ELSEVIER

Journal of Chromatography B, 749 (2000) 101–110

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Determination of a new oral cephalosporin, cefmatilen hydrochloride hydrate, and its seven metabolites in human and animal plasma and urine by coupled systems of ion-exchange and reversed-phase high-performance liquid chromatography

I. Nishino, H. Fujitomo, T. Umeda*

Shionogi Research Laboratories, Shionogi & Co., Ltd., 5-12-4 Sagisu, Fukushima-ku, Osaka 553-0002, Japan

Received 25 February 2000; received in revised form 13 June 2000; accepted 5 July 2000

Abstract

Multidimensional HPLC systems with coupled anion-exchange, cation-exchange, and reversed-phase columns, and ultraviolet and electrochemical detectors were developed for the determination of cefmatilen and its seven metabolites in the plasma and urine of humans and animals. These target compounds with a wide range of polarities were efficiently separated and determined by HPLC assay methods with simple pretreatments, deproteinization of plasma and dilution of urine. The assay methods showed good linearity, precision and accuracy. The methods were successfully applied to pharmacokinetic and metabolic studies of cefmatilen hydrochloride hydrate administered orally to humans and animals. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Cephalosporins; Cefmatilen

1. Introduction

Cefmatilen (prop-INN) hydrochloride hydrate, (−)-(6*R*,7*R*)-7-[(*Z*)-2-(2-aminothiazol-4-yl)-2-hydroxyiminoacetamido]-8-oxo-3-(1H-1, 2, 3-triazol-4-yl)thiomethylthio-5-thia-1-azabicyclo[4. 2. 0]oct-2-ene-2-carboxylic acid monohydrochloride monohydrate (I, Fig. 1), is a new oral cephalosporin having strong in vitro antibacterial activity against a variety of gram-positive and gram-negative bacteria [1–3].

Recently, metabolism of compound I was suggested to proceed according to the proposed pathway shown in Fig. 1 [4]. The compound was well metabolized in dogs, but less in rats. To clarify species differences of metabolism in relation to safety studies, we needed to develop assay methods for the major metabolites in plasma and urine after administration of I to human and animals. In a previous study [5], we established direct injection high-performance liquid chromatography (HPLC) methods with a column-switching technique and UV detection for human plasma and urine assays of I. However, modification of the established methods and application of reversed-phase gradient HPLC methods failed to simultaneously determine I and its seven major metabolites, M2–M8, which have a wide range of polarities, without interference from

*Corresponding author. Fax: +81-6-458-0987.

E-mail address: tsuneji.umeda@shionogi.co.jp (T. Umeda).

0378-4347/00/$ – see front matter
PII: S0378-4347(00)00381-9

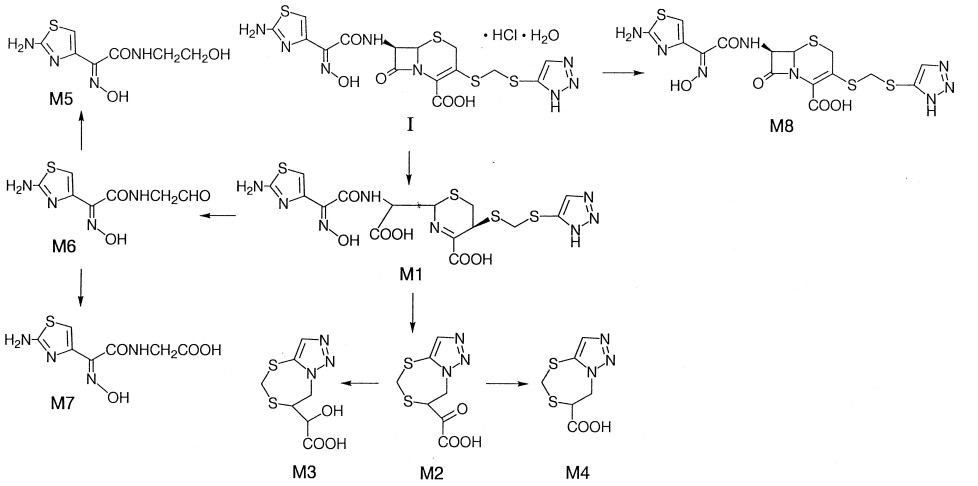

Fig. 1. Postulated metabolic pathways of I.

the many co-eluting endogenous components in the biological fluids.

To resolve this problem, we have investigated coupled ion-exchange and reversed-phase HPLC systems for the efficient separation and determination of these compounds in plasma and urine samples from humans and animals. Such coupled systems have been applied to drug analyses in biological materials [6–10].

In this paper, we will describe the HPLC methods developed for the determination of I and its seven major metabolites in plasma and urine of humans and animals by combination of ion-exchange pre-columns and octadecyl (ODS) analytical columns. The paper will also report on the successful application of the developed methods to pharmacokinetic and metabolic studies of I in humans with the results of assay validation.

