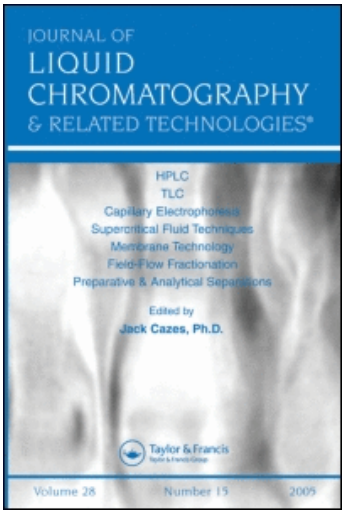

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Simultaneous Determination of the Novel Antithrombotic Agent, Acetylsalicylic Acid Maltol Ester (Aspalatone) and its Metabolites in Rat Plasma and Urine by HPLC

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Abstract: A rapid and sensitive reversed-phase high performance liquid chromatography (HPLC) method was developed for the determination of the novel antithrombotic agent acetylsalicylic acid maltol ester (aspalatone, CAS: 147249-33-0) and its four metabolites, salicylic acid maltol ester (SME), salicylic acid (SA), salicyluric acid (SUA), and gentisic acid (GA), in rat plasma and urine. After a treatment of plasma or urine sample by liquid-liquid extraction, the compounds were analyzed on an HPLC system with ultraviolet detection at 229 nm for aspalatone and SME, and at 313 nm for SA, SUA, and GA. HPLC analysis was carried out using reversed-phase isocratic elution with a C_{18} column (4.6 mm × 250 mm, 5 μm), a mobile phase of a mixture of butanol, acetic acid, doubly deionized water, and sodium sulfate (2 : 5 : 83 : 10, v/v/v/w %) at a flow rate of 1.0 mL/min. The chromatograms showed good resolution and sensitivity and no interference of plasma and urine. The calibration curves for all substances in both plasma and urine samples were linear over the concentration range of 0.05–200 μg/mL for both plasma and urine. The intra- and inter-day assay accuracies of this method were within 100 ± 11% of nominal values and the precision did not exceed 13% of relative standard deviation.

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The lower limits of quantitation were 50 ng/mL for aspalatone and its metabolites in plasma and urine, which were sensitive enough for pharmacokinetic studies.

Keywords: Acetylsalicylic acid maltol ester, (Aspalatone), Salicylic acid maltol ester, Salicylic acid, Salicyluric acid, Gentisic acid, HPLC

INTRODUCTION

The antithrombotic effect of acetylsalicylic acid (aspirin) has been a topic of considerable interest for a number of years.[1,2] Aspirin prevents thrombosis at low doses (100–300 mg daily), primarily due to its inhibitory effect on platelet cyclooxygenase.[2] Additionally, aspirin induces gastric mucosal ulceration and gastric bleeding.[3–7] In an attempt to alleviate this adverse effect, prodrugs of aspirin with low ulcerogenic activity have been developed.[8,9] However, detailed in vivo metabolic studies of the prodrugs have not been conducted, and their antiplatelet activity is unknown. It is suggested that antioxidant agents may inhibit arterial thrombosis by scavenging lipid peroxides.[10,11] Recently, a novel antithrombotic compound, acetylsalicylic acid maltol ester (aspalatone, CAS: 147249-33-0), was synthesized by esterification of aspirin and 3-hydroxy-2-metyl-Γ-pyrone (maltol) (Figure 1).[12,13] Maltol was first isolated as an active antioxidant component from Korean red ginseng.[10]

Prolongation of bleeding times by aspalatone is superior to aspirin.[14] In addition, aspalatone caused negligible gastric mucosal damage, compared to the ulcerogenic effects of aspirin.[14] These results suggest that aspalatone might be a potential antithrombotic agent with low ulcerogenicity. Aspalatone is rapidly deacetylated to salicylic acid maltol ester (SME), which, in turn,

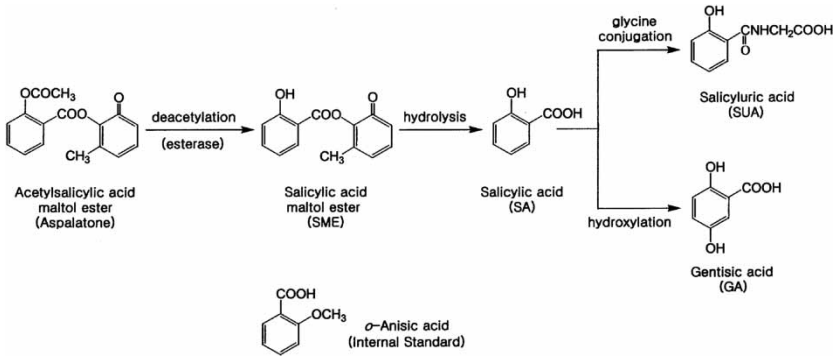

Figure 1. Structural formula of acetylsalicylic acid maltol ester (aspalatone) and internal standard (*o*-anisic acid). Aspalatone is rapidly metabolized to salicylic acid maltol ester (SME), which is, in turn, metabolized to salicylic acid (SA). SA is further metabolized to salicyluric acid (SUA) or gentisic acid (GA).

