Journal of Chromatography, 490 (1989) 450–457
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4680

Note

High-performance liquid chromatographic assay with fluorometric detection for flecainide and its major metabolites in urine and serum

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(First received June 8th, 1988; revised manuscript received January 13th, 1989)

Flecainide is a class Ic antiarrhythmic agent, available for oral and intravenous administration as an acetate salt (Tambocor, Riker Labs., Loughborough, U.K.). It is particularly useful in the treatment of ventricular arrhythmias. Its disposition in humans is known to follow a two-compartment model with a rapid distribution phase, almost invisible after oral absorption [1]. Terminal half-life averages 12–14 h in normal subjects and rises to ca. 20 h in congestive heart failure or arrhythmic patients. Oral bioavailabilities of 70% to 90 and 95% [1–3] have been reported. Flecainide is eliminated by both renal and metabolic routes. Renal excretion depends on urinary pH [4,5]. Biotransformation has been described after administration of [^{14}C]flecainide in humans where two major metabolites (as well as their glucuronides and sulpho conjugates) were characterized in urine [6,7]. The metabolism is suggested to be sequential, the first step being O-dealkylation in the *m*-position of the benzamide ring, followed by formation of a lactam on the piperidine ring. Both phase I metabolites are subsequently conjugated on the phenolic hydroxy group. Owing to the relatively insensitive and non-selective technique used [thin-layer chromatography (TLC) for the separation, difference between total radioactivity and unchanged flecainide for the plasma levels], the authors could neither present the plasma profiles nor calculate the excretion rates for the two metabolites. To our knowledge, this has never been done.

Several procedures for the determination of flecainide have been proposed:

0378-4347/89/$03.50

gas chromatography [8,9], an extraction–spectrofluorometric method [10], high-performance liquid chromatography (HPLC) [11–17] and recently a fluorescent polarization immunoassay [18]. However, they were not designed with the aim of determining flecainide metabolites. Besides the TLC method for labelled flecainide and its metabolites, only one paper in the literature presented a technique for the determination of the first of the two major metabolites, the dealkylated one [19]. This HPLC method, however, had several weaknesses, the two most troublesome being that it is valid solely for one metabolite, neither for the other nor even for the parent drug, and that three different mobile phases are necessary, depending on the sample type: untreated urine, plasma and dialysate and enzyme-treated urine and dialysate. This last point may be a real drawback if one wishes to compare urine concentrations with and without enzymic hydrolysis.

The purpose of the present paper is to offer an analytical technique combining a high sensitivity with the possibility of individually measuring the metabolites and the parent drug in a single run.

EXPERIMENTAL

Reagents

Flecainide acetate (F), *m*-O-dealkylated flecainide (MODF), *m*-O-dealkylated lactam of flecainide (MODLF) and the positional isomer of flecainide, N-(2-piperidinylmethyl)-2,3-bis(2,2,2-trifluoroethoxy)benzamide hydrochloride, used as internal standard (I.S.) in HPLC, were kindly furnished by Riker Labs. (St. Paul, MN, U.S.A.). Sodium carbonate, triethylamine (TEA) and ethyl acetate were quality puriss. p.a., supplied by Fluka (Buchs, Switzerland). Acetonitrile (LiChrosolv), chloroform (Uvasol) and disodium hydrogenphosphate dihydrate (pro analysi) were purchased from Merck (Darmstadt, F.R.G.) and acetic acid (HPLC grade) and citric acid monohydrate (Baker Analyzed reagent) from Baker (Phillipsburg, NJ, U.S.A.). 1-Pentanesulphonic acid sodium salt (PSA) and abalone β-glucuronidase/sulphatase (733 000:15 200 u/g, G 0258) were obtained from Sigma (St. Louis, MO, U.S.A.). Bidistilled water was used, for the mobile phase after filtration through 0.45-μm pore size HA filters (Millipore, Bedford, MA, U.S.A.).

Apparatus

An 8820 Series gradient liquid chromatographic system (DuPont, Wilmington, DE, U.S.A.) was used with a WISP 710B automatic injector (Waters Assoc., Milford, MA, U.S.A.). Fluorescence was measured with an LS-5 luminescence spectrometer (Perkin-Elmer, Norwalk, NY, U.S.A.). The column was a Zorbax 250 mm$\times$4.6 mm I.D. Phenyl column (DuPont).

Chromatographic conditions

The mobile phase was acetonitrile–water (40:60, v/v) with TEA and PSA (200 μl and 1000 mg/l of mobile phase, respectively) adjusted to pH 3.0 with glacial acetic acid. The column was kept in an oven at room temperature. The mobile phase was delivered isocratically at a 1.5 ml/min flow-rate, and the injection volume was 100 μl. Excitation and emission wavelengths were set at 300 and 370 nm, respectively; the corresponding slits were set at 10 and 20 nm bandwidths.