2. Experimental

2.1. Materials

Compound I and its seven metabolites, M2–M8, were synthesized in our laboratories to give HPLC purity more than 97%. Sodium 1-nonanesulfonate and tetra-*n*-butylammonium bromide were of special grade for ion-pair HPLC (Tokyo Kasei Kogyo, Tokyo, Japan). Methanol, acetonitrile (MeCN), and water were of HPLC grade. All other reagents were of analytical-reagent grade.

2.2. HPLC system

The assay of I and its six metabolites, M2–M5, M7 and M8, in human urine was performed with a liquid chromatograph composed of eight solvent delivery pumps, LC-6A (Shimadzu, Kyoto, Japan) and its equivalents, an SIL-6B autosampler (Shimadzu) kept at 7°C by a WIG-7000G thermostat (Ishido, Chiba, Japan), all of which were controlled automatically by three controllers, SCL-6B (Shimadzu) and its equivalents, three C-R7A integrators (Shimadzu), four UV detectors, SPD-10A (Shimadzu) and its equivalents, set at 260 nm, a Coulochem 5100A electrochemical detection (ED) system (Environmental Sciences Associates, Bedford, MA, USA) to control a 5010 analytical cell and a 5020 guard cell set at +0.55 V vs. Pd/H_2 and +0.65 V vs. Pd/H_2, respectively, an NPOT-2501 potentiostat (Nikko Keisoku, Kanagawa, Japan) to control another 5020 guard cell set at +0.65 V vs. Pd/H_2, and two DG-1300 degassers (Uniflows, Tokyo, Japan).

Analytical separation was done using a multidemensional LC system with an anion-exchange Nucleosil 5SB pre-column 1 (5 μm, 100 mm×4.0 mm I.D., Chemco Scientific, Kyoto, Japan), a cation-exchange Nucleosil 5SA pre-column 2 (5 μm, 150

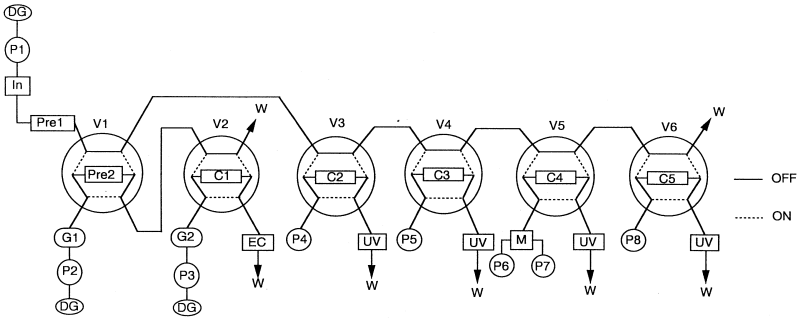

Fig. 2. Schematic diagram of multidimensional LC system for urine assay. P1–P8, pump; In, autoinjector; DG, degasser; M, mixing block; V1–V6, six-port switching valve; Pre1 and Pre2, pre-column; C1–C5, analytical column; EC, electrochemical detector; G1 and G2, guard cell; UV, UV detector; W, waste.

mm×4.0 mm I.D., Chemco), an Inertsil ODS-2 analytical column C1 (5 μm, 250 mm×6.0 mm I.D., GL Science), and four YMC-Pack ODS-AM AM-312 analytical columns, C2–C5 (5 μm, 150 mm× 6.0 mm I.D., YMC, Kyoto, Japan), which were placed in a CS-300C column heater (Chromatoscience, Osaka, Japan) kept at a temperature between 20 and 25°C. As shown in Fig. 2, the system was connected through the three PT-8000 self-controllable valve actuators equipped with six six-port switching valves, V1–V6 (Tosoh, Tokyo, Japan). Each valve at position ''off'' or ''on'' was initially set at position ''off'' for the sample injection and then switched according to the time program shown in Table 1. The mobile phase for the two pre-columns was 0.1 *M* phosphate buffer (NaH_2PO_4–H_3PO_4, pH 2.0) containing 0.075 *M* sodium nitrate. The mobile phases for columns C1–C3 were 0.03 *M* phosphoric acid–MeCN (87:13) containing 3 m*M* sodium 1-nonanesulfonate, 0.01 *M* phosphate buffer (pH 4.0)–MeCN (87:13) containing 3 m*M* tetra-*n*-butylammonium bromide, and 0.1 *M* phosphate buffer (pH 2.0)–MeCN (90:10) containing 0.075 *M* sodium nitrate, respectively. Analysis on column C4 was performed by a step-gradient method using 0.1 *M* phosphate buffer (pH 2.0)–MeCN (95:5) containing 0.075 *M* sodium nitrate as the A solution and 0.1 *M* phosphate buffer (pH 2.0)–MeCN (70:30) containing 0.075 *M* sodium nitrate as the B solution according to the following time program: A–B (90:10) between 0 and 45.0 min, A–B (78:22) between 45.1 and 75.0 min, and A–B (90:10) between 75.1 and 90.0 min. The mobile phase for column C5 was 0.1 *M* phosphate buffer (pH 2.0)–MeCN (85:15) containing 0.075 *M* sodium nitrate. The flow-rates of all of the mobile phases were kept constant at 1.0 ml/min. The mobile phases were filtered with a Type FR-70 membrane filter (0.7 μm, Fuji Photo Film, Tokyo, Japan) and degassed under reduced pressure before use.

