Quantitation of Flecainide Acetate, A New Antiarrhythmic Agent, in Biological Fluids by Gas Chromatography with Electron-Capture Detection

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Abstract □ A sensitive and selective gas chromatographic (GC) method for the quantitation of flecainide acetate, a new antiarrhythmic agent, was developed. The unchanged drug and internal standard were separated from biological fluids by a sequence of solvent extractions and then the drug was derivatized. The pentafluorobenzamide derivatives were chromatographed on a 3% SP-2250 glass column and detected with a nickel-63 electron-capture detector. By this method, ≥12.5 ng of flecainide/mL can be quantitated in a 1-mL sample of plasma, saliva, or urine. The intraday precision, expressed as the *RSD*, at plasma levels of 12.5, 25, 50, 100, 200, 300, 400, and 600 ng/mL was 3.4, 6.2, 5.3, 6.4, 1.0, 1.6, 2.0, and 0.5%, respectively; the accuracy, expressed as relative error at these levels was −24.6, −6.9, −6.0, +0.6, +3.8, −0.3, +2.4, and −1.4%, respectively. The interday precision at these levels was 13.8, 13.0, 5.7, 7.6, 8.3, 6.1, 9.0, and 5.4%, respectively. Several other antiarrhythmic agents and one β-blocker, which might be administered concurrently with flecainide acetate, do not interfere with the assay.

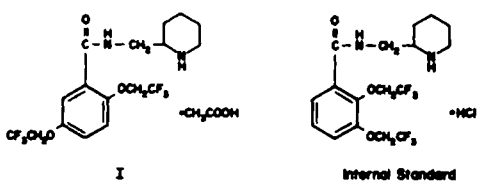
Keyphrases □ Flecainide acetate—quantitation in biological fluids, GC-ECD □ GC—quantitation of flecainide acetate in biological fluids □ Antiarrythmic agents—flecainide acetate, quantitation in biological fluids, GC-ECD

Flecainide acetate [*N*-(2-piperidylmethyl)-2,5-bis(2,2,2-trifluoroethoxy)benzamide monoacetate] (I) is a new antiarrhythmic agent currently undergoing clinical evaluation worldwide. Pharmacological, pharmacokinetic, and efficacy information have been reported previously (1–9). Sensitive fluorometric methods (10, 11) have also been reported but have suffered from a lack of adequate specificity due to interference. Recently, HPLC methods have been reported (12, 13). In this report, we describe a highly selective and sensitive gas chromatographic (GC) method for the quantitation of the drug in plasma, urine, or saliva following the administration of single or multiple doses.

EXPERIMENTAL SECTION

Reagents—All reagents were analytical reagent grade. Ether was distilled in glass[1]; all other solvents were nanograde[2]. Fresh 0.2 M trimethylamine in benzene was made daily by dilution from a concentrated stock solution stored at −10°C. The derivatization reagent, pentafluorobenzoyl chloride (PFBC)[3], was stored at 4°C. Aqueous solutions of 0.1 M NaOH, 1.0 M NaOH, and 0.5 M HCl were prepared fresh daily in distilled water.

Blank Plasma, Urine, and Saliva—Human plasma, urine, and saliva samples were obtained from volunteers who had not taken any medication during the previous week. Donors were asked to fast for 12 h prior to giving blood. Human saliva was obtained by paraffin stimulation followed by expectoration into a disposable sputum collecting cup.



I Internal Standard

Gas Chromatography—A gas chromatograph[4] equipped with an electron-capture detector (15 mCi of nickel-63) and an automatic liquid sampler[5] was used. The glass column (180 cm × 2 mm i.d.) was silanized, packed with 3% SP-2250 on 100–120 mesh Supelcoport[6], and conditioned for 48 h at 275°C. The operating conditions were as follows: injection port temperature, 275°C; detector temperature, 310°C; oven temperature, 268°C (isothermal); carrier gas (5% methane in argon) flow rate, 20 mL/min.

