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Stereospecific and simultaneous high-performance liquid chromatographic assay of flosequinan and its metabolites in human plasma

Eiji Kashiyama*, Masaaki Odomi, Takefumi Shimizu

Department of Drug Metabolism, Tokushima Research Institute, Otsuka Pharmaceutical Co., Ltd., 463-10, Kagasuno, Kawauchi-cho, Tokushima 771-01, Japan

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Abstract

A high-performance liquid chromatographic method was developed for the simultaneous determination of the enantiomers of flosequinan [(±)-7-fluoro-1-methyl-3-methylsulphinyl-4-quinolone] and its metabolites, flosequinan sulphide and sulphone, in human plasma. These compounds were extracted from plasma with chloroform. The compounds were separated on a chiral stationary phase of cellulose tris-3,5-dimethylphenylcarbamate coated on silica gel, with a mobile phase of ethanol–methanol (22:78, v/v). Flosequinan enantiomers and flosequinan sulphone were determined by UV detection at a wavelength of 320 nm. Flosequinan sulphide was determined using fluorescence detection (excitation at 370 nm, emission at 430 nm). Standard curves were linear over the concentration range 5–10 000 ng/ml for both enantiomers and flosequinan sulphide, and 20–10 000 ng/ml for flosequinan sulphone. This method is adequate for pharmacokinetic studies of the enantiomers of flosequinan and its metabolites.

1. Introduction

Flosequinan, (±)-7-fluoro-1-methyl-3-methylsulphinyl-4-quinolone, is a peripheral vasodilator with effects on both arterial and venous vascular beds [1–3]. Flosequinan is rapidly absorbed in the gastrointestinal tract, reaching a peak plasma level after 30 min, and is cleared quickly from the systemic circulation with a half-life of 1.6–2.4 h. The major metabolite, flosequinan sulphone (7-fluoro-1-methyl-3-methylsulphonyl-4-quinolone), also has haemodynamic effects. Flosequinan sulphone shows high plasma concentrations and is cleared slowly with a half-life of 37.6 h, indicating longer-lasting haemodynamic effects [4,5]. The minor metabolite was flosequinan sulphide (7-fluoro-1-methyl-3-methylthio-4-quinolone).

Flosequinan has a stereogenic centre at its sulphur atom, giving two stereoisomers. It is known that enantiomers originating from the presence of an asymmetric carbon atom may have substantially different pharmacodynamic and pharmacokinetic properties [6–8]. Few pharmacokinetic studies of drugs containing chiral

* Corresponding author.

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sulphur have been reported. In the case of flosequinan, non-stereospecific techniques for pharmacokinetic studies of flosequinan have been reported [4,5,9]. Although the pharmacodynamic effects of the *S*(−)-isomer of flosequinan were several times higher than those of the *R*(+)-isomer in *in vitro* experiments, the pharmacokinetics of the two enantiomers have not yet been investigated.

This report describes a high-performance liquid chromatographic (HPLC) assay for the determination of flosequinan enantiomers. The method is also suitable for the simultaneous measurement of the metabolites, flosequinan sulphide and sulphone, in human plasma.

2. Experimental

2.1. Chemicals

Flosequinan, flosequinan sulphone (7-fluoro-1-methyl-3-methylsulphonyl-4-quinolone), flosequinan sulphide (7-fluoro-1-methyl-3-methylthio-4-quinolone) and the internal standard [(±)-7-chloro-1-methyl-3-methylsulphinyl-4-quinolone] were supplied by Boots (Nottingham, UK). *R*(+)- and *S*(−)-flosequinan were prepared in the Research Laboratory of Otsuka Pharmaceutical (Tokushima, Japan). The molecular structures of these compounds are shown in Fig. 1. HPLC-grade ethanol and methanol and analytical-grade chloroform were obtained from Wako (Osaka, Japan).

2.2. Spectra

R(+)- and *S*(−)-flosequinan, flosequinan sulphone and flosequinan sulphide were dissolved at 50 μM in ethanol–methanol (22:78, v/v). The UV–visible spectra and fluorescence spectra were measured using an UV–visible spectrophotometer, type UV-160A (Shimadzu Kyoto, Japan) and a fluorescence spectrophotometer, type RF-540 (Shimadzu), respectively.