Metabolite M6 in human urine was assayed in a similar manner to that described above, using a part of the system in Fig. 2, in which columns C2–C5, pumps P4–P8, valves V3–V6, and the four UV detectors were removed.

I and seven metabolites in the urine of rat, dog, and monkey were also determined by similar methods to those described above.

Table 1
Valve-switching time program for urine assay[a]

	V1	V2	V3	V4	V5	V6
Time ''on'' (min)	0.5	t_5−2.0	t_1−1.5	t_2−1.5	t_3−7.0	t_4−3.0
Time ''off'' (min)	3.0	t_6+2.0	t_1+1.5	t_2+1.5	t_3+4.0	t_4+6.0

[a] t_1: Retention time of M3 on pre-column 1 (ca. 6 min); t_2: retention time of M4 on pre-column 1 (ca. 10 min); t_3: retention time of I on pre-column 1 (ca. 20 min); t_4: retention time of M8 on pre-column 1 (ca. 30 min); t_5: retention time of M5 on pre-column 2 (ca. 9 min); t_6: retention time of M7 on pre-column 2 (ca. 13 min).

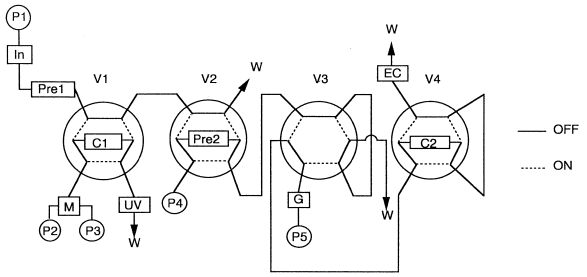

Fig. 3. Schematic diagram of multidimensional LC system for plasma assay. P1–P5, pump; In, autoinjector; M, mixing block; V1–V4, six-port switching valve; Pre1 and Pre2, pre-column; C1 and C2, analytical column; EC, electrochemical detector; G, guard cell; UV, UV detector; W, waste.

The assay of I and its six metabolites, M2–M5, M7 and M8, in human plasma was carried out with a similar system to that of the urine assay described above, except for the use of five pumps, LC-6A and its equivalents, an SIL-6B autosampler, two SCL-6B controllers, two C-R7A integrators, an SPD-6AV UV detector (Shimadzu), and a Coulochem 5100A ED system.

The analytical separation was accomplished on a Nucleosil 5SB pre-column 1 (5 μm, 150 mm×4.0 mm I.D., Chemco), a Nucleosil 5SA pre-column 2 (5 μm, 150 mm×4.0 mm I.D., Chemco), two YMC-Pack ODS-AM AM-312 analytical columns, C1 and C2 (5 μm, 150 mm×6.0 mm I.D., YMC), using a multidimensional system. As shown in Fig. 3, the system was connected in series through the two PT-8000 valve actuators equipped with four six-port switching valves, V1–V4. Each valve was switched according to the time program shown in Table 2. The mobile phase for the two pre-columns was 0.1 *M* phosphate buffer (pH 2.0) containing 0.1 *M* sodium nitrate. Analysis on column C1 was performed by a step-gradient method using 0.1 *M* phosphate buffer (pH 2.0) containing 0.1 *M* sodium nitrate as the A solution and 0.1 *M* phosphate buffer (pH 2.0)–MeCN (80:20) containing 0.1 *M* sodium nitrate as the B solution according to the following time program: A–B (60:40) between 0 and 49.0 min, A–B (40:60) between 49.1 and 64.0 min, A–B (30:70) between 64.1 and 79.0 min, and A–B (60:40) between 79.1 and 100.0 min. The mobile phase for the analysis on column C2 was 0.03 *M* phosphoric acid–MeCN (88:12) containing 3 m*M* sodium 1-nonanesulfonate.

Table 2
Valve-switching time program for plasma assay[a]

	V1	V2	V3	V4
Time "on" (min)	$t_1-2.0$	0.5	$t_3-1.0$	$t_5+4.0$
Time "off" (min)	$t_2+4.0$	3.0	$t_4+3.5$	$t_5+34.0$

[a] t_1: Retention time of M3 on pre-column 1 (ca. 5 min); t_2: retention time of M8 on pre-column 1 (ca. 18 min); t_3: retention time of M7 on pre-column 2 (ca. 5 min); t_4: retention time of M5 on pre-column 2 (ca. 8 min); t_5: retention time of M7 on column C2 (ca. 40 min).

