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Quantitation of a new potent angiotensin II receptor antagonist, TCV-116, and its metabolites in human serum and urine

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Abstract

A sensitive high-performance liquid chromatographic (HPLC) method is described for the determination of a new potent antihypertensive agent, TCV-116, and its two metabolites (M-I and M-II) in human serum or urine. After pre-treatment of the specimens, the analytes were determined using a column switching technique, except for the metabolites in urine which were determined by gradient elution mode HPLC. The quantitation limits for TCV-116, M-I and M-II were all 0.5 ng/ml in serum, and 0.5, 10 and 10 ng/ml in urine, respectively. The methods were applied to clinical trials of TCV-116.

Keywords: TCV-116; Angiotensin II receptor antagonist

1. Introduction

TCV-116, (±)-1-(cyclohexyloxycarbonyloxy)ethyl 2-ethoxy-1-[[2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl] methyl]-1*H*-benzimidazole-7-carboxylate, a new angiotensin II receptor antagonist, showed a potent and long-lasting antihypertensive effect in several animal models [1–5]. Drug metabolism studies using dogs and rats [6] revealed the existence of an active metabolite (M-I) and another metabolite (M-II) in the plasma and urine (Fig. 1).

TCV-16 is currently under extensive clinical trials as a potent orally active antihypertensive agent [7]. To determine the pharmacokinetic profile of TCV-

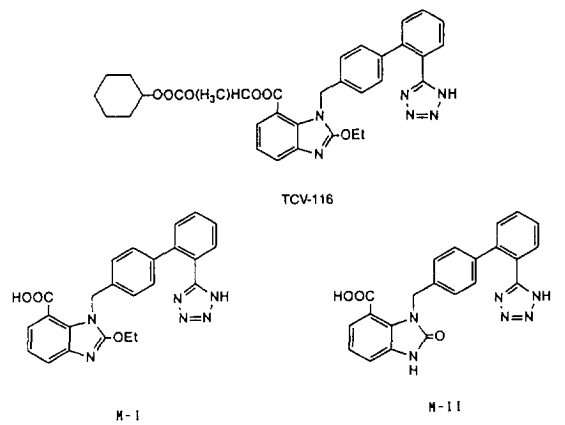

Fig. 1. Structures of TCV-116 and its metabolites M-I and M-II.

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116 in clinical studies, it is necessary to develop analytical methods for these compounds. This paper describes sensitive analytical methods for TCV-116 and its metabolites in human serum and urine using column switching or gradient elution mode. At the beginning of the clinical trials, ultraviolet (UV) absorption was used as the detection method. Alteration of the detection method from UV to fluorescence (FL) provided higher sensitivity with a smaller amount of specimen.

2. Experimental

2.1. Apparatus

The high-performance liquid chromatographic (HPLC) system consisted of two LC-6A pumps, an SIL-6A or SIL-6B autosampler, a CTO-6A column oven, all of which were controlled by an SCL-6A controller (all from Shimadzu, Kyoto, Japan). Detection was carried out with an F-1050 fluorescence detector (Hitachi, Tokyo, Japan) or an SPD-10A UV detector (Shimadzu). For column switching, an FCV-2AH six-port valve (Shimadzu) was used, which was controlled by the SCL-6A. A C-R4AX integrator (Shimadzu) or Pantos U-228 dual pen recorder (Nippon Denshi Kagaku, Kyoto, Japan) was used for quantitation. All mobile phases were degassed with a DG-980-51 on-line degasser (Jasco, Tokyo, Japan).

2.2. Reagents and materials

TCV-116, M-I, M-II and 2-ethoxy-1-[[2′-(5-oxo-2,5-dihydro-1,2,4-oxadiazol-3-yl) biphenyl-4-yl]-methyl]-1*H*-benzimidazole-7-carboxylic acid (T-57536), which was used as an internal standard (I.S.), were all synthesized by the Pharmaceutical Development Division, Takeda Chemical Industries (Osaka, Japan). Acetonitrile, methanol, *n*-hexane, dichloromethane and ethyl acetate were of HPLC grade, and diethyl ether was of pesticide residue analysis grade (all from Wako, Osaka, Japan). Deionized water was further purified using a Milli-Q laboratory water purification system (Nihon Millipore, Yonezawa, Japan). All other reagents were of analytical-reagent grade (Wako) and used without further purification. Bovine serum albumin (BSA) powder fraction V-cohn was purchased from Amour Pharmaceutical (IL, USA). Drug-free serum and urine were obtained from healthy male volunteers.

