

## Simultaneous determination of a novel anticancer drug, TAS-103, and its N-demethylated metabolite in monkey plasma by high-performance liquid chromatography using solid-phase extraction

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### Abstract

A simple and rapid method for the analysis of a novel anticancer drug, TAS-103, and its metabolite demethyl-TAS-103 in monkey plasma has been developed. This method is based on high-performance liquid chromatography with visible detection at 460 nm after solid-phase extraction with a Sep-Pak Vac PS-2 cartridge. The extraction recoveries of each compound, including the internal standard TAS-1-018, were from 88 to 102%. The quantitation limit of each compound was 5.0 ng/ml in 0.5 ml of plasma. The coefficients of variation for each compound ranged from 0.9 to 4.9%, and relative errors for each compound ranged from -3.8 to 4.6%. Both compounds in monkey plasma were stable at -80°C for 39 days and the extracts were stable at ambient temperature for 24 h. This method has been demonstrated to be useful for the pharmacokinetic study of TAS-103 in monkey plasma after intravenous administration.

**Keywords:** TAS-103; 6-[[2-(Dimethylamino)ethyl]amino]-3-hydroxy-7H-indeno[2,1-c]quinolin-7-one dihydrochloride; Demethyl-TAS-103

### 1. Introduction

TAS-103, 6-[[2-(dimethylamino)ethyl]amino]-3-hydroxy-7H-indeno[2,1-c]quinolin-7-one dihydrochloride (Fig. 1, I), has antitumor activity and has been developed as an anticancer drug intended for administration by infusion. This compound, which is a novel condensed quinoline derivative, is a potent dual-inhibitor of topoisomerases I and II and is highly effective against solid tumors [1]. These enzymes are associated with cleavage of DNA, and

passage and recombination of DNA in the processes of DNA and RNA synthesis [2,3].

In preclinical investigations, I was found to be metabolized to demethyl-TAS-103 (DM-TAS-103) by oxidative N-demethylation and also to N-oxide by oxidation. The structure of DM-TAS-103 (II) is shown in Fig. 1. Moreover, I and II form a glucuronide (GlcU) by conjugation. Among these four metabolites, II retains the original antitumor activity. This metabolite may exhibit pharmacological effects after administration of I. Therefore, to characterize both I and II pharmacokinetically in monkey plasma, we have established a method for the simultaneous determination of I and II.

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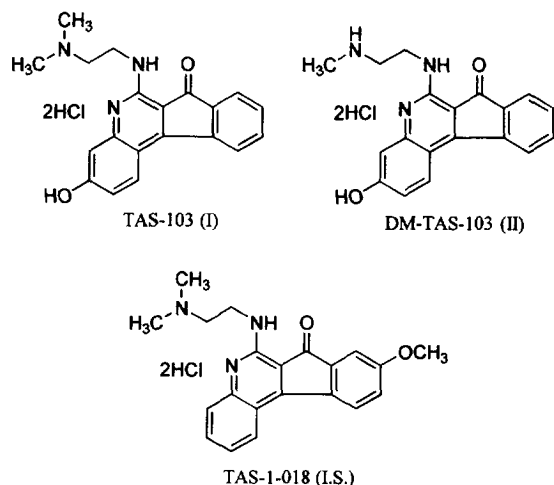


Fig. 1. Structures of TAS-103 (I), DM-TAS-103 (II) and TAS-1-018 (I.S.).

The analytical method used was a reversed-phase high-performance liquid chromatographic assay with visible detection at 460 nm. The internal standard (I.S.) used was TAS-1-018 (Fig. 1), which is an analog of I. This method utilizes polymer-based solid-phase extraction (SPE) as a sample clean-up procedure. The polymer-based SPE does not show the secondary adsorption caused by silanol or residual metal. Thus, it is suitable for basic or coordination compounds. Recently, this SPE method has become widely used for pesticide analysis in environmental science [4,5].

In this report, we describe the development and validation of a method for the simultaneous determination of I and II in monkey plasma. The quantitation limit for both compounds in plasma was 5 ng/ml. This method was shown to be useful for pharmacokinetic studies in cynomolgus monkey plasma after intravenous administration.

## 2. Experimental

### 2.1. Reagents and materials

Compounds I, II and I.S. were synthesized by Taiho Pharmaceutical (Saitama, Japan).

HPLC-grade acetonitrile, acetone and methanol, and special-grade acetic acid and formic acid were purchased from Kanto Chemical (Tokyo, Japan). The

water was purified with the Milli-Q system (Millipore, Tokyo, Japan). Special-grade trifluoroacetic acid, glycine, perchloric acid ( $\text{HClO}_4$ ), disodium ethylenediaminetetraacetate (EDTA) and the other reagents were purchased from Wako (Osaka, Japan).

