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An isocratic HPLC method for the simultaneous determination of novel stable lipophilic ascorbic acid derivatives and their metabolites

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Abstract

2-*O*-α-D-glucopyranosyl-6-*O*-hexadecanoyl-L-ascorbic acid (6-sPalm-AA-2G), a novel stable lipophilic ascorbic acid derivative, was hydrolyzed to 2-*O*-α-D-glucopyranosyl-L-ascorbic acid (AA-2G), ascorbyl 6-palmitate (6-sPalm-AA) and ascorbic acid (AA) with α-glucosidase and lipase. An HPLC method for the simultaneous determination of AA, AA-2G, 6-sPalm-AA and 6-sPalm-AA-2G was developed using a cyanopropyl column with an isocratic solution of methanol–phosphate buffer (pH 2.1) (65:35, v/v) containing 20 mg/l of dithiothreitol at a detection wavelength of 240 nm. The calibration curves were found to be linear in the range of 10–200 μM. Linear regression analysis of the data demonstrated the efficacy of the method in terms of precision and accuracy. This method was satisfactorily applied to the determination of 6-sPalm-AA-2G and its three metabolites in a 6-sPalm-AA-2G solution treated with purified enzymes or a small intestine post-mitochondrial supernatant and to the separation of novel stable lipophilic AA derivatives other than 6-sPalm-AA-2G and their metabolites. AA, AA-2G and other well-known stable AA derivatives, ascorbic acid 2-phosphate and ascorbic acid 2-sulfate, were also separated under the same conditions. The results show that the procedure is rapid and simple and that it can be employed for in vitro metabolic analysis of various AA derivatives.

Keywords: Novel stable lipophilic ascorbic acid derivative (6-acyl-AA-2G); AA-2G; Ascorbic acid; Ascorbyl 6-palmitate; Cyano column; HPLC

1. Introduction

2-*O*-α-D-glucopyranosyl-L-ascorbic acid (AA-2G), a stable ascorbate derivative developed in our laboratory [1–3], has been approved by the Japanese Government as a quasi-drug principal ingredient in skin care and as a food additive and is currently being used as a medical additive in commercial cosmetics. After enzymatic hydrolysis to ascorbic acid (AA) by α-glucosidase, AA-2G exhibits Vitamin C activity in vitro and in vivo [4–6]. Recently, we have synthesized two types of monoacylated derivatives of AA-2G, 6-*O*-acyl-2-*O*-α-D-glucopyranosyl-L-ascorbic acids having a straight-acyl chain (6-sAcyl-AA-2G) and a branched-acyl chain (6-bAcyl-AA-2G), in order to improve the bioavailability of AA-2G [7–10]. 6-sAcyl- and 6-bAcyl-AA-2G had radical scavenging activity per se [11–13], and the derivatives with an appropriate length of the acyl chain group exhibited skin permeability superior to that of AA-2G and AA [7,10]. 6-sAcyl- and 6-bAcyl-AA-2G also showed satisfactory bioavailability as an AA supplement in rats and guinea pigs compared with AA-2G [14,15]. 6-sAcyl-AA-2G synergistically enhanced both dibutyryl cyclic AMP- and nerve growth factor-induced neurite outgrowth in PC12 cells, and the synergistic effect was stronger than that of AA-2G and AA [16].

6-sAcyl-AA-2G was hydrolyzed by liver, kidney and small intestine post-mitochondrial supernatants from guinea pigs to give two hydrolysates, AA-2G and AA, while 6-bAcyl-AA-2G was hydrolyzed by these post-mitochondrial supernatants to give three hydrolysates, 6-*O*-acyl AA, AA-2G and AA [15]. The releasing pattern of these hydrolysates suggested that 6-sAcyl-AA-2G was metabolized via AA-2G to AA and that 6-bAcyl-AA-2G was metabolized via 6-*O*-acyl AA to AA. A similar metabolic difference between 6-sAcyl-AA-2G and 6-bAcyl-AA-2G was observed in a human living skin equivalent model [10]. In these experiments, the novel lipophilic AA derivatives and their metabolites were analyzed by HPLC using both ODS and phenyl columns. Separation of water-soluble AA and AA-2G was achieved by isocratic elution from an ODS column with a phosphate–phosphoric acid buffer (pH 2.1). Separation

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of 6-*O*-acyl AA, 6-sAcyl-AA-2G and 6-bAcyl-AA-2G was carried out by isocratic elution from a phenyl column with an MeOH–H_2O solution containing 1% acetic acid, since these compounds with an acyl group showed an excess affinity to the ODS stationary phase and thereby were not eluted in an experimentally acceptable time. It is therefore necessary to develop more effective HPLC method for in vitro metabolic analysis of 6-sAcyl- and 6-bAcyl-AA-2G.