Metabolite M6 in human plasma was assayed in a similar manner to that described above, using a part of the system shown in Fig. 3, in which column C1, pumps P2 and P3, valves V1 and V3, and the UV detector were removed and another column C2 (Inertsil ODS-2, 5 μm, 250 mm×6.0 mm I.D., GL Science) was used.

I and seven metabolites in dog plasma were also assayed by similar methods to those described above.

2.3. Analytical procedure

Human plasma and urine samples were stored at −80°C until analysis. A 300–400-μl portion of human urine was filtered with an Ultrafree C3HV membrane filter (0.45 μm, Nihon Millipore, Tokyo, Japan) by centrifugation at 9000 rpm (1940 *g*) for 5 min with a Kaiten-kun portable centrifuge (Funakoshi, Tokyo, Japan). To 200 μl of the urine filtrate transferred to a 2-ml silicon-coated tube (Nipro, Osaka, Japan), 20 μl of 0.05 *M* phosphate buffer (KH_2PO_4–H_3PO_4, pH 2.5)–methanol (75:25) and 800 μl of 0.5 *M* phosphate buffer (pH 2.5) were added for the assay of I and metabolites, M2–M5, M7 and M8, in human urine. After the resulting mixture was well mixed with an NS-8 vortex mixer (Pasolina, Tokyo, Japan), a 50-μl portion was injected onto the HPLC system shown in Fig. 2.

For the assay of metabolite M6 in human urine, urine filtrate was obtained by treating another 150–200-μl portion of human urine according to the same procedure as described above. To 100 μl of the urine filtrate in a 2-ml silicon-coated tube (Nipro), 10 μl of 0.05 *M* phosphate buffer (pH 2.5)–methanol (75:25), 100 μl of 0.1 *M* phosphate buffer (NaH_2PO_4–Na_2HPO_4, pH 7.5), and 800 μl of tetrahydrofuran

were added. After the resulting mixture was well mixed with a vortex mixer (Pasolina), a 50-μl portion was injected.

To 100 μl of human plasma in a 1.5-ml polypropylene test tube (Treff, Switzerland), 10 μl of 0.05 *M* phosphate buffer (pH 2.5)–methanol (75:25) and 140 μl of 10% perchloric acid–methanol (2:5) were added for the assay of I and metabolites, M2–M5, M7 and M8, in human plasma. The resulting mixture was well mixed with a vortex mixer (Pasolina), and centrifuged at 9000 rpm for 5 min with a portable centrifuge (Funakoshi). A 150-μl portion of the resulting supernatant solution was injected onto the HPLC system shown in Fig. 3.

For the assay of metabolite M6 in human plasma, 10 μl of 0.05 *M* phosphate buffer (pH 2.5)–methanol (75:25) and 300 μl of methanol were added to another 100 μl portion of human plasma in a polypropylene test tube (Treff). The resulting mixture was treated according to the same procedure as described above to obtain a supernatant solution. A 100-μl portion of the solution was injected.

Plasma and urine samples of animals were also treated according to similar procedures to those described above for human plasma and urine samples.

2.4. Standard curve

Four points of the standard solutions mixed with I and six metabolites, M2–M5, M7 and M8, were prepared in the range of 20–1000 μg/ml of I, 10–500 μg/ml each of M2 and M3, 10–100 μg/ml of M4, 5–300 μg/ml each of M5 and M7, and 5–100 μg/ml of M8 in 0.05 *M* phosphate buffer (pH 2.5)–methanol (75:25). A 300–400-μl portion of human blank urine was filtered by centrifugation as described in Section 2.3. Urine standard solutions were prepared at four points with duplicates for each point in the range of 2–100 μg/ml of I, 1–50 μg/ml each of M2 and M3, 1–10 μg/ml of M4, 0.5–30 μg/ml each of M5 and M7, and 0.5–10 μg/ml of M8 by spiking 200 μl of the blank urine filtrate with 20 μl of the standard solution prepared above. Urine standard solutions for the assay of M6 in human urine were also prepared in the range of 0.5–30 μg/ml in a similar manner to that described above.

Plasma standard solutions for the assay of I and seven metabolites in human plasma were prepared in a similar manner to the urine standard solutions: 0.05–5 μg/ml of I, 0.1–1 μg/ml each of M2–M4, 0.03–1 μg/ml each of M5–M7, and 0.05–1 μg/ml of M8.

Plasma and urine standard solutions for the assay of I and its metabolites in animal plasma and urine were also prepared using the individual biological matrix of each animal species similarly to the methods described above for human plasma and urine standard solutions.

Plasma and urine standard curves were constructed using the plasma and urine standard solutions described above by plotting the peak area versus the concentration of I and its metabolites in plasma and urine and by determining the best-fit line from the weighted linear regression analysis by the method of Aarons et al. [11].

2.5. Validation study

The assay validation method for the assay of I and metabolites in human plasma and urine is described below.