rapidly hydrolyzed to salicylic acid (SA), both in vivo and in vitro (Figure 1).[15] Orally administered aspirin is primarily hydrolyzed during its first passage through the liver, while metabolism in the gut wall is negligible.[15] The catabolism of aspalatone also occurs primarily in both its first passage through the liver and the blood circulation.[16]

Aspalatone, the maltol ester of aspirin, is currently being evaluated as a possible substitute for ulcerogenic aspirin as an antithrombotic agent. However, a thorough pharmacokinetic analysis of aspalatone has not yet been reported. Consequently, we have developed and validated a high performance liquid chromatography (HPLC) based method in order to analyze aspalatone and its metabolites in biological samples. We have also investigated the pharmacokinetic characteristics of aspalatone after both intravenous (i.v.) bolus and oral administration to rats.

EXPERIMENTAL

Chemicals and Reagents

Acetylsalicylic acid maltol ester (aspalatone), salicylic acid maltol ester (SME), salicylic acid (SA), salicyluric acid (SUA), and gentisic acid (GA) were obtained from Bukwang Pharmaceutical Co. (Kyongki, Korea). *o*-Anisic acid used as an internal standard was obtained from Sigma Chemical Co. (St Louis, MO, USA). Solvents used in the HPLC analysis were of HPLC grade and were filtered and degassed immediately prior to use. All other chemicals used in this study were of analytical reagent grade.

Animals

Animals were handled in accordance with the guidelines of the Korea Food & Drug Administration (KFDA). The experimental and surgical protocols were approved by the Chungbuk National University Animal Welfare Committee. Adult male Sprague Dawley rats (230–250 g; Sam Tac Co. Ltd., Kyunggi, Korea) were used for the pharmacokinetic studies. They were housed in individual metabolic cages during and after administration of the drug. Prior to oral dosing, the rats were fasted overnight, as well as for 4 h after the drug was administered. The animals were maintained under a 12 h light/dark cycle with free access to water at all times.

Instruments

Aspalatone, SME, SA, SUA, and GA levels were assayed by reverse phase HPLC on an Capcell Pak C_{18} column (4.6 mm × 250 mm, 5 μm,

Model: UG120, Shiseido Co., Tokyo, Japan) that was interfaced with a Jasco HPLC system consisting of a model PU-980 pump, a model AS-950-10 auto-injector, an UV-VIS detector, and an LC-Net II control borwin integrator (Jasco Co. Ltd., Tokyo, Japan). The mobile phase was a mixture of butanol, acetic acid, doubly deionized water, and sodium sulfate (2 : 5 : 83 : 10, v/v/v/w%). The flow rate was 1 mL/min. We used two sets of UV-VIS detectors for determination of aspalatone and its 4 metabolites. The detection wavelength of the detector was set at 229 nm for aspalatone and SME and 313 nm for SA, SUA, and GA.

Standard Solutions and Quality Control Samples

Stock solutions were prepared by dissolving aspalatone and its metabolites, SME, SA, SUA, and GA in methanol (5 mg/mL) and then stored at 4°C. These stock solutions were mixed and further diluted with methanol to prepare working standard solutions at the following seven concentrations: 0.05, 0.1, 1, 5, 10, 50, 100, and 200 μg/mL. The internal standard was also dissolved in methanol (1 mg/mL) and the solution stored at 4°C (stock solution). The stock solution of internal standard was then diluted with methanol to the final concentration of 100 μg/mL (working solution).

Standard curves for aspalatone and its metabolites were obtained at the following seven concentrations: 0.05, 0.1, 1, 5, 10, 50, 100, and 200 μg/mL. Quality control (QC) samples were prepared at the following concentration 0.05, 1, 10, and 200 μg/mL. To produce the range of concentrations described above, 0.1 mL of each working standard solution (0.5–2000 μg/mL) in a disposable glass culture tube (16 × 125 mm, Iwaki Glass, Tokyo, Japan) was evaporated, and the residue was dissolved in 0.1 mL of blank plasma or urine.

