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Analytical chiral separation of a new quinolone compound in biological fluids by high-performance liquid chromatography

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Abstract

Two methods for the separation of a new racemic quinolone compound, temafloxacin (TMFX), in biological fluids by high-performance liquid chromatography (HPLC) were studied. The first method was coupling of TMFX to *S*-(−)-N-1-(2-naphthyl sulfonyl)-2-pyrrolidine carbonylchloride (L-NSPC). The diastereomeric derivatives were separated on a silica gel column. The second method was separation on a chiral stationary phase with an ovomucoid conjugated to aminopropyl silica gel. Two enantiospecific methods gave a satisfactory result concerning both accuracy and precision, and the second method was superior to the first one for chromatographic separation. Furthermore, the pharmacokinetics of the enantiomers after oral administration of racemic TMFX to healthy volunteers was investigated by the second method.

Keywords: Derivatization, LC; Enantiomer separation; Temafloxacin; Quinolones

1. Introduction

Temafloxacin (TMFX), [(±)-1-(2,4-difluorophenyl)-6-fluoro-1, 4-dihydro-7-(3-methyl-1-piperazinyl)-4-oxo-quinolinecarboxylic acid monohydrochloride] (Fig. 1), is a potent member of the 4-pyridone-3-carboxylic acid class of antibacterial agents and is currently under clinical development as a broad-spectrum antimicrobial agent. It is a racemate, having a chiral centre at the C_3 of the 7-piperazinyl group. The two enantiomers were tested for their antibacterial activities. While no difference was found in in vitro antibacterial activities between them, there was a small difference in in vivo antibacterial activities [1]. For determination of the enantiomers' stability in the body and monitoring of their pharmacokinetic behaviour, it is desirable to determine the two enantiomers simultaneously in biological fluids. For chromatographic separation of enantiomers of drugs, it is always necessary to introduce a chiral environment to the analytical compounds. This can be done by various methods, for example, by using a chiral stationary phase [2],

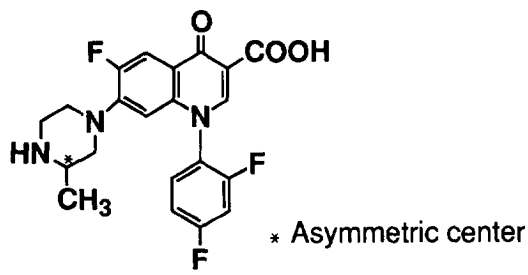

Fig. 1. Chemical structure of TMFX.

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by adding a chiral moiety to the mobile phase [3] or by derivatization of the analytical compounds with a chiral reagent [4,5].

This paper describes the chromatographic separation and quantification of racemic TMFX by two enantiospecific HPLC methods for the pharmacokinetic study of the enantiomers in biological fluids. The first method involved coupling of TMFX to L-NSPC as a chiral derivative reagent. The diastereomeric derivatives were separated successfully on a silica gel column. The second method consisted of separation on a chiral stationary phase with an ovomucoid conjugated to aminopropyl silica gel. The analysis of serum and urine samples from volunteers was carried out by the second method.

2. Experimental

2.1. Material and reagents

Methylene chloride, ammonium acetate, potassium carbonate, sulfuric acid, methanol, hexane, diethyl ether and triethylamine were purchased from Katayama Chemicals (Osaka, Japan). Thionyl chloride and isobutylchloroformate were supplied by Wako Pure Chemical Industries (Osaka, Japan). Phosphoric acid and potassium phosphate (analytical grade) were purchased from Katayama Chemicals. Phosphate buffer (pH 7.0) was prepared from 0.02 *M* dipotassium hydrogen phosphate solution and 0.02 *M* phosphoric acid solution.

Racemic TMFX was supplied by Abbott Laboratories (Chicago, IL, USA). Methylated TMFX, acetylated and methylated TMFX, acetylated and carbonylamidated TMFX were synthesized at Tanabe Seiyaku. The chiral reagent (*S*)(−)-N-1-(2-naphthylsulfonyl)-2-pyrrolidinecarbonyl chloride (L-NSPC) was synthesized at Tanabe Seiyaku.