The aim of the present study was to develop an HPLC method for the simultaneous determination of these AA derivatives and their metabolites, 6-*O*-acyl AA, AA-2G and AA in α-glucosidase and/or lipase solutions and in a post-mitochondrial supernatant from rat small intestine. Recently, simultaneous analyses of drugs and their metabolites using cyano columns have been reported [17–20]. Sottofattori et al. [21] reported that ascorbic acid 2-phosphate, imidazolidinylurea, a mixture of parabens and ascorbyl palmitate in a commercial cosmetic cream were simultaneously separated by a cyano-propyl column using a methanol gradient. However, to our knowledge, an HPLC method for the simultaneous determination of a lipophilic AA derivative and AA as its hydrophilic metabolite under isocratic conditions has not been reported. In this study, we developed a rapid and simple HPLC isocratic method for the simultaneous determination. We also examined whether this method can be applied to the separation of AA, AA-2G and other well-known stable AA derivatives, ascorbic acid 2-phosphate and ascorbic acid 2-sulfate.

2. Experimental

2.1. Chemicals

2-*O*-α-D-glucopyranosyl-L-ascorbic acid (AA-2G) was a gift from Hayashibara Biochemical Laboratories (Okayama, Japan). 6-*O*-Dodecanoyl-2-*O*-α-D-glucopyranosyl-L-ascorbic acid (6-sDode-AA-2G), 2-*O*-α-D-glucopyranosyl-6-*O*-(2-pentylheptanoyl)-L-ascorbic acid (6-bDode-AA-2G) and 2-*O*-α-D-glucopyranosyl-6-*O*-hexadecanoyl-L-ascorbic acid (6-sPalm-AA-2G) were synthesized by the methods described in our previous reports [7,9]. L-Ascorbic acid (AA), L-ascorbyl 6-palmitate (6-sPalm-AA) and lipase F-AP15 were purchased from Wako Pure Chemical Industries (Osaka, Japan). L-Ascorbic acid 2-phosphate sesquimagnesium salt, L-ascorbic acid 2-sulfate dipotassium salt and α-glucosidase from rice were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The structures of AA derivatives are shown in Fig. 1. All purchased reagents were of the highest commercial available purity. All water used was Milli-Q grade.

2.2. Apparatus and chromatographic conditions

A Shimadzu liquid chromatographic system (Kyoto, Japan) consisting of an SCL-10A system controller, LC-10AD pump, SPD-10AV UV–Vis spectrophotometric detector, SIL-10AD auto injector, CTO-6A column oven, DGU-14A degasser and C-R7A Chromatopac was used. The samples (10 μl) were injected using a refrigerated autosampler kept at 4 °C. The chromatographic analyses were carried out on an Inertsil CN-3 (4.6 i.d. × 250 mm, 5 μm, GL Sciences, Tokyo, Japan) with a guard column of Inertsil CN-3 (4.0 i.d. × 10 mm, 5 μm, GL Sciences) kept at 40 °C, using methanol: 28.6 mM H_3PO_4–NaH_2PO_4 buffer (pH 2.1) (65:35, v/v) containing 20 mg/l of dithiothreitol as a mobile phase. The flow rate was 0.7 ml/min and the absorbance at 240 nm was monitored.