Standard Solutions—All stock solutions of the drug and the internal standard, a positional isomer of flecainide [*N*-(2-piperidylmethyl)-2,3-bis(2,2,2-trifluoroethoxy)benzamide hydrochloride] (14), were made in distilled water. Standard solutions containing 800, 600, 400, 300, 200, 100, 50, 25, and 12.5 ng of flecainide acetate per 0.5 mL were made by diluting a 10-μg/mL primary standard solution. The 10-μg/mL primary standard solution of the internal standard was diluted to give a concentration of 100 ng/0.5 mL. The primary standard solutions of the drug and the internal standard were stored at 4°C.

Extraction—*Plasma*—To glass culture tubes (16 × 150 mm) with polyethylene-lined screw caps, 1-mL aliquots of unknown samples of human plasma and 0.5 mL of distilled water were added. Along with each set of unknown samples, 10 standards in blank human plasma were prepared by adding 0, 12.5, 25, 50, 100, 200, 300, 400, 600, and 800 ng of flecainide acetate in 0.5 mL of distilled water to 1-mL aliquots of blank human plasma. Next, 0.5 mL of internal standard (100 ng) solution, 0.2 mL of 0.2 M trimethylamine (TMA) in benzene, 1 mL of 1 M NaOH, and 10 mL of diethyl ether were added, in that order, to all tubes. The tubes were capped and shaken in a horizontal position for 30 min on a reciprocal mechanical shaker and then centrifuged for 5 min at 700×*g*. Then, 9.0 mL of the organic layer was transferred to a glass tube containing 2 mL of 0.5 M HCl. All tubes were shaken for 15 min and then centrifuged for 3 min. The ether was aspirated and discarded. The remaining aqueous layer was decanted into a 12-mL conical glass centrifuge tube for subsequent derivatization.

Urine—Because of the relatively high concentrations of flecainide in the urine samples, usually less than 1 mL of urine was analyzed. When less than

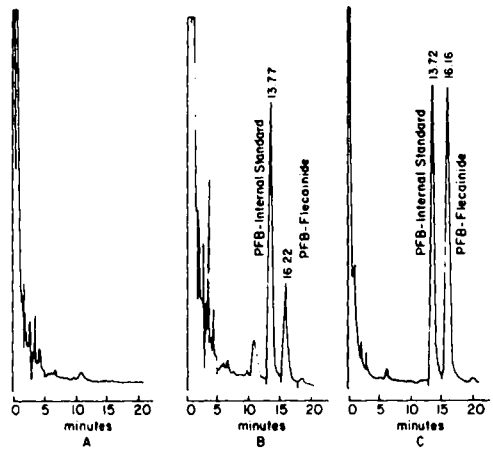


Figure 1—*Chromatograms from human plasma. (A) Blank plasma. (B) Plasma from a human subject dosed with flecainide (I) with the internal standard added (I, 42 ng/mL; internal standard, 100 ng/mL). (C) Mixture of reference pentafluorobenzamide (PFB) derivatives of I and internal standard; amount injected: PFB–I, 1.5 ng; PFB–internal standard, 0.9 ng.*

[1] Burdick-Jackson Laboratories, Inc., Muskegon, Mich.
[2] Mallinkrodt, Inc., St. Louis, Mo.
[3] Aldrich Chemical Co., Milwaukee, Wis.
[4] Model 5840A; Hewlett-Packard, Avondale, Pa.
[5] Model 7671A; Hewlett-Packard.
[6] Supelco, Inc., Bellefonte, Pa.

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Table I—Intraday Precision and Accuracy for Determination of Flecainide (I) in Human Plasma [a]

I Added, ng/mL	I Measured, ng/mL (mean ± *SD*)	*RSD*, %	Relative Error, %
12.5	9.4 ± 0.3	3.4	−24.6
25	23.3 ± 1.4	6.2	−6.9
50	47.0 ± 2.5	5.3	−6.0
100	100.6 ± 6.4	6.4	+0.6
200	207.6 ± 2.0	1.0	+3.8
300	299.1 ± 4.7	1.6	−0.3
400	409.6 ± 8.2	2.0	+2.4
600	591.8 ± 3.1	0.5	−1.4

[a] $n = 5$.