2.2. Apparatus

HPLC analysis was carried out using a LC-6A system (Shimadzu Corporation, Kyoto, Japan). The secondary ion mass spectrometry (SIMS) was measured using a Hitachi M-80A mass spectrometer equipped with a SIMS system (Hitachi, Tokyo, Japan).

2.3. Chromatographic conditions

The column used for the diastereomeric chromatography was Zorbax Sil (150 mm×4.6 mm I.D., Dupont Instruments, Orsay, France). Separation of TMFX diastereomers was achieved using a mobile phase composed of hexane–methyl acetate–methanol–ammonia water (150:100:10:1), at a flow-rate of 0.8 ml/min at room temperature. The UV spectrometer was operated at 280 nm.

The column used for the chiral stationary-phase chromatography was ovomucoid conjugated to aminopropyl silica gel (150 mm×4.6 mm I.D., Shinwa Chemical Industry, Kyoto, Japan). Separation of racemic TMFX was achieved using the mobile phase composed of 0.02 *M* phosphate buffer (pH 7.0)–acetonitrile (92:8), at a flow-rate of 1.0 ml/min at room temperature. The UV spectrometer was operated at 280 nm.

2.4. Serum and urine samples

Blood samples obtained from healthy volunteers were collected and centrifuged to obtain the serum. The serum and urine samples were stored at −20°C until the time of analysis.

2.5. Extraction procedure

Racemic TMFX was extracted from serum and urine according to the method proposed by Granneman and Vargas [6]. First, 0.5 ml of 1 *M* ammonium acetate buffer and 5 ml of methylene chloride were added to 1.0 ml of human serum or 0.5 ml of urine. The mixture was shaken vigorously for 10 min and centrifuged at 1500 *g* for 5 min. The aqueous supernatant was aspirated and discarded, then 4 ml of the organic phase was transferred to a screw-cap vial, and the dichloromethane was evaporated to dryness at 40°C under a stream of nitrogen gas. The residue was used for the derivatization by the two enantiospecific methods.

2.6. Derivatization for the diastereomeric method

The residual sample extracted with organic solvent was taken up in 0.5 ml of methanol–thionyl chloride mixture (9:1). The solution was heated at 60°C for 30 min to obtain the methylesters of racemic TMFX and evaporated to dryness at 40°C under a stream of nitrogen gas. To the residue, 0.5 ml of 0.5 *M* sulfuric acid and 1 ml of hexane were added; the mixture was shaken vigorously for 10 min and centrifuged for 5 min. The organic layer was aspirated and discarded, 1 ml of 2 *M* sodium carbonate and 3 ml of ether were added to the aqueous layer containing the methylated racemic TMFX, the mixture was shaken vigorously for 10 min and centrifuged for 5 min again. Then, the organic layer was transferred to a screw-cap vial, and the ether was evaporated to dryness at 40°C under a stream of nitrogen gas. The residue was taken up in 20 μl of L-NSPC methylene chloride solution (2 mg/ml); 5 μl of triethylamine were added to the solution, and the mixture was reacted at room temperature for 10 min to obtain the TMFX diastereomers. Subsequently, the solution was evaporated to dryness under a stream of nitrogen gas. The residue was dissolved in 0.5 ml of methylene chloride, and 50 μl of sample solution was injected into the HPLC system equipped with the autoinjector. Fig. 2 represents the derivatization of TMFX for the diastereomeric method.