2.3. Analytical procedures and HPLC conditions

In the clean-up procedures described below, solvent extraction was undertaken by freezing the aqueous layer in a dry-ice acetone bath, and decanting the upper organic layer to another vessel.

2.3.1. TCV-116 in serum

A 0.2-ml volume of 0.1 *M* HCl was added to 0.2 ml of serum and the resulting mixture was extracted with 5 ml of a mixture of diethyl ether and *n*-hexane (1:1, v/v). A 0.2-ml aliquot of 10% (v/v) propylene glycol in methanol solution (PG solution) was added to the separated organic layer and the mixture was evaporated to dryness under a stream of nitrogen at 40°C. The residue was dissolved with 0.3 ml of the mobile phase for the first column 1 (C1) described below and an aliquot of 0.1 ml was injected into the HPLC system.

A heart-cut column-switching technique using two columns [8–10] was adopted as the HPLC system. Inertsil ODS-2 (5 μm, 150 × 4.6 mm I.D., GL Science, Tokyo, Japan) was used for both C1 and the second column (C2). The mobile phase for C1 (MP1) was 0.02 *M* potassium dihydrogenphosphate–acetonitrile (40:60, v/v) adjusted to pH 4.0 with 85% orthophosphoric acid. The mobile phase for C2 (MP2) was 0.02 *M* potassium dihydrogenphosphate–

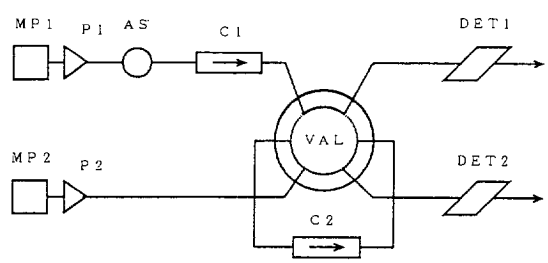

Fig. 2. Schematic diagram of the HPLC system. P1 and P2= pumps 1 and 2; AS=autosampler; VAL=switching valve; C1 and C2=columns 1 and 2; DET1 and DET2=UV or FL detectors A and B; MP1 and MP2=mobile phases for C1 and C2. The solid and dotted lines in the switching valve indicate valve positions A and B, respectively.

acetonitrile (40:60, v/v) adjusted to pH 6.0 with 1 *M* sodium hydroxide (NaOH). Detection was carried out by fluorescence (FL) at the excitation and emission maxima of 270 and 390 nm. The column temperature was 40°C and the flow-rate was 1.0 ml/min for this analysis and for all HPLC described hereafter.

A schematic diagram of the HPLC system for the column switching is shown in Fig. 2. The analytical system and procedure were similar to those reported previously [8–10], which are briefly described below. The retention time of the analyte on C1 with MP1 was checked each day before analysis to determine the precise time program for the column switching. The injected sample was first analyzed on C1 with MP1 (valve position A). Just before the elution of an analyte from C1, the valve was switched to position B and the eluted fraction containing the analyte was introduced into C2. After the elution of the analyte, the valve was switched back to position A. This eluted fraction was further separated on C2 with MP2, and the UV or FL response was monitored. The valve operation was carried out automatically by the SCL-6A controller according to the predetermined time program. A typical time schedule of valve operation for TCV-116 in serum is shown in Table 1, indicating that the heart-cut time of the compound is between 12.0 and 13.6 min after injection. The system and procedure were the same in principle for all column-switching methods described hereafter. The heart-cut time for all compounds was in the range of 1.5–2.0 min.

2.3.2. M-I and M-II in serum

An 0.5-ml volume of 0.2 *M* HCl was added to 0.5 ml of serum and the mixture was extracted with 5 ml of diethyl ether. The organic layer was back-extracted with 0.5 ml of 0.01 *M* sodium hydrogen carbonate. An 0.1-ml volume of 0.2 *M* HCl was added to the aqueous layer and the analytes were re-extracted with 5 ml of diethyl ether. An 0.1-ml volume of PG solution was added to the separated organic layer and the mixture was evaporated to dryness under a stream of nitrogen at 40°C. The residue was dissolved with 0.4 ml of 0.02 *M* potassium dihydrogenphosphate–acetonitrile (78:22, v/v), and each 0.1 ml of the solution was injected into the individual HPLC systems for the analysis of M-I and M-II separately.