Sample preparation

Serum samples were stored at −20°C until analysis. Serum (500 μl) or 100-fold diluted urine was alkalinized with 100 μl of 0.2 *M* sodium carbonate after addition of 100 μl of I.S. (10 μg/ml). Extraction with 500 μl of ethyl acetate was performed on a Model 151 multi-purpose rotator (Scientific Industries, Bender and Hobein, Zürich, Switzerland) at room temperature for 20 min. The extract was separated from the aqueous layer after centrifugation for 3 min at 10 500 *g* on a TDx centrifuge (Abbott Labs., Irving, TX, U.S.A.), then dried under nitrogen. Before injection, the sample was reconstituted in 250 μl of acetonitrile–water (1:1, v/v).

Enzymic hydrolysis

Experimental conditions were adapted from McQuinn et al. [6]. To 50 μl of urine diluted ten-fold (or to the standards and controls, see below) were added 25 μl of 1.36 mg/ml β-glucuronidase/sulphatase. Buffering at pH 4.4 was obtained with 100 μl of citrate buffer (1.185 g of citric acid monohydrate and 1.552 g of $Na_2HPO_4 \cdot 2H_2O$ in 100 ml). Finally, 10 μl of chloroform and 100 μl of I.S. (10 μg/ml) were added, and the mixture was incubated at 37°C for ca. 20 h. The reaction was stopped and the mixture alkalinized with 100 μl of 3 *M* sodium carbonate. Extraction with 1 ml of ethyl acetate and subsequent treatment were performed according to the sample preparation procedure, except that the injection volume was increased to 150 μl.

Calibration

Six standard solutions containing F (calculated for the free base), MODF and MODLF at 12.5, 25, 50, 100, 200 and 400 ng/ml were prepared in pooled blank plasmas (or water for urine with and without hydrolysis), in increasing order for F, decreasing order for MODLF, and alternate order for MODF. Concentrations were doubled when enzymic hydrolysis was performed. Pooled blank plasma (or water) were also run in parallel. In addition, a control containing F at 200 ng/ml, and MODF and MODLF each at 100 ng/ml (from independent stock solutions) was processed in duplicate every time. The standards and the controls were subsequently treated according to the sample preparation procedure, undergoing the enzymic hydrolysis step when justified.

RESULTS

Chromatograms

Typically, retention times were ca. 3, 5, 15 and 18 min for MODLF, MODF, I.S. and F, respectively (Figs. 1 and 2).

Standard curves

Calibration curves were linear over the concentration range used here (twelve calibrations, $r^2 > 0.994$ on plasma, $r^2 > 0.996$ on water) for flecainide as well as for the metabolites. Linearity remained excellent for concentrations up to 1000 ng/ml. Concentration calculations were performed using a transformed (1/concentration) least-squares regression [20].

Recoveries

Recovery was determined by comparison of peak areas for spiked samples subject to extraction or by-passing this procedure. Extraction efficacy can be estimated at 59, 46, 59 and 60% for MODLF, MODF, I.S. and F respectively [with coefficients of variation (c.v.) of 8%]. Accordingly, peak-area ratios of MODLF, MODF and F to the I.S. are, respectively, 99,78 and 101% after extraction, compared with those without extraction. Extraction efficiency is in-

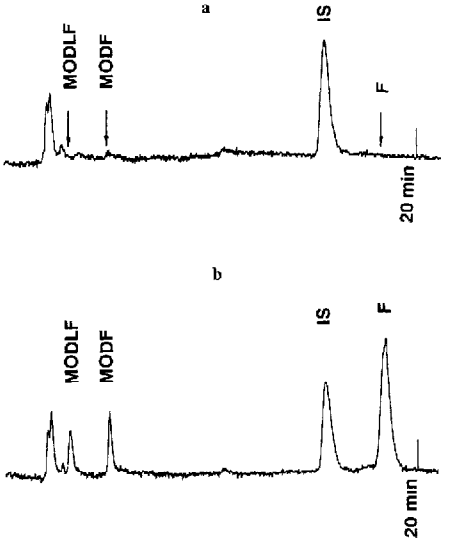

Fig. 1. HPLC profiles of a blank plasma spiked (a) with I.S. and (b) with I.S., MODLF (100 ng/ml), MODF (100 ng/ml) and F (200 ng/ml). The retention times relative to that of flecainide are 0.16 (MODLF), 0.27 (MODF) and 0.83 (I.S.). For abbreviations, conditions and sample preparation, see Experimental.