Sample Preparation

To 0.1 mL of plasma sample in a disposable glass culture tube, 20 μL phosphoric acid and *o*-anisic acid (internal standard, 50 μL of 100 μg/mL) were added. Ethyl ether (5 mL) was then added to precipitate the proteins and to extract the compounds of interest. These mixtures were vortexed for 15 min and then centrifuged for 15 min at 1500 g. The organic layer (upper phase) was transferred into a clean test tube. The organic layer was dried under a stream of dry nitrogen gas, and reconstituted in 150 μL mobile phase. A 100 μL of the supernatant was then injected onto the HPLC system. The levels of aspalatone and its metabolites in the urine were determined by the same procedure as described above.

Method Validation

Specificity

The interference of endogenous compounds was assessed by analyzing standard aspalatone and its metabolites, drug-free plasma and urine samples, plasma and urine spiked with aspalatone and its metabolites, and serum and urine samples obtained from rats given aspalatone.

Sensitivity

The lower limit of quantitation (LOQ) was defined as the lowest concentration yielding a precision of less than 20% (coefficient of variation, CV) and an accuracy between 80 and 120% of the theoretical value. It was determined at 0.05 μg/mL for both plasma and urine in five replicate samples.

Linearity

The linearity of the calibration curve was assessed by preparing plasma or urine samples containing aspalatone, SME, SA, SUA, and GA at concentrations ranging from 0.05 to 200 μg/mL. The peak area ratios of the compounds to internal standard were fit to straight lines by linear regression. The equations were treated statistically (weighting factor: 1/concentration) and are presented with their correlation coefficients.

Precision and Accuracy

In order to assess the intra- and inter-day precision and accuracy of the assay, QC samples were prepared as described above. The intra-day precision of the assay was assessed by calculating the coefficients of variation for the analysis of QC samples in five replicates, and inter-day precision was determined through the analysis of QC samples on five consecutive days. Accuracy was determined by comparing the calculated concentration using calibration curves to known concentration.

Recovery

The recoveries from plasma or urine were assessed by comparison of the peak area from extracted QC samples to the area of standard corresponding to respective concentrations, followed by application of a correction factor. The mean recoveries were determined at four concentrations of QC samples in five replicates.

Stability

To test the short- and long-term stability of aspalatone and its metabolites, four QC samples of plasma or urine were stored under different conditions: at 4°C for 0, 1, 2, 4, 12, and 24 h; at 0°C for 0, 1, 2, 4, 12, and 24 h; at −20°C for 1 month; at −70°C for 1 month.

Pharmacokinetic Studies

Under light sodium pentobarbital anesthesia, the femoral vein and artery were cannulated with PE-50 polyethylene tubing (Intramedic, Clay Adams, U.S.A.) for aspalatone administration and blood sampling, respectively. Aspalatone, suspended in 0.25 mL of 0.5% carboxymethylcellulose (CMC) solution, was administered at a dose of 80 mg/kg into the femoral vein or orally at a dose of 160 mg/kg. After i.v. bolus or oral dose, blood (200 μL) was collected into heparinized tubes containing 5% KF and 3% citrate solution (20 μL) from the femoral artery at 10 sec, 30 sec, 50 sec, 70 sec, 1.5 min, 2 min, 5 min, 15 min, 30 min, 1 h, 2 h, and 3 h for the determination of aspalatone and SME, and at 0.5, 1, 2, 3, 6, 9, 12, 18, 24, 36, 48 for the determination of SA, SUA, and GA, respectively. Blood samples were centrifuged for 15 min at 1500 g and the plasma (100 μL) was harvested. Urine was collected by the use of metabolic cages over a 48 h period following administration of aspalatone and stored at −70°C until analysis. The levels of aspalatone, SME, SA, SUA, and GA in the plasma and urine were determined as described above.

Pharmacokinetic parameters were calculated by non-compartmental analysis of plasma concentration–time curve data following i.v. bolus or oral administration.[17] The peak concentration (C_{max}) and the time to reach C_{max} (t_{max}) were determined by the inspection from individual serum concentration–time profiles for aspalatone or its metabolites. The area under the plasma concentration–time curve from time zero to infinity (AUC) was calculated from the equation $AUC = AUC_t + C_t/k$, where C_t is the last quantifiable concentration and k was calculated from the slope of a straight-line in the terminal phase of plasma disappearance. The area under the plasma concentration–time curve from time zero to the time of the last quantifiable concentration (AUC_t) was calculated by linear trapezoidal approximation. The following parameters were calculated using standard methods: the mean residence time, (MRT) = AUMC/AUC, where AUMC represents the area under the moment curve; the bioavailability (BA %) value for the oral dose was calculated from the dose-adjusted ratio of AUC_{oral} to $AUC_{i.v.}$.

