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THERAPEUTIC DRUG MONITORING OF FLECAINIDE USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND COMPARISON WITH FLUORESCENCE POLARISATION IMMUNOASSAY

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SUMMARY

The therapeutic monitoring of flecainide, like the other antiarrhythmic agents, has become an integral part of the administration of this type of drug. A full description is given here of a high-performance liquid chromatographic method which has been developed and used over the past three years for the routine monitoring of flecainide. This method uses extraction into toluene using the non-fluorinated analogue of flecainide as an internal standard and then direct back-extraction into a small volume of aqueous acid without the need for solvent evaporation. A fluorescence detection instrument is chosen which avoids the use of high-cost fluorometers. Ample sensitivity is accomplished with a simple fluorometer by selecting a combination of Schott bandpass filters with high-transmittance characteristics at appropriate wavelengths. A comparison is made between this method and the newly introduced fluorescence polarisation immunoassay. The comparison shows a good agreement between the independent technologies.

INTRODUCTION

Flecainide acetate (Tambocor) is a class Ic antiarrhythmic agent that has gained acceptance in the suppression and prevention of a variety of ventricular and supraventricular tachycardias [1]. Since its introduction into clinical use in this country, therapeutic monitoring of flecainide levels has been offered by our department on a routine basis to both hospitalised and outpatients in order to optimise their treatment.

The most popular technique for flecainide measurements has to date been high-performance liquid chromatography (HPLC) which has been the subject of a few recent publications [2–11]. We have relied upon HPLC in the past for our routine monitoring. The method we developed differed in a few respects from those published previously and so details are given in this text. More recently an interest

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has centred on the development of fluorescence polarisation immunoassay [12] and this has culminated in the introduction of a procedure by Abbott Labs. for use with their TDX analysers. These are fully automated instruments which are capable of batch analysis of a variety of drugs, flecainide being the most recent. Kit sets are available for each drug which are supplied ready to use. The tests can be done with push button ease at a faster turnover than is possible with HPLC which obviously suits the clinical environment. The ready availability of the TDX equipment and its ease of use in clinical laboratories will mean that peripheral laboratories will be able to perform the assay and relieve the burden on the centralised facilities which previously relied on HPLC for this assay. It is therefore desirable to compare the HPLC and TDX methods to see if they are compatible. It is the purpose of this paper to describe the HPLC method which has been in use at this laboratory for over three years and compare it with the new fluorescence polarisation technique.

EXPERIMENTAL

High-performance liquid chromatography

Apparatus. Chromatography was performed using a Waters Assoc. Model 510 pump, a Rheodyne Model 7125 syringe loading injector, an LDC Fluoromonitor III with a Nippo GL 4 lamp, a Schott UG 11 excitation filter and a Schott GG 395 emission filter (both 24 mm × 3 mm thick) at gain 10. The bandpass Schott UG 11 filter has a maximum transmittance of 92% at 320 nm and a bandwidth at 50% transmittance from 270 to 375 nm. The high-pass Schott GG 395 filter has a half maximal transmittance at 395 nm. Recordings were made on an Omniscribe strip chart recorder (Houston Instruments, Houston, TX, U.S.A.) at 10 mV full scale and 0.5 cm/min.

The column used was a Waters Assoc. Novapak C_{18} Radial Pak cartridge (4 μm particle size, spherical, end-capped) in a Z-Module radial compression system.

The mobile phase was a 60:40 mixture of 0.1 *M* disodium hydrogenphosphate–acetonitrile at pH 3.0. The flow-rate was 3 ml/min. The column was washed and stored in 50% aqueous methanol between batches.

The borosilicate glassware used for sample preparation consisted of 150 mm × 15 mm screw-capped test tubes and 130 mm × 15 mm screw-capped centrifuge tubes, both supplied by Kimax (Montelo, NJ, U.S.A.). The screw-caps were PTFE-lined. All glassware was washed by soaking in non-ionic Decon 90 detergent (BDH, Poole, U.K.) followed by multiple washings, rinsings and a final wash with a 2% solution of diethylamine in methanol.

