Journal of Chromatography, 564 (1991) 235–241
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5695

High-performance liquid chromatographic method for simultaneous determination of enantiomers of 5-dimethylsulphamoyl-6,7-dichloro-2,3-dihydrobenzofuran-2-carboxylic acid and its N-monodemethyl metabolite in monkey plasma and urine after chiral derivatization

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(First received August 14th, 1990; revised manuscript received October 24th, 1990)

ABSTRACT

A quantitative method for the simultaneous high-performance liqud chromatographic (HPLC) resolution and determination of the enantiomers of 5-dimethylsulphamoyl-6,7-dichloro-2,3-dihydrobenzofuran-2-carboxylic acid, a new diuretic, and its N-monodemethylated metabolite in monkey plasma and urine is described. The method includes diethyl ether extraction of the samples and *S*-(−)-α-methylbenzylamide derivatization of the extract, followed by reversed-phase solid-phase extraction and injection of the resulting diastereoisomers onto a reversed-phase HPLC column. Baseline separation was obtained. The assay showed linearity over the range 0.1–50 μg/ml of plasma and 0.25–500 μl of urine, with a lower limit of detection of *ca.* 0.01 μg/ml for each of the enantiomers. The method is adequate for pharmacokinetic and enantioselective disposition studies of both the diuretic and its metabolite.

INTRODUCTION

5-Dimethylsulphamoyl-6,7-dichloro-2,3-dihydrobenzofuran-2-carboxylic acid (I, Fig. 1) is a new diuretic [1–4]. Diuretic thiazides and other loop diuretics are widely used for treating hypertension. Hyperuricaemia is one of the well known side-effects of such drugs. Compound I causes no elevation of the plasma uric acid level at a dose that produces diuretic effects in chimpanzees [2]. The N-monodemethylated metabolite (M-1, Fig. 1), which exhibits weaker a diuretic activity than the parent compound, has been found in chimpanzees [2].

Interestingly, the *S*-(−)-enantiomer of I shows more potent diuretic effects than the *R*-(+)-enantiomer, whereas uricosuric effects are limited to the latter [2]. The racemate may offer a desirable ratio of activities. In the case of several racemic compounds, significant differences in the pharmacokinetics of the enantiomers, based on differences in the metabolic rate between enantiomers, have been described [5–7]. A precise and sensitive stereospecific method for the

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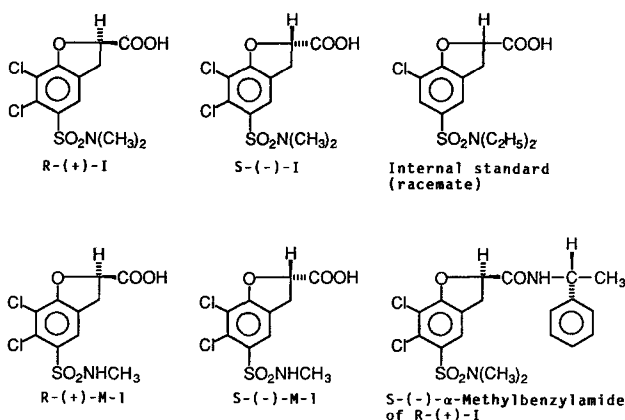

Fig. 1. Enantiomeric structures of I, M-1, the internal standard and the *S*-(−)-α-methylbenzylamide of I.

separation and quantitation of the drug and its metabolites is essential for determining the stereospecificity in metabolism and disposition of the drug.

The separation of drug enantiomers is normally carried out efficiently by high-performance liquid chromatography (HPLC) [8]. Direct chromatographic separation on chiral phases is an attractive alternative to indirect chiral separation of the enantiomers via formation of diastereoisomeric derivatives. Unfortunately, the enantiomer of I and its N-demethylated metabolite could not be simultaneously separated by chromatography on chiral stationary phases (Ultron ES-OVM, Resolvosil BSA-7).

