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SIMPLE GAS–LIQUID CHROMATOGRAPHIC METHOD FOR THE MEASUREMENT OF DISOPYRAMIDE IN BLOOD-PLASMA OR SERUM AND IN URINE

ANGELA M. HAYLER and ROBERT J. FLANAGAN
Poisons Unit, Guy's Hospital, London, SE1 (Great Britain)
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SUMMARY

A simple method has been developed for the measurement of disopyramide in blood-plasma or serum at the concentrations attained during therapy. A relatively small (200 μl) sample volume is made basic and extracted with 50 μl of chloroform containing an internal standard, and the extract is analysed directly by gas–liquid chromatography with flame-ionization detection. The instrument calibration is linear and passes through the origin of the graph. Neither solvent transfer nor evaporation steps are used in the extraction procedure, which takes less than 3 min to complete, and urine specimens may be analysed by an analogous technique. No interference from either endogenous sample constituents or other drugs has been observed, although a simple back-extraction procedure is described which eliminates potential interference from a small number of basic and neutral drugs.

INTRODUCTION

Disopyramide is reported to suppress ventricular arrhythmias in patients who have suffered acute myocardial infarction[1], and it may prove of value in the prophylactic treatment of such patients. Plasma drug concentrations between 2.8 and 7.5 mg/l are thought to be required for optimal clinical effect, but adverse reactions may occur at concentrations greater than 3.6 mg/l[2]. The apparent ineffectiveness of disopyramide in trials[3,4] in which the plasma drug concentrations attained were not monitored may have been due to inadequate dosage.

The spectrophotofluorimetric assay[5] for plasma disopyramide does not differentiate between the drug and its mono-*N*-dealkylated metabolite (MND), and variable "blank" values are produced by some specimens[6]. Of the published gas–liquid chromatographic (GLC) methods[6–8], all incorporate solvent evaporation steps, and that of Hutsell and Stachelski[6] required prolonged extraction times and an extensive extract purification procedure. Moreover, the calibration graphs obtained had non-zero intercepts, indicating that either adsorption or decomposition of the drug had occurred on-column. This latter problem has been avoided by use of liquid chromatography (LC)[9], although the extraction procedure used in this method was still

relatively long. Disopyramide and an internal standard were extracted into diethyl ether at an alkaline pH and subsequently back-extracted into dilute acid. In the LC method this extract was analysed directly using ion-paired chromatography on an octadecylsilane reversed-phase column. However, we have found that a simple re-extraction into a small volume of chloroform followed by the direct analysis of this latter extract using GLC with flame ionization detection can be easily performed. On-column decomposition of disopyramide has been prevented by prior treatment of the column with γ-glycidoxypropyltrimethoxysilane, and thus the instrument calibration obtained was linear with zero intercept.

Subsequently, it was found that the extraction procedure could be simplified considerably with no loss of sensitivity. Thus, a 200 μl volume of plasma or serum was made basic, extracted with 50 μl of chloroform containing the internal standard and analysed directly. Urinary disopyramide concentrations, which are approximately 10-fold higher than those found in plasma[9], may be measured by a similar technique, but only a 1:1 ratio of sample to solvent is required. No interference has been observed with either type of extraction procedure, but the capacity to perform the back-extraction procedure was retained in view of potential interference from a small number of basic or neutral drugs.

EXPERIMENTAL

Materials and reagents

Disopyramide free base (4-diisopropylamino-2-phenyl-2-(2-pyridyl)butyramide), an aqueous solution of disopyramide phosphate (equivalent to 200 mg/l disopyramide base), its MND; 4-isopropylamino-2-phenyl-2-(2-pyridyl) butyramide) and the internal standard, *p*-chlorodisopyramide (CDP; 4-diisopropylamino-2-*p*-chlorophenyl-2-(2-pyridyl)butyramide) (200 g/l in methanol) were all supplied by Roussel Laboratories, Wembley, Great Britain. The aqueous disopyramide solution was used to prepare a "quality control" sample in heparinized human plasma at a concentration of 4.0 mg/l. γ-Glycidoxypropyltrimethoxysilane (A-187) was supplied by Union Carbide U.K., Southampton, Great Britain. Diethyl ether, chloroform, sodium hydroxide, sulphuric acid and tris(hydroxymethyl)aminomethane (tris) were all analytical-reagent grade; the last three compounds were used as 2.0, 0.05 and 2.0 mole/l aqueous solutions, respectively.