Reagents. All reagents were of analytical grade. Acetonitrile, toluene and methanol were purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.), phosphoric acid from May and Baker (Dagenham, U.K.) and diethylamine from BDH. Flecainide acetate (Tambocor) and the internal standard, 2,5-biethoxy-N-(2-piperidylmethyl)benzamide (S-15277), were kindly donated by Riker Labs. (Loughborough, U.K.). The internal standard was supplied as the free base.

Standards. A 10^{-3} *M* stock standard solution of flecainide was prepared by

dissolving 47.6 mg flecainide acetate in 100 ml methanol. This was indefinitely stable when stored tightly sealed at −20°C. A 10^{-5} *M* working standard solution of flecainide was prepared by diluting 1 ml stock standard in 100 ml water. This was stored at 4°C and replaced every six months.

No commercial controls were available. To prepare control serum samples to be run with each batch of samples, 200, 100 and 50 μl working flecainide standard solutions were made up to 1 ml with blank serum to give concentrations of 2000, 1000 and 500 nmol/l, respectively. These were then treated identically to samples.

A stock solution of internal standard was prepared by dissolving 32 mg in 100 ml methanol. This was indefinitely stable when stored tightly sealed at −20°C. A working internal standard solution was prepared by diluting 1 ml stock internal standard into 100 ml water. This was stored at 4°C and replaced every six months.

Sample preparation. Samples (1 ml) or controls (see previous section) were placed into 150 mm × 15 mm screw-cap test tubes. A 200-μl volume of 1 *M* sodium hydroxide was added together with 20 μl of working internal standard. Following the addition of 5 ml toluene, the tubes were stoppered and mixed by rotation for 10 min. They were then centrifuged at about 600 *g* for 5 min and the top layer was transferred by pipette to a 130 mm × 10 mm screw-cap centrifuge tube containing 200 μl of 10 *M* phosphoric acid. The tubes were vigorously mixed by vortexing for 1 min and then briefly centrifuged. The top layer was aspirated to waste by suction and 100 μl of the lower layer were injected into the chromatograph.

Fluorescence polarisation immunoassay

Analyses were performed on an Abbott TDX analyser (Abbott Labs., Diagnostic Division, Irving, TX, U.S.A.) according to the manufacturer's instructions. Early analyses were done with the instrument in pipetting mode 1 but on advise from the technical service division of the company all subsequent testing was done in pipetting mode 4.

Sample collection

All blood samples were collected in plain Vacutainer tubes. No special handling procedures were involved apart from the normal consideration given to non-urgent specimens arriving at this laboratory. They were separated on receipt and the serum was subsequently stored frozen awaiting analysis. The time from sampling to analysis varied but was normally one to three days with the maximum being six days. We advised that the blood be taken before the next dose (trough level) although this was not always supervised and could not be guaranteed in such situations. When time of sampling was important it was properly supervised.

Experiments were conducted to test the stability of flecainide in serum before and after it had been extracted. These results clearly indicated that flecainide was stable in either environment when left for one week at room temperature on a bench surface. The stability of flecainide therefore presents no problems in this study, even for those transported from other locations.

RESULTS AND DISCUSSION

Chromatography

One important feature to be considered in the analysis of flecainide by HPLC is the highly dipolar nature of the drug caused by the presence of the two aromatic trifluoroethoxy groups contained within its molecular structure. This polarity has led to the use in the earlier publications of more polar trimethylsilane [2] and phenyl [3,4] columns. Methods using the less polar octadecylsilane [5–7,9] or octylsilane [10] columns have more recently been reported. A method using adsorption chromatography has also been described [8]. Chang et al. [11] expressly measured the less active dealkylated metabolite unlike the other papers.

A reversed-phase system was chosen as more convenient for our clinical situation. The mobile phase was a mixture of aqueous phosphate and acetonitrile. This style of mobile phase is widely used and by simply varying the pH and the proportion of organic solvent an impressively large number of basic drugs can be chromatographed.