Sep-Pak Vac PS-2 columns (200 mg/3.0 ml) were purchased from Nihon Waters (Tokyo, Japan).

### 2.2. Chromatography

The HPLC system (Shimadzu, Kyoto, Japan) consisted of an Erma ERC-3512 on-line degasser (Erma, Tokyo, Japan), two LC-10AD pumps, an SIL-10A auto-injector, a CTO-10A column oven, an SPD-10AV UV-Vis detector and a workstation CLASS-LC10.

Separation was performed at 40°C on J'sphere ODS-L80 (150×4.6 mm I.D., 4 μm, YMC, Kyoto, Japan) with a YMC guard cartridge. The visible wavelength was set at 460 nm. Gradient elution was performed at a flow-rate of 1.0 ml/min using a mobile phase of 0.07% trifluoroacetic acid–acetonitrile. The linear gradient ran from 17 to 35% acetonitrile in 18 min. The total run time was 30 min with a post-run time of 11 min for re-equilibration. The HPLC rinse solution used consisted of acetic acid–water–acetonitrile (2:48:50, v/v/v). Prior to use, these solutions were degassed using an aspirator.

### 2.3. Extraction

Sep-Pak Vac PS-2 columns were mounted on a Vac-Elut SPS 24 (Varian, Harbor City, CA, USA). Prior to use, the columns were prewashed with 2 ml of methanol, water, 0.2 M EDTA and 0.2 M glycine– $\text{HClO}_4$  (pH 2.0). Samples (0.5 ml) of centrifuged (1200 g, 15 min) monkey plasma were mixed with 0.1 ml of 4 μg/ml I.S. and 1 ml of 0.2 M glycine– $\text{HClO}_4$  (pH 2.0). The mixtures were loaded onto PS-2 columns and drawn through under vacuum. The columns were rinsed with 2 ml of water, followed by 3 ml of water–methanol (6:4, v/v). The analytes were eluted with 3 ml of formic acid–acetone–methanol (1:50:50, v/v/v). The eluates were dried under nitrogen at 40°C and the residues were reconstituted with 0.2 ml of 0.07% trifluoroacetic acid–acetonitrile (83:17, v/v). These solutions were centrifuged at 1200 g for 5 min, and 60-μl

volumes of the supernatant were injected into the HPLC system.

#### 2.4. Standards, calibration curve and quality control samples

Stock solutions of each of the compounds were initially prepared at a concentration of 100 µg/ml in 50 mM glycine-HClO<sub>4</sub> (pH 3.0). From these solutions, calibration standard solutions containing I and II were prepared by sequential dilution to give 10 000, 2000, 500, 100, 50 and 25 ng/ml solutions of each compound. The I.S. solution was prepared using the same solvent and diluted to 4 µg/ml. A calibration curve was prepared by spiking plasma with 0.1 ml of standard solutions ranging from 5 to 2000 ng (as the salt form)/ml of plasma. Quantification was accomplished based on the peak-height ratio of drug to I.S. using CLASS-LC10. The fitted line was determined by least squares regression using a weighting of  $1/\text{conc}^2$ .

Quality control (QC) samples were prepared with plasma and standards to give concentrations of 20, 200 and 1600 ng/ml in plasma.

#### 2.5. Recovery, precision and accuracy

The absolute extraction recovery was calculated at three concentrations of QC samples for each compound, at one concentration for I.S. The peak heights of extracted samples were compared with those of unextracted standard solutions that were prepared using 0.07% trifluoroacetic acid-acetonitrile (73:17, v/v).

Intra- and inter-assay recovery, precision and accuracy (each,  $n=5$ ) were evaluated by spiking plasma with four different concentrations of three QC samples and a quantitation limit. The precision was expressed as coefficient of variation (C.V.) by calculating the standard deviation as a percentage of the mean concentration found. The accuracy was expressed as relative error (R.E.) by calculating the concentration found as a percentage of the concentration added. Linear regression analysis was performed to calculate the slope, the intercept and the correlation coefficient ( $r$ ) of the calibration curve.

#### 2.6. Stability study

The stability test was evaluated by comparing the R.E. with the theoretical value and C.V. of triplicate QC samples. The work-up stability samples were incubated at 37°C for 1 h. The freeze-thaw stability samples were frozen at -80°C, thawed the next day and then frozen again. After two days, these samples were thawed and prepared for analysis. The stability in auto-injector vials was examined by injecting the extract in reconstituted solvent into the HPLC after 24 h. The storage stability samples were stored at -80°C. After 39 days, these samples were thawed and prepared for analysis.