Chromatograms of blank plasma and urine from several sources before administration of I were examined to evaluate the selectivity of the HPLC assay methods. The linearity between the peak area versus the concentration was evaluated using the plasma and urine standard solutions. The precision and accuracy of the plasma and urine assays were examined by testing six replicate plasma and urine standard solutions in the same batch and by assaying each sample over six different batches. Absolute analytical recoveries were calculated over three different batches using plasma and urine standard curves and the corresponding standard curves which were constructed from aqueous methanol solutions prepared by spiking 100 μl of water with 10 μl each of the standard solutions described earlier. Quality control samples in duplicate at three concentrations each of I and seven metabolites in plasma and urine were stored at −80°C and included in each batch in plasma and urine assays. The stability of I and metabolites in plasma and urine was tested over 3 months at −80°C.

Validation studies were also carried out for the assay of I and metabolites in animal plasma and

urine in a similar manner to those described above for human plasma and urine.

3. Results and discussion

3.1. HPLC system

I, M2–M4 and M8, having a carboxylic group, were well retained and preseparated on anion-exchange pre-column 1, while M5–M7 were eluted without retention, due to protonation of their aminothiazole group under acidic conditions, followed by preseparation with cation-exchange pre-column 2. These preseparated fractions were separated by ODS analytical columns. Metabolite M6 was assayed alone after sample preparation under neutral condition because of the increase by rapid degradation of an unstable metabolite M1 to M6 under acidic conditions.

In the urine assay, samples were injected directly onto the HPLC system (Fig. 2) after filtration and pH adjustment. Five fractions (1: M5 and M7, 2: M3, 3: M4, 4: I and M2, 5: M8) separated by pre-column 1 were sent to pre-column 2 and columns C2–C5, respectively. After preseparation of fraction 1 on pre-column 2, M5 and M7 were further separated by ODS analytical column C1, followed by ED. I and metabolites, M2–M4 and M8, were separated by analytical columns C2–C5 and determined with UV detection. Metabolite M6 was determined by the same procedure as M5 and M7 using a much simpler system than that shown in Fig. 2.

In the plasma assay, samples were injected onto the HPLC system (Fig. 3) after deproteinization. I, M2–M4, and M8 were transferred from pre-column 1 to ODS analytical column C1 as one fraction and analyzed by a step-gradient method because interfering peaks in plasma were much less than those in urine. On the other hand, M5–M7 were assayed according to similar methods used for their urine assays.

The wavelength selected for UV detection was 260 nm where I as well as M2–M4 and M8 showed high responses. At the same noise level, M5–M7 had nearly five-times higher responses in ED than those in UV detection at 260 nm, as Fabre et al. reported high detectability of the ED method for the aminothiazole group [12]. Each of the peak areas in the hydrodynamic voltammograms for M5–M7 almost reached the same plateau level at an applied potential of more than +0.5 V vs. Pd/H_2. Therefore, +0.55 V vs. Pd/H_2 was selected as the potential for ED of M5–M7.

No internal standards were needed for the established HPLC methods which gave highly reproducible results with quantitative recoveries as described below.

3.2. Assay validation

The results of assay validation for I and metabolites in human plasma and urine were as follows.

Typical chromatograms under the plasma and urine assay conditions are shown in Figs. 4 and 5. These assay methods were selective as demonstrated by the lack of interfering peaks in the blank plasma and urine samples following a visual inspection of the chromatograms.

Plasma and urine standard curves were found to be linear with correlation coefficients of more than 0.99. Assay precision and accuracy were determined by analyzing six replicate blank plasma and urine samples each spiked with three concentrations of I and metabolites over assay ranges beyond the limits of quantitation on the same batch and by analyzing each sample over six batches. All of the relative standard deviations for within-batch and between-batch assays were less than 8% with relative errors of −5 to +13% for plasma samples and were less than 12% with relative errors of −11 to +15% for urine samples.

The limits of quantitation for plasma and urine assays were 0.05 and 2 μg/ml for I, 0.1 and 1 μg/ml for M2–M4, 0.03 and 0.5 μg/ml for M5–M7, and 0.05 and 0.5 μg/ml for M8, respectively, where the relative standard deviations for within-batch and between-batch assays were less than 17% with acceptable low relative errors.

The absolute analytical recoveries of I and metabolites were 91–103% and 96–106% for plasma and urine assays, respectively.