The pH had essentially no effect on the retention times of flecainide or internal standard below 5 (Fig. 1). Changes in pH offered no selectivity between the compounds over the usable pH range which implies that their acidity constants must be similar owing to the piperidine functional groups being removed from the inductive effects of the aromatic substituents.

A higher degree of selectivity was afforded by a variation in acetonitrile content (Fig. 2). The substantial difference in the polarisation of these two molecules owing to the two heavily electronegative trifluoroethoxy groups on flecainide must account for this by making the latter more sensitive to solvation effects.

On the basis of the above effects, separation of flecainide from the internal standard in the shortest time was favoured at lower pH and lower acetonitrile

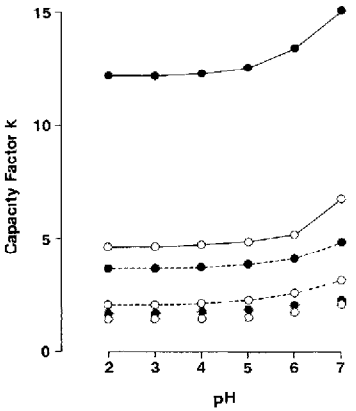

Fig. 1. Effect of pH on retention times. Column, radially compressed Waters Novapak C_{18}, 4 μm particle size; detection, fluorescence; temperature, ambient; flow-rate, 3 ml/min; mobile phase, 0.1 *M* aqueous phosphate-acetonitrile (·····, 50:50; – – –, 65:35; —, 75:25); (●) flecainide; (○) internal standard.

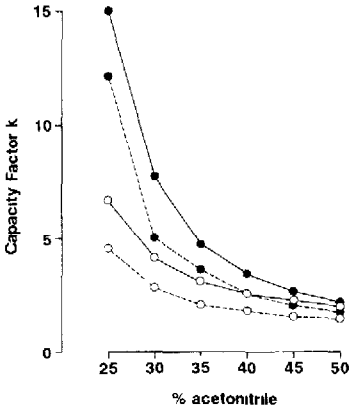

Fig. 2. Effect of acetonitrile content on retention times. Conditions as in Fig. 1. Mobile phase, 0.1 *M* aqueous phosphate–acetonitrile. (------) pH 3.0, (—) pH 7.0; (●) flecainide; (○) internal standard.

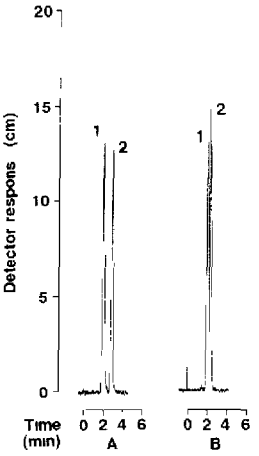

Fig. 3. Chromatograms under different conditions. Column, radially compressed Waters Novapak C_{18}, 4 μm particle size; detection, fluorescence (see text); flow-rate, 3 ml/min; mobile phases, 0.1 *M* phosphate–acetonitrile (60:40), pH 3.0 (A); 0.1 *M* phosphate–acetonitrile (50:50), pH 7.0 (B). Peaks 1 = internal standard; 2 = flecainide.

content rather than at higher pH and higher acetonitrile proportions. This is demonstrated in Fig. 3.

The addition of an amine modifier such as diethylamine reduced the retention times of flecainide and its internal standard but studies on its effect on peak shape as determined by asymmetry factors were equivocable. It afforded essentially no improvement in peak shape when tested on a new column with the mobile phase at the normal operating pH of 3. However, it was capable of preventing the loss of peak shape at higher pH commonly seen in the absence of the amine modifier. By contrast, a definite improvement was evident on a well used column of the same type. It was therefore included in the mobile phase in order to assist in the maintainance of column efficiency as the columns aged and hence extend their operational life.

Internal standard

Most of the reversed-phase methods published to date have used either the non-fluorinated analogue of flecainide [2,5] or a postional isomer of flecainide [3,4,7,10] as an internal standard. In all cases, the resolution between the former and flecainide has been superior and hence it was preferred for our work.