Individual enantiomers of a racemic compound containing a carboxylic acid group in the molecule can be separated by diastereoisomeric ether formation or amide formation. The latter is preferable because of simple preparation of the reagents and stability of the derivative [8]. For example, individual enantiomers of ibuprofen have been separated after diastereoisomeric amide derivatization [9]. This paper describes a procedure for amide formation of racemic I with a chiral α-methylbenzylamide. The resulting diastereomers can be readily separated on a reversed-phase HPLC column.

EXPERIMENTAL

Chemicals

5-Dimethylsulphamoyl-6,7-dichloro-2,3-dihydrobenzofuran-2-carboxylic acid (I), 5-monomethylsulphamoyl-6,7-dichloro-2,3-dihydrobenzofuran-2-carboxylic acid (M-1) [1], their stereoisomers, and 5-diethylsulphamoyl-7-chloro-2,3-dihydrobenzofuran-2-carboxylic acid, which was used as the internal standard, were synthesized at Shionogi Research Labs. 5-Dimethylsulphamoyl-6,7-dichloro-2-[^{14}C],3-dihydrobenzofuran-2-carboxylic acid ([^{14}C]I), with 273.8

kBq/mg (7.40 μCi/mg) of specific activity was also synthesized in these laboratories. Its radiochemical purity was greater than 97.5% on thin-layer chromatography (TLC)–autoradiography [silica gel HPTLC plate 60 F_{254}, Merck, dichloromethane–methanol–acetic acid (10:1:0.2, twice, v/v)] and liquid scintillation counting [2000 CA (Packard)]. *S*-(−)-α-Methylbenzylamine and 1,1′-carbonylbis-2-methylimidazole were purchased from Aldrich (Milwaukee, WI, U.S.A.). Acetonitrile, distilled water and molecular sieves were purchased from Wako (Osaka, Japan). Acetonitrile was dried on NRG molecular sieve for several days prior to use.

Extraction

Plasma (0.1–0.5 ml) and urine (0.1–1.0 ml) obtained from cynomolgus monkeys were transferred to test-tubes and made up to 1 ml with distilled water. The samples were also spiked with a mixture of various amounts of enantiomeric I and M-1 dissolved in 50 μl of acetonitrile, together with 1 μg (for plasma) or 10 μg (for urine) of the I.S. dissolved in 50 μl. The samples were adjusted to pH 1.0 with 0.5 ml of 1 *M* HCl and 0.5 ml of 0.25 *M* sodium acetate plus HCl solution (pH 1), and then extracted with 7 ml of diethyl ether by vortex-mixing and centrifuging (1300 *g*). The upper layer was transferred to another test-tube. The samples were back-extracted by vortex-mixing into 1.5 ml of 0.2 *M* phosphate buffer (pH 7.5, KH_2PO_4 plus Na_2HPO_4) for plasma and 1% $NaHCO_3$ (pH 8.6) for urine. After washing with 6 ml of diethyl ether, the aqueous layer was adjusted to pH 1 with 0.5 ml of 1 *M* HCl and 0.25 *M* sodium acetate plus HCl solution (pH 1.0), and then extracted with 6 ml of diethyl ether (plasma) or 6 ml of chloroform (urine). The organic layers were removed to another test-tube and then dried thoroughly.

Formation of the α-methylbenzylamide of I

Organic solvent extracts from the plasma were mixed with 100 μl of 0.2% 1,1′-carbonylbis-2-methylimidazole in acetonitrile and allowed to stand for 30–60 min at room temperature. The sample was mixed with 50 μl of 0.2% *S*-(−)-α-methylbenzylamine in acetonitrile, and 10 μl of 1% acetic acid in acetonitrile. For urine samples, five volumes of reagents were used.