Table II—Interday Precision for Determination of Flecainide (I) in Human Plasma [a]

Sample Concentration, ng/mL	Response Ratio (Mean ± *SD*)	*RSD*, %
12.5	0.32 ± 0.04	13.8
25	0.49 ± 0.06	13.0
50	0.76 ± 0.04	5.7
100	1.32 ± 0.10	7.6
200	2.57 ± 0.21	8.3
300	3.62 ± 0.22	6.1
400	4.93 ± 0.44	9.0
600	6.95 ± 0.37	5.4

[a] $n = 6$.

1 mL of a urine sample was used, blank urine was added to bring the total sample volume to 1 mL. The extraction, derivatization, and analysis procedures were the same as described above for plasma.

Saliva—A saliva sample of 1 mL or less was analyzed in the same manner as described above for plasma; when less than 1 mL was used, distilled water was added to bring the total volume to 1 mL. It was shown that a standard curve (12.5–800 ng/mL) in saliva was the same as that in distilled water; thus, in all subsequent saliva analyses, a flecainide standard curve in distilled water was used for the determination of flecainide concentrations in saliva samples.

Derivatization—For derivatization of both the drug and the internal standard, 2 μL of neat pentafluorobenzoyl chloride was added to each aqueous extract with a 10-μL syringe[7]. Immediately after the addition of pentafluorobenzoyl chloride, the tube was vortexed for 5 s to disperse and suspend the derivatizing reagent. Next, 1 mL of 1 M NaOH was added, and the tube was vortexed for 15 s. Finally, 1 mL of hexane was added and the tube was again vortexed for 15 s. The derivatization sequence was carried out with pairs of tubes.

After the derivatization procedure was completed for all samples, the tubes were capped and centrifuged for 3 min at 700×*g*. Then, ~0.8 mL of each hexane phase was transferred to a clean conical glass tube containing 3 mL of 0.1 M NaOH. The tubes were vortexed for 15 s and centrifuged for 3 min. The hexane phase was then transferred to the glass autosampler vial, and a 5-μL aliquot of each sample was injected into the glass chromatograph.

Calculation—The area ratios (the drug–internal standard) were calculated with an integrating GC terminal[8]. The slope and intercept from the least-squares line of the concentrations of the drug standards *versus* the area ratios of the drug were used to calculate the concentrations in unknown samples.

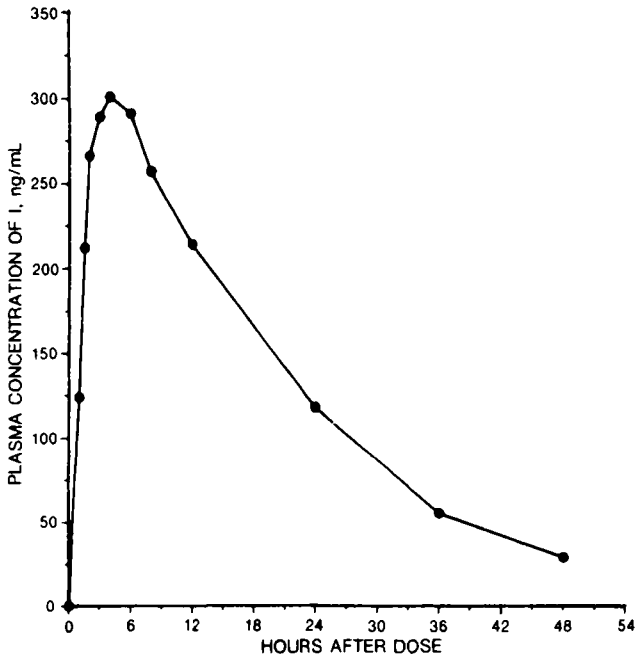

Figure 2—*Plasma concentrations of flecainide (I) in a healthy human subject following a single oral 200-mg dose of I.*

[7] Hamilton.
[8] Model 18850A; Hewlett-Packard.