2.3. Instrumentation and chromatographic conditions

The HPLC system consisted of a pump (Model 510, Waters Division of Millipore Japan, Tokyo, Japan), an autosampler (Model 712 WISP, Waters Division), a variable-wavelength UV absorbance detector (Model 484, Waters Division), fluorescence detector (RF-535, Shimadzu) and integrators (C-R3A, Shimadzu). The analytical column, Chiralcel OD (250 mm × 4.6 mm I.D., 10-μm particle size, Daicel Chemical Industries, Tokyo, Japan) was used at 30°C. The mobile phase was ethanol–methanol (22:78, v/v). The

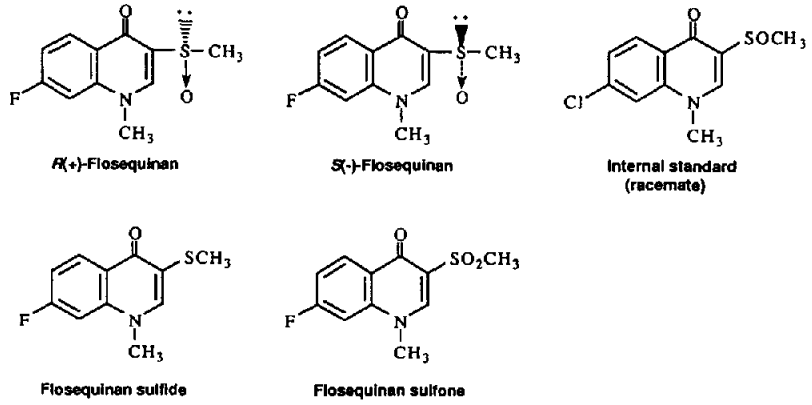

Fig. 1. Molecular structures of *R*(+)- and *S*(−)-flosequinan, flosequinan sulphide, flosequinan sulphone and the internal standard.

flow-rate was 0.7 ml/min, with *ca.* 50 kg/cm^2 back pressure.

2.4. Sample extraction

A 1-μg amount of the internal standard in 10 μl of acetonitrile–methanol (1:1, v/v) and 5 ml of chloroform was added to 1 ml of plasma in a 10-ml glass tube. After shaking for 10 min at room temperature and centrifugation at 1800 *g* for 10 min, 4 ml of the organic layer were transferred to another tube and evaporated under a gentle stream of nitrogen gas at 40°C. The residue was dissolved in 100 μl of methanol, and part of this was injected into the HPLC column.

2.5. Standards and calibration

A stock solution (100 μg/ml) of *R*(+)- and *S*(−)-flosequinan, flosequinan sulphide, flosequinan sulphone and the internal standard was dissolved in acetonitrile–methanol (1:1, v/v). Standard plasma samples were prepared at concentrations from 5 to 10 000 ng/ml, by the addition of the stock solution to blank plasma. Calibration curves were prepared by plotting the relationship between the peak-height ratio of each enantiomer and metabolite against the internal standard and their concentrations.

2.6. Pharmacokinetic study

Peripheral blood was drawn 3.5 h after oral administration of *RS*-flosequinan at 25 mg/body once a day for 10 days from six healthy male volunteers. Plasma was collected by centrifugation and stored at −20°C. Concentrations of *R*(+)- and *S*(−)-flosequinan, flosequinan sulphide and flosequinan sulphone in plasma were measured until 7 days after final administration.

3. Results

UV–visible spectra of *R*(+)- and *S*(−)-flosequinan, flosequinan sulphide and flosequinan sulphone at 50 μM are shown in Figs. 2A and B. The spectra of *R*(+)- and *S*(−)-flosequinan were exactly the same, showing absorption maxima at 213, 257, 320 and 332 nm. Absorption maxima were at 210, 270 and 348 nm for flosequinan sulphide, and at 210, 245 and 320 nm for flosequinan sulphone. A wavelength of 320 nm was adopted for quantitative analysis, because of the minimum interference from endogenous components. Flosequinan sulphide has

