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Determination of sodium 5,6-benzylidene-L-ascorbate and related compounds by high-performance liquid chromatography

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ABSTRACT

A simple high-performance liquid chromatographic process with ultraviolet detection was established to determine ascorbic acid, benzoic acid, benzaldehyde and the two different diastereomers of sodium 5,6-benzylidene-L-ascorbate. The five substances were reproducibly separated on a reversed-phase C_{18} column with gradient elution with an acetate buffer (pH 6.85)–methanol system. This method was applied to the determination of the concentrations of these substances in various biological fluids after their extraction and deproteinization with acetonitrile.

INTRODUCTION

Benzaldehyde, an antitumour substance, can be isolated from the volatile fraction of figs [1]. Oral administration of sodium 5,6-benzylidene-L-ascorbate (SBA) (see Fig. 1 for the structure of its two diastereomers) to patients with advanced, inoperative carcinoma induced remarkable necrotic change of the tumours [2]. We have previously shown that intravenous administration of SBA induced degeneration of 3'-methyl-4-dimethylaminoazobenzene-induced rat hepatocellular carcinoma (vacuolar, eosinophilic degeneration, nuclear debris) [3,4]. In cultured L-929 cells, SBA induced chromatin condensation, DNA fragmentation into nucleosomal oligomers and disappearance of cell surface microvilli, which are characteristic of apoptotic cell death [5]. However, the process of induction of the antitumour action of SBA is still unclear. We report here a method for the determination of SBA and related compounds using high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Chemicals and reagents

The following chemicals and reagents were used: SBA (ChemiScience, Tokyo, Japan); acetonitrile (for HPLC) and methanol (for HPLC) (Kanto Chemical, Tokyo, Japan); RPMI 1640

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medium (Gibco, Grand Island, NY, USA); foet-al bovine serum (FBS) (Filtron, Brooklyn, Australia); L-(+)-ascorbic acid (Wako, Osaka, Japan); benzoic acid (Koso Chemical, Tokyo, Japan); benzaldehyde (Kanto Chemical); bovine serum albumin (Fraction V) (Wako).

Preparative separation of SBA diastereomers

One gram of SBA diastereomers was separated by chromatography using a column pre-packed with Toyopearl HW-40S (850 × 30 mm I.D.) (Tosoh, Tokyo, Japan), eluted with methanol–water (10:90, v/v) delivered by a peristalic pump. After the fractionated solutions had been checked by analytical HPLC, all solutions including the two diastereomers, compounds A and B, were combined. The methanol in them was evaporated *in vacuo* at 40°C or lower. Lyophilization of the resulting aqueous solutions shielded from light gave 0.2 and 0.6 g of each diastereomeric SBA, both of which were pale-orange amorphous compounds.

The structures of the two SBA diastereomers,

separated by preparative HPLC, were confirmed from the NMR chemical shifts, coupling constants and nuclear Overhauser effect (NOE) (Fig. 1). To confirm the configuration of the benzylidene site by NMR, compounds A and B were subjected to permethylation of the hydroxy groups by, respectively, excess of methyl iodide and sodium hydride in dimethylformamide (DMF).

^{1}H NMR spectroscopy in C^2HCl_3 of per-methylated compound A (A-Me) showed the presence of a signal at δ 5.78 (s) due to the benzylic proton of the benzylidene group, whereas permethylated compound B (B-Me) showed the presence of a signal at δ 5.86 (s) due to that of the benzylic proton. The configuration at the benzylidene centre was not clear from these data alone. A notable difference between A-Me and B-Me was revealed by an NOE difference of 1.52–2.03% between the benzylic proton of the benzylidene group and C_5–H of A-Me. On the other hand, no NOE was observed between the benzylic proton of benzylidene group and C_5–H

Fig. 1. Structures, chemical shifts and NOE value for the two SBA diastereomers, A-Me (*S* configuration) and B-Me (*R* configuration) (δ ppm, C^2HCl_3, Varian VXR-400, 400 Mz).

of B-Me. Therefore, we concluded that the stereochemistry of the benzylic site of A-Me is S and that of B-Me is R (Fig. 1).

