)	48	73	76
Sex	M	M	F
Weight (kg)	70	73	51
Indication for therapy	VPB* after myocardial infarction	Sick sinus syndrome	Paroxysmal atrial tachycardia
Preparation and dosage**	Norpace® 150 mg twice each day	Rythmodan® 100 mg/day	Norpace® 100 mg three times each day
Duration of therapy	9 days	1 week	2.5 weeks
Concomitant diseases	Coronary heart disease Hyperthyroidism	Hypertension	Cerebral thrombosis
Concurrent medications	Isosorbide dinitrate Oxprenolol Bromazepam	Digoxin Debrisoquine Tienilic acid Cloxacillin Flunitrazepam	Digoxin Oxazepam
Serum creatinine (μmol/l)	94	147	71
Creatinine clearance (ml/min)		61	
Disopyramide serum concentration (μg/ml)	1.3	2.2	1.7
MND metabolite serum concentration (μg/ml)	—	—	0.5

*VPB = ventricular premature beats.
**Norpace® = disopyramide phosphate; Rythmodan® = disopyramide.

values obtained are consistent with the low disopyramide dosage they were receiving [1, 21]. A detectable concentration of MND was found in only one case, an elderly woman with the longest duration of therapy and with normal renal function.

In view of these data, the HPTLC method described here is suitable for routine analyses of disopyramide and MND. In comparison with a conventional TLC method such as that developed by Gupta et al. [19], it differs by the following features.

(1) The use of high-performance layers in conjunction with the band application technique contributes to achieve a high resolution and sensitivity and makes a UV spectrophotometric determination possible even with very small samples. A first advantage associated with UV spectrophotometry is that it does not require the step of dipping the plate into sulfuric acid and waiting for the appearance of fluorescence. The UV response remains constant in the course of time. In these conditions, the plates may be scanned again with the same accuracy. Moreover, there is a second advantage, in the sense that there is no difficulty in using an internal standard, such as *p*-chlorodisopyramide, that has an intense fluorescence, much greater than that of disopyramide or MND. The conditions described offer a good separation of *p*-chlorodisopyramide from disopyramide and MND.

(2) The use of chloroform instead of benzene allows a rapid application by bands, under safer conditions and without heating.

(3) Finally, the use of plates with fluorescent indicator contributes to easily optimizing the positioning of the plate in the scanner and to ensure constant chromatographic conditions from plate to plate.

CONCLUSION

The method described in this paper may prove suitable for the routine serum level determination of disopyramide and its mono-N-dealkylated metabolite, firstly, because it is rapid, simple, reliable and relatively inexpensive, and secondly because the serum sample is very small (100 μl). This assay requires only a single serum extraction and eighteen samples may be applied semi-automatically on the same HPTLC plate. Another advantage is the use of a new chromatographic support after every 18 samples, avoiding those problems related to column ageing and clogging encountered with the other methods. The low plate cost per sample analysed may rapidly compensate for the initial investment in the HPTLC scanner.

The possibility of simultaneously determining the concentration of disopyramide and its mono-N-dealkylated metabolite makes this method especially useful for monitoring disopyramide therapy in patients with impaired renal function. In addition, it may also prove to be a useful tool for the detection of those other clinical situations where there is a high concentration of metabolite, such as in hepatic microsomal enzyme induction.

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