Detection

The previously published papers have used either UV [2,4,5,9,10] or fluorescence [3,4,6–8,10] detection. Fluorescence was considered by us to be the most desirable method mainly because of its greater selectivity for patients on multiple drug therapies. Flecainide is not a strongly fluorescent compound and therefore may not give as good a sensitivity as low-wavelength UV detection unless special measures are taken. The fluorescence detectors used in several of the cited methods normally employ about 300 nm excitation with 370 nm emission which are not sensitive enough to avoid the use of 1-ml samples without a preconcentration step during sample preparation. An exception to this is the method of Bhamra et al. [8] that gained sensitivity by excitation at 200 nm with no emission filter allowing the use of only 0.1-ml samples which were diluted 1:2 during extraction. In addition, De Jong et al. [6] used only 0.2 ml which was also diluted 1:2 during processing. The latter authors achieved their sensitivity by the use of an expensive fluorometer with fairly wide slit widths.

The fluorescence spectra of flecainide and internal standard are presented in Fig. 4. The excitation and emission maxima of the internal standard were located at longer wavelengths. Neither fluorescence spectra were altered by pH between 3 and 7 nor by a variation in acetonitrile proportion between 40 and 50%. This placed no constraints on the mobile phase chosen.

In order to achieve sensitivity, a filter was sought which could make most of the excitation energy available. The Schott UG 11 was chosen because of its broad bandpass and high throughput in the desired wavelength range (see Fig. 4). A suitable emission cut-off filter was a Schott GG 395 which captured most of the internal standard-emitted light but unfortunately less than half that from flecainide. A lower-wavelength filter such as the GG 385 could not be used because of excessive spectral overlap with the UG 11 filter. The use of 280- or 313-nm interference filters for excitation gave less sensitivity even with emission cut-off filters down to 360 nm.

The above combination of filters permitted the detection of flecainide down to 50 nmol/l using 1 ml serum which is compatible with low-wavelength UV detection [5,9] and much better than detection at the absorption maximum at about 308 nm [2,4,10]. This sensitivity is low enough for therapeutic purposes.

Sample preparation and recovery

The sample preparations developed by the previous authors have utilised protein precipitation [6], column extraction [4,7] and solvent extraction with evaporation [2,3,8–10] or back extraction [5]. We decided on a method similar to that of Boutagy et al. [5] namely extraction followed by back-extraction into a small volume of dilute aqueous acid. Provided the serum was alkalinised, efficient ex-

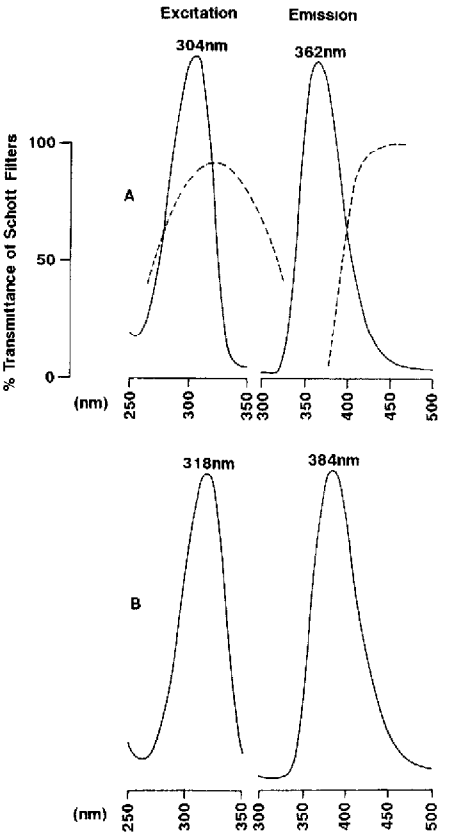

Fig. 4. Fluorescence spectra of flecainide and internal standard in aqueous phosphate–acetonitrile (60:40), pH 3.0. (A) Flecainide; (B) internal standard; (----) transmittance of the Schott UG 11 and GG 395 filters.

traction was possible with a number of organic solvents. Those tried were diethyl ether, diisopropyl ether, hexane, ethyl acetate and toluene. Hexane had the characteristic emulsification problems experienced when extracting from basic serum as well as giving the slightly lower recoveries noted in the earlier published work [2,3]. Ethyl acetate had to be discarded because back-extraction into the aqueous acid was only about 10% efficient. The ethers and toluene were equally acceptable but toluene was chosen because it was easy to handle and could be used without interference with a fluorescence detector. Toluene could not be used with UV detection because the dissolved component would absorb strongly. The recoveries observed in the different solvents are summarised in Table I.