Radiometric Analysis of Carbon-14—For the extraction recovery, an aqueous solution of ^{14}C-labeled drug (15) was used (9.2 μCi/mg, 10 ng/μL). Aliquots of 0, 5, 10, 40, and 60 μL were added to 1-mL blank human plasma samples in triplicate, and these samples were extracted by standard procedures. After the drug was extracted again into 0.5 M HCl, 1 mL of the aqueous phase was mixed with 0.4 mL of 1 M NaOH and counted in 15 mL of liquid scintillation solution[9]. Triplicate aliquots of the standard solution in 0, 5, 10, 40, and 60 μL were also directly counted in 15 mL of the same liquid scintillation solution. After cold and dark adaptation, carbon-14 was measured in a liquid scintillation counter[10]. Counting data were converted to disintegrations per minute by computer using the BIOAES program[11]. The percent extraction was calculated from the total disintegrations per minute in the aqueous extract compared with the disintegrations per minute in the spiked sample.

Tests for Other Drugs—A 500-ng/0.5 mL deionized water solution was prepared for propranolol hydrochloride, procainamide hydrochloride, disopyramide phosphate, quinidine sulfate, mexiletine hydrochloride, and lidocaine. Of each of these solutions, 0.5 mL was added to 1 mL of human plasma; the mixture was then extracted and derivatized by the procedure described above for flecainide. A positive control, a 600-ng flecainide standard with internal standard added, was run in parallel with the samples described above.

Gas Chromatographic–Mass Spectrometric Analysis—GC–MS analysis was performed on a gas chromatograph–mass spectrometer system which was comprised of a gas chromatograph[12], a magnetic double-focusing mass spectrometer[13], and a data system[14]. A stainless steel column (180 cm × 3.2 mm o.d.) packed with the same material as that for the glass column was used. The operating conditions were as follows: for the gas chromatograph, oven temperature, 245°C; injection port temperature, 230°C; carrier gas flow rate, 25 mL/min (He); for the mass spectrometer, resolving power, 500; source temperature, 250°C; for the data system, acquisition rate, 6 kHz, scan time, 2 s/decade. Unknown samples were extracted and derivatized by standard procedures. Aliquots of the final hexane extract were injected into the GC–MS system. Unknown samples were compared qualitatively with the synthetic pentafluorobenzamide of the drug at *m/z* 195, 278, 301, and 331, which are characteristic of the pentafluorobenzamide derivative of the drug.

RESULTS AND DISCUSSION

Derivatization—Based on the results from earlier studies[15] of ^{14}C-labeled flecainide in animals, it was anticipated that the plasma levels of the drug in humans would be in the low nanogram per milliliter range. Thus, it was decided at the outset that a GC–electron capture detector (GC–ECD) approach would be investigated. Flecainide contains six fluorine atoms which should provide enough ECD sensitivity; however, the material proved to be relatively difficult to chromatograph at the picogram to low nanogram amounts, possibly due to the presence of the piperidyl group. Thus, the piperidyl group was derivatized with pentafluorobenzoyl chloride. Although the perfluorobenzamide derivative, in comparison with its corresponding perfluorobutyramide, has a relatively low vapor pressure, thus requiring a higher column temperature for elution, this disadvantage is counterbalanced by the greater ECD sensitivity of the perfluorobenzamide derivative (16, 17) and the lack of any noticeable interferences.

Chromatography and Peak Separation—Although the drug and the internal

[9] New England Nuclear Corp., Boston, Mass.
[10] Model 3385; Packard Instrument Co., Downers Grove, Ill.
[11] BIOAES: Biological Automatic External Standardization; computer program on file at Riker Laboratories, Inc., St. Paul, Minn.
[12] Model 2740; Varian, Walnut Creek, Calif.
[13] Model 21-491C; Dupont, Wilmington, Del.
[14] Model 21-094; Dupont.
[15] Unpublished results.

standard differ only in the position of the trifluoroethoxy group on the aromatic ring, sufficient separation was achieved by using a 3% SP-2250 column. Under the chromatographic conditions used, there was no interference with the drug or the internal standard by any extractable endogenous materials present in plasma, urine, or saliva. Typical GC tracings of derivatized extracts from blank human plasma, from human plasma of a subject dosed with the drug, and reference flecainide acetate and internal standard derivatives are shown in Fig. 1. The retention times of the pentaflurobenzoyl derivatives of I and internal standard under these experimental conditions are 16.2 and 13.7 min, respectively.