#### 2.7. Application to cynomolgus monkey

Three male cynomolgus monkeys, weighing 6.6–7.3 kg, each received a single dose of I (10 mg/kg i.v.; 10 mg/5 ml saline with 10% Tween 80, adjusted with 1 M sodium hydroxide to pH 3.6). Blood was collected from the forearm vein with a heparinized syringe. The samples were transferred into polypropylene tubes at the following times after dosing: 0, 0.083, 0.25, 0.5, 1, 2, 4, 6, 8, 12 and 24 h. Plasma was immediately separated by centrifugation at 1200 g for 15 min and stored frozen at -80°C until analysis.

### 3. Results and discussion

#### 3.1. Selection of extraction column

To obtain good reproducibility, it is favorable to obtain an extraction efficiency of 80% to 90%. Various solid-phase extraction columns were tested. Silica-based columns, even with weak cation-exchangers such as carboxylic acid derivatives, or short chain reversed-phase columns such as C<sub>2</sub> did not give sufficient recovery with a best result of 60%. When more hydrophobic columns such as cyclohexyl or phenyl resins were used, lower recoveries (less than 25%) were obtained. However, with the use of polymer-based columns, we obtained comparatively good recoveries. Best results were obtained with

Sep-Pak Vac PS-2 extraction columns. The samples at pH 2.0 were placed on the extraction columns. At pH 5.0, at neutral pH and basic pH, the recovery of I was somewhat lower than at pH 2.0. The eluate was therefore composed of a mixture of acid and organic solvent, i.e., formic acid–acetone–methanol. Although addition of acid was needed to obtain good recovery, inclusion of hydrochloric acid in the eluate was not suitable because one of the metabolites, TAS-103 N-oxide, decomposed and this affected the determination of the parent drug. As shown in Table 1, the extraction recoveries of both compounds from monkey plasma were from 88.7 to 101.7%. Also, the recoveries of other metabolites, TAS-103 N-oxide (III), TAS-103 GlcU (IV) and DM-TAS-103 GlcU (V) were more than 90%.

### 3.2. Optimization of the chromatographic conditions

The phosphate buffer mobile phase was tested over the pH range of 2.0–3.5 at intervals of 0.5 pH units. The concentration of I at the range 10–2000 ng/ml was in proportion to the peak height at pH 2.0 and 2.5, but at pH 3.0 and 3.5 the peak height at low concentrations decreased beyond the proportional relationship. Accordingly, to obtain sufficient linearity, a strongly acidic mobile phase was required. To obtain good chromatographic resolution between the parent drug and all its metabolites, which included conjugates, the mobile phase selected was 0.07% trifluoroacetic acid instead of phosphate buffer. The

Table 1  
Extraction recoveries of I, II and I.S. from monkey plasma

Compound	Concentration added (ng/ml)	Recovery (mean $\pm$ S.D.) (%)
I (n=5)	20	101.7 $\pm$ 2.8
	200	98.1 $\pm$ 1.8
	1600	93.9 $\pm$ 1.5
II (n=5)	20	94.7 $\pm$ 2.7
	200	93.3 $\pm$ 1.7
	1600	88.7 $\pm$ 1.4
I.S. (n=15)	800	100.2 $\pm$ 2.1

HPLC rinse solution used included acetic acid to avoid memory effects after injection [6].

HPLC chromatograms for blank plasma and monkey plasma spiked with I, II and I.S. are shown in Fig. 2A,B, respectively. The chromatogram from blank plasma was free of endogenous interferences at

