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Simultaneous determination of tiopronin and its metabolites in rat blood by LC-ESI-MS-MS using methyl acrylate for stabilization of thiol group

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Abstract

A sensitive and reproducible HPLC-electrospray tandem mass spectrometric method has been developed for the analysis of tiopronin (TP) and its metabolites, 2-mercaptopropionic acid (2-mpa) and S-methylated TP (SA13), in rat blood using methyl acrylate (MA) for the stabilization of a thiol group. The thiol groups of TP and 2-mpa in rat blood were immediately derivatized by the addition of MA-acetonitrile solution in 0.1 M Tris–HCl (pH 9.1). The purification of the derivatives was accomplished by a simple liquid-liquid extraction procedure involving protein precipitation step. The analysis was performed on a Zorbax SB-C18 analytical column by a gradient elution with methanol-0.05 M acetic acid (15:85 and 7:3, v/v). Negative ion electrospray ionization with selected reaction monitoring was employed for the detection of analytes. Linearity of calibration was observed over the range of 0.5–1000 ng/ml for TP and 2-mpa, and 2–1000 ng/ml for SA13. The intra- and inter-assay variability for all analytes at the limit of quantitation (LOQ) level ranged from 5.47 to 16.75% and 4.95 to 7.23%, respectively. The LOQs estimated for TP, 2-mpa and SA13 were 0.5, 0.5 and 2 ng/ml, respectively. This assay method was successively applied to a pharmacokinetics study after an oral administration of TP (10 mg/kg) to rats. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Tiopronin; Methyl acrylate; Thiol; Determination; Electrospray; Liquid chromatography-mass spectrometry

1. Introduction

Tiopronin (2-mercaptopropionylglycine, TP) is a thiol compound, which has been used as a hepatoprotective and anti-cataract agent [1,2]. It also modifies rheumatoid arthritis [3] and has been applied in the treatment of cystinuria [4]. Early studies indicated that TP was hydrolyzed to

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2-mercaptopropionic acid (2-mpa) or S-methylated to SA13 (Fig. 1). The thiol compounds can be easily oxidized to disulfides either as a dimer or mix forms with endogenous thiols [5]. Therefore, immediate stabilization for thiol group should be carried out to determine the actual concentrations after obtaining blood sample. The simultaneous determination of TP and its metabolite, 2-mpa, in biological samples together with the thiol stabilization has been developed by pre-column derivatization on HPLC due to the absence of a chromophore [6,7]. Several methods have been reported for the determination of thiol compounds and all methods depend on reagents with sufficient absorption or fluorescent functional groups for sensitive detection [8–10]. N-substituted maleimides or active halogens are the most commonly used for thiol stabilization [11]. The maleimides react with thiol group by Michael addition reaction, however a new asymmetric carbon is formed on the maleimide portion of the derivative. TP is a racemic compound, yielding diastereoisomers by reaction with the maleimide, which then results in several peaks arising from the thiol derivatization on the reverse-phase HPLC system [6]. The active halides are generally

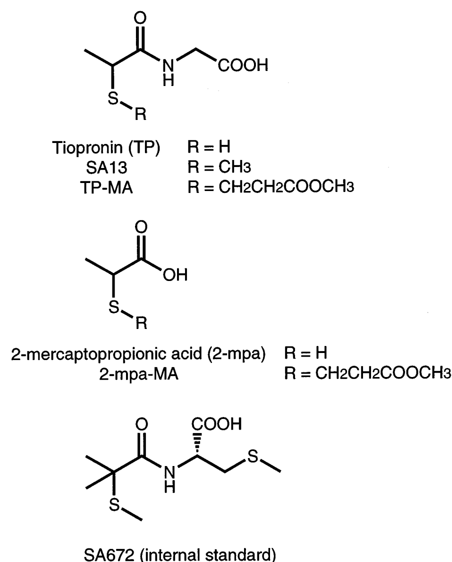

Fig. 1. Chemical structures of TP, 2-mpa, SA13, SA672 (internal standard) and corresponding derivatives with MA.