Extraction and deproteinization of samples by acetonitrile

Cell lysate was prepared from human myeloblastic leukaemic ML-1 cells [6] by sonication (1 min at 0°C) in 50 mM acetate buffer (pH 6.85) and centrifugation for 10 min at 13 000 g. An extract from the liver of 5-month-old Donryu rats (Sankyo Laboratory Service) was prepared by sonication in the same buffer.

To investigate the efficiency of acetonitrile extraction, a mixture of SBA (786 μg), ascorbic acid (105 μg), benzoic acid (1313 μg) and benzaldehyde (179 μg) was added to 0.45 ml of each of the following: regular culture medium (RPMI 1640 medium supplemented with 10% of FBS), FBS, cell lysate (containing 10.8 mg/ml of protein) or rat liver extract (containing 62.2 mg/ml of protein). After allowing the mixtures to stand for 30 s at room temperature, they were vigorously mixed with 1.05 ml of acetonitrile (final acetonitrile concentration = 70%) and centrifuged for 1 min at 4200 g. The supernatant (80 μl) was diluted with 200 μl of 50 mM acetate buffer containing 15% of methanol and 20 μl of the diluent was applied to HPLC.

Apparatus

Chromatographic analyses were performed at 25°C on a Hitachi HPLC system (D-2500 chromato-integrator, L-4200 UV–Vis detector, L-6200 intelligent pump) with an ODS-80T$_M$ column (150 × 4.6 mm I.D., 5 μm) (TSK-Gel, Tosoh) and a Guardgel ODS-80T$_M$ column (15 × 3.2 mm I.D.) (Tosoh). The sample was loaded and separated with a linear gradient of 15–40% (v/v) methanol in 50 mM sodium acetate buffer (pH 6.85). The flow-rate was 1.0 ml/min and the elution profile, monitored by the absorbance at 255 nm, was recorded at attenuation 6. The gradient elution was run for 30 min.

Protein determination

The amount of protein was determined by the method of Lowry et al. [7], using bovine serum albumin as standard.
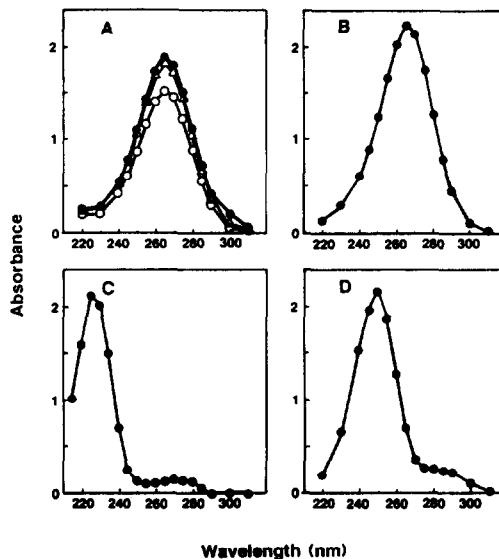

Fig. 2. Absorption spectra of (A) SBA and its two diastereomers, (B) ascorbic acid, (C) benzoic acid and (D) benzaldehyde. SBA or its two diastereomers ($\triangle = S$ configuration; $\bigcirc = R$ configuration (50 μg/ml) and ascorbic acid (25 μg/ml) were dissolved in 50 mM acetate buffer (pH 6.85). Benzoic acid (25 μg/ml) was dissolved in methanol. Benzaldehyde (15.9 μg/ml) was dissolved in 50 mM acetate buffer (pH 6.85) containing 0.086% (v/v) of methanol.

