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Double column-switching high-performance liquid chromatographic method for the determination of TAK-603 and its metabolites in human serum

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Abstract

A double column-switching high-performance liquid chromatographic (HPLC) method for the determination of concentrations for TAK-603 (T) and its metabolites, T-72258 (M-I) and T-72294 (M-III), in human serum was developed. The analytes were extracted with ethyl acetate from human serum samples treated with triethylamine and injected into the HPLC system. Separation of the analytes was performed on the HPLC system with double column-switching technique. The mobile phases A and B for the first column and the mobile phase C for the second column used were a mixture of methanol–10 m*M* aqueous ammonium acetate solution (1:1, v/v), methanol and a mixture of methanol–10 m*M* aqueous ammonium acetate solution (11:9, v/v), respectively. The eluate was monitored with a UV detector at a wavelength of 253 nm. The work-up procedure was reproducible and more than 90% of the analytes could be recovered from human serum. The lower limits of quantitation were all 1 ng/ml for the analytes when 0.5 ml of human serum was used. Standard curves were linear with a correlation coefficient (*R*) of more than 0.999 in the range of 1–500 ng/ml for T, M-I and M-III in human serum. The intra- and inter-day precision of the method for the various analytes were below 4.8%. The accuracy was good with the deviations between spiked and calculated concentrations of the analytes being within 11.0%. The method was successfully applied to analyze serum samples after an oral administration of T to healthy male volunteers.

Keywords: TAK-603

1. Introduction

TAK-603, ethyl 4-(3,4-dimethoxyphenyl)-6,7-dimethoxy-2-(1,2,4-triazol-1-ylmethyl)-quinoline-3-carboxylate, (T) (Fig. 1) is currently under development as an antirheumatic agent in Japan and the USA [1,2]. In the preclinical studies using ^{14}C-labeled-T chemical structures for metabolites of T were elucidated by liquid-chromatography–tandem mass spectrometry (LC–MS–MS) in rats and dogs [3]. In the plasma of rats and dogs after oral dosing of T, M-I [ethyl 4-(4-hydroxy-3-methoxyphenyl)-6,7-dimethoxy-2-(1,2,4-triazol-1-ylmethyl)-quinoline-3-carboxylate] (Fig. 1), which has the same pharmacological potency as T, was detected as the major metabolite with a minor one, M-III [ethyl 4-(3,4-dimethoxyphenyl)-7-hydroxy-6-methoxy-2-(1,2,4-triazol-1-ylmethyl)-quinoline-3-carboxylate]

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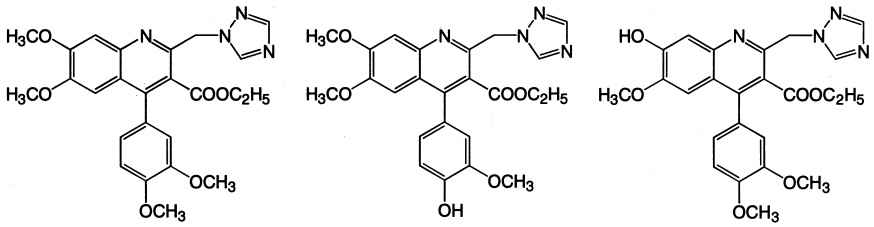

Fig. 1. Chemical structures of T and its metabolites (M-I and M-III).

(Fig. 1) [4]. To determine simultaneously the concentrations of T, M-I and M-III in a phase I study, a double column-switching high-performance liquid chromatographic (HPLC) method was developed.

2. Experimental

2.1. Materials and reagents

T, M-I and M-III with chemical purities of 99.8, 100.0 and 99.2%, respectively, were synthesized by Takeda (Osaka, Japan). HPLC-grade methanol was purchased from Wako (Osaka, Japan). All the other chemicals were of reagent grade. Water was purified with a Milli-Q water purification unit (Millipore, Bedford, MA, USA).