used for blocking reagent of thiols for proteins and peptides, such as iodoacetic acid and iodoacetamide [12]. However, the reaction is much slower than that with the maleimides and not sufficient for the small amount of thiol compound in complicated matrixes like plasma or blood. The acrylate derivatives react with thiol under the same reaction mechanism as maleimide without the formation of a new asymmetric carbon. Therefore, the acrylate derivatives substantially have some advantages over the maleimides and the active halides. Meanwhile, the derivatization reaction does not function to determine the S-methylated metabolite, SA13, because of its unreactivity with the acrylates.

Electrospray ionization-mass spectrometry (ESI-MS) is a proven technique for the rapid and sensitive determination of drugs and metabolites [13]. The formation of a particular ion depends on the ion affinity and the molecular structure of the analyte [14,15]. The polar functional groups incorporated into molecule are particularly relevant to the response of the molecular ion species produced. TP and all its metabolites have a polar functional group of a carboxylic acid and would be sensitively ionized in ESI-MS. We have demonstrated that acrylate derivatives are highly reactive to a thiol group and are suitable for the stabilization of a thiol group [16,17]. Furthermore, those derivatives that have structurally diverse polar functional groups have been proven to enhance the sensitivity of MS detection in ESI-MS [18,19]. The acrylate derivatives having the functional group with a high proton affinity especially increase the ion intensity of protonated molecule in the positive ion mode. On the other hand, the derivatives with a strong acid or an electronegative group give high ion responses in the negative ion mode [18]. Therefore, the combination of stabilization of a thiol group using the optimum acrylate derivative and analysis by LC-ESI-MS allows development of the sensitive and simultaneous determination of TP and its metabolites.

This paper describes a rapid, sensitive and selective LC-ESI-MS-MS assay for the simultaneous determination of TP and its metabolites, 2-mpa and SA13, using a methyl acrylate for thiol

Table 1
Precursor and product ions and collision voltages used for the selected reaction monitoring of all analytes

Compound	Precursor ion (m/z)	Product ion (m/z)	Collision voltages (eV)
TP-MA	248	162	20
2-mpa-MA	191	105	20
SA13	176	74	25
SA672 (IS)	250	158	20

stabilization and its practical application to the pharmacokinetic study of TP after oral administration to rats.

2. Experimental

2.1. Materials

Methyl acrylate (MA) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Tris(hydroxymethyl)aminomethane (Tris) and 2-mercaptopropionic acid (2-mpa) were from Nacalai Tesque (Kyoto, Japan). Ethylenediaminetetraacetic acid disodium salt (EDTA · 2Na), acetic acid, hydrochloric acid (HCl), methanol (HPLC grade) and ethyl acetate were from Wako (Osaka, Japan). All sorbents and reagents without methanol were guaranteed reagent grade. Water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). Tiopronin (TP), SA13 and SA672 were products of Santen Pharmaceutical (Osaka, Japan).

2.2. LC-MS-MS conditions

A Hewlett-Packard 1050 HPLC system (Palo Alto, CA, USA) was used for solvent delivery. The analysis column used was Zorbax SB-C18 (5-μm particle size, 150 × 2.1-mm i.d., Hewlett-Packard) at a setting of 40°C and the flow-rate was fixed at 0.2 ml/min. The elution was accomplished by a linear gradient system of two kinds of mobile phases following methanol-50 mM acetic acid — (A), 15:85, v/v and (B), 7:3, v/v. The proportion of mobile phase (A) was changed as follows: $t = 0$, 3 and 13 min, (A)% = 85, 5 and 5, respectively.