RESULTS AND DISCUSSION

Absorption spectrum

Fig. 2 shows the UV absorption maxima (A) at 265 nm for unseparated SBA and its two diastereomers, (B) at 265 nm for ascorbic acid, (C) at 225 nm for benzoic acid and (D) at 250 nm for benzaldehyde. Therefore, their elution profiles were monitored by HPLC at 255 nm. The molar absorption coefficient (577) of benzoic acid, measured at this wavelength, was ca. one order of magnitude lower than those of SBA, ascorbic acid and benzaldehyde (8294, 11 765, 12 533).

Effect of pH on retention time

The optimum pH of the running buffer for HPLC separation was determined. At pH < 3, elution of two SBA diastereomers was relatively slow, as indicated by the longer retention time (Fig. 3A). The elution time was a minimum at pH 6–9. The peak area of SBA diastereomers, determined with a D-2500 chromato-integrator,
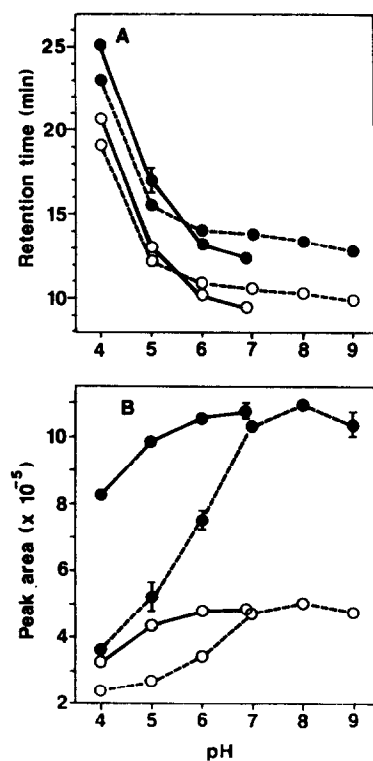
Fig. 3. Effect of pH of running buffer on the retention time and peak area of SBA diastereomers. SBA (3 μg) was equilibrated with 50 mM phosphate buffer (broken line) or 50 mM acetate buffer (solid line), adjusted to the indicated pH, and (A) the retention time and (B) peak area of two SBA diastereomers (● = R configuration; ○ = S configuration) were then determined. Each point is the mean from two independent experiments.

decreased with decrease in the pH of the running buffer (Fig. 3B). This was more apparent when the pH was adjusted with phosphate buffer than when it was adjusted with acetate buffer. Therefore, acetate buffer (adjusted to pH 6.85) was used in subsequent HPLC experiments. SBA was found to be extremely unstable at low and high pH: the degradation half-life of SBA in 50 mM phosphate buffer at pH 2 and 9 at 37°C was 4 and 5 min, respectively. The major degradation products of SBA at pH 2 were ascorbic acid and benzaldehyde (data not shown).

HPLC separation

When SBA was subjected to HPLC with elution with a 15–40% linear gradient of metha-

nol in 50 mM acetate buffer (pH 6.85), it was separated into two diastereomers, one with S configuration (retention time 9.24 min) and the other with R configuration (retention time 12.18 min) (Fig. 4A). These peaks were confirmed by co-elution with purified diastereomers (Fig. 4D). The ratios of S and R diastereomers to total SBA, determined with the chromato-integrator, were $31.04 \pm 0.27\%$ and $68.96 \pm 0.27\%$ ($n = 13$), respectively. Two small peaks that eluted at 1.70 and 27.27 min, representing about 5% of the total peak, were superimposed with the ascorbic acid (peak I) and benzaldehyde (peak III in Fig.
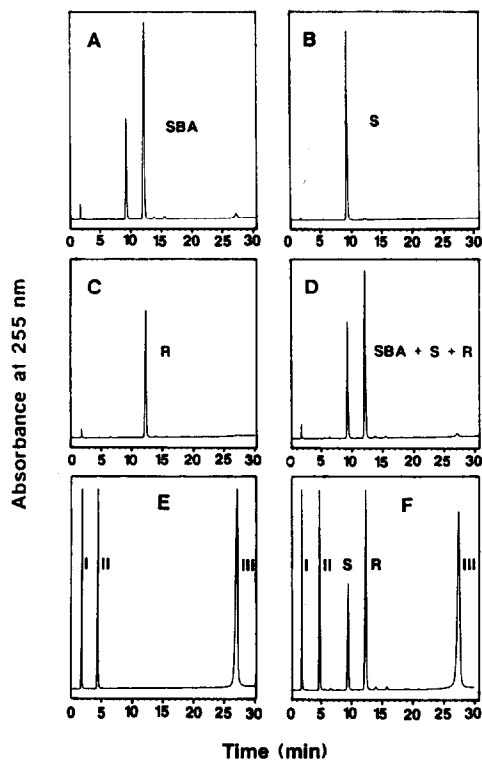