To determine the yield of the α-methylbenzylamide of I, 3 μg of [^{14}C]I, combined with 100 μg of unlabelled racemic I and M-1, were subjected to the above procedure. An aliquot of the reaction mixture was spotted on a TLC plate (HPTLC plate, silica gel 60 F_{254}, Merck) and then chromatographed twice with benzene–dichloromethane–ethyl acetate–2-propanol (8:1:0.6:0.4, v/v). Zones of radioactive enantiomer were scraped off the plates and transferred to a counting vial, followed by the addition of 10 ml of Pico-fluor 40 (Packard). The radioactivity was measured by a liquid scintillation spectrometer. The yield of the amide obtained was 94%. No decrease of the peak area on HPLC of the synthesized amide of I and M-1 was observed four days after the derivatization.

Clean-up by Bond Elut C_{18}

After evaporation of the reaction mixture, the samples were dissolved in 2.7 ml of 45% methanol containing 0.5% acetic acid. The solution was filtered through a Millipore filter (Type FH, 0.5 μm) and then the filtrate was applied to Bond Elut C_{18} (3 ml, Analytichem International, Harbor City, CA, U.S.A.), which had been washed with 2.5 ml of methanol five times followed by washing once with 45% methanol containing 0.5% acetic acid. The Bond Elut was washed twice with 2.5 ml of 45% methanol containing 0.5% acetic acid and eluted with 2.5 ml of 70% acetonitrile (recovery, over 99%). After thorough evaporation of the solvent, the sample was dissolved with 150 μl of acetonitrile, and 15 μl of the solution were injected onto the HPLC system.

Chromatographic system

The chromatographic system consisted of a Model LC-6A HPLC pump (Shimadzu, Kyoto, Japan), a Model SIL-6A autoinjector (Shimadzu) and a UV detector (Shimadzu) set at 223 nm for plasma and 254 nm for urine. Nucleosil C_{18} columns (250 mm × 4.6 mm I.D., pore size 100 Å for plasma; 200 mm × 4.6 mm I.D. connected with 150 mm × 4.6 mm I.D., pore size 120 Å for urine; 5 μm particle size; Chemco-packed, Macherey-Nagel, Düren, Germany) was used at room temperature. The mobile phase was acetonitrile–methanol–water (45:5:50) for plasma and acetonitrile–2-propanol–water (40:5:55) for urine, delivered at flow-rate of 1 ml/min.

RESULTS AND DISCUSSION

Representative chromatograms for I and M-1 in cynomolgus monkey plasma and urine are shown in Fig. 2. Fig. 3 shows the plasma concentration–time curve for the enantiomers of I and M-1 in a cynomolgus monkey after oral administration of I (10 mg/kg).

During method development, several precautions were found to be necessary. Because the sample volume of plasma was limited and compound concentrations were low, the wavelength chosen was 223 nm, at which the *S*-(−)-α-methylbenzylamides of I and M-1 show stronger absorbance than at 254 nm. The wavelength of 223 nm produced much interference, which was minimized using chloroform extraction and 0.2 *M* phosphate buffer (pH 7.5) back-extraction. With these preparations, baseline separation was obtained for the diastereoisomers of the α-methylbenzylamides of I and M-1. The baseline was clean and free from interfering peaks. The method we developed to simultaneously measure I and M-1 showed very long retention times, but attempts to shorten the retention times were not successful because of the introduction of interfering compounds during derivatization.