LC-MS-MS systems consisted of a Finnigan TSQ-7000 triple stage quadruple mass spectrometer (Thermo Quest, San Jose, CA, USA) equipped with a Finnigan ESI (API I) interface. The ESI spray voltage was set to 4.5 kV. Nitrogen was used as a sheath gas with a pressure setting of 70 psi and an auxiliary gas was not used. The heated capillary temperature was set to 250°C. For collision-induced dissociation (CID), argon was used as a collision gas at a pressure of 1.6 mTorr. Quantification was performed by selected reaction monitoring (SRM, dwell time 500 ms) of the deprotonated molecules (precursor ions, $[M-H]^-$) and their corresponding product ions in the negative ion mode. The monitoring ions and the collision energy settings for SRM detection are shown in Table 1. An electron multiplier voltage of 1500 V was used. Data processing was carried out using an LC QUAN data analysis program (Thermo Quest, San Jose, CA, USA).

2.3. Standard solution

A stock solution of TP, SA13 and 2-mpa (0.2 mg/ml for each) was prepared in 2 mM EDTA-0.05 M HCl. This solution was further diluted with 2 mM EDTA-0.05 M HCl to give a series of standards with concentrations of 4, 16, 40, 160, 640, 2000 and 8000 ng/ml (0.5, 2, 5, 20, 80, 250 and 1000 ng/ml in blood, respectively). A stock solution of SA672 (0.2 mg/ml) for internal standard (IS) was prepared in methanol and diluted to a concentration of 500 ng/ml with methanol.

2.4. Sample preparation

A 0.2-ml aliquot of blood was added into a glass test tube containing 0.4 ml of 0.1 M Tris–

HCl buffer (pH 9.1, 5 mM EDTA). A 25-µl aliquot of the standard solution and 0.1 ml of 7% MA in acetonitrile were immediately added to the tube. The sample mixture was vortex-mixed, and then left at room temperature for 30 min. After 25 µl of IS solution was added, the mixture was deproteinized with 1 ml of acetone and the precipitate was removed by centrifugation at 3000 rpm for 10 min. The supernatant was transferred into a glass-stoppered test tube and dried under a stream of nitrogen at 40°C. Then 0.5 ml of water and 0.2 ml of 1 M HCl were added to the residue and extracted with 2 ml of ethyl acetate. The organic layer was transferred into a glass test tube and evaporated to dryness. The residue was dissolved in 0.1 ml of mobile phase (A) and 10 µl of the sample solution was subjected to LC-MS-MS analysis.

2.5. Method validation

The precision and the accuracy of the method were determined by the replicate analysis of blood sample containing the analytes at blood concentrations of 0.5, 2, 20 and 250 ng/ml for TP and 2-mpa, and 2, 20 and 250 ng/ml for SA13 in the calibration range. The calibration line was constructed by weighted ($1/x$) least-square linear regression analysis of the peak area ratio of analytes to IS versus the concentrations. The calibration equation was used to calculate the concentration of analytes in the replicated and administered samples.

2.6. Storage stability

The storage stability of TP, 2-mpa and SA13 in rat blood was studied by replicate analysis of blood containing known amounts of analytes stored at −80°C for 4 weeks at blood concentrations of 5, 80 and 1000 ng/ml. As described in Section 2.4, the test samples prepared by mixing the standard solutions and MA solution in blank rat blood and 0.1 M Tris–HCl buffer were left at room temperature for 30 min, and then stored at −80°C for 4 weeks until use.

2.7. Animal study

Female Lewis rats weighing 142–167 g were used. The rats were allowed free access to water and food. After oral administration of TP at a dose of 10 mg/kg, blood samples were drawn at 0.25, 0.5, 1, 1.5, 2, 3, 4, 6 and 8 h ($n = 3$ at each sampling point) and collected in polyethylene tubes containing EDTA · 2Na. The blood samples were immediately stabilized with MA as described in Section 2.4 and left at room temperature for 30 min. Resulting sample mixtures were stored at −80°C until analysis.

The terminal elimination rate constant (k_{el}) was determined by linear regression of the log blood concentrations from data points of 3–4 h. The $T_{1/2}$ was calculated by the relationship, $T_{1/2} = \ln 2/k_{el}$. The area under the curve (AUC) was calculated by the linear trapezoidal rule up to the last point.