Fig. 4. Separation of the two SBA diastereomers using a linear 15–40% gradient of methanol in 50 mM acetate buffer (pH 6.85). Flow-rate, 1.0 ml/min; detection wavelength, 255 nm; attenuation, 6 (100% absorbance on ordinate = 0.16). Applied sample amounts: (A) SBA (3 μg); (B) diastereomer of SBA with S configuration (1.5 μg); (C) diastereomer of SBA with R configuration (1.5 μg); (D) mixture of SBA (1.5 μg) and its two diastereomers (0.75 μg each); (E) mixture of ascorbic acid (0.4 μg) (peak I), benzoic acid (5 μg) (peak II) and benzaldehyde (0.68 μg) (peak III); (F) mixture of ascorbic acid (0.4 μg), benzoic acid (5 μg), SBA (3 μg) and benzaldehyde (0.68 μg).

4F) peaks, respectively. SBA showed no detectable amount of benzoic acid (peak II in Fig. 4E).

Evaluation of present method

Repeated experiments showed that ascorbic acid (peak I), benzoic acid (peak II), the S and R diastereomers of SBA and benzaldehyde (peak III) could be reproducibly eluted at retention times of 1.693 ± 0.013 ($n = 24$), 4.497 ± 0.105 ($n = 18$), 9.061 ± 0.212 ($n = 25$), 11.826 ± 0.337 ($n = 25$) and 26.992 ± 0.290 min ($n = 24$), respectively. This indicates the reproducibility of the gradient formation, as judged by the elution profiles and retention times.

Linearity of quantification of the peak areas on the chromatogram was investigated (Fig. 5).

When the amounts of ascorbic acid, benzoic acid, SBA and benzaldehyde subjected to HPLC were within the range 0.001–10 μg, their peak areas increased linearly. At a higher level (100 μg), the peak areas were slightly smaller than expected.

Extraction and deproteinization by acetonitrile

The efficiency of acetonitrile as a deproteinizing agent was examined. Acetonitrile precipitated serum proteins, cellular proteins and rat liver proteins in a concentration-dependent manner (Fig. 6). Addition of 70% acetonitrile precipitated 97–99% of these proteins. When the 70% acetonitrile-soluble fraction was subjected to HPLC, several peaks, referred to as back-

TABLE I

EXTRACTION OF SBA AND RELATED COMPOUNDS WITH 70% ACETONITRILE

Compound	Peak area (recovery, %)				
	Added[a]	Recovered from[b]			
		RPMI 1640 +10% FBS	FBS	Cell lysate	Liver extract
Ascorbic acid	626 391 ±17 285 (98)	613 625 ±14 133 (98)	500 248 ±98 729 (80)	671 397 ±57 431 (107)	512 253 ±20 054 (82)
Benzoic acid	704 648 ±26 736 (109)	768 416 ±1844 (109)	792 467 ±4590 (112)	748 744 ±2846 (106)	781 080 ±25 078 (111)
(S)-SBA	494 631 ±10 535 (102)	504 941 ±22 602 (102)	475 020 ±35 705 (96)	497 634 ±2950 (101)	480 986 ±15 537 (97)
(R)-SBA	1 094 309 ±24 458 (102)	1 118 257 ±45 465 (102)	1 052 998 ±78 777 (96)	1 098 017 ±6326 (100)	1 060 961 ±33 368 (97)
Benzaldehyde	1 796 129 ±31 463 (96)	1 726 382 ±91 435 (96)	1 911 461 ±57 661 (106)	1 767 409 ±38 912 (98)	1 713 563 ±124 982 (95)

[a] A mixture of ascorbic acid (0.4 μg), benzoic acid (5 μg), SBA (3 μg) and benzaldehyde (0.68 μg) in 20 μl was subjected to HPLC.