Quality control samples were subjected to two analytical batches of a series of samples. Both

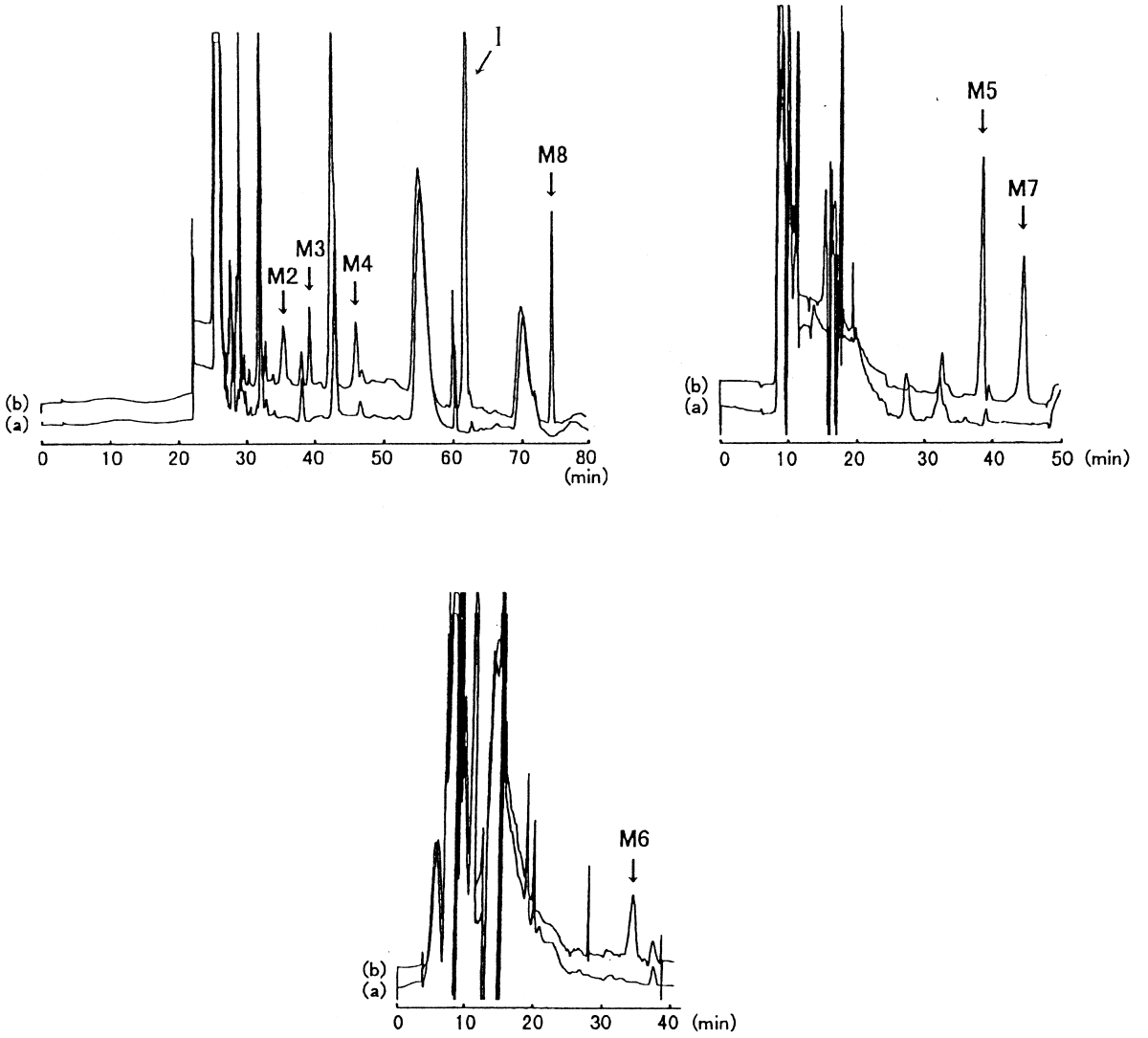

Fig. 4. HPLC chromatograms for plasma assay. (a) Blank plasma, (b) blank plasma spiked with 5 μg/ml of I, 1 μg/ml each of M2–M4 and M8, and 0.1 μg/ml each of M5–M7.

plasma and urine assays over the two batches were demonstrated to be acceptable, because all of the quality control samples gave relative errors less than 20%.

Plasma and urine samples were stable for at least 2 months at −80°C.

Assay validation for the assay of I and metabolites in animal plasma and urine gave similar results to those described above for human plasma and urine.

3.3. Application to human and animal plasma and urine samples

The HPLC methods developed were successfully applied to pharmacokinetic and metabolic studies after oral administration of I to human and animals.

Figs. 6 and 7 show the representative plasma concentration–time and urinary excretion rate–time profiles of I and metabolites after oral administration