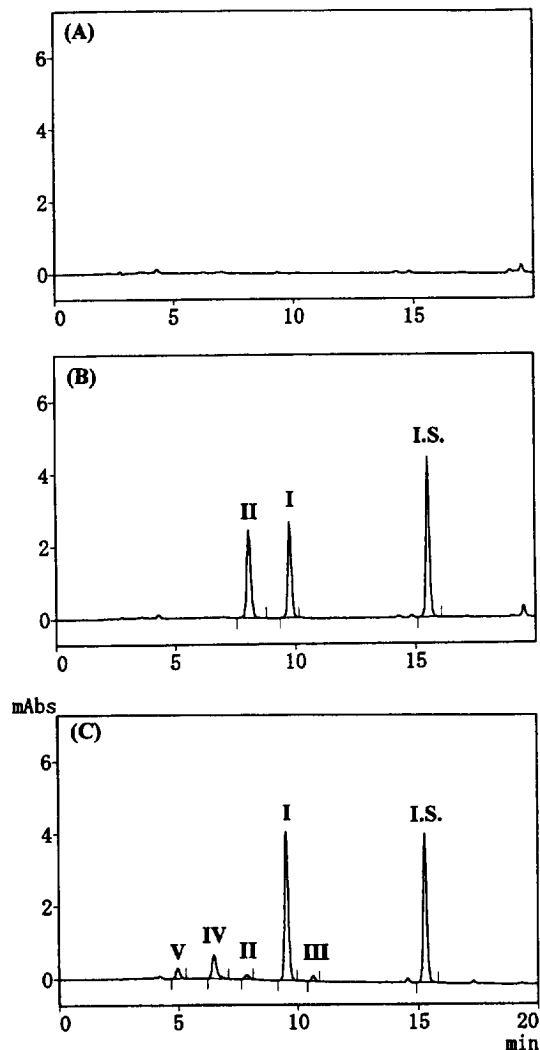


Fig. 2. Representative chromatograms of a blank plasma sample (A), a plasma sample spiked with 200 ng/ml of I, 200 ng/ml of II and 800 ng/ml of I.S. (B), and a plasma sample obtained 4 h after intravenous administration (C). Peaks: I=TAS-103, II=DM-TAS-103, III=TAS-103 N-oxide, IV=TAS-103 GlcU, V=DM-TAS-103 GlcU.

retention times corresponding to I, II and I.S. As shown in Fig. 2B, the retention times for II, I and I.S. were approximately 8.0, 9.8 and 15.5 min, respectively.

### 3.3. Linearity, precision and accuracy

The calibration curves obtained from six assays showed a linear relationship between the peak-height ratio of compound to I.S. and the compound concentration. The linear regression equations for I and II were  $y=(317.55\pm 12.21)x-(0.1795\pm 0.4091)$  and  $y=(341.33\pm 16.06)x-(0.2422\pm 0.1476)$ , respectively, where  $y$  is the drug concentration and  $x$  is the peak-height ratio of drug to I.S. The intercepts for I and II were very close to zero. The mean  $r$  values for I and II were 0.99949 and 0.99907, respectively, over the range of 5–2000 ng/ml.

The intra- and inter-assay precision and accuracy for I and II are shown in Table 2. The precision and accuracy of the HPLC method were evaluated by analysing five replicates of four concentrations (5, 20, 200 and 1600 ng/ml) in plasma containing I and

II. The intra- and inter-assay C.V. for both compounds ranged from 0.7 to 4.9%. Similarly, the intra- and inter-assay mean R.E. of both compounds ranged from -3.8 to 4.6%.

### 3.4. Stability study

Table 3 shows the stabilities of I and II in monkey plasma after freezing–thawing, at 37°C for 1 h and after storage for 39 days at -80°C. These studies were performed with QC samples at concentrations of 20, 200 and 1600 ng/ml. The C.V. of each compound varied from 0.7 to 4.1%, which was within the range of intra- and inter-assay variability. Similarly, the mean R.E. of each compound ranged from -4.6 to 3.2%. These results suggested that freezing–thawing and the blood sampling process had no effect upon the determination of I and II. In addition, both compounds were stable during storage at -80°C for 39 days. The stability of the extracts in reconstituted solvent was not a problem because the mean R.E. of each compound was from 4.7 to -0.5%.

Table 2  
Precision and accuracy of the determination of I and II in monkey plasma ( $n=5$ )

Compound	Concentration added (ng/ml)	Concentration found (mean $\pm$ S.D.) (ng/ml)	C.V. (%)	R.E. (%)	
I	Intra-assay	5	4.91 $\pm$ 0.09	1.8	-1.8
		20	19.55 $\pm$ 0.18	0.9	-2.3
		200	199.08 $\pm$ 1.36	0.7	-0.5
		1600	1605.08 $\pm$ 12.48	0.8	+0.3
	Inter-assay	5	5.23 $\pm$ 0.14	2.7	+4.6
		20	19.44 $\pm$ 0.67	3.4	-2.8
		200	198.42 $\pm$ 8.45	4.3	-0.8
		1600	1607.52 $\pm$ 49.43	3.1	+0.5
II	Intra-assay	5	5.10 $\pm$ 0.21	4.1	+2.0
		20	19.24 $\pm$ 0.23	1.2	-3.8
		200	198.37 $\pm$ 1.48	0.7	-0.8
		1600	1594.01 $\pm$ 21.35	1.3	-0.4
	Inter-assay	5	5.10 $\pm$ 0.21	4.1	+2.0
		20	19.31 $\pm$ 0.21	1.1	-3.5
		200	197.85 $\pm$ 9.74	4.9	-1.1
		1600	1619.62 $\pm$ 54.10	3.3	+1.2