2.7. Derivatization for the chiral stationary-phase method

The residual sample extracted with organic solvent was taken up in 100 μl of acetic anhydride–pyridine mixture (1:1). The solution was heated at 60°C for 30 min to obtain the acetylated racemic TMFX, and was evaporated to dryness at 40°C under a stream of nitrogen gas. The residue was taken up in 300 μl of THF; 5 μl of triethylamine was then added to the solution. The mixture was treated with 50 μl of isobutylchloroformate for 30 min in ice water saturated with sodium chloride (at −10°C), and 100 μl of ammonia hydroxide (28% ammonia solution) was added to the solution. After reacting at −10°C for 10 min, the solution was evaporated at room temperature under a stream of nitrogen gas. To the residue, 0.5 ml of 0.5 *M* sulfuric acid and 1 ml of hexane were added; the mixture was shaken vigorously for 10 min and centrifuged for 5 min. The organic layer was aspirated and discarded, 1 ml of 2 *M* sodium carbonate and 3 ml of ether were added to the

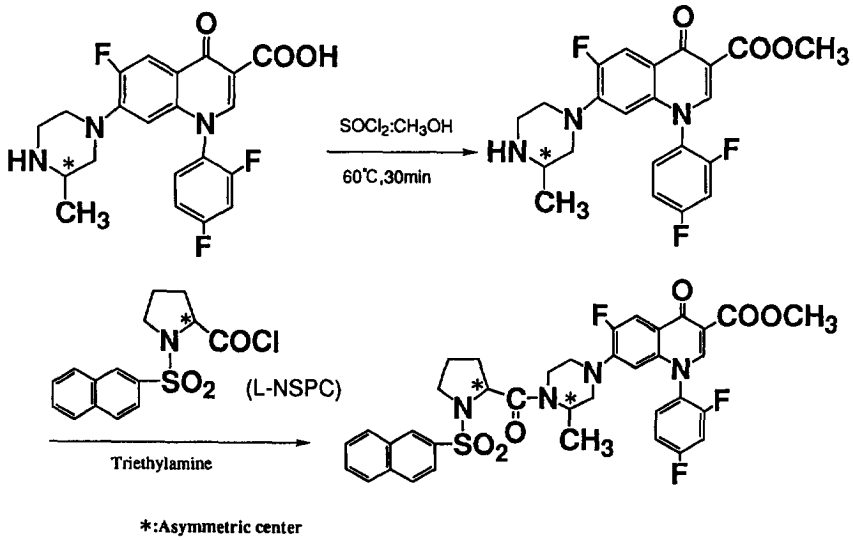

Fig. 2. Derivatization of TMFX for the diastereomeric method.

aqueous layer containing the derivatives of racemic TMFX, the mixture was shaken vigorously for 10 min and centrifuged again for 5 min. Then, the organic layer was transferred to a screw-cap vial, and the ether was evaporated to dryness at 40°C under a stream of nitrogen gas. The residue was dissolved in 0.3 ml of mobile phase, and 50 μl of sample solution were injected into the HPLC system equipped with the autoinjector. Fig. 3 represents the derivatization of TMFX for the chiral stationary method.

2.8. Calibration curves, accuracy and precision

The calibration curves for determination of racemic TMFX were prepared by the addition of TMFX (0, 5, 10, 25, 50, 100, 250, 500, 1000, 2500 and 5000 ng/ml) in serum and urine. These spiked samples were analyzed by the procedure described above.

The accuracy and precision of the proposed two enantiospecific methods were determined by assaying control serum and urine containing known amounts of racemic TMFX.

Aliquots of the sample solutions were injected precisely into the HPLC system because they did not contain an internal standard.

3. Results and discussion

3.1. Extraction procedure

All recently developed quinolone compounds have both a carboxylic acid group and a strongly basic centre; they are ionized throughout the pH range. The common sample preparation methods are deproteinization [7] or extraction with organic solvent [8–10], usually followed by HPLC.

For the preparation procedure of TMFX in biological fluids, two preparation procedures were reported by Granneman and Vargas [6]. The first method was ultrafiltration, and the second one was extraction with organic solvent. The ultrafiltration method is very simple but less sensitive, and the sample solutions treated by this method were not suitable for injection into the HPLC system using a normal-phase column. Thus, we chose the extraction method with methylene chloride for the preparation procedure of racemic TMFX in biological fluids. The recoveries of TMFX from spiked serum and urine were more than 90% against the known amounts of TMFX under neutral pH conditions with 1 *M* ammonium acetate buffer, and the residue containing racemic TMFX was derivatized for the two enantiospecific methods.

