Journal of Chromatography, 527 (1990) 381–388
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 5198

Determination of a new calcium antagonist, sesamodil fumarate (SD-3211), and its metabolite in plasma by liquid chromatography with electrochemical detection

KENJI MORISHIMA*, RITSUKO TAHARA and MASATO HORIUCHI

Central Research Laboratories, Santen Pharmaceutical Co., Ltd., 9-19 Shimoshinjo 3-chome, Higashiyodogawa-ku, Osaka 533 (Japan)

(Received October 31st 1989)

SUMMARY

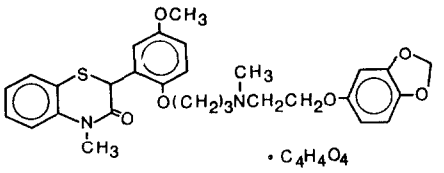
A sensitive and selective high-performance liquid chromatographic method is described for the determination of a novel calcium antagonist, (+)-(*R*)-3,4-dihydro-2-[5-methoxy-2-[3-[N-methyl-N-[2-[(3,4-methylenedioxy)phenoxy]ethyl]amino]propoxy]phenyl]-4-methyl-3-oxo-2*H*-1,4-benzothiazine hydrogen fumarate (sesamodil fumarate; JAN, SD-3211, I), and its N-desmethylated metabolite (II) in plasma. Compounds I and II and an internal standard were isolated from plasma by solid-phase and liquid-liquid extraction. The extract was chromatographed on a reversed-phase C_{18} column, and the compounds of interest were detected by dual coulometric electrodes operated in an oxidative screen mode. The limit of determination for both I and II was at least 0.4 ng/ml in plasma. The utility of the assay was demonstrated by determining plasma levels of I and II in five dogs administered an oral dose of 60 mg of the drug.

INTRODUCTION

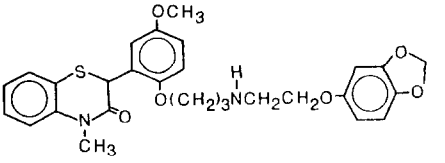
(+)-(*R*)-3,4-Dihydro-2-[5-methoxy-2-[3-[N-methyl-N-[2-[(3,4-methylenedioxy)phenoxy]ethyl]amino]propoxy]phenyl]-4-methyl-3-oxo-2*H*-1,4-benzothiazine hydrogen fumarate (sesamodil fumarate; JAN, I, Fig. 1) is a new benzothiazine derivative with calcium entry blocking activity [1,2].

In general, the quantification of calcium antagonists in plasma is difficult owing to the low levels encountered for reasons such as the low therapeutic doses, significant first-pass metabolism on oral administration, extensive me-

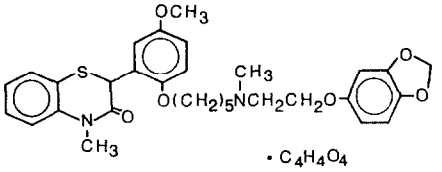
0378-4347/90/$03.50



I



II



III

Fig. 1. Molecular structures of I, II (N-desmethylated metabolite) and III (internal standard).

tabolism to numerous metabolites and a large volume of distribution [3–13]. To evaluate the pharmacokinetics and metabolic profile of these calcium antagonists, sensitive, precise and specific analytical methods are required.

In recent years, electrochemical detection (ED) has been applied to high-performance liquid chromatography (HPLC). ED was reported to be effective for the quantitative determination of drugs with a heterocyclic structure, such as phenothiazine and benzothiazepin derivatives [14–20].

This paper described a selective, accurate and precise HPLC method with ED in an oxidative screen mode for the determination in plasma of I and its N-desmethylated metabolite (II, Fig. 1), which are benzothiazine derivatives, with subnanogram sensitivity. This method can be applied to investigate the absorption, elimination and metabolism of I in dogs and humans.