2.2. HPLC system and conditions

The HPLC system consisted of an SCL-6B system controller (Shimadzu, Kyoto, Japan), three LC-6A pumps (Shimadzu), an SIL-6B autosampler (Shimadzu), a 860-CO column oven (Jasco, Tokyo, Japan) for maintaining at 40, an FCV-2AH six-port valve (Shimadzu), two SPD-10A ultraviolet detectors (Shimadzu) and a Model 805 integrator (Waters, Milford, MA, USA). Two types of analytical columns were used, Develosil ODS-UG-5 (5 μm, 150× 4.6 mm I.D., Nomura, Aichi, Japan) as the first column (C1) and YMC-Pack A-402 [phenyl (Ph) type, 5 μm, 150×4.6 mm I.D., YMC, Kyoto, Japan) as the second column (C2) for column-switching HPLC separation. A mixture of methanol–10 m*M* aqueous ammonium acetate solution (1:1, v/v), and methanol were used as mobile phase A [MP(A)] and mobile phase B [MP(B)] for C1, respectively, and a mixture of methanol–10 m*M* aqueous ammonium acetate solution (11:9, v/v) as mobile phase C [MP(C)] for C2. Time program and flow-rates of MP(A), MP(B) and MP(C) are shown in Table 1.

The column-switching procedures are as follows. The retention times of analytes in C1 were checked each day prior to analysis to determine the time program for the column-switching. After the sample solution (0.15 ml) was injected into the HPLC system, the eluate fractions containing M-I and M-III from C1 were transferred to C2 by the first valve operation (first heart-cut). The fractions were further separated on C2 and monitored at a wavelength of 253 nm. By the second valve operation (second heart-cut) after an appropriate interval, fractions containing T eluted from C1 were transferred to C2. The flow-rate of MP(A) was decreased to 0.3 ml/min between eluting of the first and second fractions, because the interval time of the valve operations for separating the first fractions on column C2 required 10 min. The C1 was washed out with a mixture of MP(A)–MP(B) (1:4, v/v) at a flow-rate of 1 ml/min

Table 1
Valve position and typical flow-rate of mobile phase for the determination of T, M-I and M-III in serum

Time after injection (min)	Valve position	Flow-rate (ml/min)		
		MP(A)	MP(B)	MP(C)
0	0	1	0	1
6.4	1	1	0	1
8.8	0	0.3	0	1
16.8	1	1	0	1
19.1	0	0.2	0.8	1
30	0	1	0	1
45	0	1	0	1

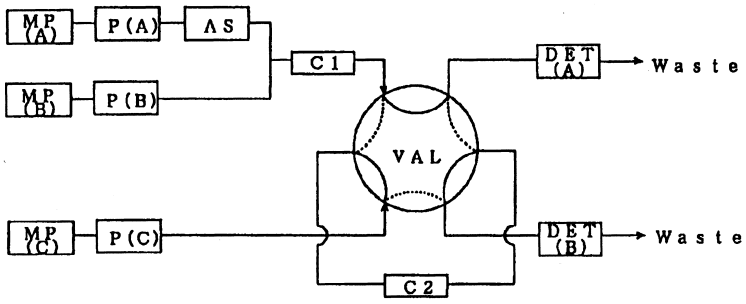

Fig. 2. Schematic diagram of HPLC for column-switching. P(A), P(B) and P(C): Pumps A, B and C. AS: Auto-sampler. VAL: Six-port valve. C1 and C2: Columns 1 and 2. DET(A) and DET(B): UV detectors A and B. MP(A), MP(B) and MP(C): Mobile phases A, B and C. ———: Valve position 0, · · ·: Valve position 1.

for removing the interfering substances from the matrix after the elution of T fraction was completed.

Fig. 2 and Table 1 show the schematic diagram of the HPLC system for column-switching and the time program, respectively.

2.3. Preparation of standard solutions

Standard solutions of T, M-I and M-III were prepared by dissolving them at 1 mg/ml each in methanol. These solutions could be stored for two months under cold (4°C) and dark conditions. Equal volumes of T, M-I and M-III were mixed, and diluted serially with methanol to prepare concentrations of each analyte at 2500, 1000, 250, 100, 25 and 5 ng/ml. Serum standards were prepared as follows. After addition of a solution of propylene glycol in methanol (1:9, w/v, 0.1 ml) and a solution of 0.01% pyrogallol in methanol (0.1 ml) to 0.1 ml each of diluted standard solutions, the mixture was evaporated under a stream of nitrogen gas below 40°C. The residue was dissolved in control human serum (0.5 ml) to prepare concentrations of each analyte in serum at 500, 200, 50, 20, 5 and 1 ng/ml in serum. Quality control (QC) samples of which each volume was 10 ml were prepared similarly so that the final concentrations were 200, 20 and 5 ng/ml.