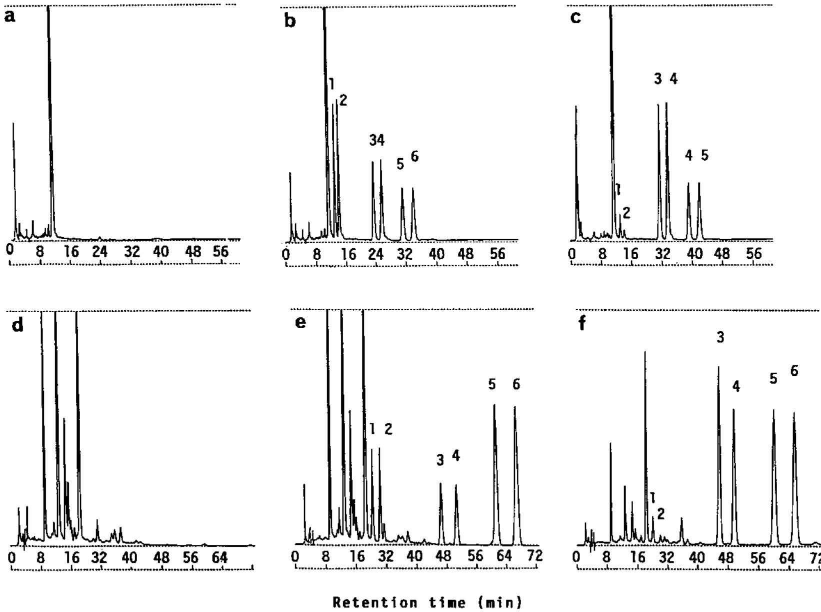

Fig. 2. Chromatograms of *S*-(−)-α-methylbenzylamide-derivatized biological samples: (a) and (d) blank plasma and urine; (b) blank plasma spiked with 0.5 μg of each enantiomer; (e) blank urine spiked with 5 μg of each enantiomer; (c) and (f) plasma and urine obtained from a monkey dosed with I. A male cynomolgus monkey was dosed orally with I (10 mg/kg). Plasma samples were collected 2 h after the administration, and the urine was pooled for 6–24 h. Peaks: 1 = *R*-(+)-M-1; 2 = *S*-(−)-M-1; 3 = *R*-(+)-I; 4 = *S*-(−)-I; 5 and 6 = internal standards.

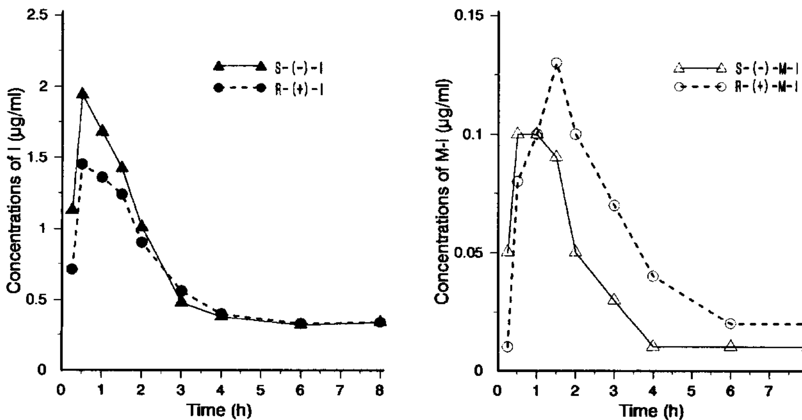

Fig. 3. Plasma concentration–time curves of I (▲ and ●) and M-1 (○ and △) in a cynomolgus monkey dosed orally with I (10 mg/kg).

Calibration curve

Blank plasma (0.1 or 0.5 ml) spiked with 0.05, 0.25, 1 or 5 μg of each enantiomer of I and M-1 showed a good linear correlation (Table I). A good linear correlation was also found with 0.1 and 1 ml of blank urine (Table I). Correlation coefficients for all linear regression plots exceeded 0.999.

Accuracy and precision

Table I shows the day-to-day variation in assays using 0.1 or 0.5 ml of plasma and 0.1 or 1.0 ml of urine. The coefficient of variation (C.V.) ranged from 0.1 to

TABLE I

DAY-TO-DAY ACCURACY AND PRECISION FOR THE DETERMINATION OF I AND M-1 IN MONKEY PLASMA AND URINE

$\bar{X}$ represents the mean of four experiments.