EXPERIMENTAL

Materials

Sesamodil fumarate (I), the oxalate of N-desmethylated metabolite (II) and the internal standard, 4-dihydro-2-[5-methoxy-2-[5-[N-methyl-N-[2-[(3,4-methylenedioxy)phenoxy]ethyl]amino]pentyloxy]phenyl]-4-methyl-3-oxo-2*H*-1,4-benzothiazine hydrogen fumarate (III, Fig. 1) were synthesized by the Central Research Laboratories of Santen Pharmaceutical (Osaka, Japan). A Bond-Elut C_{18} column (1 ml capacity), used for the extraction, was purchased from Analytichem International (Harbor City, CA, U.S.A.). All other reagents and solvents were purchased from commercial sources. Cyclohexane was distilled before use.

Instrumentation and chromatographic conditions

The chromatographic system consisted of a solvent-delivery pump (Model L-6000, Hitachi, Tokyo, Japan) with a pulse damper (Shimadzu, Kyoto, Japan), an automatic injector (Model SIL-9A, Shimadzu) and a Finepak SIL C_{18} reversed-phase column (25 cm×4.6 mm I.D., particle size 5 μm, Japan Spectroscopic, Tokyo, Japan). The column was maintained at 50°C by a column oven (Model 655-52, Hitachi). A coulometric dual-electrode electrochemical detector (Model 5100A, ESA, Bedford, MA, U.S.A.) was connected to a recorder (Model C-R4A Chromatopack, Shimadzu). This electrochemical detector was equipped with a guard cell (Model 5020, ESA) placed in-line before the injector in order to electrolyse components of the mobile phase. The dual-electrode cell (Model 5011, ESA) was operated in an oxidative screen mode. The applied cell potential of the screen electrode was set at +0.65 V and the sample electrode at +0.85 V. The guard cell was set at +0.90 V. To protect the graphite electrode, in-line filters (0.5-μm carbon filter, ESA) were placed before the guard cell and the analytical cell.

The mobile phase was a mixture of an acidic solution (30 m*M* di-*n*-butylamine and 35 m*M* phosphoric acid adjusted to pH 3 with phosphoric acid) and acetonitrile (55:45, v/v). The mobile phase was filtered through a 0.45-μm filter (Sumitomo Electric, Tokyo, Japan). The flow-rate was adjusted to 1.2 ml/min.

Sample preparation

A Bond-Elut C_{18} column was activated with 1 ml of acetonitrile and washed with 1 ml of 0.1 *M* potassium hydrogensulphate. To 1 ml of plasma, 2 ml of 0.1 *M* potassium hydrogensulphate and 0.2 ml of the internal standard solution (0.5 μg/ml) were added. The mixture was loaded onto the Bond-Elut C_{18} column. After loading, the column was washed with 1 ml of water and 1 ml of acetonitrile. Compounds I and II and the internal standard were eluted from the column with 1 ml of a mixture of an acidic solution (150 m*M* di-*n*-butylamine and 175 m*M* phosphoric acid adjusted to pH 3 with phosphoric acid) and acetonitrile (5:95, v/v). The eluate was evaporated to dryness, and the residue was dissolved in 2 ml of 0.1 *M* potassium hydrogensulphate. The aqueous solution was washed twice with 1 ml of cyclohexane and mixed with 0.5 ml of 1 *M* sodium hydroxide. Then the solution was extracted twice with 1 ml of cyclohexane. The organic phase was transferred to another glass tube and evaporated to dryness. The residue was dissolved in 200 μl of HPLC mobile phase, and 100 μl were injected onto the HPLC column.

Quantification

The plasma concentrations of I and II were determined by comparing the peak-height ratios (I/internal standard, II/internal standard) with those of the standard solution.

RESULTS AND DISCUSSION

Plasma extraction

Compounds I and II and the internal standard were efficiently adsorbed on the Bond-Elut C_{18}. The recovery rates from the Bond-Elut C_{18} with 1 ml of solvent are given in Table I. Compounds I and II and the internal standard were strongly bound unless di-*n*-butylamine was added to the eluting solvent.