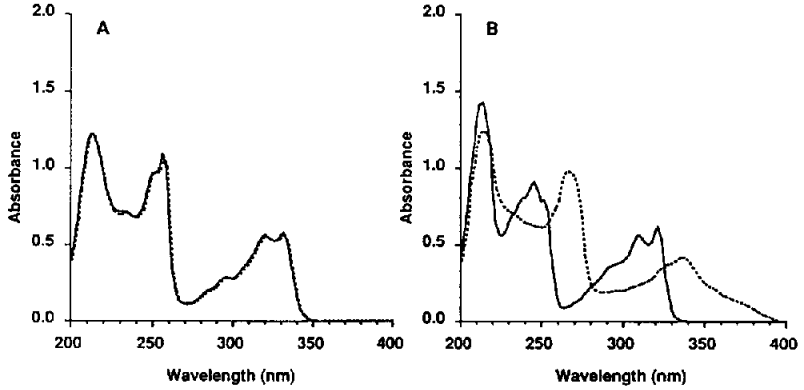

Fig. 2. UV–visible spectra of (A) 50 μM *R*(+)-flosequinan (dashed line) and *S*(−)-flosequinan (solid line), and (B) flosequinan sulphide (dashed line) and flosequinan sulphone (solid line).

a fluorescent absorption at 370-nm excitation wavelength and 430-nm emission wavelength.

HPLC chromatograms of blank plasma, spiked standard and plasma from volunteers who had been administered flosequinan racemate, are shown in Fig. 3. The peaks of *R*(+)- and *S*(−)-flosequinan, flosequinan sulphide, flosequinan sulphone and the enantiomers of the internal standard indicated a satisfactory separation. The retention times of *R*(+)- and *S*(−)-flosequinan were 12.1 and 7.9 min, respectively. Flosequinan sulphide and sulphone had retention times of 7.2 and 9.5 min, respectively. The first- and second-eluting peaks of the enantiomers of the internal standard were detected at 8.9 and 14.6 min.

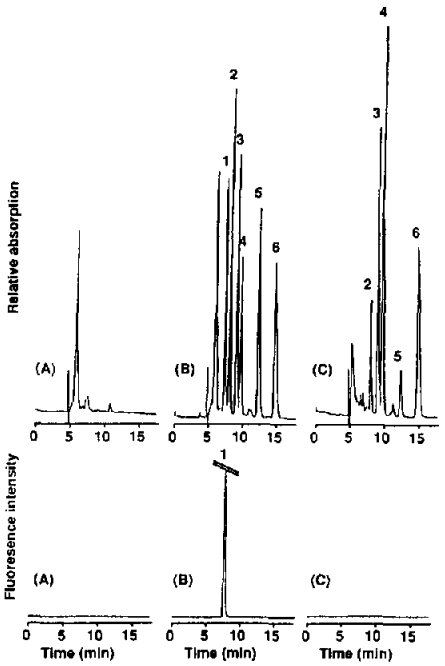

Fig. 3. Chromatograms of (A) a blank plasma extract, (B) a spiked plasma extract (concentrations of *R*(+)- and *S*(−)-flosequinan, flosequinan sulphide and flosequinan sulphone were 500 ng/ml), and (C) an extract from plasma sample of 1 ml taken 1 h after final administration of *RS*-flosequinan. Chromatograms of UV detection are shown above and of fluorescence detection below. Peaks: 1 = flosequinan sulphide; 2 = *S*(−)-flosequinan; 3 = internal standard; 4 = flosequinan sulphone; 5 = *R*(+)-flosequinan; 6 = internal standard.

A small peak of an endogenous component was observed at a retention time similar to that of flosequinan sulphide in UV detection. The fluorescence peak of flosequinan sulphide did not suffer from interference from endogenous components.

The accuracy and precision of within-day and day-to-day assays are summarized in Tables 1 and 2. The second-eluting peak of the internal standard, detected at 14.6 min, was employed for the calculation of peak-height ratios. The calibration curves showed good linearity over the range 5–10 000 ng/ml for *R*(+)- and *S*(−)-flosequinan and flosequinan sulphide, and 20–10 000 ng/ml for flosequinan sulphone. The mean R^2 was over 0.999. The accuracy and precision of the assay of all compounds were less than 13.8% in the range of calibration curves, except for 5 ng/ml flosequinan sulphone (Table 1). The extraction yields from plasma were more than 82.4%. There was no appreciable difference after two months' storage at −20°C in measurements of these compounds from plasma. No chiral inversion of either enantiomer was observed during storage.