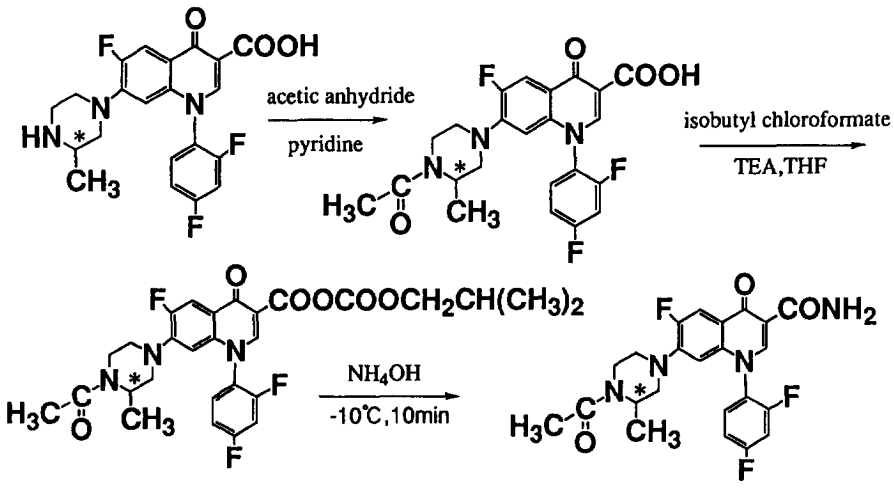

Fig. 3. Derivatization of TMFX for the chiral stationary-phase method.

3.2. Diastereomeric method

TMFX has both a carboxylic acid and a piperazine group that are able to react to form TMFX diastereomers. However, the diastereomers of the piperazine derivatives are more useful than those of carboxylic acid derivatives because the distance between asymmetric carbons of the former is shorter than that of the latter [11]. So, we investigated the diastereomeric derivatization method for coupling L-NSPC to the piperazine group.

At first, the residue containing racemic TMFX extracted from serum and urine was converted to the methylester with a thionylchloride–methanol mixture (1:9). As the TMFX methylester has basic properties, the back-extraction procedure could be used for excluding endogenous substances. The isolated TMFX methylester was derivatized to form diastereomers with L-NSPC reagent.

Optimal conditions for L-NSPC derivatization of the TMFX were investigated in terms of the amounts of L-NSPC (0, 0.1, 0.2, 0.4, 1.0, 2.0 mg), the amounts of triethylamine (0, 3, 5, 10, 25 μl) and the reaction time (0, 15, 30, 90 min) at room temperature. It was found that the optimum amounts of L-NSPC and triethylamine were 0.4 mg and 5 μl, respectively. Fig. 4 shows the reaction time for derivatization. This reaction was so rapid, that the efficiency of derivatization was not improved by increasing the reaction time (0–90 min). Thus, the optimum reaction time for the derivatization was taken to be 15 min at room temperature. It was recognized that there was no difference in the reaction yield between the *R*-diastereomer and *S*-diastereomer. Therefore, TMFX diastereomers coupling to L-NSPC under the optimal conditions were separated by the normal-phase column with a separation coefficient of 1.05.

Fig. 5 shows the mass spectra of *S*- and *R*-diastereomers using SIMS. The protonated molecular ion $[M+H]^+$ of *R*- and *S*-diastereomers were observed at *m/z* 719.

3.3. Chiral stationary-phase method

The chiral stationary phase, ovomucoid conjugated with aminopropyl silica gel (OVM column), was developed in 1987 [12], and produces chiral separations of racemic drugs [13,14]. Since the OVM column may be used for reversed-phase HPLC, it can analyze the enantiomers in biological samples.