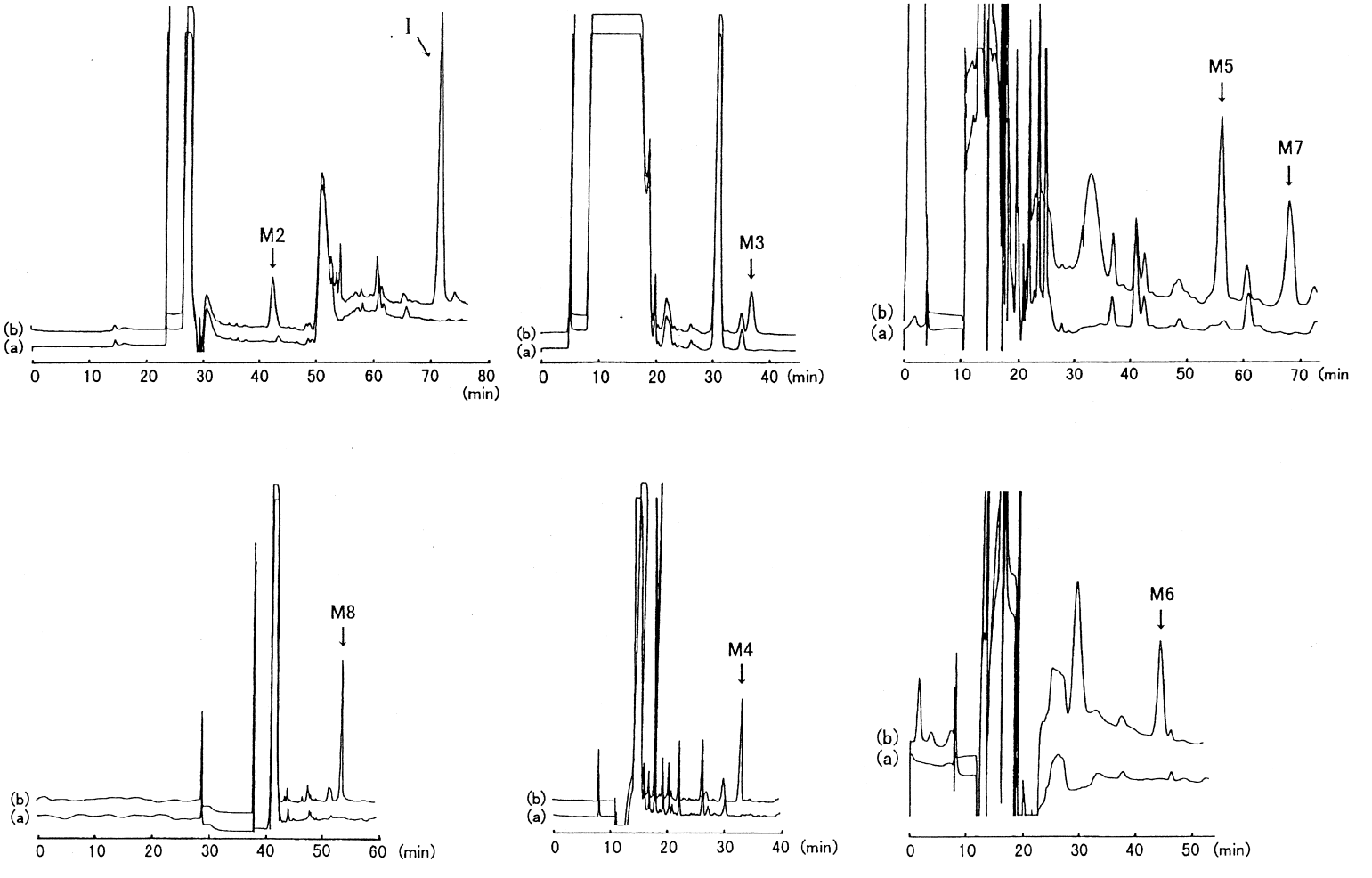

Fig. 5. HPLC chromatograms for urine assay. (a) Blank urine, (b) blank urine spiked with 20 μg/ml of I, 10 μg/ml each of M2–M4 and M8, and 1 μg/ml each of M5–M7.

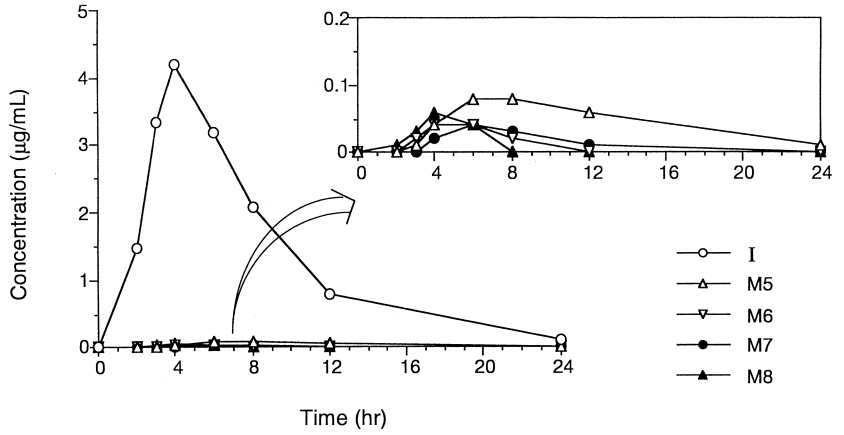

Fig. 6. Mean concentration of I and its metabolites in human plasma after oral administration of I (200 mg, n=6).

of 200 mg of I to six healthy human volunteers. The concentration of I in plasma gave almost the same time course with t_{max} at 4 h as that reported previously [5]. Although the levels of M5–M8 in plasma, which were much lower than that of I, were determined to show t_{max} at nearly 6 h and 4 h for M5–M7 and M8, respectively, the M2–M4 levels were near or below the limits of quantitation due to their lower detectability than that of M5–M7. The level of the 0–24 h urinary excretion rate of I was almost the same as that reported previously [5]. The mean 0–24 h urinary excretion levels of M2, M3, and M5–M7 were 7–10%, while M4 and M8 showed low mean levels of below 1%.