2.4. Sample preparation

Triethylamine (10 μl) was added to a serum sample (0.5 ml) and vortexed well. To the mixture were added 1 *M* hydrochloric acid (50 μl) and ethyl acetate (5 ml), and shaken for 5 min. After centrifugation for 3 min at 3000 rpm, the aqueous layer was frozen in a dry ice–acetone bath. The supernatant was transferred into another glass tube, and a solution of propylene glycol in methanol (1:9, w/v, 0.1 ml) and a solution of 0.01% pyrogallol in methanol (0.1 ml) were added to the organic layer, and evaporated under a stream of nitrogen gas below 40°C. The residue was reconstituted in mobile phase A (0.25 ml), centrifuged for 5 min at 3000 rpm, and the supernatant was used as an assay sample.

2.5. Quantitation

Each of the standard curves were obtained on the peak heights of the respective analytes versus their nominal concentrations in the standard serum by the $1/C$ weighted least-square linear regression:

$$Y = a + bC \quad (1)$$

where *Y*, *C*, *a* and *b* are the peak height, the analyte concentration, the *Y*-intercept and the slope, respectively. The concentration of the analyte in the spiked samples, QC samples, and specimen was calculated by back-solving the equation of the standard curve:

$$C = (Y - a)/b \quad (2)$$

Recovery was calculated by a comparison of the peak height of the analyte in the spiked sample at concentrations of 500, 200, 50, 20, 5 and 1 ng/ml

with that in the reference standard solution at the same concentration.

3. Results and discussion

3.1. Evaporation procedure

When a solution containing T, M-I and M-III in methanol or ethyl acetate only was evaporated under a stream of nitrogen gas, the recoveries of M-I and M-III were insufficient. Thus, by additions of pyrogallol and propylene glycol into the solution, all the analytes were recovered quantitatively.

3.2. Purification by the liquid–liquid extraction

Extraction of T, M-I and M-III from human serum were examined with various solvents. Although T and M-III were extracted quantitatively from human serum with ethyl acetate, M-I was often recovered insufficiently. Thus, by addition of triethylamine, all the analytes were extracted quantitatively from human serum with ethyl acetate after neutralizing with 1 *M* hydrochloric acid.

3.3. Column-switching HPLC

C_4, C_8, C_{18} and Ph columns were examined for HPLC separation of T, M-I and M-III with a mobile phase consisting of methanol or acetonitrile and aqueous ammonium acetate solution. An HPLC system in combination with a C_{18} or Ph column and the mobile phase containing methanol resolved T, M-I and M-III in sharp peak shapes compared with resolutions of other combinations. When a C_{18} column with a mobile phase consisting of methanol–10 m*M* aqueous ammonium acetate solution (1:1, v/v) was used, the retention times of M-III, M-I and T were 7.3, 7.9 and 12.4 min, respectively. However, the peaks of these compounds were interfered with endogenous materials in human serum. When the fraction containing M-III and M-I eluted from the C_{18} column was injected into an HPLC system using a Ph column with the same mobile phase, the endogenous materials separated completely from M-III and M-I with retention times of 15.0 and 15.9 min, respectively. For the fraction containing T, the same results were also obtained and its retention time was 30.3 min.

Column-switching techniques that exploited such advantageous characteristics have been widely used in HPLC for the separation of various drugs in biological fluids [5–10]. Although T, M-I and M-III were not completely separated from the endogenous materials using only one column, each analyte could be separated completely from interfering materials on the second column by off-line. Thus, we developed the double column-switching HPLC method to determine concentrations for M-I and M-III (first heart-cut) and that for T (second heart-cut) in combination with a C_{18} column as the first column and a Ph column as the second one. As a result, only a small amount of endogenous substances interfered with the retention times of T, M-I and M-III. Fig. 3 and Table 1 show typical chromatograms of human serum and valve position and typical flow-rate of mobile phases, respectively.

3.4. Linearity

Standard curves for spiked human serum containing each analytes were linear over the range between 1 and 500 ng/ml for T, M-I and M-III (correlation coefficient: $R>0.999$).

3.5. Stability of standard solution

Stabilities of T, M-I and M-III standard solutions were confirmed to be satisfactory during the development of the analytical method: the remaining percents were 100.0, 97.1, and 99.1%, respectively, after 86 days under refrigeration.