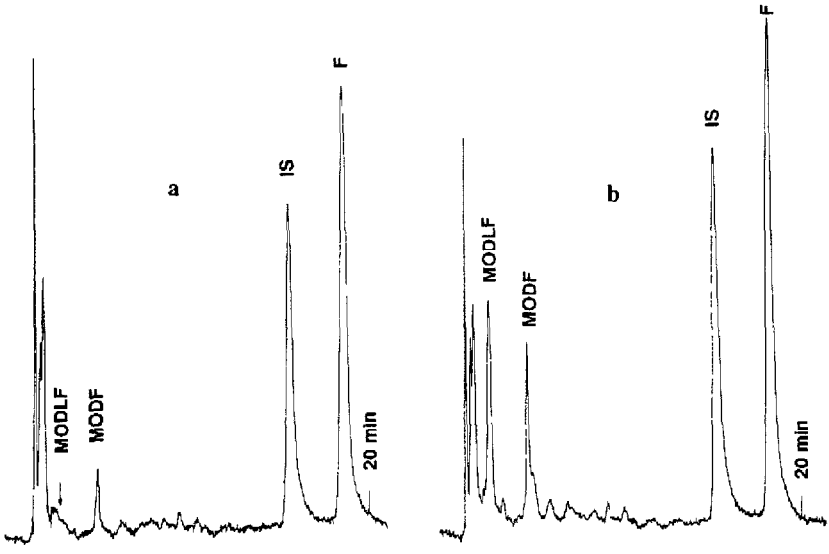

Fig. 2. HPLC profiles of a 4–6 h urine sample (a) before and (b) after enzymic hydrolysis. For abbreviations, conditions and sample preparation, see Experimental.

dependent of the concentration, and equilibrium is reached after 10–15 min extraction.

Detection limit

In the mobile phase used here, and under the described fluorescence detector conditions, the minimum detectable amount is ca. 1 ng of flecainide (giving a peak three times the height of the background noise). According to the sample preparation procedure, this corresponds to a serum concentration of 5 ng/ml. However, if the need arises, the detection limit can be lowered to 1 ng/ml by doubling the serum aliquot, the volume of ethyl acetate, and the volume of injection.

Precision

Intra-day variability is expressed as the c.v. of eight determinations of a spiked plasma. Inter-day variability has been calculated for urine and plasma on eleven determinations each, spread over two months, of spiked samples (in water and blank pooled plasma, respectively). Inter- and intra-day variabilities for the two metabolites and the parent drug are presented in Table I.

Concentration–time profile

Fig. 3 represents a concentration–time profile of flecainide and the dealkylated metabolite in one volunteer after a 30-min injection of 150 mg of flecainide

TABLE I

INTRA-DAY AND INTER-DAY VARIABILITIES FOR FLECAINIDE AND ITS METABOLITES

Compound[a]	Coefficient of variation (%)		
	Intra-day	Inter-day	
		Plasma	Urine
MODLF	6	19	11
MODF	6	9	7
F	5	7	5

[a]For abbreviations see text.

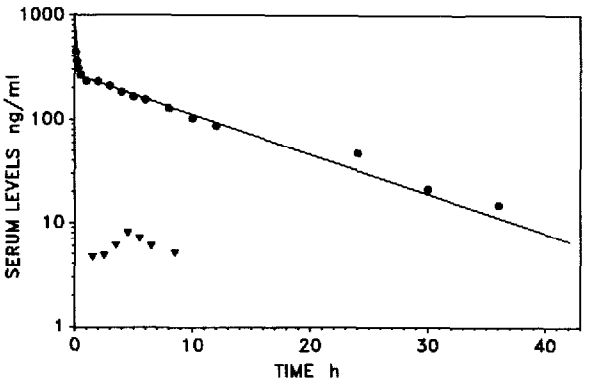

Fig. 3. Serum concentration-time profile of flecainide in a young healthy volunteer following a 150-mg 30-min constant-rate infusion. (●) Flecainide; (▼) *m*-O-dealkylated flecainide. The lactam metabolite was not detectable.

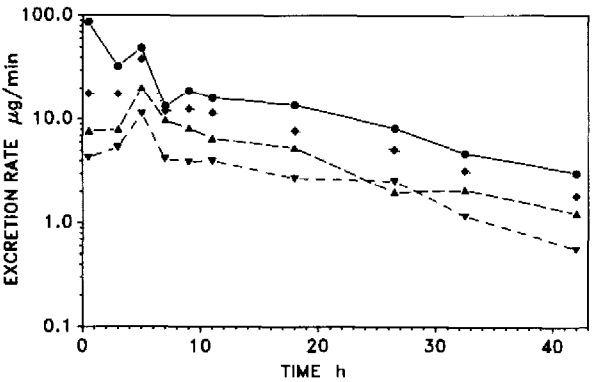

Fig. 4. Excretion rate versus the midtime point of the urine collection interval in the same volunteer as in Fig. 3 (●) Flecainide; (♦) conjugate of *m*-O-dealkylated lactam of flecainide; (▼) *m*-O-dealkylated flecainide; (▲) conjugate of *m*-O-dealkylated flecainide.

acetate. Fig. 4 is a plot of the excretion rate versus midtime point for F, MODF, and the conjugates of MODF and MODLF. Urine was treated with and without hydrolysis, and the concentrations of the conjugated metabolites were calculated by difference.