It is known that basic drugs interact with residual silanol groups [21–23]. The retention of I, II and the internal standard on the Bond-Elut C_{18} is explained by a complex mechanism, possibly involving a hydrophobic interaction with C_{18} groups and an ionic interaction with silanol groups. The very strong interaction between the compounds of interest and silanol groups makes it possible to wash the column with acetonitrile to remove interfering endogenous compounds. Compounds I and II and the internal standard were eluted with a mixture of an acidic solution (150 m*M* di-*n*-butylamine and 175 m*M* phosphoric acid adjusted to pH 3 with phosphoric acid) and acetonitrile (5:95, v/v). Di-*n*-butylamine deactivated the silanol groups. It can be concluded that I, II and the internal standard are isolated with high selectivity from plasma using the Bond-Elut C_{18} for extraction.

In addition, the eluate was concentrated by cyclohexane extraction.

Chromatography

As a mobile phase in the HPLC, the mixture of an acidic solution described in Experimental gave a good separation of I, II and the internal standard. A typical chromatogram of the standard solution is shown in Fig. 2A. The retention times of I, II and internal standard were 10.5, 8.5 and 14.5 min, respectively.

With the aim of finding the potential of ED with a low background current and high sensitivity for the determination of I and II, various conditions of oxidation potential were examined with certain drugs with a heterocyclic

TABLE I

ELUTION RECOVERIES BY SOLID-PHASE EXTRACTION

Eluting solvent	Recovery (%)		
	I	II	I.S.[a]
Water	0	0	0
Methanol	0	0	0
Acetonitrile	0	0	0
0.2 *M* Phosphoric acid–acetonitrile (5:95)	0	0	0
Acidic solution[b]–acetonitrile (5:95)	96 ± 5	93 ± 7	92 ± 8

[a]Internal standard.

[b]150 m*M* Di-*n*-butylamine and 175 m*M* phosphoric acid, pH 3.

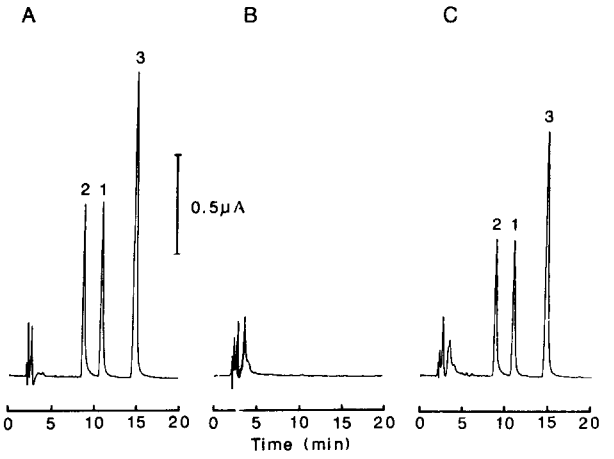


Fig. 2. Chromatograms of (A) standard solution, (B) drug-free dog plasma and (C) dog plasma spiked with I (40.5 ng/ml), II (40.0 ng/ml) and the internal standard (100.7 ng/ml). Peaks: 1 = I, 2 = II (N-desmethylated metabolite); 3 = III (internal standard).

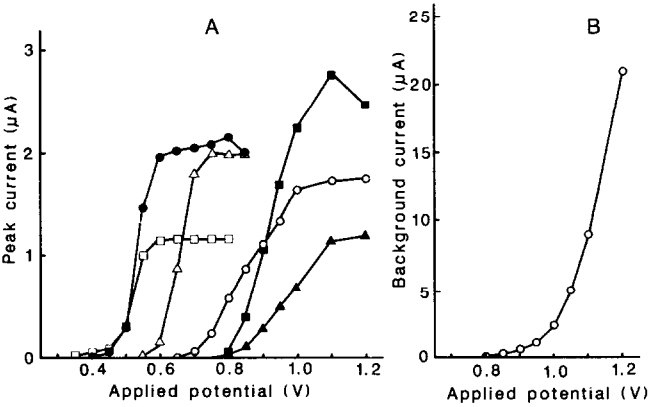


Fig. 3. (A) Hydrodynamic voltammograms of 20 ng each of I (○), diltiazem (▲), tiaramide (■), dimetotiazine (△), promethazine (●) and ethopropazine (□). (B) Background current–voltage curve.