[b] The samples were added to culture medium, FBS, cell lysate or liver extract and then extracted with 70% acetonitrile. The acetonitrile-soluble fraction was diluted and amounts equivalent to those in footnote a were subjected to HPLC, as described under Experimental. The peak areas of these samples were determined, after subtraction of the background peaks (indicated by the lower chromatograms in Fig. 7A–D). Each value is the mean ± S.D. from 2–4 independent experiments. Numbers in parentheses are percentage recovery.
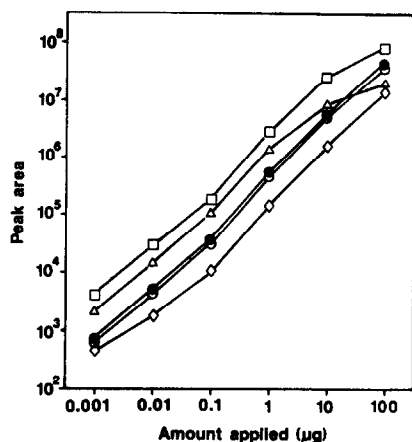
Fig. 5. Relationship between amount applied and peak area. The indicated amount of (○) the S diastereomer of SBA, (●) the R diastereomer of SBA, (△) ascorbic acid, (◇) benzoic acid or (□) benzaldehyde was subjected to HPLC. Attenuation, 6; detection wavelength, 255 nm. Each point is the mean of two independent experiments.

ground peaks, appeared at retention times from 1.7 to 5.0 min (lower chromatograms in Fig. 7B–D).
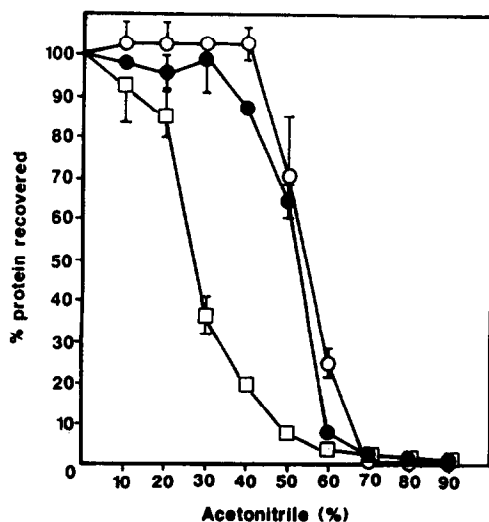

Fig. 6. Deproteinization by acetonitrile. Acetonitrile was added to the (○) FBS, (●) ML-1 cell lysate or (□) rat liver extract to the indicated final concentrations and the protein concentration of the supernatant was determined. Initial concentrations of FBS, cell lysate, and rat liver were 3819 ± 101, 1080 ± 4, and 6219 ± 934 μg/ml, respectively. Each value is the mean ± S.D. from three independent experiments.
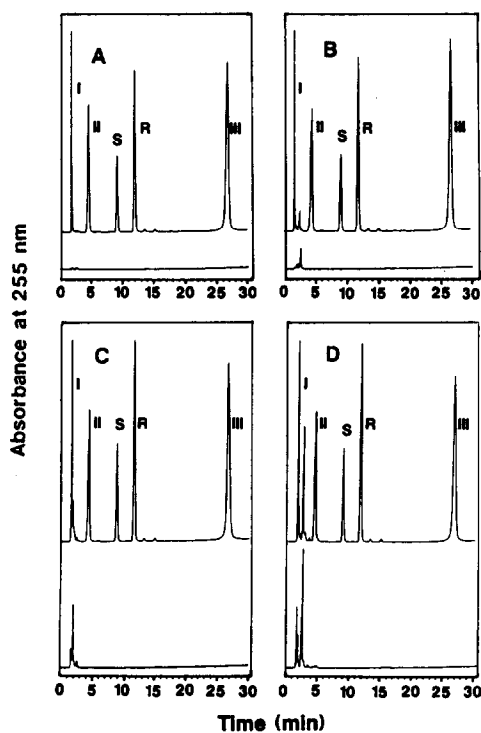