2.3. Preparation of post-mitochondrial supernatant from rat small intestine

Wistar/ST rats (male, 8 weeks) were obtained from Japan SLC (Hamamatsu, Japan). Rats were killed by drawing blood

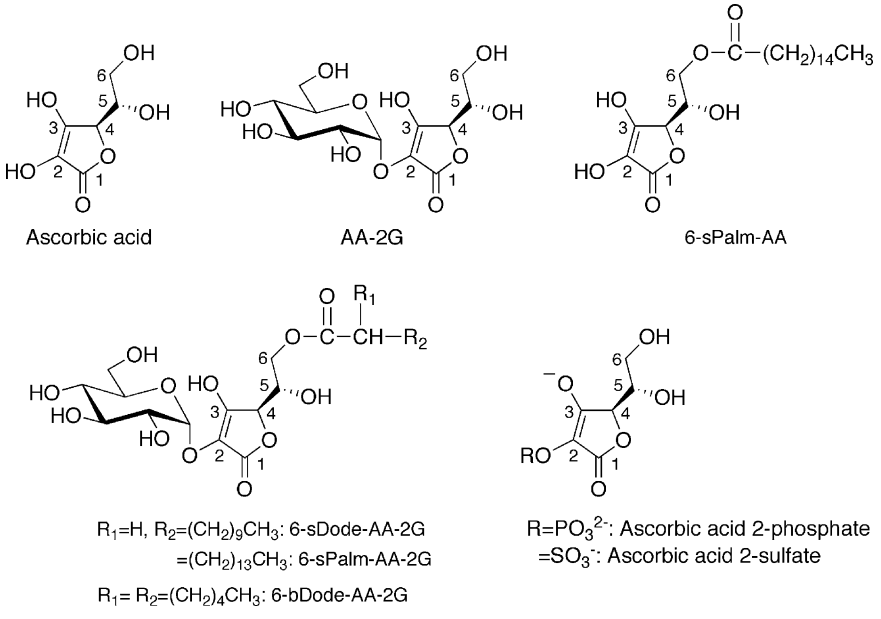

Fig. 1. Chemical structures of ascorbic acid and its derivatives.

from the heart under diethyl ether anesthesia. All of the subsequent manipulations were done at around 4 °C. The small intestine was removed, rinsed with cold saline and homogenized in four volumes of a 0.1 M sodium phosphate buffer (pH 7.4) containing 1 mM DTT with an Ultra-Turrax T 25 basic disperser (IKA Labortechnik, Germany). The homogenate was centrifuged at 700 × *g* for 10 min, and the supernatant was further centrifuged at 12,000 × *g* for 20 min. The post-mitochondrial supernatant was dialyzed overnight against a 10 mM sodium phosphate buffer (pH 7.4), before being centrifuged at 12,000 × *g* for 20 min for use as an enzyme source.

2.4. Linearity, accuracy and precision

Stock solutions (1 mM) were freshly prepared by dissolving AA, AA-2G and 6-sPalm-AA-2G in the HPLC mobile phase. A stock solution of 6-sPalm-AA (1 mM) was made by dissolving in MeOH and diluting with the mobile phase. Standard solutions of 10, 25, 50, 100 and 200 μM were prepared by mixing 900 μl of appropriately diluted stock solutions with 100 μl of 10 mM sodium phosphate buffer (pH 7.4). In general, the final solvent solution for standards and samples was the HPLC mobile phase—10 mM phosphate buffer (pH 7.4) (9:1, v/v). Calibration curves were constructed in the range of 10–200 μM to encompass the expected concentrations in measured samples. Calibration curves were constructed by plotting the peak areas of each compound versus concentration and gave the values of slope along with the intercept and correlation coefficient for each calibration curve. Each point was analyzed three times. The calibration curves were used for the quantification of AA, AA-2G, 6-sPalm-AA and 6-sPalm-AA-2G in enzymatic hydrolysis experiments of 6-sPalm-AA-2G.

Intra-assay validation was used to determine the accuracy and precision of the method in α-glucosidase and lipase solutions or in small intestine post-mitochondrial supernatants with known amounts of 6-sPalm-AA-2G and its three metabolites. Samples were prepared by mixing 900 μl of appropriately diluted stock solutions with 100 μl of 1 units/ml α-glucosidase and 0.1 mg/ml lipase in 10 mM phosphate buffer (pH 7.4) or with 100 μl of 10% (v/v) post-mitochondrial supernatant in 10 mM phosphate buffer (pH 7.4). For the intra-assay validation, six replicates at each concentration were processed. Relative difference and relative standard deviation (R.S.D.) were calculated from the theoretical and experimental concentrations in order to determine the precision and accuracy of the method.