3. Results and discussion

3.1. LC-MS-MS optimization

Chemical structures of TP, its metabolites (SA13 and 2-mpa), SA672 (internal standard, IS) and derivatives of TP and 2-mpa obtained by the reaction with MA are shown in Fig. 1. To determine SA13 and the derivatives using the SRM mode, full scan and product ion spectra of the analytes were investigated under the present HPLC conditions. All analytes had a carboxylic acid and were essentially strongly ionized in the negative ion mode. The deprotonated molecule ($[M-H]^-$) was the most abundant ion as the base peak and the $[M+AcO]^-$ adduct ion was also observed with weak abundance in the ESI full scan spectra of all analytes in the negative ion mode. The $[M-H]^-$ ion of each derivative was therefore selected as the precursor ion for product ion spectrum. The product ion spectra of all analytes are shown in Fig. 2. According to the assignments shown in the mass spectra, both dominant product ions from TP-MA and 2-mpa-MA derivatives are formed by a bond cleavage between a sulfur atom and a methyl acrylate

moiety. The most abundant product ion from SA13 is produced by a cleavage at the amide bond in the molecule. The collision voltages were changed from 15 to 35 eV and the best settings giving the intense product ions were determined. The precursor and product ions chosen for the SRM detection and the best collision voltage settings are summarized in Table 1.

The chromatographic conditions were optimized in view of giving symmetrical peak shapes and achieving maximum peak responses, namely by not interfering with the ionization process of the analytes in the ESI source. The retention property of SA13 on a reverse-phase column was far different from those of TP-MA and 2-mpa-MA derivatives due to the presence of the additional moiety from MA. Therefore, a gradient elution using two mobile phases was attempted. The types of ions observed in the ESI source depend on the solutes and solvents which are present in the mobile phase. Particularly, modifiers to improve the LC separation properties and their concentrations significantly affect the formation of ion species and the signal responses observed. To achieve the symmetrical peak separation, the acidic modifiers should be added to the mobile phase due to a carboxylic acid in all the analyte molecules. Acetic acid was one of the most commonly used modifiers to give acidity in the LC-MS analysis, and did not cause inhibition of ion production in the previous study [18]. Therefore, the optimum concentration and the composition were investigated. As a result, mobile phase systems consisting of methanol-0.05 M acetic acid (15:85 and 7:3, v/v) were found to be optimal. The SRM chromatograms of TP-MA, 2-mpa-MA, SA13 and SA672 (IS) obtained from rat blood spiked with 20 ng/ml of each analyte are shown in Fig. 3.

