Sensitive and Simple Analysis of Sorbic Acid Using Liquid Chromatography with Electrospray Ionization Tandem Mass Spectrometry

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Sorbic acid (SA: CH₃–CH=CH–CH=COOH) and its salts are widely used as preservatives in foodstuff because of their growth inhibitory effects on mold, yeast and a wide range of bacteria. However, it is still unclear whether SA and its salts are actually incorporated in these organisms and a higher organisms like mammalian cells. Acidic compounds such as SA are usually analyzed by HPLC with eluents containing acetic acid, formic acid and their ammonium acetates, but such acidic buffers may suppress the ionization efficiency of the acidic compounds in negative-mode electrospray ionization (ESI). In this study, we present a sensitive and simple method for analysis of SA by HPLC with non-acidic solvents such as CH₃CN/CH₃OH–H₂O by negative ion mode ESI-LC/MS. As a result, SA at less as 30 fmol was selectively determined by the selected reaction monitoring (SRM) mode. It was defined as the peak area with a signal-to-noise ratio (S/N) of 3. Good linearity was obtained in the range from 55 fmol (S/N 3) to 500 fmol (r²=0.9968) for SA by using LC/MS with the SRM mode. We also show that the method is useful to analyze SA level in the cytosol of mastocytoma cells, which were pretreated with SA. These results suggest the applicability of this method for the highly sensitive determination of SA in the mammalian tissues and cells.

Key words sorbic acid; LC/MS/MS; electrospray ionization; selected reaction monitoring

Sorbic acid (SA) and its salts are commonly used as food additives because of their antibacterial and growth inhibitory activities against yeast and fungi. They are used in cosmetics, pharmaceuticals and tobacco products.1) Since SA and its salts are classified as “Generally Recognized as Safe” (GRAS) additives by U.S. FDA,2) their use in food preservation is considered safe for human consumption. On the other hand, previous reports have shown that SA and its salts exhibit a weak genotoxic potential3–5) including damage to DNA6) and alkylating activity on nucleophile 4-(p-nitrobenzyl)pyridine (NBP).7) Also, Soschin and Leyden reported that SA induced erythema and edema of human skin,8) but the mechanism was not clarified.

We are interested in understanding the actions of SA and its salts in terms of the possible cytostatic or cytotoxic effects in mammalian tissues and cells, although few studies have been performed to consider the effect of SA on cultured mammalian cells.9,10)

Incidentally, SA has been analyzed by HPLC with acetic acid or formic acid buffer, but the detection limit for SA was less than 1 pmol (S/N 3) which is not enough to determine the cellular SA level. Thereafter, Negri et al. reported that SA in urine could be measured by selected ion monitoring (SIM) of m/z 111.13 with ESI-LC/MS in the negative ion mode with the detection limit of 4 μmol/l.11) and Cartwright et al., reported that SA derivative at less than 4 fmol was detected by using ESI-LC/MS/MS using a mixture of acetonitrile and 0.05% (v/v) formic acid in the positive ion mode, and using detection of the SRM mode.12) Generally, the SRM mode is more selective and sensitive than the SIM mode, and the technique of SA derivative requires extra time for analysis owing to unwanted side products.

In the present study, we have developed a simple and sensitive method for the determination of SA in cells by using LC/MS in the SRM mode. We performed three experiments. First, to evaluate the optimum detection conditions for trace analysis of SA, we compared the performance of this method with those using ESI and atmospheric pressure chemical ionization (APCI) and positive and negative ion modes based on the peak intensities of the protonated or deprotonated molecular ion. In the analysis of acidic compounds, acid and buffer solvents are usually selected for the mobile phase on an ODS column, but this can suppress the ionization efficiency of these target compounds in the negative ion mode. Second, we compared the use of acid solvents with the use of ammonium acetate and neutral solvents (CH₃OH/CH₃CN–H₂O). Third, to evaluate the matrix effect for the analysis of SA using LC/MS with the SRM mode we measured the recovery of SA from the cytosol of SA-preloaded mastocytoma P-815 cells, which are used as a proper model of growing mammalian cells because P-815 cells are favorable for examining growth and differentiation, and also for evaluation of various compounds on cytotoxicity, phototoxicity and immunotoxicity.13–19)

Experimental

Materials SA, guaranteed grade, and LC/MS-grade of H₂O, CH₃OH and CH₃CN were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Guaranteed grades of HCOOH, HCOONH₄ and CH₃COONH₄ were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Argon gas (99.99%) used as the collision gas of the SRM mode was obtained from Neriki Gas Co., Ltd. (Hyogo, Japan).