Metabolism of I in animals and human will be reported elsewhere in detail.

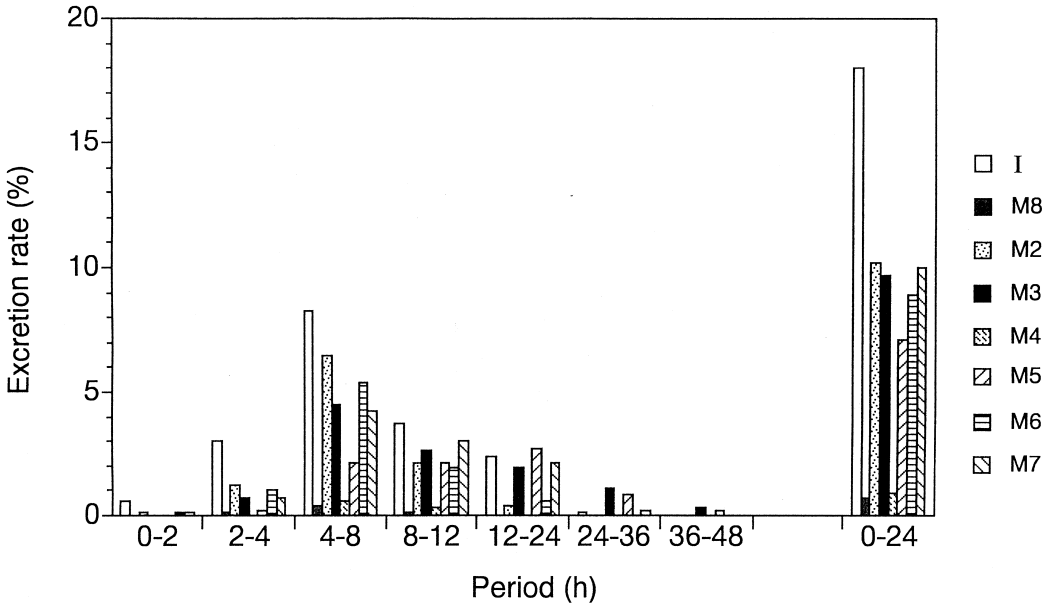

Fig. 7. Mean urinary excretion rate of I and its metabolites after oral administration of I (200 mg, n=6).

4. Conclusions

The HPLC methods developed enabled accurate and precise determination of I and its metabolites in plasma and urine of humans and animals. The methods could be successfully applied to pharmacokinetic and metabolic studies of I in humans and animals.

References

[1] M. Kume, T. Kubota, Y. Kimura, H. Nakashimizu, K. Motokawa, M. Nakano, J. Antibiot. 46 (1993) 177.
[2] M. Kume, T. Kubota, Y. Kimura, H. Nakashimizu, K. Motokawa, Chem. Pharm. Bull. 41 (1993) 758.
[3] M. Tsuji, Y. Ishii, A. Ohno, S. Miyazaki, K. Yamaguchi, Antimicrob. Agents Chemother. 39 (1995) 2544.
[4] K. Sakamoto, S. Futaguchi, K. Nakamoto, S. Iwashita, K. Inazawa, S. Sakamoto, H. Fujitomo, I. Nishino, T. Umeda, M. Koike, in: AAPS Annual Meeting, 1999, Abstract S-33.
[5] H. Fijitomo, T. Nagaoka, I. Nishino, T. Umeda, J. Chromatogr. B 728 (1999) 125.
[6] Y. Tokuma, Y. Shiozaki, H. Noguchi, J. Chromatogr. 311 (1984) 339.
[7] R.M. Mader, B. Rizovski, G.G. Steger, H. Rainer, R. Proprentner, R. Kotz, J. Chromatogr. 613 (1993) 311.
[8] A.J. Szuna, T.E. Mulligan, B.A. Mico, R.W. Blain, J. Chromatogr. 616 (1993) 297.
[9] K.A. Wang, R.W. Blain, A.J. Szuna, J. Pharm. Biomed. Anal. 12 (1994) 105.
[10] J.G. Dorsey, W.T. Cooper, B.A. Siles, J.P. Foley, H.G. Barth, Anal. Chem. 68 (1996) 515R.
[11] L. Aarons, S. Toon, M. Rowland, J. Pharmacol. Methods 17 (1987) 337.
[12] H. Fabre, M.-D. Blanchin, W.Th. Kok, Analyst 113 (1988) 651.