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Note

Simplified high-performance liquid chromatographic determination of flosequinan and its metabolite in plasma, serum and urine

MARY BETH SLEGOWSKI*, CYNTHIA MILLER and R. STEPHEN PORTER

Pharmacokinetics and Drug Metabolism, Likoff Cardiovascular Institute, Hahnemann University, Broad and Vine Streets, Philadelphia, PA 19102 (U.S.A.)

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Flosequinan is a new peripheral vasodilator with effects on both arterial and venous vascular beds [1]. Studies indicate dose-dependent reductions in blood pressure and increases in heart rate at doses ranging from 50 to 400 mg [2,3]. Flosequinan has a short half-life of 2.4 h and its major metabolite (BTS 53554) has a prolonged half-life of 38 h, which is probably responsible for the sustained duration of response. The therapeutic range has not yet been established but in clinical trials the concentration of drug and metabolite in both plasma and urine has ranged from less than 0.05 to 70 μg/ml. Flosequinan is still undergoing clinical trials in the U.S.A.

The previously published technique for the measurement of flosequinan and its metabolite required a relatively large sample volume and a long extraction procedure which involved the use of chloroform and a dry down step [2]. The method presented here is based on the high-performance liquid chromatographic (HPLC) analysis of an extract that is obtained from a small sample volume and a safe, rapid extraction technique. This method can be used for the analysis of flosequinan and metabolite in plasma, serum* or urine. The assay shows excellent linearity for plasma within the range 0.05–5.0 μg/ml and for urine within the range 2.5–50 μg/ml.

*Serum and plasma will be used interchangeably within the text.

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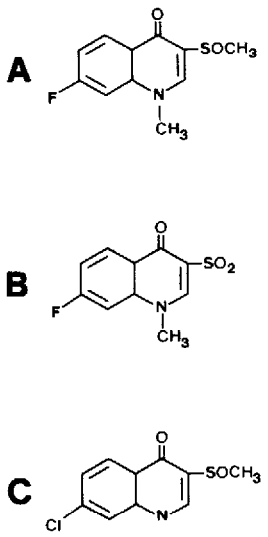

Fig. 1. Chemical structures of flosequinan (A), its major metabolite (B) and the internal standard (C).

EXPERIMENTAL

Materials

Flosequinan, its major metabolite (BTS 53554) and the internal standard (BTS 49037; 7-chloro-1-methyl-3-methylsulphinyl-4-quinolone) were supplied by Boots Pharmaceuticals (Shrevesport, LA, U.S.A.). The structures are shown in Fig. 1. Methanol and acetonitrile were HPLC grade (J.T. Baker, Phillipsburg, NJ, U.S.A.). The Baker-10 solid-phase extraction (SPE)^{TM} columns (octadecyl C_{18}, 1 ml) were also obtained from J.T. Baker. Institutionally supplied deionized water was further processed using a Barnstead nanopure filtration system (Barnstead, Boston, MA, U.S.A.). Blank human plasma was obtained from Utak Labs. (Canyon Country, CA, U.S.A.). The blank urine was collected from laboratory personnel and was assumed to contain caffeine but was deemed free from other chemical substances.

Instrumentation and chromatographic conditions

The solvent delivery system was a constant-flow reciprocating pump (Model 510, Waters Assoc., Milford, MA, U.S.A.) with a flow-rate of 1.2 ml/min. Injections were made using a Waters WISP^{TM} autosampler equipped with a 96-sample carriage. The analytical column was a Waters C_{18} Nova-Pak^{TM} (15 cm×3.9 mm, 5 μm spherical particles). The column effluent was monitored using a Waters Lambda-Max^{TM} (Model 481) variable-wavelength detector set at 254 nm. A Hewlett-Packard integrator (Model 3390A) was used to calibrate. The mobile phase consisted of filtered water–methanol–acetonitrile (73:20:7) which was filtered and degassed under vacuum. The system pressure was approximately 130 bar (1900 p.s.i.) at a flow-rate of 1.2 ml/min.

Sample extraction

The samples were extracted using a Baker-10 solid-phase extraction system with C_{18} 1-ml extraction columns. The extraction apparatus was connected to a vacuum source and maintained at 7–10 mmHg. The plasma or urine (100 μl) along with 25 μl of internal standard (2 μg/ml for plasma, 10 μg/ml for urine) were added to the column, which had been primed with methanol, then with filtered water. The sample and internal standard were aspirated through the column followed by two column volumes of filtered water. A 200-μl aliquot of methanol was added to each column, and the eluate was collected in 75 × 10 mm disposable borosilicate glass tubes (Fisher Scientific, King of Prussia, PA, U.S.A.). The analyte injection volume was 5 μl for plasma and 2 μl for urine.