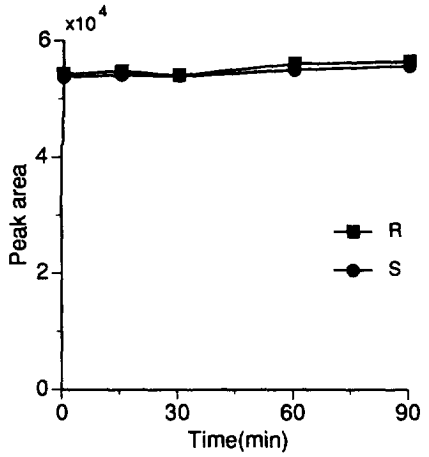

Fig. 4. Effect of reaction time for diastereomeric derivatization of racemic TMFX with L-NSPC.

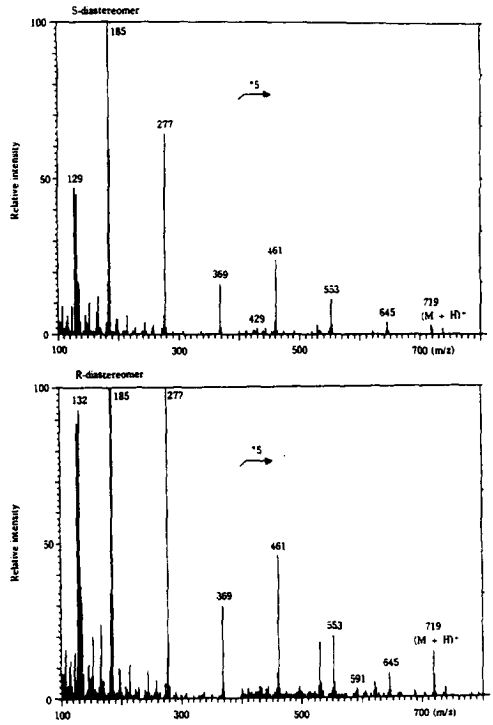

Fig. 5. Mass spectra of *S*- and *R*-diastereomers for TMFX.

The resolution factor of racemic compounds using this column depends on pH, ionic strength and organic solvent concentration of the mobile phase.

Thus, the effect of pH on resolution and selectivity was investigated with an authentic compound: racemic TMFX and three derivatives of racemic TMFX (Table 1). Buffers used were in the pH range 4–7, and their concentration was 0.02 *M*. Resolution of underivatized racemic TMFX was not achieved with this pH range. However, the three derivatives of TMFX were resolved by masking the carboxylic acid, and the separation coefficient increased at higher pH values. It was thought that the resolution of the basic derivatized TMFX tended to improve as the value of pH rose. As a result, acetylated and carbonylamidated TMFX enantiomers could be baseline separated by using a mobile phase containing 8% of acetonitrile adjusted to pH 7.0, and a separation coefficient of 1.50 was obtained. Thus, the chiral stationary-phase method was superior to the diastereomeric method for the chromatographic separation of the derivatized TMFX enantiomers. We therefore investigated the derivatization procedure for converting to N-acetylated and carbonylamidated TMFX.

At first, the residue containing racemic TMFX was acetylated with an acetic anhydride–pyridine mixture. After evaporation, the residue was converted to a carbonylamide type by the mixed anhydride method with isobutylchloroformate [15]. To avoid interference from biological samples, all derivatives of racemic TMFX employed the back-extraction procedure.

The mass spectra of *S*- and *R*-TMFX derivatives were analyzed using the SIMS method. The protonated molecular ion $[M+H]^+$ of *R*- and *S*-TMFX derivatives was observed at *m/z* 459.

Table 1
Effect of pH and type of TMFX derivatives on the value of the separation coefficient of racemic TMFX

pH	TMFX derivatives			
	None	Methyl ester	N-Acetyl methyl ester	N-Acetyl carbonylamide
4.0	1.00	1.00	1.00	1.08
6.5	1.00	1.00	1.00	1.33
7.0	1.00	1.10	1.03	1.50

Mobile phase: 0.02 *M* phosphate buffer–acetonitrile (92:8).