2.5. Sample preparation

For metabolic experiments using α-glucosidase and lipase, 6-sPalm-AA-2G dissolved in DMSO at 1.0 M was diluted with 10 mM sodium phosphate buffer (pH 7.4) to give a 1.25 mM solution. Reaction mixtures were composed of 800 μl of 1.25 mM 6-sPalm-AA-2G solution, 100 μl of 2 mg/ml dithiothreitol in the phosphate buffer and 100 μl of 10 units/ml α-glucosidase and/or 1.0 mg/ml lipase in the phosphate buffer. Since AA and 6-sPalm-AA generated by the enzyme reaction were very unstable under this condition, dithiothreitol was added as a stabilizer at the above concentration. Each mixture was incubated in a thermoregulated shaker (M·BR-022, TAITEC, Saitama, Japan) and was stirred at 1000 rpm for 1 h at 37 °C. For metabolic experiments using a small intestine post-mitochondrial supernatant, each AA derivative dissolved in DMSO at 1.0 M was diluted with 10 mM phosphate buffer (pH 7.4) to give a 1.25 mM solution. Reaction mixtures were composed of 800 μl of 1.25 mM sample solution, 100 μl of 2 mg/ml dithiothreitol and 100 μl of the post-mitochondrial supernatant. Each mixture was incubated for 4 h at 37 °C. The reaction mixture was diluted 5–10 times with the HPLC mobile phase and then filtered through a PTFE membrane of 0.45 μm pore size. The filtrate was subjected to HPLC analysis.

A mixture solution (10 mM) of AA and well-known stable AA derivatives was freshly prepared by dissolving AA, AA-2G, ascorbic acid 2-phosphate and ascorbic acid 2-sulfate in water and was diluted with the mobile phase to obtain the final concentration of 100 μM. The resulting solution was injected into the HPLC system.

3. Results and discussion

3.1. Chromatography

In order to develop an isocratic HPLC method to separate a series of novel stable lipophilic AA derivatives, 6-sAcyl-AA-2G and 6-bAcyl-AA-2G, and their metabolites in a single run, it was necessary to employ a stationary phase with high retention of polar compounds and lower retention of hydrophobic compounds. When the possibilities of C_8 and phenyl columns were explored, it was found that these columns were not suitable for the simultaneous determination (data not shown). A cyanopropyl column can provide a more efficient analysis of samples that contain a wide range of polarities by reducing analysis time and minimizing the necessity of gradient elution, compared to C_{18}, C_8 and phenyl columns. This improvement is achieved by a combination of increased retention of polar compounds, which are retained away from the solvent front, and decreased retention of nonpolar compounds, which reduces the total analysis time.

An Inertsil CN-3 (4.6 i.d. × 250 mm, 5 μm, GL Sciences) was employed for the simultaneous separation of 6-sAcyl-AA-2G and 6-bAcyl-AA-2G and their metabolites. 6-sAcyl-AA-2G and 6-bAcyl-AA-2G could be metabolized to AA, AA-2G and the corresponding 6-*O*-acyl AA with a different acyl group. Optimization of the separation conditions was carried out using 6-sPalm-AA-2G and its metabolites, AA, AA-2G and 6-sPalm-AA (Fig. 1), since 6-*O*-acyl AAs other than 6-sPalm-AA are not commercially available. The optimal mobile phase and its flow rate were found to be methanol–phosphate buffer (pH 2.1) (65:35, v/v) containing 20 mg/l of dithiothreitol and 0.7 ml/min, resulting in a maximum run time of 15 min. Fig. 2 shows a typical chromatogram of a standard mixture of AA, AA-2G, 6-sPalm-AA and 6-sPalm-AA-2G. The retention times were: AA, 4.5 min; AA-2G, 5.1 min; 6-sPalm-AA, 10.1 min; and 6-sPalm-AA-2G, 11.9 min.