Standards and calibration

Stock solutions of flosequinan, metabolite and internal standard were diluted with acetonitrile–methanol (1:1, v/v) to a concentration of 100 μg/ml. The internal standard was diluted further with filtered water to a concentration of 2 μg/ml for plasma samples and to 10 μg/ml for urine samples. Standards were prepared by drying down under nitrogen appropriate amounts of the stock solutions of drug and metabolite. These were reconstituted with blank plasma or urine and ranged from 0.05 to 5.0 μg/ml for the plasma assay and from 2.5 to 50 μg/ml for the urine assay. The standards were stored at −20 °C in 500-μl aliquots. The frozen standards were found to be stable for at least six months. New stock solutions were prepared each time more spiked plasma or urine standards were needed. A control sample was prepared by spiking 10 ml of blank plasma or blank urine to a concentration of 3.0 and 15.0 μg/ml, respectively. This was then aliquoted into 500-μl samples and frozen at −20 °C. A control was run with each standard curve and batch of samples. A midrange standard was used to calibrate based on peak-area ratios. For the plasma assay, the attenuation was 0 (0.001 a.u.f.s.) and for the urine samples the attenuation was 2 (0.004 a.u.f.s.).

RESULTS

Chromatograms resulting from the HPLC analysis of plasma and urine blanks, spiked standards and actual patient samples are shown in Figs. 2 and 3. The retention times of flosequinan and metabolite were 3.4 and 3.0 min, respectively, and the internal standard had a retention time of 7.8 min. The drug, its metabolite and the internal standard eluted as symmetrical peaks which were satisfactorily resolved from each other and from endogenous components in the plasma or urine; no interfering peaks were observed when blank plasma or urine was analyzed.

The standard curve for the plasma assay passed through the origin and was found to be linear over the range 0.05–5.0 μg/ml. The urine assay also passed through the origin and was linear over the range 2.5–50 μg/ml. After eighteen plasma standard curves, the mean r^2 for the drug was 0.9981 and for the metabolite the mean r^2 was 0.9958. The mean r^2 for the drug after eleven urine standard curves was 0.9960 and for the metabolite it was 0.9961.

Intra-run reproducibility was assessed by extracting ten aliquots each of blank

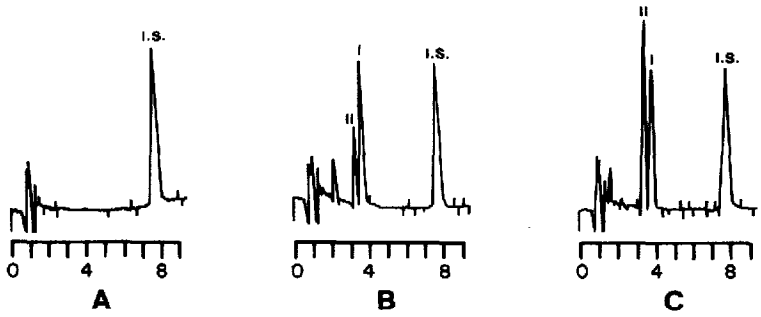

Fig. 2. Chromatograms resulting from the analysis of blank plasma (A), plasma spiked with 1.0 μg/ml flosequinan (I) and the metabolite (II) (B), and an actual patient plasma sample with concentrations of I and II of 1.0 and 2.6 μg/ml, respectively (C).

plasma and blank urine that had been spiked with 2.0 and 15.0 μg/ml, respectively, of both the drug and metabolite. The coefficient of variation of the plasma assay for both the drug and metabolite was 2.9%. The urine assay also had an intra-run coefficient of variation of 2.9% for both the drug and metabolite. For inter-run reproducibility, the plasma and urine standard curves and controls were prepared by spiking blank plasma or urine with the appropriate amount of drug and metabolite, aliquoting and freezing at −20 °C, and then extracting and analyzing a curve and control with each batch of patient samples over the course of one year. The results are presented in Tables I and II.

To evaluate potential sources of interference, blank plasma spiked with normal to high concentrations of various drugs was analyzed under the conditions of this assay. The drugs tested were acetominophen, amiodarone, diazepam, digoxin, furosemide, lidocaine, nitroglycerin, procainamide, propranolol and quinidine. No interfering peaks in the chromatograms were observed from the analysis of any of these samples.