Plasma concentrations of *R*(+)- and *S*(−)-flosequinan, flosequinan sulphide and flosequinan sulphone were measured after multiple oral administrations of *RS*-flosequinan to healthy volunteers, as described in Experimental. The plasma concentration profiles are shown in Fig. 4. No sample was observed with more than 1 ng/ml flosequinan sulphide. During the administration period, plasma concentrations of *R*(+)- and *S*(−)-flosequinan were kept almost constant, and the contents of *S*(−)-flosequinan were higher than those of *R*(+)-flosequinan. Although the concentrations of *R*(+)- and *S*(−)-flosequinan were varied among individual volunteers, their proportion showed a much smaller difference. The *R*(+)- and *S*(−)-flosequinan showed maximum levels at a ratio of four to six in plasma after multiple oral administration of *RS*-flosequinan (Fig. 5). The concentrations of flosequinan sulphone were *ca.* ten times higher than the concentrations of *R*(+)- and *S*(−)-flosequinan in plasma (Fig. 4).

Table 1
Within-day accuracy, precision and extraction yields

Concentration (ng/ml)		Accuracy (error, %)	Precision (C.V., %)	Extraction yield (%)
Added	Measured			
R(+)-Flosequinan				
5	5.1	3.0	3.8	112
20	20.5	10.3	12.2	110
100	96.8	3.6	3.6	97.8
500	495	1.0	0.6	97.1
2000	1996	1.0	1.3	101
10 000	10 180	3.0	2.3	86.8
S(−)-Flosequinan				
5	5.0	2.7	3.6	108
20	20.5	11.4	13.6	110
100	98.3	3.5	4.0	99.4
500	496	0.4	0.3	98.8
2000	2001	1.7	2.2	103
10 000	10 059	2.3	2.8	88.3
Flosequinan sulphide				
5	5.0	3.6	4.1	87.0
20	20.6	5.2	9.4	91.0
100	97.0	3.0	2.3	84.0
500	505	2.0	2.2	88.5
2000	2008	2.4	3.2	93.8
10 000	9992	3.2	3.7	82.4
Flosequinan sulphone				
5	–[a]	–[a]	25.5	150
20	21.7	12.7	13.8	132
100	92.5	7.5	2.6	103
500	482	3.6	0.5	101
2000	2028	2.4	2.3	106
10 000	10 295	3.8	2.8	88.4

[a] Not calculated, owing to high C.V. value.

4. Discussion

The direct resolution of many kinds of compound containing an asymmetric sulphur atom has been reported using different HPLC chiral phases. Protein [10,11], chiral peptide derivatives [12,13] and various polysaccharide derivatives [14,15] were used as chiral stationary phases on silica gel. Chiralcel OD, cellulose tris-3,5-dimethylphenylcarbamate coated on silica gel [16,17], was selected from many chiral phases, owing to its ability to resolve the enantiomers in this study. This column is widely used for the direct resolution of compounds containing an asymmetric carbon atom, such as β-adrenergic blocking drugs and anticholinergic drugs [16,17]. In the present study, several kinds of mobile phase were investigated, and the conditions for the resolution of $R(+)$- and $S(-)$-flosequinan, flosequinan sulphide and flosequinan sulphone were established. An internal standard used in a non-stereospecific assay [9] also showed good separation of the enantiomer. It should be noted that the internal standard is also a racemic