structure. Fig. 3 shows the hydrodynamic voltammograms for these drugs and the background current–voltage curve. It was found that dicyclic compounds, such as tiaramide and diltiazem, exhibited higher potentials than tricyclic compounds, such as dimetotiazine, promethazine and ethopropazine. Compounds I and II and the internal standard, which are benzothiazine derivatives, exhibited a relatively high potential. It was necessary to use an ED system with dual coulometric electrodes in the oxidative screen mode for the trace determination of I and II in plasma. In this mode, the potential of the first electrode

was set at +0.65 V in order to reduce the interfering peaks derived from endogenous substances in plasma without a decrease in the response of I, II and the internal standard. The electrochemical response of I reached a plateau at ca. +1.0 V. But the potential of the second electrode was chosen at +0.85 V, because significant background current occurred at the plateau value. The detection limit, based on a signal-to-noise ratio of 3:1, was found to be 50 pg (ca. 100 fmol) for both I and II.

Typical chromatograms of drug-free plasma and plasma containing I, II and the internal standard are shown in Fig. 2B and C, respectively. No peak derived from endogenous substances in plasma interfered significantly with the compounds of interest.

Precision and accuracy

Known amounts (0.4–400 ng/ml) of I and II were added to dog and human plasma, and these were determined by the present method. The precision and the accuracy in this determination are shown in Tables II and III. The coefficients of variation (C.V.) of dog and human plasma samples were between 1.0 and 6.2% for I and between 0.5 and 5.9% for II. The accuracy was determined by comparing the nominal concentrations of I and II with those observed. The relative error ranged from −5.6 to 2.5% for I and from −7.5 to 2.6% for II.

Stability

The stability of I and II in plasma was assessed by spiking a known amount of each compound into dog plasma and storing at −80°C. No significant drug degradation in the frozen samples was observed during sixteen days.

Application of the method

The method was used in a pharmacokinetic study in which a 60-mg single oral dose of I was given to five dogs. Fig. 4 shows the time-course of the plasma

TABLE II

PRECISION AND ACCURACY IN THE DETERMINATION OF I AND II IN DOG PLASMA

Each value is the mean ± S.D. of five determinations.

Compound	Theoretical concentration (ng/ml)	Measured concentration (ng/ml)	C.V. (%)	Relative error (%)
I	0.40	0.41 ± 0.02	4.9	2.5
	40.45	39.63 ± 0.82	2.1	−2.0
	404.51	385.77 ± 8.88	2.3	−4.6
II	0.40	0.37 ± 0.02	5.4	−7.5
	39.96	39.03 ± 0.29	0.7	−2.3
	399.65	410.15 ± 4.30	1.0	2.6

TABLE III

PRECISION AND ACCURACY IN THE DETERMINATION OF I AND II IN HUMAN PLASMA

Each value is the mean ± S.D. of three determinations.

Compound	Theoretical concentration (ng/ml)	Measured concentration (ng/ml)	C.V. (%)	Relative error (%)
I	0.40	0.40 ± 0.01	2.5	0.0
	4.05	3.97 ± 0.06	1.5	−2.0
	40.45	40.57 ± 2.51	6.2	0.3
	404.51	381.72 ± 3.89	1.0	−5.6
II	0.40	0.38 ± 0.02	5.3	−5.0
	4.00	3.72 ± 0.06	1.6	−7.0
	39.96	40.97 ± 2.43	5.9	2.5
	399.65	389.92 ± 1.87	0.5	−2.4

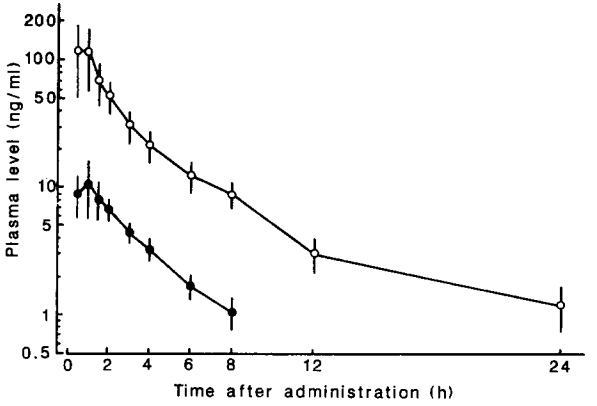


Fig. 4. Plasma levels of I (○) and II (●) after single-dose oral administration of 60 mg of I to beagle dogs. Each point presents the mean ± S.D. of five dogs.