3.6. Recovery, precision and accuracy

Table 2 shows mean recoveries of T, M-I and M-III. The recoveries for T, M-I and M-III from human serum were satisfactorily high: those were over 90%. Intra- and inter-day precisions and accuracies of this method shown in Table 3 were high enough, however, those met the requirement for the lower limit of quantitation [“both the coefficient of

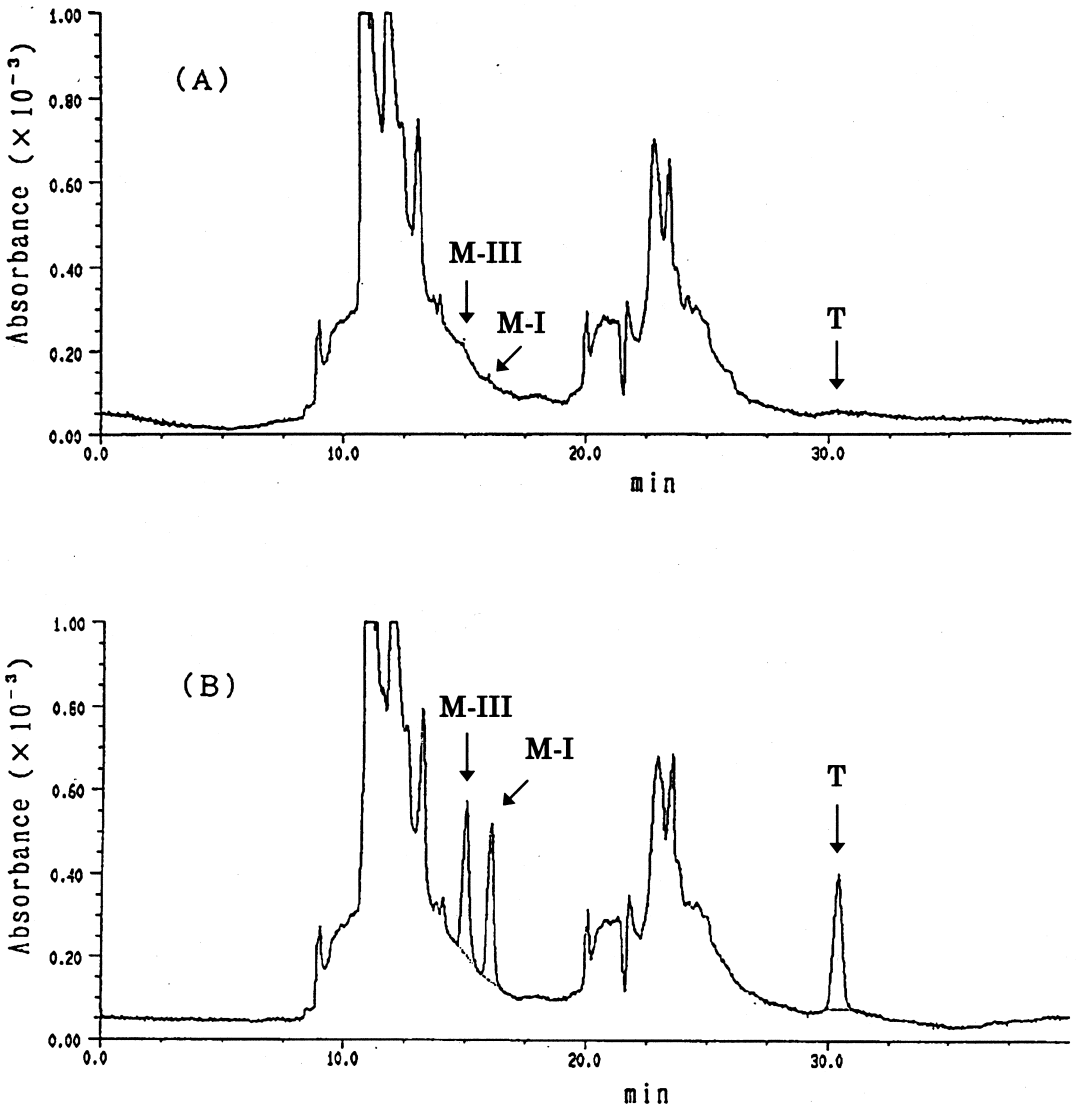

Fig. 3. Typical chromatograms of control serum (A), and serum spiked with T, M-I and M-III (B). The concentrations of each compound in serum were 5 ng/ml.

variation (C.V.) and absolute value of relative errors (R.E.) should not be higher than 20%''] [11].