Statistical Analysis

The unpaired Student's t-test was used to compare two groups. One-way analysis of variance was used to test for significant differences between multiple groups. Statistical significance was defined as $P < 0.05$.

RESULTS AND DISCUSSION

Specificity

Typical chromatograms of aspalatone, salicylic acid maltol ester (SME), salicylic acid (SA), salicyluric acd (SUA), gentisic acid (GA), and the internal standard (IS) in plasma or urine are shown in Figure 2. Control

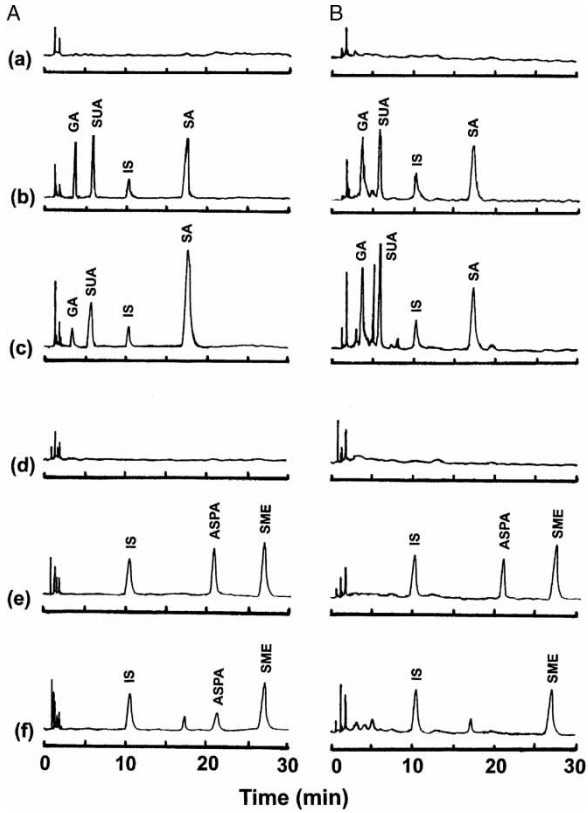

Figure 2. Representative chromatograms of plasma (A) and urine samples (B). The detection wavelength of the detector was set at 313 nm for gentisic acid (GA), salicyluric acid (SUA), and salicylic acid (SA) (a, b, c), and 229 nm for aspalatone and salicylic acid maltol ester (SME) (d, e, f). (a) blank plasma and urine; (b) blank plasma and urine spiked with 5 μg/mL of GA, SUA, and SA containing 5 μg/mL internal standard (IS); (c) plasma sample obtained 30 min after i.v. bolus administration of 80 mg/kg aspalatone, and urine sample obtained 6 h after i.v. bolus administration of 80 mg/kg aspalatone; (d) blank plasma and urine; (e) blank plasma and urine spiked with 5 μg/mL of aspalatone and SME containing 5 μg/mL internal standard (IS); (f) plasma sample obtained 30 sec after i.v. bolus administration of 80 mg/kg aspalatone, and urine sample obtained 2 h after i.v. bolus administration of 80 mg/kg aspalatone.

plasma or urine samples taken prior to drug treatment show that there are no peaks that interfere with aspalatone, its metabolites or IS signals. The retention times of GA, SUA, SA, aspalatone, and SME were 3.6 ± 0.27, 5.1 ± 0.56, 17.6 ± 1.63, 21.7 ± 1.24, and 27.3 ± 1.29 min (mean ± S.D., n = 5), respectively. The retention times of IS were 10.2 ± 0.64 min for determining GA, SUA, and SA at 313 nm, and 10.7 ± 1.16 min for determining aspalatone and SME at 229 nm (mean ± S.D., n = 5). The total run time was within 30 min. The isocratic elution with mobile phase afforded good separation of aspalatone, its metabolites and IS from endogeneous constituents in the plasma or urine.

Sensitivity

The limit of detection (LOD) of aspalatone or its metabolites was determined to be 25 ng/mL, as defined by the concentration of the analyte giving a signal to noise ratio of 3 : 1. The limit of quantitation (LOQ) of aspalatone or its metabolites was defined as those that could be estimated with an intra- and inter-day precision less than 20% (CV), and accuracy between ±20% (bias). The LOQ was found to be 50 ng/mL for both plasma and urine samples, as defined by the lowest concentration in linear range that can be detected with variation less than 12.5%. The relative standard deviation (RSD) of five replicate determinations was in the range of 8.18–11.65% for plasma or 7.85–12.42% for urine (Tables 1, 2).