Table 1
Typical valve operation schedule for the column switching system for TCV-116 analysis in serum

Time (min)	Valve position
0.0 (start)	A
12.0	B
13.6	A
30.0 (stop)	A

Both systems for M-I and M-II used the column-switching technique similar to that for TCV-116 in serum described above. YMC-PACK ODS A-302 (5 μm, 150 × 4.6 mm I.D., YMC, Kyoto, Japan) was used for both C1 and C2. MP1 was 0.02 *M* potassium dihydrogenphosphate–acetonitrile (68:32 for M-I and 78:22 for M-II, v/v) adjusted to pH 3.5 with 85% orthophosphoric acid. MP2 was 0.02 *M* potassium dihydrogenphosphate–acetonitrile (68:32 for M-1 and 78:22 for M-II, v/v). UV detection was carried out at 210 nm.

2.3.3. TCV-116 in urine

To 0.2 ml of a urine sample treated with 0.5% (w/v) BSA, as described in Section 3, was added 0.2 ml of 0.02 *M* disodium hydrogenphosphate, and the mixture was extracted with 5 ml of a mixture of ethyl acetate and dichloromethane (6:1, v/v). After the addition of 0.2 ml of PG solution, the extract was evaporated under a stream of nitrogen at 40°C. The residue was dissolved in 0.3 ml of MP1 and an aliquot of 0.1 ml was injected into the HPLC system.

A similar column-switching technique was adopted. Inertsil ODS-2 (5 μm, 150 × 4.6 mm I.D.) was used for both C1 and C2. MP1 was 0.02 *M* potassium dihydrogenphosphate–acetonitrile (40:60, v/v) adjusted to pH 4.0 with 85% orthophosphoric acid. MP2 was 0.02 *M* potassium dihydrogenphosphate–acetonitrile (40:60, v/v) adjusted to pH 6.0 with 1 *M* NaOH. Fluorescence detection was carried out at the excitation and emission maxima of 270 and 390 nm.

2.3.4. M-I and M-II in urine

An 0.2-ml volume of 0.2 *M* HCl and 0.01 ml of I.S. solution (20 μg/ml in methanol) was added to 0.2 ml of urine sample, without the treatment of BSA

and the mixture was extracted with 5 ml of diethyl ether. The analytes were back-extracted from the organic layer with 0.5 ml of 0.01 *M* sodium hydrogen carbonate. The aqueous layer was acidified with 0.1 ml of 0.2 *M* HCl and the analytes were re-extracted with 5 ml of diethyl ether. After the addition of 0.1 ml of PG solution, the extract was evaporated under a stream of nitrogen at 40°C. The residue was dissolved in 0.5 ml of a mixture of 0.02 *M* potassium dihydrogenphosphate and acetonitrile (80:20, v/v), and 0.02 ml of the solution was injected onto the HPLC system.

The gradient elution mode was adopted. The column was Inertsil ODS-2 (5 μm, 150 × 4.6 mm I.D.). Mobile phases A and B were as follows; 0.02 *M* potassium dihydrogenphosphate–acetonitrile (80:20 for A and 50:50 for B, v/v) adjusted to pH 3.5 with 85% orthophosphoric acid. Fluorescence detection was carried out at the excitation and emission maxima of 260 and 395 nm. The time program for the gradient elution is presented in Table 2.

2.4. Validation

Drug-free serum or urine spiked with known amounts of TCV-116, M-I and M-II were analyzed according to the analytical methods described above. Peak heights for each compound were plotted against the respective concentrations to give the calibration graphs, except for M-I and M-II in urine, in which peak-height ratio was used. The percentage recoveries of these compounds from biological specimens were calculated from the peak heights or peak-height ratios of spiked samples relative to directly injected standard solutions. Precision and accuracy were assessed by back-calculating the concentrations of the analyte from the peak height (or peak-height ratio) in the calibration graph of the spiked samples.