To determine the analytical recovery of flosequinan and the metabolite, the standard stock solutions were diluted with methanol to 0.5 and 2.5 μg/ml and

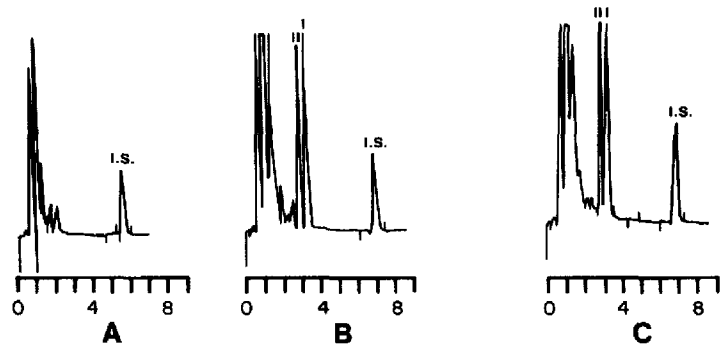

Fig. 3. Chromatograms resulting from the analysis of blank urine (A), urine spiked with 5.0 μg/ml flosequinan (I) and the metabolite (II) (B), and an actual patient urine sample with concentrations of I and II of 5.6 and 7.2 μg/ml, respectively (C).

TABLE I

INTER-RUN PRECISION OF THE PLASMA ASSAY

Concentration (μg/ml)	n	Coefficient of variation (%)	
		Flosequinan	Metabolite
0.05	13	13.2	10.4
0.50	17	3.6	3.7
5.00	15	5.8	4.8
Control	18	4.0	4.6

TABLE II

INTER-RUN PRECISION OF THE URINE ASSAY

Concentration (μg/ml)	n	Coefficient of variation (%)	
		Flosequinan	Metabolite
2.5	19	3.8	4.7
10.0	15	2.6	2.3
50.0	13	3.5	5.5
Control	17	5.3	5.5

TABLE III

ANALYTICAL RECOVERY OF FLOSEQUINAN AND METABOLITE FROM PLASMA

Concentration (μg/ml)	Recovery (mean, $n=5$) (%)	
	Flosequinan	Metabolite
0.5	96	98
2.5	83	89

injected directly. The peak areas were compared to those of freshly extracted plasma standards of 0.5 and 2.5 μg/ml. The results are presented in Table III.

DISCUSSION

Because the plasma standard curve had such a wide range (0.05–5.0 μg/ml) there was some loss of precision at either end but especially at the low end of the curve. The precision of the lower standards would be improved if the standard curve had a narrower range, perhaps 0.05–0.50 μg/ml. This would be only a 10-fold increase rather than the 100-fold increase that was used. Changing the attenuation or increasing the injection volume would result in larger peaks which would be more reproducible. The wide range standard curve was used for the

analysis of blinded clinical trial samples but in routine clinical use a narrower range curve could be used.

The mobile phase used is very simple to prepare and because no buffers are used, it is not damaging to the HPLC system. It is similar in composition to other mobile phases used in this laboratory so it is easy to switch from one assay to another.

The main advantage of this method is that the sample extraction is extremely simple and rapid. Using the Baker-10 SPE apparatus, ten samples can be processed in approximately 15 min and if two systems are available, twenty samples can be extracted in 20 min. Also the use of chloroform, as described in the previous method [2], has been eliminated. This assay can be adapted easily to analyze plasma or urine by simply changing the attenuation and the injection volume. All other parameters remain the same.

The method described here for the extraction and analysis of flosequinan and its metabolite, in serum, plasma and urine, is an assay that has been used in this laboratory to analyze over 3000 samples over the course of one year. The advantages include small sample volume, a safe, rapid extraction, short analysis time, and good recovery, precision and linearity.

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REFERENCES

1 A.J. Cowley, R.D. Wynne and J.R. Hampton, Hypertension, 2 (Suppl. 3) (1984) 547–549.
2 R.D. Wynne, E.L. Crampton and I.D. Hind, Eur. J. Clin. Pharmacol., 28 (1985) 659–664.
3 R.S. Porter, E. Paran, D.T. Lowenthal, S. Saris, M.B. Slegowski, C. Miller, C. Bies and C. Defesche, Acta Pharm. Toxicol., 59 (Suppl. 5) (1986) 178.