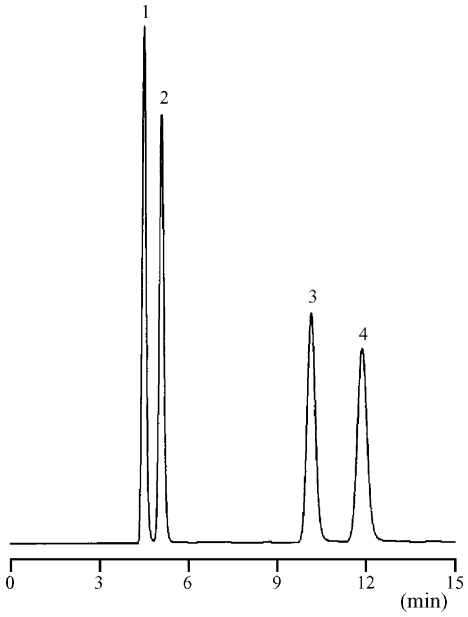

Fig. 2. A typical chromatogram of a standard mixture of AA, AA-2G, 6-sPalm-AA and 6-sPalm-AA-2G. The concentration of each standard was 100 μM. Peaks: 1, AA; 2, AA-2G; 3, 6-sPalm-AA; 4, 6-sPalm-AA-2G.

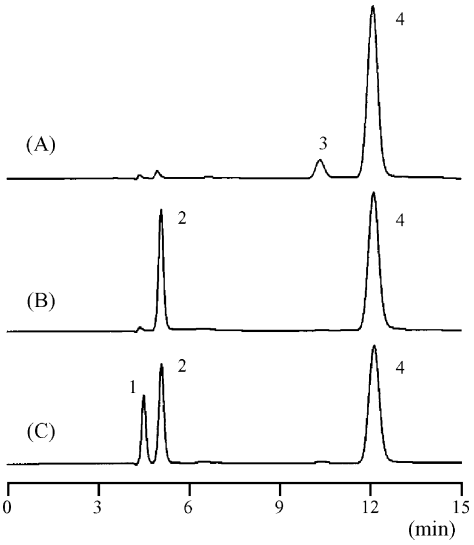

Fig. 3. Chromatograms of 6-sPalm-AA-2G hydrolyzed with α-glucosidase and/or lipase: (A) α-glucosidase treatment; (B) lipase treatment; (C) α-glucosidase and lipase treatment. Peaks: 1, AA; 2, AA-2G; 3, 6-sPalm-AA; 4, 6-sPalm-AA-2G.

3.2. Validation

The method was validated for linearity, accuracy and precision. Linear curve fitting was applied to calculate the calibration curves for each standard in the range of 10–200 μM. The results are given in Table 1. Excellent linearity was obtained between the peak area (y) and the corresponding concentrations (x) on the standard curve of 6-sPalm-AA-2G or its three metabolites. The correlation coefficients of the calibration curves were 1.0000 for the four analytes. The intra-day accuracy and precision of this assay method in α-glucosidase and lipase solutions or in small intestine post-mitochondrial supernatants are also shown in Table 1. The precision of the method was calculated as the relative standard deviation (R.S.D.) of assays containing AA, AA-2G, 6-sPalm-AA and 6-sPalm-AA-2G in the same range of concentration. The R.S.D. ranged from 0.5 to 2.9%. The accuracy was assessed by calculating relative recoveries of AA, AA-2G, 6-sPalm-AA and 6-sPalm-AA-2G. The analytical recoveries were 97.3–106.3%. The reproducibility of this method was considered to be satisfactory for the determination of 6-sPalm-AA-2G and its three metabolites.

3.3. Application

This method was applied to the determination of 6-sPalm-AA-2G and its three hydrolysates in enzymatic hydrolysis of 6-sPalm-AA-2G (Fig. 3 and 4). Hydrolyses of 6-sPalm-AA-2G by α-glucosidase, lipase and both α-glucosidase and lipase were used as models for the metabolic experiments. 6-sPalm-AA-2G was hydrolyzed by α-glucosidase to give 6-sPalm-AA (Fig. 3A) and was hydrolyzed by lipase to give AA-2G (Fig. 3B). 6-sPalm-AA-2G was also hydrolyzed by both of these enzymes to give two hydrolysates, AA and AA-2G, but no 6-sPalm-AA was observed (Fig. 3C). Next, a metabolic experiment of 6-sPalm-AA-2G was carried out using a post-mitochondrial supernatant from rat small intestine (Fig. 4). AA and AA-2G were detected as metabolites of 6-sPalm-AA-2G. Quantitative results for these