Table 2
Between-day accuracy, precision and extraction yields

Concentration (ng/ml)		Accuracy (error, %)	Precision (C.V., %)
Added	Measured		
R(+)-Flosequinan			
5	5.3	6.0	2.0
20	19.3	3.7	0.9
100	97.4	2.6	0.9
500	490	2.0	0.7
2000	2021	1.2	1.1
10 000	10 205	2.0	0.5
S(−)-Flosequinan			
5	5.5	10.6	2.8
20	18.7	6.7	2.1
100	95.9	4.1	1.9
500	489	2.4	2.5
2000	2012	2.4	3.1
10 000	10 362	3.6	1.1
Flosequinan sulphide			
5	5.0	1.2	1.5
20	19.8	1.5	1.2
100	101.7	1.8	1.1
500	508.2	2.5	2.4
2000	1975	2.5	3.8
10 000	9987	1.5	1.9
Flosequinan sulphone			
20	19.4	4.0	3.5
100	102.8	2.8	1.6
500	507	1.8	1.5
2000	2021	1.3	1.4
10 000	9781	2.2	1.0

Data from three sets of four experiments are shown.

mixture of isomers for monitoring chiral resolution.

Absorption at 320 nm was adopted for quantitative HPLC analyses. Although the compounds in this study showed approximately half the absorption at 320 nm compared with that at 210 and 250 nm, interfering absorption by endogenous components was observed at 210 and 250 nm. However, the chromatogram of blank plasma showed an endogenous component at the same retention time as flosequinan sulphide. However, the fluorescence detection of flosequinan sulphide showed no interference from endogenous components.

Plasma concentrations of flosequinan and its metabolites differed individually over a wide range after oral administration of *RS*-flosequinan [8,9], therefore linearity over a wide range (5–10 000 ng/ml) was achieved in this study. The precision of the quantitative analysis of flosequinan sulphone was relatively low in the lower concentration range, because of the partial overlapping of the first-eluted peak of the internal standard, which eluted immediately before flosequinan sulphone. Therefore, the standard curve of flosequinan sulphone was prepared in the range from 20 to 10 000 ng/ml. The plasma concentrations of flosequinan sulphone were always higher than those of flosequinan and other metabolites after oral administration of

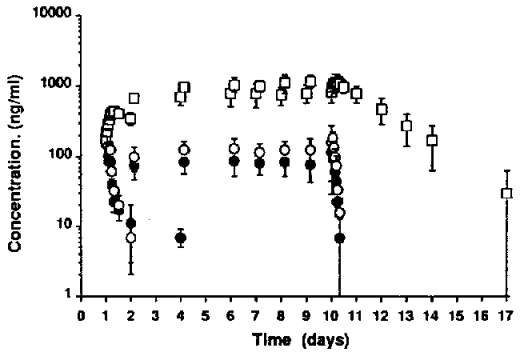

Fig. 4. Plasma concentrations of (●) *R*(+)-flosequinan, (○) *S*(−)-flosequinan and (□) flosequinan sulphone following multiple oral administration of *RS*-flosequinan at 25 mg/day to six healthy volunteers. Points represent mean values ± S.D.

RS-flosequinan [4,5], therefore the sensitivity of this assay for flosequinan sulphone is considered sufficiently high. The total plasma concentrations of *R*(+)- and *S*(−)-flosequinan and flosequinan sulphone determined in the present study were almost the same as those measured using a non-stereospecific determination method (data not shown).

In this study, it became apparent that the pharmacokinetics of the enantiomers varied from individual to individual, and the ratio of the enantiomers in the plasma of volunteers receiving multiple administrations was constant. The present method enables stereospecific, rapid, precise and sensitive assay of both enantiomers of flosequinan and its metabolites.

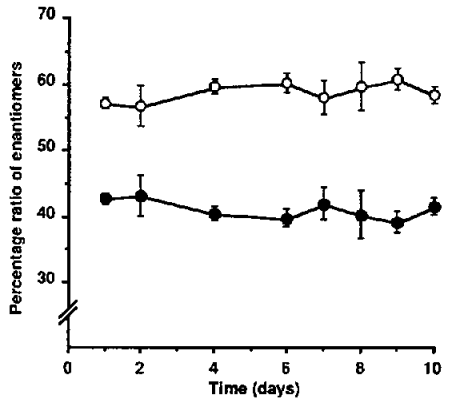

Fig. 5. Percentage ratio of (●) *R*(+)-flosequinan and (○) *S*(−)-flosequinan concentrations in plasma following multiple oral administration of *RS*-flosequinan at 25 mg/day to six healthy volunteers. Points represent mean values ± S.D.

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6. References

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