Gas–liquid chromatography

A Pye Series 204 gas chromatograph equipped with a flame ionization detector and linked to a 10 mV recorder was used. Integration of peak areas was performed using a Hewlett-Packard 3352 data system. The column and detector oven temperatures were 240° and 300°, respectively; injection block heaters were not employed. The nitrogen (carrier gas) flow-rate was 40 ml/min, and the flame was supplied by air and hydrogen at inlet pressures of 16 and 21 p.s.i., respectively, giving flow-rates of approximately 440 and 40 ml/min.

A coiled glass column (1.5 m × 4 mm I.D.) was silanized by immersion in 5% dichlorodimethylsilane in toluene for 1 h, rinsed in methanol and dried at 100°. The column was packed with 3% OV-1 on 80–100 mesh Supelcoport, purchased ready-prepared from Chromatography Services, Merseyside, Great Britain. The

packed column was conditioned at 300° with a nitrogen flow of 40 ml/min for 15 h, and was treated subsequently by injection of from 10 to 20 μl of A-187. Thereafter, occasional injections of 5–10 μl of this compound were performed to maintain the column in the deactivated form.

The retention times of disopyramide and of some other compounds on this system measured relative to CDP are given in Table I. The chromatography of a chloroform solution containing both disopyramide and CDP is illustrated in Fig. 1.

TABLE I

RETENTION DATA OF DISOPYRAMIDE AND SOME OTHER COMPOUNDS ON THE OV-1 COLUMN SYSTEM

Compound	*Retention time (relative to CDP)*
Procainamide*	0.27–0.31
MND**	0.48
Diazepam	0.48
Dipipanone	0.56
Chlorprothixene	0.58
Chlorpromazine	0.58
Methixine	0.60
Disopyramide	0.62
Methotrimeprazine	0.64
Pecazine	0.64
Trimethoprim*	0.67–0.80
N-Acetylprocainamide*	0.76–0.80
Chloroquine	0.80
Metoclopramide	0.89
Trifluoperazine	0.95
Acepromazine	0.98
CDP	1.00
Phenazocine	1.04
Propiomazine	1.04
Quinine	1.24
Quinidine	1.26

* Positively skewed peak —retention times measured on analysis of 5 and 0.05 μg, respectively.
** Principal peak; other compounds eluted at relative retention times of 0.26 and 0.30 (*cf.* Fig. 5).

Extraction procedures

Direct extraction of plasma or serum. Sample (200 μl), tris solution (20 μl) and internal standard solution (50 μl of 20 mg/l CDP in chloroform) were added to a clean Dreyer tube (Poulten, Selfe and Lee, Wickford, Great Britain). The last two additions were performed using Hamilton repeating mechanisms fitted with 1.0 ml and 2.5 ml Hamilton gas-tight luer fitting glass syringes, respectively (Field Instruments, Richmond, Great Britain). Everett stainless-steel needles (No. II serum) were affixed to these syringes.

The contents of the tube were mixed thoroughly on a vortex mixer for 30 sec and the tube was centrifuged for 2 min at 9950 *g* in an Eppendorf centrifuge 5412 (obtained from Anderman and Co., East Molesey, Great Britain, and modified to accept Dreyer tubes by slight drilling-out of the 0.4 ml test tube centrifuge adaptors).

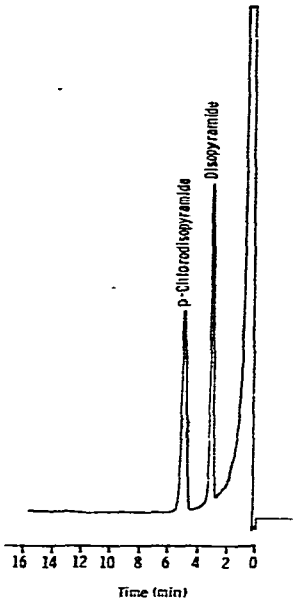


Fig. 1. Chromatogram obtained on analysis of a solution of disopyramide (16 mg/l) and CDP (20 mg/l) in chloroform; 3-μl injection.

Subsequently, a 1–5 μl-portion of the chloroform phase was obtained and injected on to the column of the gas chromatograph. This latter portion was obtained by taking 5 μl of air into a gas-chromatographic syringe and passing the syringe needle through the basic layer into the chloroform. The air was expelled, and 1–5 μl of the extract were taken up for injection.

The extraction was performed in duplicate and the mean result was taken. If the difference between the duplicates was greater than 10%, both the extractions and the analysis were repeated.