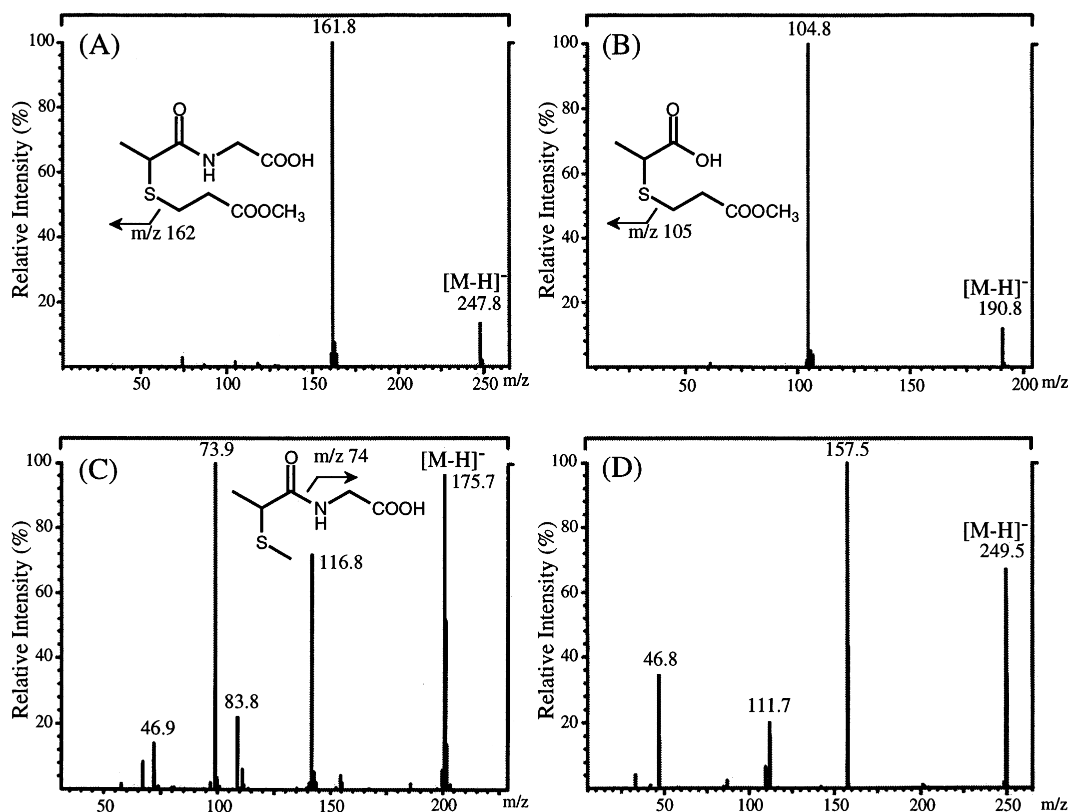

Fig. 2. Negative product ion spectra of TP-MA (A), 2-mpa-MA (B), SA13 (C) and SA672 (IS, D) with each deprotonated molecule ($[M-H]^-$) as a precursor ion.

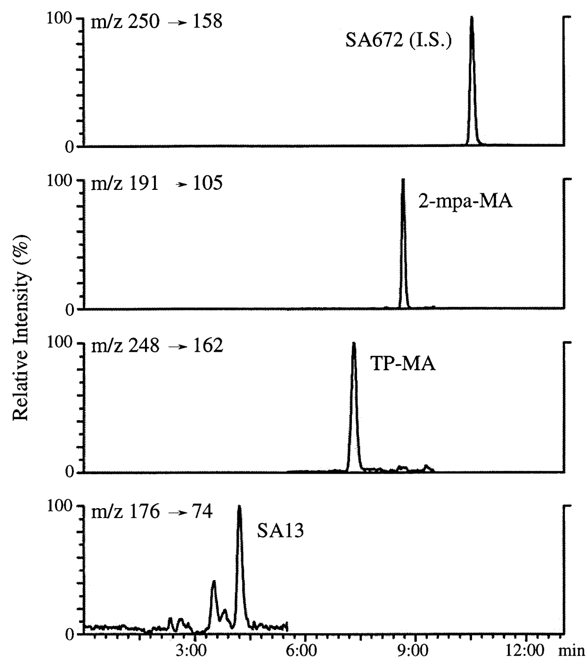

Fig. 3. Typical SRM chromatograms of TP-MA, 2-mpa-MA, SA13 and SA672 (IS) obtained from rat blood spiked with 20 ng/ml of each analyte.

3.2. Derivatization of thiol group

The quantitative reaction of TP with acrylate derivatives which have structurally diverse functional groups in Tris–HCl buffer (pH 9.1) has been demonstrated [18]. The reaction proceeded quickly due to the Michael addition reaction and was completed within 10 min at room temperature in basic aqueous solution such as Tris–HCl buffer. Among the acrylate derivatives which showed the quantitative reactivity, MA, isobutyl acrylate (IBA) and trifluoroethyl acrylate (TFEA) particularly enhanced the ionization response of the TP-derivative resulting from thiol stabilization in the negative ESI-MS. The response of $[M-H]^-$ ion of TP-TFEA showed the highest sensitivity followed by TP-IBA and TP-MA. However, MA was chosen in the present study for the following reasons: (1) to shorten the total analysis time, the chromatographic profile of corresponding derivatives of TP and 2-mpa which are produced by the reaction with acrylates should be close to SA13. The acrylate derivative used for the stabilization should be a small molecule and as hydrophilic as possible. MA was the most polar acrylate derivative among the candidates; and (2) the detection sensitivity of TP-MA and 2-mpa-MA in the ESI-MS was sufficient to evaluate the pharmacokinetics of TP and metabolites on rats.