Fig. 7. HPLC separation of SBA and related compounds after extraction from various biological fluids with acetonitrile. Ascorbic acid, benzoic acid, SBA and benzaldehyde was added to (A) RPMI 1640 medium supplemented with 10% of FBS, (B) FBS, (C) ML-1 cell lysate or (D) rat liver extract. Acetonitrile was added at a final concentration of 70%, and then the acetonitrile-extractable fraction, equivalent to 0.4 μg ascorbic acid, 5 μg benzoic acid, 3 μg SBA and 0.68 μg benzaldehyde, was subjected to HPLC. The lower chromatogram in each part is for a control without test compounds. I = Ascorbic acid; II = benzoic acid; III = benzaldehyde; S = S diastereomer of SBA; R = R diastereomer of SBA.

Ascorbic acid, benzoic acid, two diastereomers of SBA and benzaldehyde were added to the culture medium, FBS, cell lysate or liver extract and then extracted with 70% acetonitrile. When one of the acetonitrile extracts was subjected to HPLC, these compounds were separated as distinct peaks (upper chromatograms in Fig. 7A–D). It was necessary to subtract the absorbance of the background peaks in order to calculate the concentrations of these compounds accurately. These compounds, except for ascorbic acid in FBS and liver extract, were quantitatively recovered in the acetonitrile supernatant (Table I).

CONCLUSIONS

The proposed HPLC technique is useful for the determination of the two SBA diastereomers (compounds A and B), which were separated from the major metabolites, and for ascorbic acid and benzaldehyde. To extract the sample present in culture medium or serum, the use of acetonitrile is recommended (Table I). The use of an acidic solution, such as perchloric acid or trichloroacetic acid, for extraction and deproteinization should be avoided, as SBA is extremely unstable at low pH (Fig. 3B). This study has demonstrated the applicability of the proposed technique to the study of the metabolism of SBA.

ACKNOWLEDGEMENTS

REFERENCES

1 M. Kochi, *Jpn. Pat.*, 560 349 (1969).
2 M. Kochi, S. Ueda and T. Hagiwara, in F. Bresciani, R.J.B. King, M.E. Lippman and J.P. Raynaud (Editors), *Hormones and Cancer 3 (Progress in Cancer Research and Therapy*, Vol. 35), Raven Press, New York, 1988, p. 338.
3 H. Sakagami, K. Asano, K. Fukuchi, K. Gomi, H. Ota, K. Kazama, S. Tanuma and M. Kochi, *Anticancer Res.*, 11 (1991) 1533.
4 H. Sakagami, M. Takeda, A. Utsumi, S. Fujinaga, A. Tsunoda, N. Yasuda, M. Shibusawa, T. Koike, H. Ota, K. Kazama, D. Shiokawa, S. Tanuma and M. Kochi, *Anticancer Res.*, 13 (1993) 65.
5 S. Tanuma, D. Shiokawa, Y. Tanimoto, M. Ikekita, H. Sakagami, M. Takeda, S. Fukuda and M. Kochi. *Biochem. Biophys. Res. Commun*, 194 (1993) 29.
6 H. Sakagami, R. Hromchak and A. Bloch, *Cancer Res.*, 44 (1984) 3330.
7 O.H. Lowry, N.J. Rosenbrough, A.L. Farr and R.J. Randall, *J. Biol. Chem.*, 19 (1951) 265.