Preparation of Cytosol and HPLC Analysis The procedure for preparation of cytosol from mastocytoma P-815 cells, treated with 2.5 mmol/l SA for 0.5 h is summarized in Fig. 1. SA was extracted and analyzed by HPLC and LC-MS/MS. The HPLC system LC-9A (Shimadzu Co., Kyoto, Japan).
with UV detector SPD-6A at 251 nm (Shimadzu Co., Kyoto, Japan) was used. Separation was performed on a Handy ODS (4.6 mm i.d.×150 mm, 5 μm) from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) with 0.05% HCOOH-CH₃OH (40:60) as the eluent at a flow rate of 0.8 ml/min. SA (10 mmol/l, 10 μl) was added to filtered cytosol (10 μl), and then the mixture (10 μl) was injected to HPLC.

**Triple-Quadruple Mass Spectrometer Conditions** A Quattro premier triple-quadruple LC-MS (Micromass, Manchester, U.S.A.), equipped with an ESI source and an APCI source was used for the MS/MS analyses coupled to the alliance HT Waters 2795 separation module (Waters Co., Milford, MA, U.S.A.). All chromatographic separation using TSKgel ODS coupled to the alliance HT Waters 2795 separation module (Waters Co., Milford, MA, U.S.A.). All chromatographic separation using TSKgel ODS-150V (4.6×150 mm, 3 μm) from TOSOH Co. (Tokyo, Japan) was performed at a flow of 0.4 ml/min. The mass spectrometer was operated at low resolution for both Q1 and Q3 in the SRM mode. Both positive ionization and negative ionization modes were used for analysis. The parameters of the ionization efficiency were optimized by evaluating the sensitivity based on flow injection analysis. SA, 1 mmol/l, was injected at 10 μl/min by syringe and connected with a line of 0.4 ml/min mobile phase of CH₃OH-H₂O system via a T-joint. The instrumental parameters were optimized with regard to the maximum signal intensity of the protonated molecular ion [M+H]+ and deprotonated molecular ion [M−H]− by injections of 10 mmol/l standard solutions, and the parameters were determined. MS/MS was performed based on the collision-induced dissociation (CID) of the specific precursor ion and the generation of characteristic fragment ions. Subsequently, product ion spectra were obtained by scanning Q3 over the mass range of m/z 45—130. Both Q1 and Q3 quadrupoles’ LM and HM resolution values were 15. The flow rate of argon collision gas for fragmentation in the SRM mode was 0.3 ml/min (3.37—3.39×10⁻³ mbar) by which the collisional energy was optimized for the fragment ion of SA. During the SRM analysis, both Q1 and Q3 quadrupoles’ LM and HM resolution values were 10.

**Results and Discussion**

**Optimization of MS Conditions** To obtain the most sensitive ionization condition for analysis, the ESI and APCI modes were used in both positive and negative modes. In the positive ion mode ESI and APCI experiments, one peak due to the deprotonated molecule [M−H]− m/z 111 was obtained as the base peak and lesser peaks appeared (Figs. 2C, D). The peak intensity at m/z 111 in the negative ion mode was higher than m/z 113 in the positive ion mode. As SA includes a carbonyl group, it is not a good proton acceptor but is a good proton donor compound.

Our preliminary experiments indicated that the signals of [M+H]+ and [M−H]− increased significantly when the voltage of the cone increased from 17 to 20 for ESI and to 17 for APCI study. The parameters of the analyzer were optimized when the source and desolvation temperatures were 120°C and 350°C for ESI, 150°C and 200°C for APCI, respectively, and the probe was 200°C for APCI. The gas flow rate (l/h) of the cone and desolvation were 100 and 1000 for ESI and 50 and 200 for APCI, respectively. The capillary voltage was 3.5 kV for the positive ion mode and 3.2 kV for the negative one.

**Optimization of MS/MS Conditions** In MS/MS studies, both ESI and APCI interfaces were used in conjunction with the flow injection system for optimization of the MS/MS parameters. The product ion mass spectra of protonated and deprotonated molecular ion, [M+H]+, m/z 113 and [M−H]−, m/z 111 of SA were obtained in the scan range of m/z 35—150. As can be seen in Fig. 3, the major ions at m/z 67 and 95 appeared in the positive ion mode, and m/z 67 was absorbed in the negative ion mode ESI and APCI production mass spectra. Therefore, the mass transition patterns, m/z 113→m/z 67 and 95 were selected to monitor SA in the positive ion mode, and m/z 111→m/z 67 was selected to monitor SA in the negative ion mode and in SRM analyses under ESI and APCI modes. The extent of the fragmentation of the precursor ion depends on the collisional energy. The effects of
this energy on the fragmentation efficiencies were also investigated to generate the maximum MS/MS performance of this mass analysis. When the collisional energies of 5—20 eV were examined, the maximum performance was reached at the collisional energy of 11 for the positive ion ESI mode, 9 for the negative ion one, 13 for the positive ion APCI mode and 11 for the negative ion one. SRM chromatograms of SA were obtained under these conditions. The maximum peak area was obtained in the negative ion ESI mode and less than 1/20 times of area was obtained in negative ion APCI mode. Under the positive ion ESI and APCI modes, no desirable results were obtained, and therefore the negative ion ESI mode was used for SRM analysis of SA.