Sample (ml)	Added (μg)	*R*-(+)-M-1		*S*-(−)-M-1		*R*-(+)-I		*S*-(−)-I	
		$\bar{X}$ (μg)	C.V. (%)	$\bar{X}$ (μg)	C.V. (%)	$\bar{X}$ (μg)	C.V. (%)	$\bar{X}$ (μg)	C.V. (%)
Plasma									
0.1	0.05	0.051	4.9	0.052	6.0	0.051	4.9	0.049	8.2
	0.25	0.249	1.4	0.248	1.4	0.249	1.5	0.252	2.1
	1	0.998	3.0	0.998	3.2	1.009	1.8	1.005	1.8
	5	5.001	0.1	5.000	0.1	4.999	0.1	4.999	0.1
0.5	0.05	0.049	6.6	0.051	6.1	0.051	5.2	0.054	1.8
	0.25	0.251	1.7	0.249	1.8	0.249	1.2	0.245	0.4
	1	1.038	2.2	1.042	2.5	1.029	1.3	1.009	1.3
	5	4.993	0.1	4.993	0.1	4.995	0.1	5.016	0.7
Urine									
0.1	0.25	0.249	1.4	0.247	2.9	0.249	2.2	0.249	1.4
	1	1.005	0.8	1.002	1.2	1.005	0.7	1.004	0.5
	2	2.040	2.2	2.030	1.9	2.039	1.8	2.036	1.4
	5	4.917	2.0	4.906	1.8	4.935	1.5	4.931	1.4
	10	9.942	2.2	9.925	1.9	9.957	0.7	9.948	0.4
	20	19.876	1.2	19.894	1.0	19.927	0.8	19.940	0.5
	35	35.061	0.7	35.062	0.7	34.992	0.3	34.995	0.3
	50	50.023	0.2	50.020	0.2	50.048	0.1	50.042	0.1
1.0	0.25	0.254	4.0	0.246	2.1	0.247	1.2	0.248	2.0
	1	0.989	1.0	1.003	1.3	1.002	0.3	0.987	1.5
	2	2.014	1.1	1.998	0.8	2.009	0.3	2.013	0.5
	5	4.959	0.8	4.954	1.1	4.966	0.4	4.963	0.5
	10	10.007	1.3	9.995	1.2	9.989	0.6	9.960	0.5
	20	19.849	0.9	19.862	0.8	19.986	0.5	19.987	0.5
	35	34.989	0.9	35.006	0.9	35.188	0.4	35.223	0.3
	50	50.069	0.6	50.055	0.6	49.880	0.3	49.861	0.2

8.2% at 0.1–50 μg/ml of I and from 0.1 to 8.2% at the same concentration range of M-1. The limit of sensitivity for an enantiomer of I or M-1 was 0.01 μg/ml (signal-to-noise ratio 10:1).

REFERENCES

1 H. Harada, Y. Matsushita, M. Yodo, M. Nakamura and Y. Yonetani, *Chem. Pharm. Bull.*, 35 (1987) 3215.
2 Y. Yonetani, K. Iwaki, T. Shinosaki, A. Kawase-Hanafusa, H. Harada and A. A. van Es, *Jpn. J. Pharmacol.*, 43 (1987) 389.
3 Y. Yonetani, K. Iwaki, M. Ishii and H. Harada, *Jpn. J. Pharmacol.*, 43 (1987) 399.
4 M. Nakamura, T. Shimizu, K. Miyata, T. Kawabata and H. Harada, *Drug Develop. Res.*, 12 (1988) 41.
5 F. Jamali, R. Mehvar and F. M. Pasutto, *J. Pharm. Sci.*, 78 (1989) 695.
6 D. E. Drayer, in I. W. Wainer and D. E. Drayer (Editors), *Drug Stereochemistry*, Marcel Dekker, New York, 1988, p. 206.
7 S. K. Pratt, M. J. Winn and B. K. Park, *J. Pharm. Pharmacol.*, 41 (1989) 743.
8 B. Testa, *Xenobiotica*, 16 (1986) 265.
9 G. J. Vangiessen and D. G. Kaiser, *J. Pharm. Sci.*, 64 (1975) 798.