The optimum concentration of MA to be added was determined from the relationship between the concentration added and the linearity of calibration of TP and 2-mpa in rat blood. Addition of a 0.1-ml aliquot of 7% MA in acetonitrile and reaction time of 30 min at room temperature gave excellent linearity in the calibration range of TP and 2-mpa.

3.3. Linearity of calibration

The calibration range was based on the concentration expected in the blood sample to be analyzed. The concentration range of 0.5–1000 ng/ml proved to be sufficient for the analysis in rat blood. Blank rat blood samples spiked with the corresponding compounds to give concentrations of 0.5, 2, 5, 20, 80, 250 and 1000 ng/ml for TP and 2-mpa, and 2, 5, 20, 80, 250 and 1000 ng/ml for SA13 were analyzed. For all analytes, excellent linearity was obtained in the specified concentration range. The correlation coefficients for the calibration regression line were 0.997 or greater. The equations of the calibration lines were as follows: $y = 0.00512 + 0.00627x$ for TP-MA, $y = 0.00048 + 0.00306x$ for 2-mpa-MA and $y = -0.00038 + 0.00071x$ for SA13.

3.4. Precision and accuracy

The intra-assay precision and accuracy for the assay method were determined by analyzing five replicates at 0.5, 2, 20 and 250 ng/ml for TP and 2-mpa, and 2, 20 and 250 ng/ml for SA13. The accuracy was determined by calculating the relative error (RE) and the precision was by calculating the coefficient of variation (CV). The intra-assay precision and accuracy data are summarized in Table 2. The RE of TP and 2-mpa at

0.5 ng/ml were 12.23 and −1.22%, respectively and that of SA13 at 2 ng/ml was 0.60%. The CV of TP and 2-mpa at 0.5 ng/ml were 16.75 and 7.75%, respectively and that of SA13 at 2 ng/ml was 5.47%. The limit of quantitation (LOQ) is defined as the lowest concentration that can be determined with an acceptable accuracy of ±20% and a precision below 20%. Therefore, the LOQs of TP, 2-mpa and SA13 were determined as 0.5, 0.5 and 2 ng/ml, respectively.

The intermediate precision and accuracy were studied by 13 replicates at 2, 20 and 250 ng/ml on each of 3 days. The precision and accuracy data are shown in Table 3. The accuracy for all analytes ranged from −6.74 to 4.32% of nominated concentrations with precision ranging from 2.87 to 12.35% over the concentrations evaluated.

3.5. Storage stability

The storage stability of TP-MA, 2-mpa-MA and SA13 in rat blood was evaluated at −80°C for 4 weeks using blood samples spiked with the compounds at blood concentrations of 5, 80 and 1000 ng/ml. The concentrations of these compounds in replicate samples were determined. The recoveries are summarized in Table 4. The recoveries ranged from 91.3 to 103.3% for all analytes, indicated that they were stable for at least 4 weeks in rat blood stored at −80°C.

Table 2
Intra-assay precision and accuracy for determination of TP and its metabolites in rat blood

Compound	Nominal concentration (ng/ml)	Mean measured concentration (ng/ml)	CV (%)	Accuracy (%)	*n*[a]
TP	0.48	0.42	16.75	12.23	5
	1.92	1.77	4.08	7.97	5
	19.20	21.08	3.30	−9.81	5
	240.0	241.0	9.65	−0.41	5
2-mpa	0.49	0.50	7.75	−1.22	5
	1.96	2.05	8.54	−4.67	5
	19.60	18.93	4.17	3.40	5
	245.0	244.4	2.23	0.26	5
SA13	2.02	2.01	5.47	0.60	5
	20.20	21.14	3.90	−4.66	5
	252.5	257.0	1.82	−1.78	5

[a] Number of analyses conducted.