Linearity

To determine the linearity of the HPLC method, quality control samples were prepared for aspalatone, SME, SA, SUA, and GA. Seven concentrations ranging from 0.05 to 200 μg/mL were used for the quality control samples and five identical sets of samples were prepared for each drug. These samples were assayed on the day of preparation and on the following five consecutive days, generating 10 calibration curves for each compound. Mean regression equations were calculated from the calibration curves. For the samples prepared in plasma, the mean regression equations for aspalatone, SME, SA, SUA, and GA were $y = 0.0158x + 0.00135$ ($r^2 = 0.992$), $y = 0.0112x + 0.00306$ ($r^2 = 0.997$), $y = 0.0110x + 0.00132$ ($r^2 = 0.994$), $y = 0.0219x + 0.00205$ ($r^2 = 0.997$), and $y = 0.0105x + 0.00149$ ($r^2 = 0.992$), respectively, where y is the peak area ratio of each compound to IS and x is the concentration. The mean regression equations for aspalatone, SME, SA, SUA, and GA in urine were $y = 0.0156x + 0.00172$ ($r^2 = 0.993$), $y = 0.0125x + 0.00285$ ($r^2 = 0.994$), $y = 0.0128x + 0.00107$ ($r^2 = 0.992$), $y = 0.0226x + 0.00183$ ($r^2 = 0.995$), and $y = 0.0109x + 0.00121$

Table 1. Precision and accuracy of HPLC analysis of aspalatone, salicylic acid maltol ester (SME), salicylic acid (SA), salicyluric acid (SUA), and gentisic acid (GA) in rat plasma

		Intra-day (n = 5)			Inter-day (n = 5)		
Compounds	Nominal concentration (μg/mL)	Determined concentration (mean ± S.D.)	Precision (% RSD)	Accuracy (%)	Determined concentration (mean ± S.D.)	Precision (% RSD)	Accuracy (%)
Aspalatone	0.05	0.056 ± 0.0048	8.57	111.96	0.054 ± 0.0052	9.63	108.83
	1	0.098 ± 0.0060	6.11	97.56	0.098 ± 0.0055	5.62	97.71
	10	10.22 ± 0.386	3.78	102.20	10.10 ± 0.6128	6.07	100.96
	200	200.9 ± 4.760	2.37	100.43	200.4 ± 5.311	2.65	100.21
SME	0.05	0.055 ± 0.0048	8.73	109.61	0.047 ± 0.0039	8.45	93.23
	1	0.096 ± 0.0072	7.58	95.58	0.103 ± 0.0076	7.37	103.18
	10	10.43 ± 0.388	3.72	104.31	10.70 ± 0.4845	4.53	106.96
	200	196.2 ± 4.258	2.17	98.11	200.3 ± 4.748	2.37	100.16
SA	0.05	0.054 ± 0.0050	9.14	108.69	0.054 ± 0.0052	9.61	107.73
	1	0.105 ± 0.0060	5.65	105.42	0.097 ± 0.0091	9.36	97.08
	10	9.575 ± 0.355	3.71	95.75	10.38 ± 0.7329	7.06	103.81
	200	197.2 ± 7.453	3.78	98.59	198.8 ± 10.852	5.46	99.38
SUA	0.05	0.045 ± 0.0053	11.65	90.87	0.053 ± 0.0049	9.3	106.15
	1	0.104 ± 0.0081	7.86	103.56	0.093 ± 0.0070	7.52	93.09
	10	10.13 ± 0.462	4.56	101.29	9.489 ± 0.5655	5.96	94.89
	200	200.2 ± 7.847	3.92	100.09	200.1 ± 8.242	4.12	100.03
GA	0.05	0.054 ± 0.0044	8.18	108.31	0.054 ± 0.0045	8.42	107.51
	1	0.107 ± 0.0074	6.89	106.91	0.110 ± 0.0057	5.14	109.93
	10	9.661 ± 0.385	3.98	96.61	10.56 ± 0.5165	4.89	105.62
	200	205.8 ± 4.281	2.08	102.91	199.4 ± 2.472	1.24	99.69

Table 2. Precision and accuracy of HPLC analysis of aspalatone, salicylic acid maltol ester (SME), salicylic acid (SA), salicyluric acid (SUA), and gentisic acid (GA) in rat urine