The advantages of back-extraction into aqueous acid as compared with evaporation of the solvent are that it allows the use of high-boiling solvents such as toluene and does not concentrate solvent impurities or coextracted lipids. The latter effect may lead to more rapid column deterioration and an increased risk of interferences. In order to obtain the greatest preconcentration of drug the aqueous volume must be as small as possible. The limiting factor to volume is efficiency of back-extraction from a large organic volume. Back-extraction is en-

TABLE I

RECOVERY OF FLECAINIDE USING DIFFERENT SOLVENTS

Solvent	Recovery ($n=12$) (%)	
	Range	Average
Toluene	86–92	89
Hexane	77–83	80
Diethyl ether	87–92	90
Diisopropyl ether	87–91	89
Ethyl acetate	5–13	8

hanced by acid which cannot be too strong because it can damage the column. Taking these constraints into account flecainide was quantitatively back-extracted into 200 μl of 0.1 *M* phosphoric acid with no perceivable chromatographic changes or column damage. The use of a smaller volume or weaker acid resulted in losses of drug.

The most serious problem encountered during sample preparation was due to losses caused by adsorption onto the glassware surfaces. This seemed to be a consequence of residual detergent after washing. To avoid this, all glassware was cleaned by rinsing with methanolic diethylamine and allowing it to dry be draining. This feature has not been experienced with any of the other antiarrhythmics which are measured here but has been observed with the tricyclic antidepressant drugs.

Precision and linearity

The precision of the test method on a within-day and between-day basis is shown in Table II. It is comparable to the earlier reports. The standard deviation of duplication ($n=58$) is 43.2.

The method is linear up to 3000 nmol/l which is well above the expected therapeutic levels. Some curvature was evident at greater levels and so dilution of samples is necessary if overdoses are being quantitated.

All results are reported to the nearest 10 nmol/l.

TABLE II

PRECISION DATA FOR THE DETERMINATION OF FLECAINIDE

Concentration (nmol/l)	Coefficient of variation ($n=15$) (%)	
	Within-day	Between-day
500	4.3	6 8
1000	3.6	5.2
1500	3.6	5 6
2000	2.9	4.9
2500	3.1	4 7

Interferences

In the course of three years over 2000 routine samples have been analysed from patients on a large number of therapies. Our experience has shown only three drugs which are potential interferents. None of these would cause an erroneous flecainide level to be inadvertantly issued nor completely prevent a reasonable result to be calculated. The drugs in question are quinidine, triamterene and dipyridamole which all fluoresce strongly under the detection conditions (see Fig. 5). Quinidine poses only minor problems as it elutes on the void volume but it can tail so that the internal standard lies on a sloping baseline. It is not often prescribed with flecainide but can be present during changeover from one drug to the other. Triamterene has been observed in two patients on diuretic therapy and also causes tailing in the region of the internal standard. It elutes later than quinidine but the peak height is generally not sufficient to completely mask the internal standard. The most difficult interference is from dipyridamole which generates a tall peak slightly before the internal standard and prevents its measurement. Five patients have been analysed with this drug but flecainide results were still issued using an external standard quantitation by taking greater care with extraction and injection volumes. The possibility always exists for cardiac patients to be administered antithrombotics but dipyridamole is obvious when present and would not go unnoticed.