DISCUSSION

The procedure described here represents the first selective and sensitive assay for flecainide and its metabolites. Furthermore, compared with previously published methods [8–19], its sensitivity has been increased by a factor of 2–10. These are obvious advantages when assessing changes in flecainide metabolism, which up to now have been deduced only by variations in systemic clearance.

Preliminary data suggest that flecainide is metabolized through the cytochrome P-450 enzymic system. The determination of metabolites is mandatory for investigating the influence of cytochrome P-450 inhibitors on flecainide metabolism [21] and the potential genetic polymorphism in flecainide dealkylation.

Among the drawbacks of the procedure is the necessity of resorting to an organic liquid extraction step. The great polarity difference between the metabolites and the parent drug, as well as the ageing of serum or plasma [14], preclude the use of solid-phase extraction columns. In addition, measuring conjugated metabolites in urine necessitates lengthy enzymic hydrolysis.

Although not dedicated to routine analysis, our method allows selective and sensitive measurement of flecainide and its two major metabolites in a single run. It thus is a useful tool in research studies on the treatment and prevention of severe cardiac arrhythmias by flecainide and in elucidating possible population polymorphism.

REFERENCES

1 T.B. Tjandra-Maga, R. Verbesselt, A. Van Hecken, A. Mullie and P.J. De Schepper, Br. J. Clin. Pharmacol., 22 (1986) 309–316.
2 A.M. Gillis and R.E. Kates, Clin. Pharmacokin., 9 (1984) 375–403.
3 B. Holmes and R.C. Heel, Drugs, 29 (1985) 1–33.
4 K.A. Muhiddin, A. Johnston and P. Turner, Br. J. Clin. Pharmacol., 17 (1984) 447–451.
5 A. Johnston, S. Warrington and P. Turner, Br. J Clin. Pharmacol., 20 (1985) 333–338.
6 R.L. McQuinn, G.L. Quarfoth, J.D. Johnson, E.H. Banitt, S.V. Pathre, S.F. Chang, R.E. Ober and G.J. Conard, Drug Metab. Dispos., 12 (1984) 414–420.
7 G.J. Conard and R.E. Ober, Am. J. Cardiol , 53 (1984) 41B–51B.
8 G.J. Conard, G.L. Carlson, J.W Frost and R.E. Ober, Clin. Pharmacol. Ther., 25 (1979) 218 (Abstract).
9 J.D. Johnson, G.L. Carlson, J.M. Fox, A.M. Miller, S.F. Chang and G.J. Conard, J. Pharm. Sci , 73 (1984) 1469–1471.
10 S.F. Chang, A.M. Miller, M.J. Jernberg, R.E. Ober and G.J. Conard, Arzneim. Forsch , 33 (1983) 251–253.

11 J.W. de Jong, J.A.J Hegge, E. Harmsen and P.Ph. de Tombe, J. Chromatogr., 229 (1982) 498–502.
12 S.F. Chang, T.M. Welscher, A.M. Miller and R.E. Ober, J. Chromatogr., 272 (1983) 341–350.
13 R K Bhamra, R.J. Flanagan and D.W. Holt, J. Chromatogr., 307 (1984) 439–444.
14 J.U. Becker, J. Clin. Chem. Clin. Biochem., 22 (1984) 389–393.
15 S.F. Chang, A.M. Miller, J.M. Fox and T.M. Welscher, J. Liq. Chromatogr., 7 (1984) 167–174.
16 J. Boutagy, F.M. Rumble and G.M. Shenfield, J. Liq. Chromatogr., 7 (1984) 2579–2590
17 T.A. Plomp, H.T. Boom and R.A.A. Maes, J. Anal. Toxicol., 10 (1986) 102–106.
18 R.J. Straka, T.J. Hoon, R.L. Lalonde, J.A. Pieper and M.B. Bottorf, Clin. Chem., 33 (1987) 1898–1900.
19 S.F. Chang, T.M. Welscher, A.M. Miller, R.L. McQuinn and J.M. Fox, J. Chromatogr., 343 (1985) 119–127.
20 J. Neter and W. Wasserman, Applied Linear Statistical Models, R.D. Irwin, Homewood, IL, 1974, p. 131.
21 A. Munafo, G. Reymond, A. Borgeat and J. Biollaz, Clin. Res., 36 (1988) 368A (Abstract).