		Intra-day (n = 5)			Inter-day (n = 5)		
Compounds	Nominal concentration (μg/mL)	Determined concentration (mean ± S.D.)	Precision (% RSD)	Accuracy (%)	Determined concentration (mean ± S.D.)	Precision (% RSD)	Accuracy (%)
Aspalatone	0.05	0.046 ± 0.0057	12.42	92.21	0.045 ± 0.0040	8.87	89.53
	1	0.093 ± 0.0081	8.75	93.11	0.108 ± 0.0105	9.67	108.34
	10	10.17 ± 0.492	4.84	101.69	9.362 ± 0.6048	6.46	93.62
	200	199.5 ± 10.992	5.51	99.75	190.6 ± 7.356	3.86	95.29
SME	0.05	0.046 ± 0.0054	11.67	91.69	0.046 ± 0.0036	7.78	91.59
	1	0.093 ± 0.0070	7.58	92.91	0.092 ± 0.0079	8.59	92.22
	10	10.33 ± 0.642	6.21	103.32	10.24 ± 0.6787	6.63	102.37
	200	204.4 ± 4.763	2.33	102.21	202.7 ± 5.413	2.67	101.36
SA	0.05	0.046 ± 0.0044	9.62	92.21	0.054 ± 0.0047	8.77	108.06
	1	0.093 ± 0.0081	8.75	93.11	0.092 ± 0.0060	6.46	92.13
	10	10.07 ± 0.487	4.84	100.67	10.45 ± 0.5843	5.59	104.53
	200	203.4 ± 4.698	2.31	101.69	198.4 ± 4.741	2.39	99.18
SUA	0.05	0.054 ± 0.0042	7.85	108.21	0.055 ± 0.0052	9.56	109.42
	1	0.094 ± 0.0079	8.44	93.76	0.108 ± 0.0084	7.78	108.31
	10	10.14 ± 0.539	5.32	101.37	9.218 ± 0.3398	3.69	92.08
	200	201.5 ± 6.570	3.26	100.76	199.6 ± 3.733	1.87	99.82
GA	0.05	0.047 ± 0.0045	9.57	93.46	0.047 ± 0.0037	7.86	93.13
	1	0.096 ± 0.0078	8.11	95.82	0.095 ± 0.0057	5.95	95.34
	10	10.24 ± 0.493	4.81	102.44	9.821 ± 0.3594	3.66	98.21
	200	203.4 ± 6.815	3.35	101.71	204.5 ± 10.142	4.96	102.24

($r^2 = 0.998$), respectively. These equations show that there is significant linearity ($P < 0.005$) over the concentration range of 0.05 to 200 μg/mL.

Precision and Accuracy

The intra- and inter-day precision and accuracy of aspalatone, SME, SA, SUA, and GA are summarized in Tables 1 and 2. The intra-day accuracies for plasma and urine samples were 90.9–112% and 91.7–108.2% at QC samples with the precision (CV) less than 11.65 and 12.42%, respectively. The inter-day accuracies for plasma and urine samples ranged from 93.1 to 109.9% and 89.5 to 109.4% at QC samples with the precision (CV) less than 9.63 and 9.67%, respectively. These results indicated that the present method has a satisfactory accuracy, precision, and reproducibility for the determination of aspalatone, SME, SA, SUA, and GA in both plasma and urine samples.

Recovery

The extraction recoveries of aspalatone, SME, SA, SUA, and GA were determined at four concentrations of QC samples in five replicates (Table 3). The mean absolute recoveries of aspalatone or its metabolites were found to be more than 87.6% for plasma, and more than 88.2% for urine. The simple liquid–liquid extraction method has been successfully applied to the extraction of aspalatone or its metabolites from plasma or urine samples.

Stability

Plasma and urine samples were stored at −70°C until analysis. After thawing the plasma or urine, the samples were kept in ice (approximately 0°C) until being extracted with organic solvent. Four QC samples of aspalatone, its metabolites, and IS were stable at both 4°C and 0°C with quantitation variation less than 8.1% and 5.7% during 24 h, respectively. It was confirmed that these drugs would not decompose in organic solvent at 4°C during the HPLC assay. They were also stable at −70°C during 1 month in plasma and urine (CV was less than 6.3%). Finally, the storage of stock solutions at 4°C during 6 h produced no significant decrease of each aspalatone, its metabolites, and IS in peak areas.