Table 2
Time program for gradient elution for M-I and M-II in urine

Time (min)	Mobile phase B concentration (%)	
0.0 (start)	0	linear
18.0	60	
18.0	60	constant
23.5	60	
23.51	0	constant
29.0 (stop)	0	

3. Results and discussion

Because the retention times of all analytes were strongly affected by the pH of the mobile phase (Fig. 3), a heart-cut column-switching technique [8–10] using the pH change from MP1 to MP2 was considered to be effective for separation of the analyte from the endogeneous compounds. Therefore, this technique was adopted to obtain a high sensitivity for the analysis of TCV-116 and its metabolites in serum and urine. Only the method for the metabolites in urine used the gradient mode in place of the column-switching technique, because the sensitivity obtained

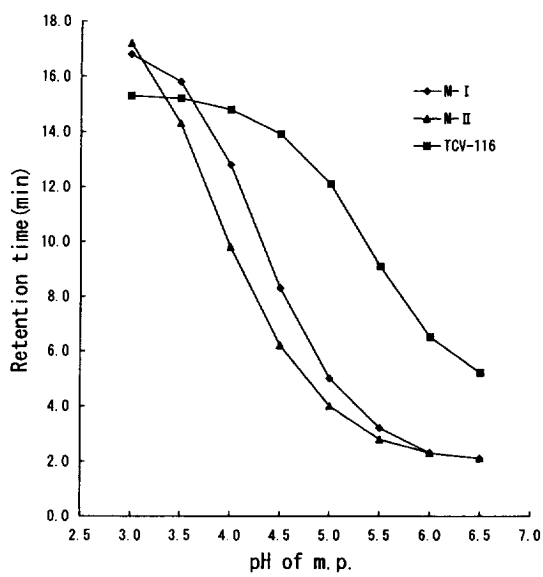

Fig. 3. Relationship between the retention times of TCV-116, M-I and M-II and the pH of the mobile phase (MP). HPLC conditions: column, YMC-pack ODS A-302 (5 μm particle size, 150 × 4.6 mm I.D.); MP, acetonitrile–potassium dihydrogenphosphate (60:40, v/v for TCV-116; 32:68, v/v for M-I; 22.78, v/v for M-II) adjusted to pH 3–6.5 with 85% orthophosphoric acid or 1 *M* NaOH; column temperature, 40°C; flow-rate, 1.0 ml/min; detection, UV at 210 nm.

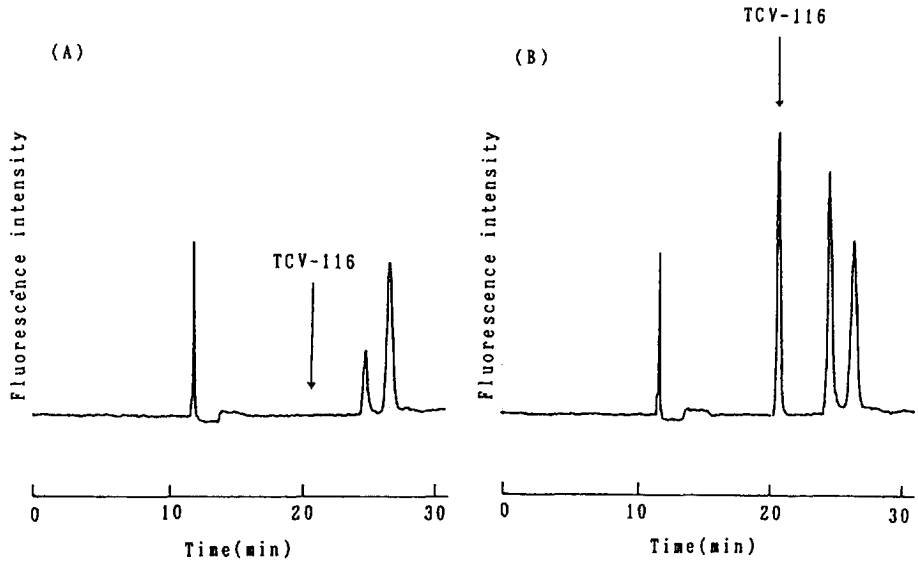

Fig. 4. Typical chromatograms of (A) a drug-free human serum and (B) a drug-free human serum spiked with TCV-116 (10 ng/ml)

was sufficient for present purposes and the simultaneous analysis for M-I and M-II was also possible.

Typical chromatograms of a drug-free human serum and the serum spiked with TCV-116, M-I and M-II are shown in Figs. 4–6, respectively. Fig. 7 shows the chromatograms of a drug-free human urine containing 0.5% (w/v) BSA, and the urine spiked with TCV-116. Fig. 8 also shows typical chromatograms of a drug-free human urine and the urine spiked with M-I and M-II. No endogeneous peaks were observed at the retention time of each compound.

Addition of PG to the extraction solvent was essential to prevent a reduction in the recovery of analytes during evaporation of the solvent under a nitrogen stream (data not shown).