Table 3  
Stability of I and II in monkey plasma ( $n=3$ )

Compound	Concentration added (ng/ml)	Concentration found (mean $\pm$ S.D.) (ng/ml)	C.V. (%)	R.E. (%)	
I	Freeze–thaw	20	19.32 $\pm$ 0.29	1.5	–3.4
		200	196.51 $\pm$ 7.20	3.7	–1.7
		1600	1580.70 $\pm$ 25.18	1.6	–1.2
	37°C for 1 h	20	19.79 $\pm$ 0.19	1.0	–1.1
		200	202.64 $\pm$ 1.40	0.7	+1.3
		1600	1621.28 $\pm$ 32.46	2.0	+1.3
	Storage for 39 days	20	19.81 $\pm$ 0.61	3.1	–1.0
		200	201.43 $\pm$ 2.75	1.4	+0.7
		1600	1595.30 $\pm$ 30.86	1.9	–0.3
II	Freeze–thaw	20	19.08 $\pm$ 0.32	1.7	–4.6
		200	195.18 $\pm$ 7.92	4.1	–2.4
		1600	1557.32 $\pm$ 15.17	1.0	–2.7
	37°C for 1 h	20	19.84 $\pm$ 0.52	2.6	–0.8
		200	205.39 $\pm$ 1.99	1.0	+2.7
		1600	1651.31 $\pm$ 27.43	1.7	+3.2
	Storage for 39 days	20	19.92 $\pm$ 0.45	2.3	–0.4
		200	200.38 $\pm$ 2.83	1.4	+0.2
		1600	1583.59 $\pm$ 29.51	1.9	–1.0

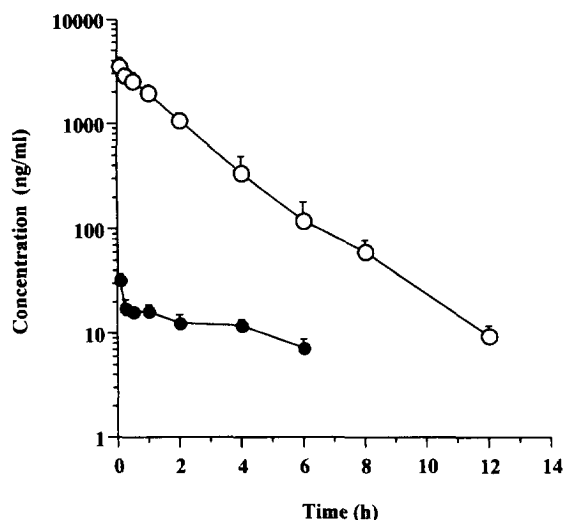


Fig. 3. Plasma concentration–time profiles of I (○) and II (●) in monkey plasma after intravenous administration of 10 mg/kg of TAS-103.

### 3.5. Application to a pharmacokinetic study

This method has been applied to the pharmacokinetic study of I in monkey plasma after intravenous administration at 10 mg/kg. After administration of I, I and II should be determined to investigate the pharmacokinetic behavior of I. A representative chromatogram obtained for a monkey plasma sample is shown in Fig. 2C. In addition to I ( $t_R$ , 9.5 min) and II (7.9 min), III (10.6 min), IV (6.5 min) and V (5.0 min) were observed on this chromatogram. Fig. 3 shows mean plasma concentration–time profiles of I and II. Compounds I and II were quantified from 5 min up to 12 h in plasma. Using 0.1 ml of plasma, we obtained values exceeding 2000 ng/ml in all 5 min samples, and some of those obtained at 15 min, 30 min and 1 h.

### 4. Conclusion

An analytical method was developed and validated for the determination of TAS-103 and DM-TAS-103

in monkey plasma by means of reversed-phase HPLC with visible detection at 460 nm. The use of polymer-based solid-phase extraction could achieve efficient recovery without irreversible adsorption, which often occurs with basic substances. Many metabolites, including polar compounds, could be extracted simultaneously. The method described in this study was specific, linear, precise and accurate over the range from 5 to 2000 ng/ml of TAS-103 and DM-TAS-103 using 0.5 ml samples of monkey plasma. All coefficients of variation and relative errors of this method were below 5%. TAS-103 and DM-TAS-103 are stable in monkey plasma at  $-80^{\circ}\text{C}$  for a period of at least 39 days.

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