Pharmacokinetics and Urinary Excretion of Aspalatone and its Metabolites

The concentrations of aspalatone, SME, SA, SUA, and GA in plasma over time after i.v. bolus (80 mg/kg) or oral administration (160 mg/kg) of aspalatone are shown in Figure 3. We could not detect aspalatone levels in the

Table 3. Recoveries of aspalatone, salicylic acid maltol ester (SME), salicylic acid (SA), salicyluric acid (SUA), and gentisic acid (GA) in rat plasma and urine[a]

	Concentration (μg/mL)	Aspalatone	SME	SA	SUA	GA
Plasma	0.05	92.3 ± 5.8	93.1 ± 3.8	90.4 ± 6.2	93.5 ± 3.2	92.1 ± 6.6
	1	89.7 ± 6.8	92.8 ± 5.2	93.1 ± 5.3	93.7 ± 6.4	89.1 ± 5.1
	10	91.4 ± 4.2	93.4 ± 6.7	89.4 ± 4.8	92.4 ± 6.1	93.4 ± 5.9
	200	88.5 ± 5.4	90.3 ± 3.3	87.6 ± 6.8	93.3 ± 5.4	88.6 ± 4.1
Urine	0.05	93.5 ± 6.4	92.7 ± 4.1	89.6 ± 5.2	91.6 ± 4.8	91.7 ± 7.3
	1	92.4 ± 4.7	91.5 ± 4.8	91.5 ± 4.8	92.7 ± 6.1	92.8 ± 5.9
	10	89.6 ± 6.3	93.7 ± 3.7	93.7 ± 4.2	90.6 ± 5.7	88.6 ± 3.6
	200	88.4 ± 5.6	91.6 ± 6.7	88.2 ± 3.3	91.2 ± 3.4	90.1 ± 3.3

[a]%, Mean ± S.D., n = 5.

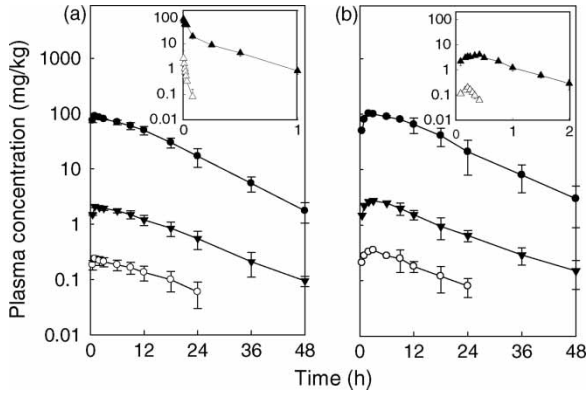

Figure 3. Aspalatone (□), salicylic acid maltol ester (SME) (▲), salicylic acid (SA) (●), salicyluric acid (SUA) (▼), and gentisic acid (GA) (○) concentrations in rat plasma over time after i.v. bolus administration of 80 mg/kg aspalatone (A) or oral administration of 160 mg/kg aspalatone (B). Each point represents the mean ± S.E. of three rats.

plasma after i.v. bolus (8 mg/kg) or oral administration (16 mg/kg). Thus, the high doses of aspalatone in the pre-clinical studies were administered for simultaneous determination of aspalatone and its metabolites. Aspalatone was rapidly metabolized to SME, and, as a result, the plasma concentration of aspalatone decreased below the quantifiable limit within 5 min for i.v. bolus and 30 min for oral administration. SME was metabolized to SA, and, thus, also disappeared rapidly from the plasma within 1–2 h. SA, SUA, and GA showed a much slower plasma disappearance rate. The pharmacokinetic parameters of aspalatone, SME, SA, SUA, and GA after i.v. bolus or oral administration of aspalatone are summarized in Table 4. The mean plasma half-lives ($t_{1/2}$) of aspalatone, SME, SA, SUA, and GA following i.v. bolus administration were 0.01, 0.2, 7.4, 9.6, and 11.2 h, respectively. The $t_{1/2}$ values of aspalatone and SME for oral dose were about 12 and 2 fold longer than the values for i.v. bolus dose. However, the $t_{1/2}$ and MRT of SA, SUA, and GA did not significantly differ between i.v. bolus and oral routes. The bioavailability of orally administered aspalatone, calculated from the dose-adjusted ratios of the AUC_{oral} relative to the $AUC_{i.v.}$, was about 66%.