3.4. Assay linearity, accuracy, precision and sensitivity

Calibration curves were obtained by plotting the peak area of the derivatives of *R*- and *S*-TMFX spiked in serum and urine.

In serum samples, the linear calibration range was 0–5000 ng/ml for *R*- and *S*-TMFX (r=0.9989 for *R*-TMFX, and r=0.9989 for *S*-TMFX) by the diastereomeric method, and 0–500 ng/ml for *R*- and *S*-TMFX (r=0.9978 for the *R*-TMFX, and r=0.9954 for the *S*-TMFX) by the chiral stationary-phase method.

In urine samples, the calibration curves in the range of 0–5000 ng/ml for *R*- and *S*-TMFX were likewise obtained with good linearity.

The accuracy and precision of the proposed method were calculated by comparing the found concentrations of *S*- and *R*-TMFX derivatives from serum and urine samples spiked with racemic TMFX with the expected concentrations of *S*- and *R*-TMFX derivatives to which the added racemic TMFX was converted according to the proposed derivative procedure (Table 2). The found concentrations of *R*- and *S*-TMFX were above 90% compared with their added concentrations, and the values of the coefficient of variation indicated good precision. It is evident from the data in Table 2 that the proposed two methods were almost satisfactory in both accuracy and precision. The concentrations in working samples were calculated with the concentrations of standard solutions spiked in control serum and urine spiked with known amounts of racemic TMFX using the proposed preparation. Therefore, it was considered that the proposed two enantiomeric methods were suitable for pharmacokinetic and bioavailability studies in humans.

The detection limits of both the diastereomeric method and the chiral stationary-phase method were 5 ng/ml in serum and urine. Typical chromatograms of racemic TMFX by the two enantiospecific methods are shown in Fig. 6.

3.5. Applications

The described chiral stationary-phase method was applied to the determination of TMFX enantiomers in serum and urine of healthy volunteers orally dosed

Table 2
Accuracy and precision for the determination of *S*- and *R*-TMFX in serum and urine using the chiral stationary-phase method and the diastereomeric method ($n=3$)

	Amount added (μg/ml)		Amount found (mean±S.E.) (μg/ml)		Coefficient of variation (%)	
	S-Isomer	*R*-Isomer	*S*-Isomer	*R*-Isomer	*S*-Isomer	*R*-Isomer
Diastereomeric method						
Serum	0.125	0.125	0.121±0.006	0.124±0.005	7.8	7.1
	0.500	0.500	0.539±0.019	0.528±0.016	6.1	5.0
Urine	0.125	0.125	0.113±0.001	0.115±0.002	2.1	2.6
	0.500	0.500	0.497±0.009	0.511±0.006	3.6	2.1
Chiral stationary-phase method						
Serum	0.125	0.125	0.117±0.005	0.115±0.005	6.7	7.7
	0.500	0.500	0.499±0.019	0.492±0.016	6.5	5.6
Urine	0.125	0.125	0.120±0.004	0.120±0.003	5.0	4.1
	0.500	0.500	0.513±0.004	0.507±0.007	1.4	2.5

with four 150-mg tablets of TMFX. The serum concentrations of enantiomers are shown in Fig. 7. The time-course of the *S*-form serum level was similar to that of the *R*-form. The pharmacokinetic parameters of *S*- and *R*-TMFX are shown in Table 3. The AUC value of *S*-TMFX was almost equal to that of *S*-TMFX. The urinary excretions of *S*- and *R*-TMFX are shown in Fig. 8. The concentration ratios of the enantiomers were 0.96–1.2 in urine, which suggested that the pharmacokinetics of the enantiomers differed slightly from each other in humans.