Direct extraction of urine. The procedure was identical to that described above except that (i) 50 μl of sample were taken, and (ii) 50 mg/l CDP in chloroform was used as the extraction solvent.

Back-extraction of plasma or serum. Sample (500 μl), sodium hydroxide solution (100 μl) and diethyl ether (5 ml) were added to a 10 ml tapered glass tube. Subsequently, 50 μl of the internal standard solution (50 mg/l aqueous CDP) were added using a Hamilton repeating mechanism. The tube was sealed using a ground-glass stopper, the contents were vortex-mixed for 20 sec and the tube was then centrifuged in a windshielded instrument at 1800 *g* for 4 min. The ether layer was transferred by aspiration to a second tapered tube containing sulphuric acid (500 μl), and the contents of this tube were similarly vortex-mixed and centrifuged. Subsequently, the organic layer was removed by aspiration and any residual solvent eliminated under a stream of air. The acidic solution was made basic with sodium hydroxide solution (300 μl), and chloroform (50 μl) was added. After vortex mixing and centrifugation at 1250 *g* for 4 min, a portion of the chloroform extract was injected on to the column of the gas chromatograph. This portion was obtained by an analogous method to

that described for direct-extraction analyses. Plasma samples were assayed in duplicate and the mean results taken.

Instrument calibration and calculation of results

In the case of the direct-extraction analyses, standard solutions containing both disopyramide and CDP were prepared in chloroform and were used to obtain calibration graphs of peak area ratio (disopyramide/internal standard) against disopyramide concentration. The sample disopyramide concentration was calculated from the peak area ratio obtained on analysis of the extract and by the use of a previously calculated recovery factor. In contrast, solutions prepared in heparinized human plasma and containing disopyramide at a range of concentrations were analysed by the back-extraction procedure, together with each batch of specimens, and the sample drug concentration was obtained directly from the calibration graph.

The "quality control" specimen containing disopyramide (4.0 mg/l), which was obtained from an independent source of the drug, was analysed along with each batch of plasma specimens. If a mean result was obtained which differed by more than $\pm 5\%$ from the true value, the batch of analyses was repeated.

Direct extraction: plasma or serum analyses. Standard solutions containing disopyramide at concentrations of 2.0, 4.0, 6.0, 8.0, 12.0, 16.0, 24.0 and 32.0 mg/l were prepared by dilution of a 1 g/l solution of the drug in chloroform. (*N.B.* These concentrations were four times higher than the equivalent sample concentrations since a sample–solvent ratio of 4:1 was used in the extraction). Each standard solution also contained CDP (20 mg/l) which was obtained from a similar stock source. A linear calibration graph with zero intercept was obtained on analysis of these solutions (Fig. 2); the calibration gradient (peak area ratio/plasma drug concentration) normally obtained was 0.24 l/mg. (*N.B.* Analogous graphs were obtained from the other sets of standard solutions). The results of sample analyses were multiplied by a factor of 1.13 to compensate for the incomplete extraction of the drug.

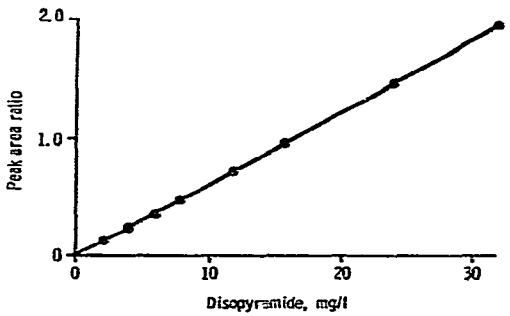


Fig. 2. Calibration graph obtained on analysis of the standard disopyramide solutions in chloroform used in conjunction with the direct extraction of plasma specimens.

Direct extraction: urinary analyses. The instrument calibration procedure was identical to that given above except that the range of disopyramide solutions ran from 20 to 100 mg/l, in increments of 20 mg/l, and each contained CDP at a concentration of 50 mg/l. The calibration gradient normally obtained was 0.023 l/mg, and the results of sample analyses were multiplied by a factor of 1.12.

Back-extraction procedure: plasma or serum analyses. Standard solutions of disopyramide at concentrations of 0.5, 1.0, 3.0, 5.0 and 8.0 mg/l were prepared in heparinized human plasma by dilution of an aqueous solution (1 g/l) of disopyramide free base. The calibration gradient normally obtained on analysis of these solutions was 0.28 l/mg.