3.7. Limit of quantitation

When 0.5 ml of human serum was used, the lower limits of quantitation of the present method were 1 ng/ml for all the analytes.

3.8. Application to phase I study

The column-switching HPLC established was applied to studies on the pharmacokinetic profiles of T in human. Fig. 4 shows the time profiles of the concentrations for T, M-I and M-III in human serum samples after oral administration of T to healthy male volunteers at a single dose of 100 mg. T and M-I were mainly detected in human serum, and

Table 2
Recovery of T, M-I and M-III from human serum

Spiked concentration (ng/ml)	T		M-I		M-III	
	Mean recovery (%)	C.V. (%)	Mean recovery (%)	C.V. (%)	Mean recovery (%)	C.V. (%)
1	93.0	1.9	91.4	4.0	90.7	5.0
5	96.3	2.5	91.8	3.7	91.0	2.9
20	100.0	1.2	98.3	4.0	96.0	1.7
50	96.7	1.8	97.2	3.8	91.5	2.2
200	98.0	1.8	97.2	2.8	92.7	1.8
500	98.5	1.0	98.0	1.1	93.5	1.2

Each value was obtained from five determinations within an assay.

M-III was hardly detected in the matrix. The pharmacokinetic parameters for T and M-I were as follows; the maximum serum concentrations (C_{max}) were 70 and 136 ng/ml, the times of C_{max} (T_{max}) were both 2.33 h, and the areas under the serum concentration–time curve (AUC_{0-24}) were 173 and 476 ng h/ml, respectively.

4. Conclusions

A selective and sensitive column-switching HPLC method for the determination of concentrations for T, M-I and M-III in human serum was developed. This method has a low quantitation limit of 1 ng/ml for three analytes, and was applied to the determination

Table 3
Intra- and inter-day accuracy and precision of determination method of T, M-I and M-III in human serum

Analyte	Spiked concentration (ng/ml)	Intra-day		Inter-day	
		C.V. (%)	R.E. (%)	C.V. (%)	R.E. (%)
T	1	1.9	4.0	–	–
	5	2.4	−1.0	0.9	−5.0
	20	1.2	−0.5	2.7	−3.0
	50	1.8	−1.6	–	–
	200	1.8	−1.0	1.6	−3.5
	500	1.0	0.4	–	–
M-I	1	3.6	11.0	–	–
	5	3.5	−4.0	3.6	−8.4
	20	4.0	−4.5	4.4	−4.5
	50	3.9	−2.2	–	–
	200	2.7	−1.5	2.5	−1.0
	500	1.1	1.2	–	–
M-III	1	4.8	5.0	–	–
	5	2.8	−1.0	2.0	−3.2
	20	1.7	−1.0	2.7	−2.0
	50	2.2	−2.2	–	–
	200	1.8	−1.5	1.0	−2.0
	500	1.2	1.0	–	–

– = Not determined. Each value in intra-day was obtained from five determinations within an assay. Each value in inter-day was obtained from assays on three different days.

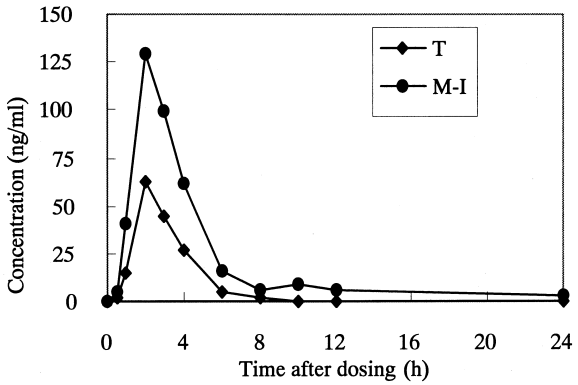

Fig. 4. Mean serum concentration of T and M-I after single oral administration of T (dose of 100 mg) in six healthy volunteers.

of concentrations for T, M-I and M-III in human serum after oral administration of T to healthy male volunteers.

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