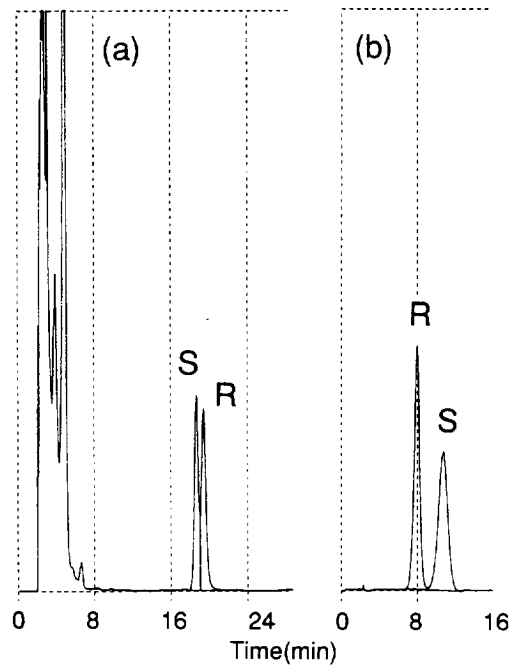

Fig. 6. Typical chromatograms of racemic TMFX obtained with (a) the diastereomeric method and (b) the chiral stationary-phase method.

4. Conclusions

The diastereomeric and the chiral stationary-phase methods showed good chromatographic separation and quantification for the determination of a new racemic quilone compound in serum and urine. The two enantiospecific methods were satisfactory in both accuracy and precision. The TMFX diastereomers coupling to L-NSPC were resolved slightly,

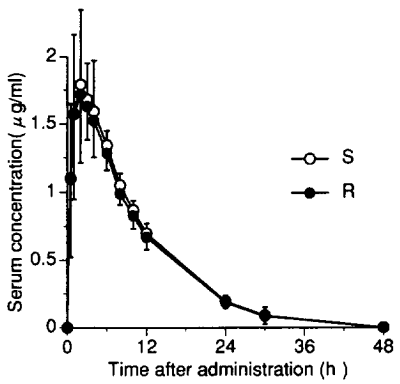

Fig. 7. Time courses of serum concentration of *S*- and *R*-TMFX after oral administration of a 600-mg dose ($n=6$, mean±S.E.).

Table 3
Pharmacokinetic parameters of *S*- and *R*-TMFX after oral administration of 600 mg ($n=5$)

Isomer		C_{max} (μg/ml)	T_{max} (h)	$t_{1/2}$ (h)	AUC_{0-30} (μg·h/ml)	AUC_{∞} (μg·h/ml)
S	Mean	1.90	2.80	6.27	20.99	21.89
	S.E.	0.21	0.86	0.68	1.02	1.15
R	Mean	1.85	2.40	6.22	20.13	20.97
	S.E.	0.21	0.93	0.72	1.00	1.16

The time elapsed to peak (T_{max}) and the maximum concentration (C_{max}) were taken directly from the observed value. The half-life ($t_{1/2}$) was calculated using the least-square method. The area under the curves was computed by the linear trapezoidal rule from 0 to 30 h (AUC_{0-30}). The value of $AUC_{\infty}=AUC_{30}+$[serum concentrations at 30 h after dosing/the elimination rate constant].

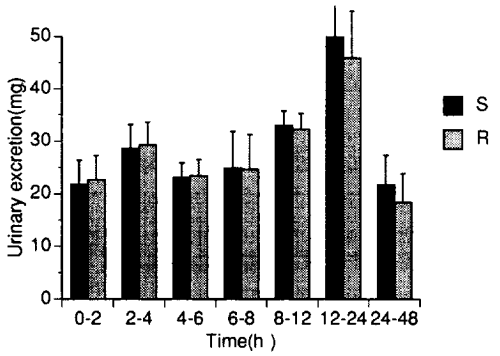

Fig. 8. Time courses of urinary excretion of *S*- and *R*-TMFX after oral administration of a 600-mg dose ($n=6$, mean±S.E.).

and the acetylated and carbonylamidated TMFX were resolved with an excellent separation coefficient on the chiral stationary-phase column. The latter method, which showed superior chromatographic separation, has been applied to the pharmacokinetic study of TMFX enantiomers in humans.

Acknowledgments

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