RESULTS AND DISCUSSION

Column deactivation

The relationship between the disopyramide concentration in the chloroform standards and the peak area ratio of drug to internal standard was non-linear, especially at low drug concentrations, if the column was not treated with A-187. Moreover, the chromatogram obtained using an untreated column on analysis of a 1 g/l solution of disopyramide in chloroform showed clear evidence of on-column decomposition of the drug; the baseline rose before the elution of the disopyramide, *i.e.* a negatively skewed peak was obtained. Column treatment with A-187 to prevent the degradation of compounds such as disopyramide during GLC analysis has been discussed by Averill[10]. This silane is used to promote the adhesion of organic materials to inorganic substrates, and it may act by promoting a relatively complete stationary phase coating of the support material, thus masking catalytically active sites.

Column treatment with a silylating agent (Rejuv-8, obtained from Chromatography Services) did not noticeably reduce the on-column decomposition of disopyramide, although the injection of 5–10 μl portions of a 5 g/l solution of DL-α-phosphatidylcholine dipalmitoyl (Sigma London, Kingston-upon-Thames, Great Britain) in chloroform was partially effective. However, daily injections of 5–10 μl of this solution were required to maintain the column in the deactivated form, and since relatively large peaks were obtained for up to 1 h after treatment, this was considered unsatisfactory. Although peaks were normally obtained following column treatment with A-187, such treatment was only required infrequently (at most, weekly with a relatively new column, and monthly thereafter). The column was maintained at 140° with nitrogen flow of 40 ml/min when not in use, in order to minimize the need for further treatment.

Recovery studies

Detailed recovery studies with the back-extraction procedure were not necessary since standard disopyramide solutions prepared in heparinized human plasma were analysed together with each batch of samples, and were used to provide the calibration graph. However, in the case of the direct-extraction analyses, recovery factors were calculated in order to facilitate calibration using standard solutions prepared in chloroform, with a consequent reduction in the total analysis time.

Plasma analyses. The calibration standards used here contained disopyramide at a concentration four-fold higher than in the corresponding plasma solutions since a sample: solvent ratio of 4:1 was used in the extraction procedure. Thus, solutions containing disopyramide at concentrations of 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0 and 8.0 mg/l were prepared in heparinized bovine plasma by dilution of a 1 g/l aqueous solution of the drug. The quintuplicate analysis of each of these solutions revealed a mean recovery of 88.5 $\pm$ 5.3 (SD)% which was uniform over the range of concentra-

tions studied. The results of sample analyses were therefore multiplied by a factor of 1.13.

In order to assess the validity of this factor when applied to human plasma assays, 50 specimens obtained from patients treated with disopyramide were analysed in duplicate by the direct- and back-extraction procedures. There was a good correlation ($r = 0.95$) between the results given by each method, and the mean of all of the analyses obtained using the back-extraction (3.87 $\pm$ 1.41 (S.D) mg/l) was the same as that obtained from the direct extraction (3.87 $\pm$ 1.38 (S.D) mg/l).

Urinary analyses. In this case, the calibration standards contained disopyramide at concentrations equivalent to the corresponding urinary solutions since a sample–solvent ratio of only 1:1 was required in the extraction. Thus, solutions containing disopyramide at concentrations of 20–100 mg/l, in increments of 20 mg/l, were prepared in drug-free human urine. The triplicate analysis of these solutions revealed a mean recovery of 89 $\pm$ 4 (S.D)%, which was uniform over the range studied; the results of sample analyses were therefore multiplied by a factor of 1.12.

Assay reproducibility

The coefficient of variation (C_v) of the plasma direct-extraction procedure assessed from the difference between duplicates of 50 sample analyses was 3.8% in the range 1.4–7.0 mg/l. The intra-assay C_v at 4.0 mg/l was 2.2% ($n = 20$).

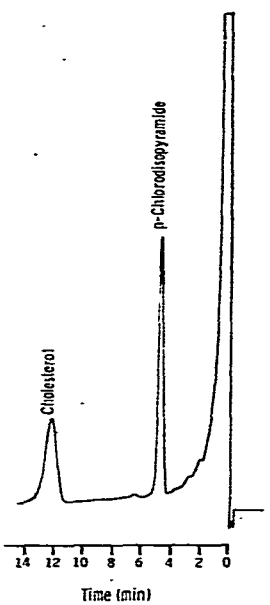
The C_v of the back-extraction procedure assessed from the difference between duplicates of 50 analyses was 4.0% in the range 1.2–7.5 mg/l. The intra-assay C_v of this procedure at 3.0 mg/l was 4.1% ($n = 10$). The inter-assay C_v at 2.6 mg/l was 3.2% ($n = 15$) and at 4.0 mg/l was 3.0% ($n = 22$). The similarity of these results to those obtained with the LC disopyramide assay[9] was not unexpected in view of the similarities between the extraction procedures used in each method.