Table 1
Validation data from calibration curves of AA, AA-2G, 6-sPalm-AA and 6-sPalm-AA-2G

Compounds	Regression equation	Correlation coefficient	Added (μM)	α-Glucosidase + lipase ($n=6$)		Post-mitochondrial supernatant ($n=6$)	
				Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)
AA	$y=15535x-15109$	1.0000	50	97.3	0.8	106.3	0.7
			100	100.5	1.0	103.7	0.6
AA-2G	$y=14699x+24494$	1.0000	50	100.4	1.0	100.2	0.5
			100	100.8	1.2	100.0	0.9
6-sPalm-AA	$y=14151x-43904$	1.0000	50	97.5	1.1	99.7	2.9
			100	99.7	1.0	99.8	0.8
6-sPalm-AA-2G	$y=13400x-11658$	1.0000	50	100.0	1.3	101.0	2.7
			100	100.0	1.1	99.8	0.8

y, peak area; x, concentration.

Table 2
Hydrolytic profiles of 6-sPalm-AA-2G (1 mM) by various enzymes

Enzymes	Concentrations (μM)					Recovery (%)
	AA	AA-2G	6-sPalm-AA	6-sPalm-AA-2G	Total	
α-Glucosidase	ND	ND	105 ± 6	942 ± 32	1047 ± 39	104.7
Lipase	ND	258 ± 24	ND	796 ± 10	1054 ± 16	105.4
α-Glucosidase + lipase	150 ± 3	228 ± 11	ND	666 ± 13	1044 ± 25	104.4
Post-mitochondrial supernatant	95 ± 4	322 ± 13	ND	573 ± 15	990 ± 18	99.0

Results are presented as mean ± S.D. ($n = 3$). ND, not detectable.

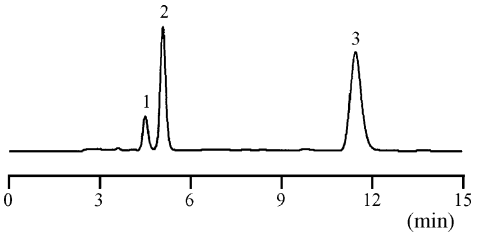

Fig. 4. Chromatogram of 6-sPalm-AA-2G hydrolyzed by post-mitochondrial supernatant from rat small intestine. Peaks: 1, AA; 2, AA-2G; 3, 6-sPalm-AA-2G.

experiments are shown in Table 2. The sum of concentrations of detected 6-sPalm-AA-2G and its metabolites was nearly in accordance with the initial substrate concentration. These results showed that this method is an excellent tool for quantitative analysis and that it can be employed for in vitro metabolic experiments of 6-sPalm-AA-2G.

This method was also applied successfully to the separation of novel lipophilic AA derivatives other than 6-sPalm-AA-2G and their metabolites in the post-mitochondrial supernatant (Fig. 5). 6-sDode-AA-2G having a straight-acyl chain of C_{12} and 6-bDode-AA-2G possessing a branched-acyl chain of C_{12} were employed as novel lipophilic AA derivatives (Fig. 1). 6-sDode-AA-2G and 6-bDode-AA-2G were hydrolyzed by the post-mitochondrial supernatant to give AA, AA-2G and the corresponding 6-*O*-acyl AA (Fig. 5). The peak of 6-*O*-acyl AA was identified by comparison with the retention time of a complete α-glucosidase hydrolysate of 6-sDode-AA-2G or 6-bDode-AA-2G. The major metabolites from 6-sDode-AA-2G and 6-bDode-AA-2G were AA-2G and 6-*O*-acyl AA, respectively. The metabolic profiles of 6-sDode-AA-2G and 6-bDode-AA-2G agree with the hydrolysis patterns of 6-sAcyl-AA-2G and 6-bAcyl-AA-2G with various tissue post-mitochondrial supernatants from guinea pigs described previously [15]. These results suggested that this method can be applied to routine analysis for in vitro metabolic experiments of a series of 6-sAcyl-AA-2G and 6-bAcyl-AA-2G.