Fluorescence polarisation immunoassay

The fluorescence polarisation immunoassay is now being routinely used. Like all immunoassays it potentially offers the best analysis because of the lower sample volume required, higher throughput, absence of sample preparation and its

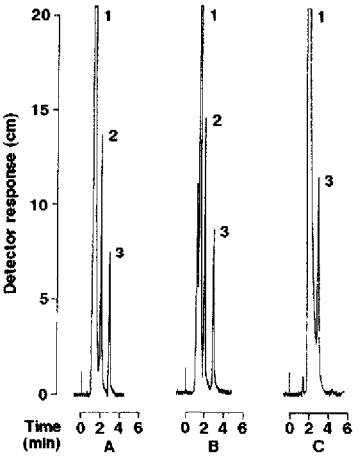

Fig. 5. Chromatograms showing the known interferences. Conditions as before. (A) Patient on flecainide (1160 nmol/l) and quinidine; peaks: 1 = quinidine; 2 = internal standard; 3 = flecainide. (B) Patient on flecainide (1270 nmol/l) and triamterene; peaks: 1 = triamterene; 2 = internal standard; 3 = flecainide. (C) Patient on flecainide (1580 nmol/l) and dipyridamole; peaks: 1 = dipyridamole; 2 = internal standard; 3 = flecainide.

push button ease of use. All these features are desirable in a clinical situation and will result in the proliferation of flecainide testing by this method.

The performance characteristics of the immunoassay has been tested and conforms to the published data accompanying the kit sets. These need not be elaborated further except to say that no obvious interferences have been detected to date (325 samples) although this does not preclude them as they may have gone unnoticed.

Comparison of HPLC and TDX

The serum flecainide levels on a number of patients were measured by the HPLC and TDX methodologies. Initially, a comparison was made between 56 samples collected and stored frozen from our routine HPLC analyses and then subsequently analysed on a large batch basis using a TDX kit set supplied by the manufacturers for this express purpose. The pipetting mode 1 was used on the TDX analyser. These initial data showed good agreement (correlation coefficient 0.94). These preliminary data are not presented here. TDX flecainide analyses were then introduced routinely and run concurrently with the established HPLC method. The data obtained in this way were on a small batch basis (total number of samples, 189; average batch size, 3.9; range 1–8) as samples arrived in order to include between-batch variation and closely match the real clinical situation. The TDX pipetting mode 4 was used on advise from the manufacturers. The comparative results are shown in Fig. 6. The correlation coefficient was 0.97 and the regression line had a slope of 1.047 and an intercept of 7.58. This shows that a good agreement exists between the two methods although the TDX gave results

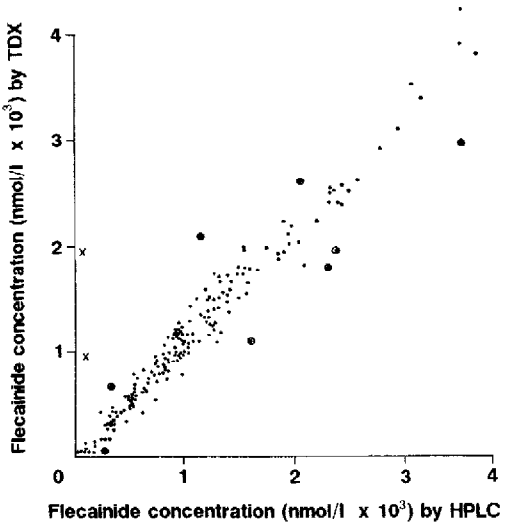

Fig. 6. Comparison of flecainide determined by HPLC and TDX (●) Data points ($n=189$); (○) data points which have been rechecked by TDX; (×) discrepant data points shown on repeat by TDX to be incorrect (not included in regression data).

about 5% lower than HPLC. This is attributable to the standardisation of the test methods because the HPLC controls (prepared in house) gave results that were also close to 5% too low when measured by TDX.

Some of the outliers were retested by TDX in order to reconfirm their original values. The ones tested are indicated in Fig. 6 and in most cases they remained unaltered. There were, however, two analyses which gave complete disagreement between the TDX and HPLC methods. On repeat analyses the TDX results decreased markedly and came into line with the HPLC values. These are also shown in Fig. 6 but they were not used in the regression analysis. The reason for this could not be explained but it seems at this stage that they were spurious isolated errors. The obvious concern is that in the absence of parallel HPLC analysis they would have been unchallenged and issued to the requesting physician.

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