3.1. TCV-116 in serum

TCV-116 is an ester pro-drug, and is easily hydrolyzed to M-I in human and animal bodies. Since the serum concentration of the parent drug was presumed to be very low, the column-switching technique was adopted to obtain a high sensitivity. At an early stage of the clinical trials, UV detection

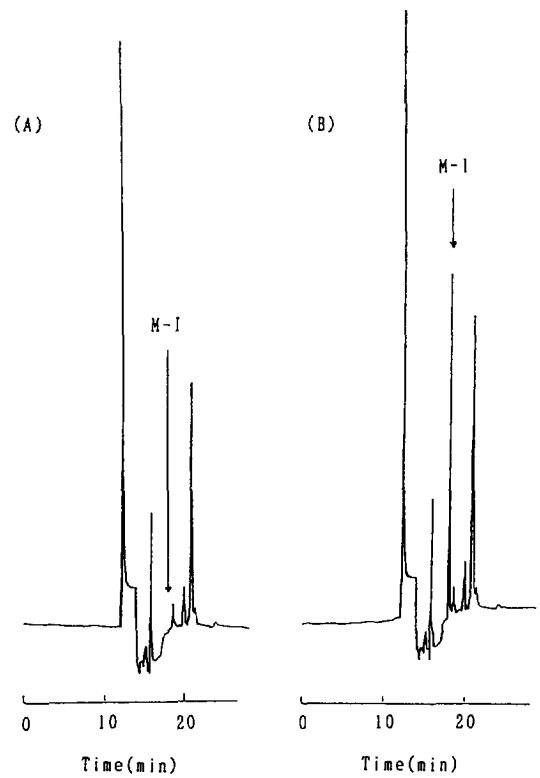

Fig. 5. Typical chromatograms of (A) a drug-free human serum and (B) a drug-free serum spiked with M-I (10 ng/ml)

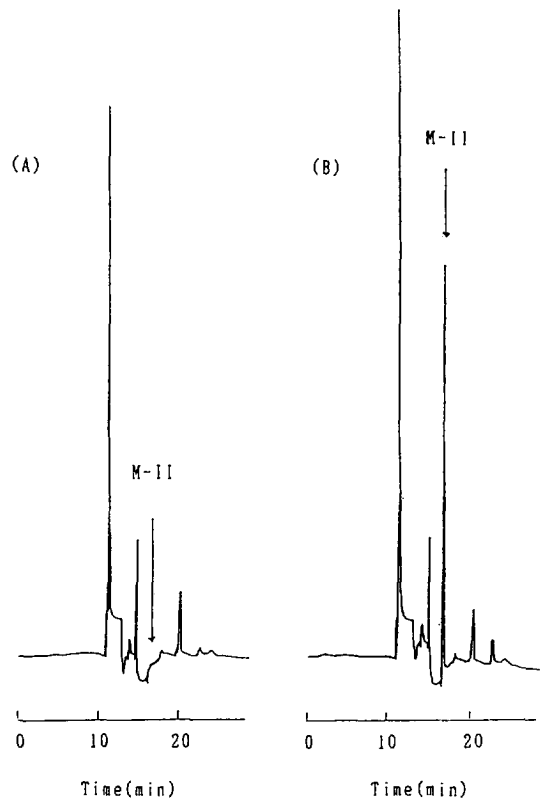

Fig. 6. Typical chromatograms of (A) a drug-free human serum and (B) a drug-free serum spiked with M-II (10 ng/ml).

was used (detection limit; 1 ng/ml at a signal to noise (*S/N*) ratio of 3 using 0.5 ml of serum) [11]. The detection method was changed from UV to FL in an attempt to obtain a higher sensitivity. As a result, the sensitivity was improved to the quantitation limit of 0.5 ng/ml with a smaller amount of serum (0.2 ml).