Table 3
Intermediate precision and accuracy for determination of TP and its metabolites in rat blood

Compound	Nominal concentration (ng/ml)	Mean measured concentration (ng/ml)	CV (%)	Accuracy (%)	*n*[a]
TP	1.92	1.84	4.95	4.32	13
	19.20	18.51	12.35	3.57	13
	240.0	244.4	7.57	−1.83	13
2-mpa	1.96	2.07	5.97	−5.51	13
	19.60	20.92	11.62	−6.74	13
	245.0	242.2	6.53	1.13	13
SA13	2.02	1.96	7.23	3.08	13
	20.20	20.78	4.08	−2.85	13
	252.5	256.1	2.87	−1.43	13

[a] Number of analyses conducted over a period of 3 days.

Table 4
Stability data for TP and its metabolites stored at −80°C for 4 weeks in rat blood

Compound	Concentration level (ng/ml)	Recovery (mean ± S.D., %)[a]
TP	5	94.4 ± 4.4
	80	91.3 ± 2.3
	1000	100.5 ± 2.0
2-mpa	5	95.7 ± 6.1
	80	102.7 ± 2.2
	1000	96.4 ± 4.1
SA13	5	102.1 ± 4.6
	80	103.3 ± 2.9
	1000	99.6 ± 2.5

[a] Determined from five replicate analyses.

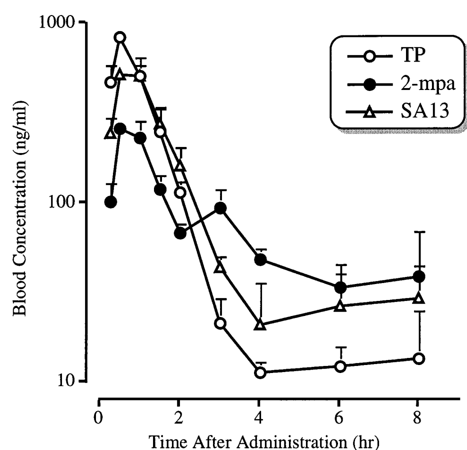

Fig. 4. Blood concentration-time curve of TP and its metabolites obtained after oral administration of TP (10 mg/kg) to female Lewis rats. Each point represents the mean + S.D. ($n = 3$).

3.6. *Blood concentration profiles of TP and its metabolites*

Blood concentration-time curves of TP and its metabolites, SA13 and 2-mpa, after oral administration of TP (10 mg/kg) to female rats are shown in Fig. 4. The concentration of TP peaked at 0.5 h and then decreased with a terminal half-life ($T_{1/2}$) of 1.10 h. The area under the curve (AUC_{0-8}) of TP was 971.8 ng/h per ml. The metabolites, SA13 and 2-mpa, immediately appeared in blood and peaked at 0.5–1.0 h. The $T_{1/2}$ and AUC_{0-8} were calculated as 0.93 h and 922.2 ng/h per ml for SA13 and 1.08 h and 617.6 ng/h per ml for 2-mpa, respectively.

4. Conclusion

A sensitive LC-ESI-MS-MS assay for TP and its metabolites, SA13 and 2-mpa, in rat blood has been developed utilizing MA as a stabilizing reagent for a thiol group and allows the simultaneous determination of the TP administered and all metabolites found in rat blood. The assay method provided sufficient sensitivity with accuracy and precision for all the analytes and was successfully applied to a pharmacokinetics study of TP in rat.

The quantitative derivatization of TP and 2-mpa into corresponding derivatives in rat blood was achieved under the derivatization conditions developed. When the same amount of the MA derivative and the intact compound was subjected to the analysis, the detection limits of TP-MA and 2-mpa-MA were far higher than that of SA13 and the unlabeled compounds in the present study. These findings suggest that MA has a rapid reactivity for a thiol group together with the enhancement of sensitivity on MS detection compared with the intact compound and should be a valuable reagent for the stabilization.

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