Interference due to excess pentafluorobenzoyl chloride was eliminated by more accurate addition of the 2 μL of this reagent and/or by increasing the amount of NaOH added (from 1.0 to 1.5 mL of 1 M NaOH) to hydrolyze any excess derivatizing reagent. Care must also be taken when transferring the 0.8-mL hexane phase to the tube containing 3 mL of 0.1 M NaOH for the washing step. If any of the aqueous phase is transferred with the hexane, the chromatogram will show a large interfering solvent front.

Adsorption sites on a new column must be saturated by repeated injections of the synthetic derivatives of flecainide and the internal standard. Saturation is adequate when approximately equal peak areas are obtained for several successive injections. Extensive use of a column results in the deterioration of the resolution of flecainide from the internal standard. Repacking the front ~5 cm of the column was found to be necessary after ~800 sample injections.

Extraction Recovery—The extraction recovery of the drug from plasma was determined by carrying ^{14}C-labeled flecainide through the extraction to the derivatization steps and then measuring the total radioactivity recovered. The extraction recovery over the concentration range of 50 to 600 ng/mL was 86 to 91%, with a mean $\pm SD$ of 88 ± 2%.

Selectivity—GC-MS (electron impact) studies of plasma samples from human subjects who received the drug, in comparison with the synthetic pentafluorobenzoyl derivative of the drug, showed identical fragmentation patterns; thus, the pentafluorobenzoyl derivative of the drug is the species actually measured in human plasma samples.

Lidocaine, mexiletine, procainamide, disopyramide, propranolol, and quinidine, drugs which may be present together with flecainide in specimens obtained from patients, were tested for interference under the extraction and derivatization conditions used for the drug and the internal standard. Lidocaine, mexiletine, procainamide, and disopyramide showed no response, whereas propranolol and quinidine showed longer retention times than the pentafluorobenzoyl derivatives of the drug and the internal standard.

Linearity—A total of 9 or 10 single standards (0–800 ng) were run daily with each set of unknown samples. The mathematical expression of the least-squares line from a typical standard curve (0–800 ng) is $y = 0.0096x - 0.0662$. The correlation coefficient is 0.9998, and the coefficient of determination is 0.9996, indicating good linear proportionality between the concentration of flecainide and the detector response. The concentration-response relationship has also been shown to be linear up to a concentration of 1400 ng/mL. However, in most cases a shorter standard curve is used; *e.g.*, for routine analysis of samples after a single dose of flecainide, a 0–600 ng/mL range is used.

Sensitivity—By this method, concentrations as low as 12.5 ng of flecainide/mL of plasma, saliva, or urine can routinely be quantitated. Although not done routinely, under ideal chromatographic conditions, samples containing ≥6 ng of flecainide/mL can be quantitated.

Precision and Accuracy—The intraday precision and accuracy of this method was assessed by carrying five replicate samples at eight concentrations (12.5, 25, 50,100, 200, 300, 400, and 600 ng/mL) through the entire procedure on one analysis day. The intraday precision, expressed as the *RSD*, was 3.4, 6.2, 5.3, 6.4, 1.0, 1.6, 2.0, and 0.5% for the eight concentrations listed above, respectively (Table I). The accuracy of this method, expressed as relative error, at these levels was −24.6, −6.9, −6.0, +0.6, +3.8, −0.3, +2.4, and −1.4%, respectively.

The interday precision, at the concentrations described above, was established by analyzing these standards on 6 separate days over a 1-month period (Table II). The response ratios from 6 separate days for each standard concentration were pooled, and the mean ± *SD* values were calculated. The *RSD* values were 13.8, 13.0, 5.7, 7.6, 8.3, 6.1, 9.0, and 5.4% for concentrations of 12.5, 25, 50, 100, 200, 300, 400, and 600 ng/mL, respectively.

Application—The method has been used to measure plasma, urinary, and salivary levels of flecainide from various metabolic, pharmacological, and therapeutic studies in humans. More than 10,000 samples have been successfully analyzed by this procedure in our laboratory, as well as in several other laboratories worldwide. A typical curve of the plasma concentration of the drug *versus* time following a single oral 200-mg dose of the drug in a healthy human volunteer is shown in Fig. 2.

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