3.2. M-I and M-II in serum

Drug metabolism studies using animal models [6] indicated that highly sensitive analytical methods were also essential for determination of the metabolites. At the beginning of the clinical trials, an attempt was made to develop an analytical method for M-I alone in serum using UV detection. Solid-phase extraction [12–14] from human serum using Sep-Pak C_{18} (Waters) could not be adopted because of background peaks at the retention time of M-I. Since M-I has a carboxylic acid moiety in the structure, extraction with an organic solvent under acid conditions, followed by back extraction under alkaline conditions was found to be effective for the clean-up. The detection limit for M-I was 0.2 ng/ml at a *S/N* ratio of 3, using 0.5 ml of serum. A later investigation showed that M-II could also be extracted by the same procedure that was used for M-I, although the HPLC conditions for column switching were different from those for M-I. Therefore, the following analytical method for M-I and M-II was developed as described in Section 2; after the simultaneous extraction of M-I and M-II from 0.5 ml of serum, M-I and M-II were analyzed separately using the different HPLC conditions. This method allowed the analysis of M-II in addition to M-I with the same sample volume that was used in the original method for M-I alone. The quantitation limits of M-I and M-II were both 0.5 ng/ml. Although the sensitivity for M-I was lowered a little compared with the original method, it was considered sufficient from the observed M-I concentrations in the clinical trials. Because the column-switching method using UV detection offered sufficient sensitivity, alteration to FL detection was not investigated.

3.3. TCV-116 in urine

During the development of the analytical method for TCV-116 in human urine, the compound spiked into a drug-free urine was found partly adsorbed onto the glass or plastic containers. On the contrary, TCV-116 spiked into a drug-free human serum was not adsorbed on these containers. These findings suggested that the addition of BSA was effective for the prevention of the adsorption of the analyte to the vessel wall. As expected, addition of 0.5% (w/v) BSA powder decreased the adsorption of TCV-116 by more than 80%. M-I and M-II spiked into a human urine were not adsorbed onto the containers. Therefore, in the clinical trials, 1–2 ml of the collected urine sample was stored for the analysis of M-I and M-II, and BSW powder (0.5%, w/v) was added to the remaining urine for the analysis of TCV-116. These samples were stored separately, at −20°C.

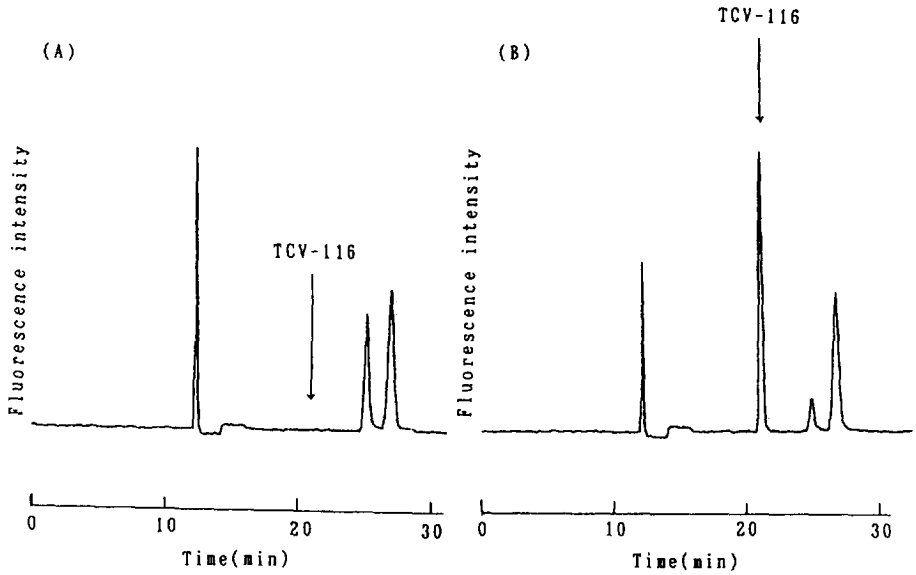

Fig. 7. Typical chromatograms of (A) a drug-free human urine containing 0.5% (w/v) BSA and (B) the urine spiked with TCV-116 (10 ng/ml).

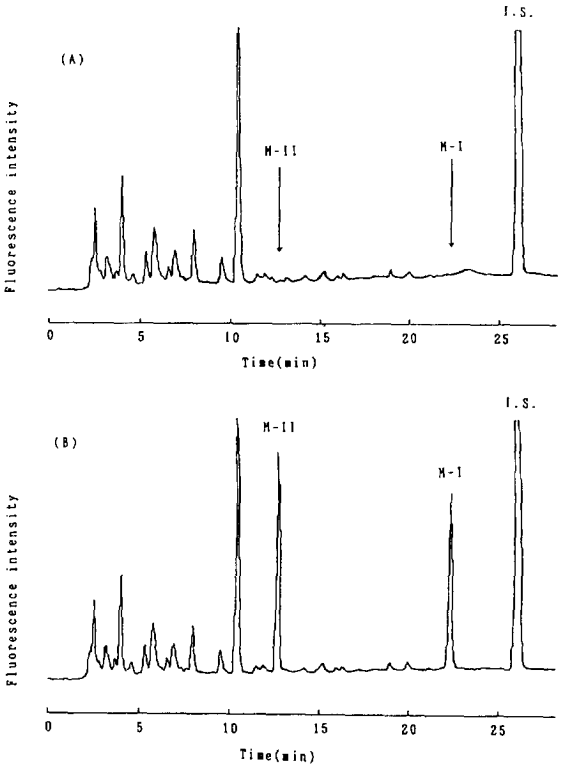

Fig. 8. Typical chromatograms of (A) a drug-free human urine and (B) a drug-free human urine spiked with M-I and M-II (250 ng/ml, each).