The cumulative amounts of SME, SA, SUA, or SA that are excreted into the urine after i.v. bolus (80 mg/kg) or oral administration (160 mg/kg) of aspalatone are shown in Figure 4. The majority of SME was excreted to the urine 3–4 h after the administration. In contrast, the excretion of SA, SUA, GA to the urine was linear up to 24 h after either i.v. bolus or oral administration. After absorption and distribution, the majority of SA is eliminated by urinary excretion and by formation of SUA and GA.[18,19] Virtually, an entire dose of aspirin can be accounted for in urine by recovery of SA,

Table 4. Pharmacokinetic parameters of aspalatone, salicylic acid maltol ester (SME), salicylic acid (SA), salicyluric acid (SUA), and gentisic acid (GA) after i.v. bolus administration (80 mg/kg) or oral administration (160 mg/kg) of aspalatone to rats[a]

Dose (mg/kg)	Parameters	Aspalatone	SME	SA	SUA	GA
i.v. (80)	$t_{1/2}$ (h)	0.0101 ± 0.0016	0.208 ± 0.0069	7.42 ± 0.729	9.63 ± 0.824	11.2 ± 1.69
	C_{max} ($\mu g \cdot mL^{-1}$)	—	—	91.2 ± 8.62	2.13 ± 0.0914	0.238 ± 0.0312
	t_{max} (h)	—	—	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
	AUC ($\mu g \cdot h \cdot mL^{-1}$)	0.0468 ± 0.0005	8.87 ± 0.440	1390 ± 147	37.7 ± 4.01	4.34 ± 0.301
	MRT (h)	0.0195 ± 0.0036	0.230 ± 0.0951	12.4 ± 2.17	14.9 ± 1.47	16.3 ± 2.53
Oral (160)	$t_{1/2}$ (h)	0.122 ± 0.0211[b]	0.435 ± 0.0746[b]	8.01 ± 0.956	10.7 ± 0.673	9.64 ± 0.925
	C_{max} ($\mu g \cdot mL^{-1}$)	0.199 ± 0.0193	3.99 ± 0.447	102 ± 5.04	2.75 ± 0.386	0.357 ± 0.0436
	t_{max} (h)	0.208 ± 0.00	0.417 ± 0.00	2.00 ± 0.00	3.00 ± 0.00	3.00 ± 0.00
	AUC ($\mu g \cdot h \cdot mL^{-1}$)	0.0620 ± 0.0064	3.43 ± 0.684	1760 ± 201	49.3 ± 4.14	5.85 ± 0.471
	MRT (h)	0.276 ± 0.0297[b]	0.741 ± 0.0093[b]	13.3 ± 1.57	15.6 ± 3.82	14.6 ± 2.72

[a]Mean ± S.E. of three rats.
[b]Significantly different from the i.v. bolus dose ($P < 0.01$).

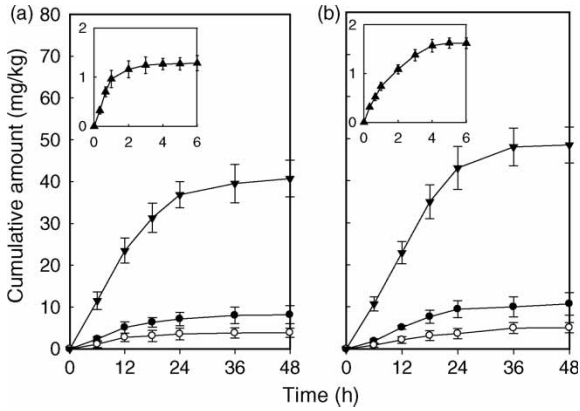

Figure 4. Cumulative urinary excretion of salicylic acid maltol ester (SME) (▲), salicyluric acid (SUA) (▼), salicylic acid (SA) (●), and gentisic acid (GA) (○) after i.v. bolus administration of 80 mg/kg aspalatone (A) or oral administration of 160 mg/kg aspalatone (B). Each point represents the mean ± S.E. of three rats.

SUA, and GA.[18,20] In the present study, SA and its further metabolites, SUA and GA, were excreted into urine up to 48 h after i.v. bolus or oral administration of asplatone (Figure 4). After i.v. bolus administration of 80 mg/kg asplatone, 94.8% of a dose of aspalatone was recovered in the form of SUA (67.8%), SA (19.2%), and GA (7.8%) excreted after 48 h into urine, calculated from the molecular weight-adjusted amounts.

CONCLUSIONS

A rapid and accurate validated HPLC based method was developed for the determination of aspalatone and its four metabolites, SME, SA, SUA, and GA in rat plasma and urine. It uses a simple solvent extraction method and chromatography on a C_{18} column with two sets of UV-VIS detectors. The HPLC based method developed and validated in this study was extremely useful for pharmacokinetic studies of aspalatone and its metabolites, such as SME, SA, SUA, and GA, following i.v. bolus or oral administration of aspalatone. It has been currently used in this laboratory for a number of years for investigating the pharmacokinetic characteristics of aspalatone in pre-clinical and clinical studies, and these results will be presented elsewhere.

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