concentration of I and II. The drug was rapidly absorbed and the maximum plasma concentration (129.8 ± 68.7 ng/ml) was reached 0.9 ± 0.2 h after administration. The areas under the concentration–time curves of I and II were 357 ± 115 and 37 ± 10 ng h/ml, respectively.

This method was also applied to clinical pharmacokinetic studies and the results will be reported elsewhere.

CONCLUSIONS

The combination of HPLC and ED makes it possible to determine I and its N-desmethylated metabolite in plasma with selectivity and subnanogram sen-

sitivity. This method can be applied to pharmacokinetic studies of I in dogs and humans.

REFERENCES

1 N. Miyawaki, K. Matsuno, Y. Tsukushi, T. Takada, H. Yamauchi and T. Iso, Jpn. J. Pharmacol. (Suppl.), 49 (1989) 210.
2 T. Takada, K. Nishimura, K. Nakata, N. Miyawaki, K. Matsuno, N. Ishida, H. Yamauchi and T. Iso, Jpn. J. Pharmacol. (Suppl.), 49 (1989) 212.
3 T. Nagao, M. Sato, Y. Iwasawa, T. Takada, R. Ishida, H. Nakajima and A. Kiyomoto, Jpn. J. Pharmacol., 22 (1972) 467.
4 T. Fujita, K. Doi and S. Sakuma, Pharmacometrics, 8 (1974) 757.
5 L.K. Evlin, M.M. Ralph and Z. Robert, J. Clin. Pharmacol., 21 (1981) 337.
6 M. Sakuma, M. Yoshikawa and Y. Sato, Chem. Pharm. Bull., 19 (1971) 995.
7 J. Sugihara, Y. Sugawara, H. Ando, S. Harigaya, A. Etoh and K. Kohno, J. Pharmacobio-Dyn., 7 (1984) 24.
8 M. Schomerus, B. Spiegelhalder, B. Stieren and M. Eichelbaum, Cardiovasc. Res., 10 (1976) 605.
9 Y. Koike, K. Shimamura, I. Shudo and H. Saito, Res. Commun. Chem. Pharmacol., 24 (1979) 37.
10 M. Eichelbaum, M. Ende, G. Remberg, M. Schomerus and H.J. Dengler, Drug Metab. Dispos., 7 (1979) 145.
11 S. Higuchi and Y. Shiobara, Xenobiotica, 10 (1980) 447.
12 S. Higuchi and Y. Shiobara, Biomed. Mass Spectrom., 7 (1980) 339.
13 S. Higuchi and Y. Shiobara and T. Sado, Xenobiotica, 7 (1977) 469.
14 J. Wallace, E. Shimek, S. Stavchansky and S. Harris, Anal. Chem., 53 (1981) 960.
15 G. McKay, J.K. Cooper and K.K. Midha, J. Chromatogr., 233 (1982) 417.
16 S. Melethil, A. Dutta, V. Chungi and L. Dittert, Anal. Lett., 227 (1983) 701.
17 K. Murakami, T. Ueno and T. Muto, J. Chromatogr., 227 (1982) 103.
18 P. Höglund and L.-G. Nilsson, J. Chromatogr., 414 (1987) 109.
19 R.F. Suckow and T.B. Cooper, J. Pharm. Sci., 70 (1981) 257.
20 J.K. Cooper, G. McKay and K.K. Midha, J. Pharm. Sci., 72 (1983) 1259.
21 D.E.M.M. Vendrig, J. Teeuwsen and J.J.M. Holthuis, J. Chromatogr., 424 (1988) 83.
22 G. Musch and D.L. Massart, J. Chromatogr., 432 (1988) 209.
23 J.W. Cox and R.H. Pullen, J. Chromatogr., 424 (1988) 285.