3.4. M-I and M-II in urine

At the beginning of the clinical trials, M-I alone was analyzed in urine using UV detection with a column-switching technique, and the limit of detection was 1 ng/ml at a *S/N* ratio of 3, using 0.5 ml of urine. Further investigation using FL detection made it possible to analyze M-I and M-II simultaneously with a gradient elution mode. Although the sensitivity was lowered to a quantitation limit of 10 ng/ml, it was enough considering the excreted amount observed in the clinical trials.

3.5. Validation data

The accuracy, precision, reproducibility, recovery and linearity data are shown in Tables 3–6. The linearity between spiked concentration and HPLC response was satisfactory as shown in Table 3. Absolute recoveries of the analytes and I.S. were more than 75% with coefficient of variation (C.V.) of not more than 5%. In the analysis of serum, intra-day C.V. and relative error (R.E.) values were not more than 10.4 and 3.8%, and inter-day values were not more than 7.8 and 2.1% respectively. In the case of

Table 3
Results of linear regression analysis for analytes in a drug-free serum and urine

Sample	Compound	Concentration range (ng/ml)	n	r^a
Serum	TCV-116	0.5–200	5	0.9999
	M-I	0.5–200	6	0.9999
	M-II	0.5–200	6	0.9999
Urine	TCV-116	0.5–200	5	0.9999
	M-I	10–5000	5	0.9998
	M-II	10–5000	5	0.9999

[a] Correlation coefficient.

Table 4
Mean absolute recovery of analytes and I.S. from a drug-free serum and urine

Compound	Concentration (ng/ml)	n	Recovery[a] (%)	
			Serum	Urine
TCV-116	10	15	93.1(3.2)	99.2(0.7)
M-I	20	15	84.5(2.0)	–
	250	15	–	75.2(5.0)
M-II	20	15	81.9(2.6)	–
	250	15	–	79.1(4.2)
I.S.	1000	15	–	84.4(3.7)

[a] Values in parentheses are coefficients of variation (%).

urine, the intra-day precision and accuracy were not more than 7.4 and 11.0%, and the inter-day values were not more than 9.8 and 5.0%, respectively.

The quantitation limits for TCV-116, M-I and M-II were all 0.5 ng/ml in serum and 0.5, 10 and 10 ng/ml in urine, respectively (C.V.<20%).

3.6. Stability

The analytes and I.S. in methanol were stable for at least three months at 4°C as shown in Table 7. The reconstituted sample solutions of the analytes and I.S. were stable for at least 24 h at room temperature.

Table 5
Precision and accuracy of TCV-116, M-I and M-II in a drug-free human serum

Compound	Nominal concentration (nm/ml)	Intra-day			Inter-day		
		n	C.V. (%)	R.E.[a] (%)	n	C.V. (%)	R.E.[a] (%)
TCV-116	0.5	5	10.4	0.0	3	7.8	2.0
	10	5	0.8	−3.0	3	4.9	−1.9
	200	5	0.6	−0.3	3	0.6	0.3
M-I	0.5	5	2.3	2.8	3	0.0	2.0
	20	5	0.8	−3.1	3	0.9	−2.1
	200	5	1.2	0.3	3	0.1	0.2
M-II	0.5	5	3.4	3.4	3	2.3	2.0
	20	5	1.6	−3.8	3	2.2	−1.3
	200	5	1.6	0.4	3	0.2	0.1

[a] Relative error.