Specificity

No interference has been observed in either direct- or back-extracts of drug-free heparinized human plasma, or in direct extracts of drug-free human urine, and an example of such an analysis is given in Fig. 3. Analogous extractions performed without the addition of CDP have not revealed the presence of compounds that could elute with this standard. In addition, specimens of either plasma or urine obtained from patients treated with disopyramide have shown a similar absence of interference in both direct- and back-extraction analyses (Figs. 4–6). Again, no compounds have been observed which could elute with CDP.

The major metabolite of disopyramide (MND)[11] is reported[6] to be unstable under similar GLC conditions to those used here. Indeed, three compounds eluting before disopyramide were represented on the chromatogram obtained on analysis of a 1 g/l solution of MND in chloroform, and an analogous pattern of peaks has been observed on analysis of urine specimens obtained from patients treated with disopyramide (*cf.* Fig. 5). Since disopyramide is excreted largely unchanged in the urine of healthy subjects[11], and MND is reported to be not only inactive against ventricular arrhythmias but also less active than disopyramide against atrial arrhythmias[12], the plasma or urinary assay of MND appears unlikely to be of clinical value.

A number of basic and neutral drugs were investigated as possible sources of interference. Mexiletine and lignocaine both eluted with the solvent under the GLC



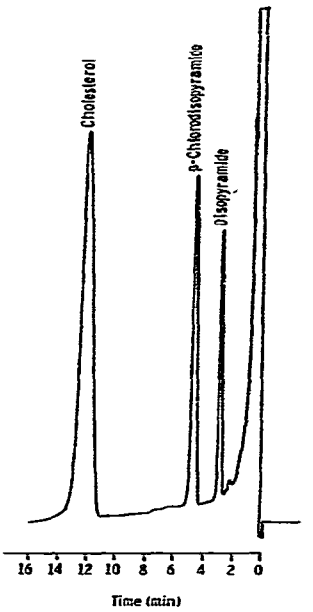
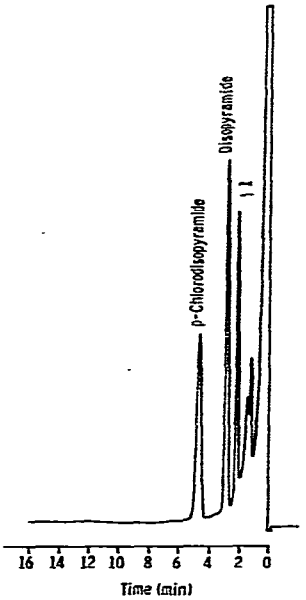


Fig. 3. Chromatogram obtained on analysis of a direct extraction of drug-free human plasma; 4-μl injection. The CDP concentration was 20 mg/l.

Fig. 4. Chromatogram obtained on analysis of a direct extract of plasma obtained immediately prior to dosage from a patient treated with disopyramide (200 mg, 8 hourly); 4-μl injection. The CDP concentration was 20 mg/l, and the plasma disopyramide concentration was found to be 2.3 mg/l.



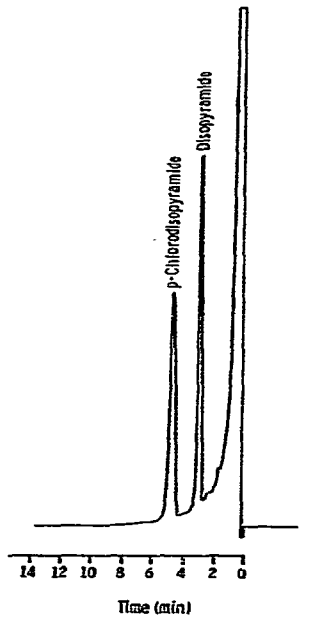


Fig. 5. Chromatogram obtained on analysis of a direct extract of urine obtained from a patient treated with disopyramide (100 mg, 6 hourly); 2-μl injection. The CDP concentration was 50 mg/l and the urinary disopyramide concentration was found to be 52 mg/l. (1 = Degradation products of MND.)