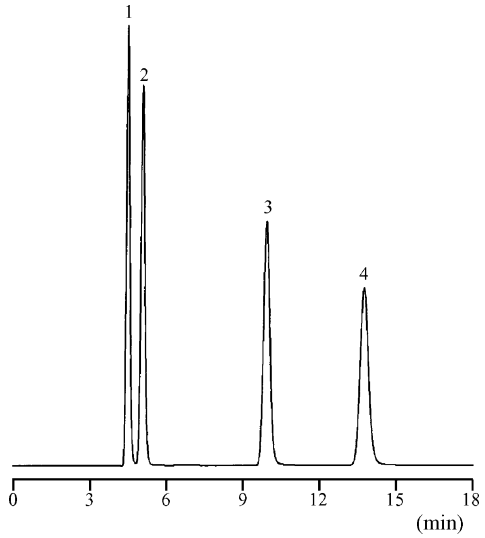

Fig. 6. A typical chromatogram of a mixture of AA, AA-2G, ascorbic acid 2-phosphate and ascorbic acid 2-sulfate. Peaks: 1, AA; 2, AA-2G; 3, ascorbic acid 2-phosphate; 4, ascorbic acid 2-sulfate.

L-Ascorbic acid 2-phosphate and L-ascorbic acid 2-sulfate are well known as stable AA derivatives that are *O*-substituted at the C-2 position of AA (Fig. 1). AA, AA-2G, ascorbic acid 2-phosphate and ascorbic acid 2-sulfate were successfully separated under the same HPLC conditions (Fig. 6). The retention times were: AA, 4.5 min; AA-2G, 5.1 min; ascorbic acid 2-phosphate, 9.9 min; ascorbic acid 2-sulfate, 13.7 min.

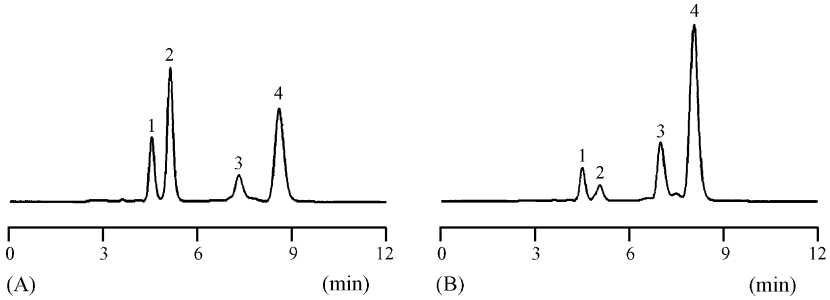

Fig. 5. Chromatograms of 6-sDode-AA-2G (A) and 6-bDode-AA-2G (B) hydrolyzed by post-mitochondrial supernatant from rat small intestine. Peaks: 1, AA; 2, AA-2G; 3, corresponding 6-*O*-acyl AA; 4, 6-sDode-AA-2G or 6-bDode-AA-2G.

Recently, a monoacylated ascorbic acid 2-phosphate, 6-*O*-palmitol-ascorbate-2-*O*-phosphate, has been developed as a stable lipophilic AA derivative [22]. This derivative, as well as 6-sAcyl- and 6-bAcyl-AA-2G, is assumed to give metabolites that contain a wide range of polarities. This method may be applied to routine analysis for metabolic experiments on a variety of AA derivatives besides 6-sAcyl- and 6-bAcyl-AA-2G.

4. Conclusions

An isocratic HPLC method has been developed for the simultaneous determination of novel stable lipophilic AA derivatives (6-sAcyl-AA-2G and 6-bAcyl-AA-2G) and their metabolites. 6-sAcyl-AA-2G and 6-bAcyl-AA-2G were mainly metabolized via AA-2G to AA and via 6-*O*-acyl AA to AA, respectively [10,15]. Since AA and AA-2G are very polar, and 6-*O*-acyl AA, 6-sAcyl-AA-2G and 6-bAcyl-AA-2G very nonpolar, they were separated by two different columns in our previous studies. The new method enables analysis of these compounds in a maximum run time of 15 min without the gradient elution or two different analyses. AA, AA-2G and other well-known stable AA derivatives, ascorbic acid 2-phosphate and ascorbic acid 2-sulfate, were also separated under the same conditions. These results show that the procedure is rapid and simple and that it can be used for metabolic analyses of a wide variety of AA derivatives.

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