Table 6
Precision and accuracy of TCV-116, M-I and M-II in a drug-free human urine

Compound	Nominal concentration (ng/ml)	Intra-day			Inter-day		
		n	C.V. (%)	R.E.[a] (%)	*n*	C.V. (%)	R.E.[a] (%)
TCV-116	0.5	5	7.4	6.0	3	9.8	2.0
	10	5	0.7	−3.0	3	5.5	−0.7
	200	5	1.0	−0.3	3	0.4	0.0
M-I	10	5	4.2	11.0	3	5.7	5.0
	250	5	3.1	−2.1	3	0.8	−2.0
	5000	5	1.4	2.6	3	2.3	0.6
M-II	10	5	3.5	9.0	3	3.8	5.0
	250	5	1.0	−0.2	3	1.1	−0.9
	5000	5	1.0	2.6	3	2.0	0.7

[a]Relative error.

Table 7
Stability of TCV-116, M-I, M-II and I.S. under various storage conditions

Sample	Storage conditions	Residual content (mean) (%)			
		TCV-116	M-I	M-II	I.S.
Methanol	4°C, 3 months	100.0	100.5	101.7	100.5
HPLC sample	RT[a], 24 h	98.4	99.8	100.0	100.1
Serum	RT, 5 h	101.2	95.4	98.4	–
	−20°C, 3 months	99.9	92.0	97.4	–
Urine	−20°C, 3 months	–	100.8	98.1	–
Urine (BSA)[b]	−20°C, 1 month	103.4	–	–	–

[a]RT: room temperature.
[b]Urine containing 0.5% (w/v) BSA.

TCV-116, M-I and M-II in serum were stable for three months at −20°C. In urine, M-I and M-II were stable for at least three months at −20°C. TCV-116 in urine containing 0.5% BSA was also stable for at least one month at −20°C.

3.7. Application of the methods to the clinical trials

Fig. 9 shows serum concentrations of TCV-116, M-I and M-II after oral administration of a tablet containing 20 mg of TCV-116 to healthy male volunteers. TCV-116 in serum (0.5–1.1 ng/ml) was very low compared with M-I, indicating that most of the parent drug (TCV-116) was hydrolyzed to the active metabolite (M-I) in the body. Fig. 10 shows the urinary excretion profile of these compounds.

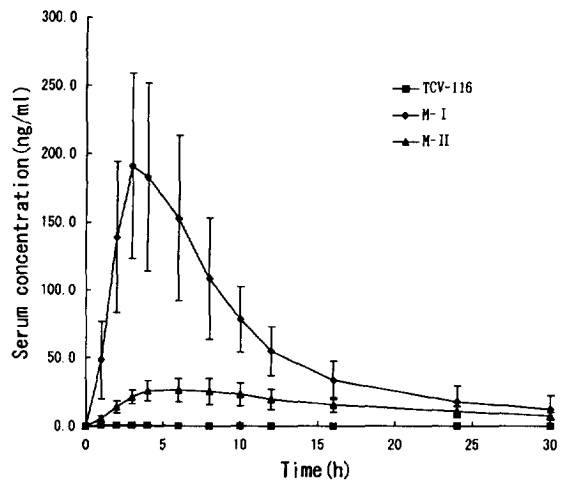

Fig. 9. Serum concentration profile of TCV-116, M-I and M-II after a single oral administration of a tablet containing 20 mg of TCV-116 to healthy volunteers. Each point and bar represent the mean and the standard deviation ($n=6$), respectively.

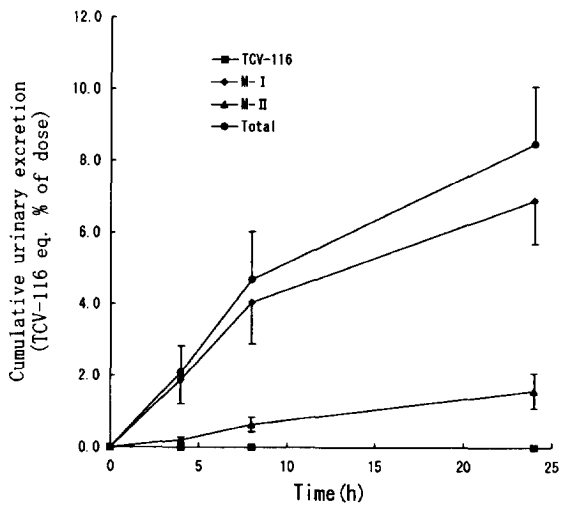

Fig. 10. Cumulative urinary excretion profile of TCV-116, M-I and M-II after a single oral administration of a tablet containing 20 mg of TCV-116 to healthy volunteers. Each point and bar represent the mean and the standard deviation ($n=6$), respectively.

M-I and M-II were excreted in urine, but TCV-116 was not detected.

The details of the results in the clinical trials will be reported elsewhere in the future.

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