Fig. 6. Chromatogram obtained on analysis of a back-extract of plasma obtained 3 h after dosage from a patient treated with disopyramide (100 mg, 6 hourly); 3-μl injection. The plasma disopyramide concentration was found to be 3.3 mg/l.

conditions used, and the retention times of the remaining compounds measured relative to the retention time of CDP are given in Table I. None of the compounds likely to be present in the plasma of patients receiving disopyramide (*i.e.* procainamide, *N*-acetylprocainamide, diazepam, trimethoprim, metoclopramide and quinidine) interfered in the assay. Most of the remaining drugs are administered at low dosage (less than 100 mg/day) or are extensively metabolized and are thus unlikely to interfere in either the direct- or back-extracts of plasma. Nevertheless, the capacity to perform the back-extraction was retained in order to counter possible interference from compounds not surveyed in the present work.

Limits of sensitivity

The limit of sensitivity of the direct-extraction plasma assay was taken to be 0.2 mg/l if 200 μl of sample were used. Although the use of a larger sample volume could produce a corresponding decrease in the limit of sensitivity of the assay, this was not thought to be necessary in view of the plasma disopyramide concentrations associated with effective therapy[2]. The use of relatively small volumes of plasma is especially useful where further analyses are to be performed using the same specimen or where sample size may be limited. The back-extraction plasma assay has a similar limit, but 500 μl of sample are required. Since the urinary disopyramide concentrations attained during therapy are approximately 10-fold higher than in plasma, the calibration range and limit of sensitivity of the direct-extract urinary assay were correspondingly higher, and this minimized the need for dilution of the specimen prior to analysis. However, increased sensitivity could easily be obtained if required by adopting the assay procedure used for plasma.

Choice of direct-extraction conditions

Initially, the conditions used in the direct-extraction procedure were similar to those used prior to back-extraction in that 2 mole/l sodium hydroxide was employed. However, emulsions were obtained on analysis of some plasma specimens under these conditions. No emulsions were obtained following the use of 2 mole/l tris in place of the sodium hydroxide solution, whilst the recovery of disopyramide was unchanged. The pH of a mixture of 1 volume of tris solution and 10 volumes of plasma was found to be 9.4.

An extraction time of 30 sec was chosen on the basis of results obtained with other direct-extraction drug assay procedures performed using Dreyer tubes[13–15].

Advantages of the direct-extraction procedures

Disopyramide has an elimination half-life of *ca.* 7 h in patients who have suffered a myocardial infarct[16]. A plasma disopyramide concentration may be accurately measured using the direct-extraction procedure within *ca.* 20 min of receipt of the specimen, provided that the instrument calibration has been accomplished previously, and thus the result may be made available with sufficient speed to have relevance to therapy. In contrast, an analysis using the back-extraction procedure takes *ca.* 40 min to complete. The direct-extract analysis of urine also minimizes the time required for the assay, especially since previous methods[6,9] advocated the dilution of the urine specimen prior to disopyramide measurement by the same procedure as for plasma.

In addition to the rapidity and small sample requirement of direct-extract drug assays, there are other advantages to the use of such procedures. These have been discussed in detail elsewhere[13–15], and include the requirement of minimal apparatus and reagents, the absence of interference derived from solvent transfer and evaporation stages or from inadequately cleaned glassware and the good accuracy and reproducibility of the techniques.

A disadvantage to the direct-extraction procedure was that cholesterol was represented on all of the chromatograms of plasma or serum extracts (*cf.* Figs. 3 and 4). Although this compound did not interfere in the analysis, its presence did reduce the rate at which these analyses could be performed. Nevertheless, the advantages of the direct-extraction procedure outweighed this latter consideration. The use of nitrogen-selective detection, or indeed LC analysis, in place of flame ionization could serve to prevent the detection of cholesterol should this be required.

CONCLUSIONS

The direct-extraction procedures described represent improvements over previously published methods for the measurement of either plasma or urinary disopyramide at the concentrations achieved during therapy. A complete quantitative analysis can be performed, in duplicate, within 20 min and with the use of a relatively small sample volume. The extraction may be completed in less than 3 min and is performed in a single tube, and thus solvent transfer and evaporation stages are not required. No interference from endogenous sample constituents, other drugs or drug metabolites has been observed.

It has proved possible to use an analogous direct-extraction procedure to that described here in the measurement by GLC of two other antiarrhythmic drugs, mexiletine and lignocaine, at the plasma concentrations attained during therapy (D. W. Holt, A. M. Hayler, M. Loizou and R. J. Flanagan, unpublished results). In addition, preliminary results suggest that the method described here may prove to be applicable to the measurement of the plasma concentrations of not only disopyramide but also procainamide, *N*-acetylprocainamide and quinidine which are achieved during therapy.

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