The mass transition patterns of \( m/z \) 111 \( \rightarrow \) 67 indicate \([M - H]/H_{11002}H\] breakdown to \([M - CO_{2}H + H - H]/H_{11002}H\].

**LC Optimization** We tried to use 0.05% HCOOH–CH$_3$OH, an eluent usually used for HPLC analysis of acid compounds. Under acid eluent conditions, we did not obtain a sufficient peak area of \( m/z \) 111 \( \rightarrow \) 67 for quantification of SA in the cells (Fig. 4A). Under ESI conditions, the ionization efficiency depends on both the chemical nature of the sample and the presence of contaminants. For a high sensitivity of analyte under the negative ion mode, such as for the acid compound SA (pK$_a$ 4.6$^{20}$), the anion form must be retained in the eluent. For this reason, the ionization efficiency of SA would be suppressed in an acid eluent. To solve this problem, we tried to use 5 mmol/l HCOONH$_4$, 5 mmol/l CH$_3$COONH$_4$ and H$_2$O as eluents, and evaluated them for ionization efficiency based on the peak area of the SRM chromatogram of SA. SRM chromatograms of SA were obtained with these eluents are shown in Figs. 4B and C. The maximum peak area of SRM was obtained under H$_2$O conditions (Fig. 4C), and 5 mmol/l HCOONH$_4$ condition (Fig. 4B), was better than 5 mmol/l CH$_3$COONH$_4$. With respect to the peak sharpness in chromatographic resolution, the mobile phase composed of CH$_3$OH/CH$_3$CN–H$_2$O was optimized in terms of both band broadening and ionization efficiency. In this study, the CH$_3$CN–H$_2$O mixture (40 : 60) gave symmetrical peaks and better detection sensitivity than the CH$_3$OH–H$_2$O (40 : 60) mixture (Fig. 4D). The effects of the eluents on ionization efficiency are shown in Table 1 as peak area ratios.

**Sensitivity and Matrix Effect** Using these optimized conditions, calibration was performed at five different concentrations by LC-ESI-MS/MS with the SRM method. The calibration graph for SA was generated from the peak areas of the mass transition pattern, \( m/z \) 110.8 \( \rightarrow \) 66.8, obtained with these eluents are shown in Figs. 4B and C. The maximum peak area of SRM was obtained under H$_2$O conditions (Fig. 4C), and 5 mmol/l HCOONH$_4$ condition (Fig. 4B), was better than 5 mmol/l CH$_3$COONH$_4$. With respect to the peak sharpness in chromatographic resolution, the mobile phase composed of CH$_3$OH/CH$_3$CN–H$_2$O was optimized in terms of both band broadening and ionization efficiency. In this study, the CH$_3$CN–H$_2$O mixture (40 : 60) gave symmetrical peaks and better detection sensitivity than the CH$_3$OH–H$_2$O (40 : 60) mixture (Fig. 4D). The effects of the eluents on ionization efficiency are shown in Table 1 as peak area ratios.

<table>
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<th>Eluent</th>
<th>pmol</th>
<th>Peak area</th>
<th>Peak area ratio</th>
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<tr>
<td>5 mmol/l CH$_3$COONH$_4$–CH$_3$OH</td>
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<td>96.244</td>
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<tr>
<td>5 mmol/l HCOONH$_4$–CH$_3$OH</td>
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<td>2.1</td>
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<td>1500.347</td>
<td>23.2</td>
</tr>
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</table>

Fig. 3. Product Ion Spectra of SA under Optimized Collisional Energies: from the Precursor Ion \( m/z \) 113 (A) ESI, (B) APCI; from the Precursor Ion \( m/z \) 111 (C) ESI, (D) APCI

Fig. 4. SRM Chromatograms of SA: (A) 0.05% HCOOH–CH$_3$OH (40 : 60), 2500 nmol/l of SA, 5 \( \mu \)l Injected; (B) 5 mmol/l HCOONH$_4$–CH$_3$OH (40 : 60), 1000 nmol of SA, 5 \( \mu \)l Injected; (C) CH$_3$OH–H$_2$O (40 : 60), 500 nmol/l of SA, 5 \( \mu \)l Injected; (D) CH$_3$CN–H$_2$O (40 : 60), 500 nmol/l of SA, 5 \( \mu \)l Injected.
limit was 30 fmol (S/N 3).

Next, the matrix effect was evaluated by comparing the peak areas of the cytosol blank spiked with SA to those prepared in the mobile phase at the corresponding concentration. A linear calibration curve was constructed using the same method mentioned above. Good linearity was obtained up to 500 fmol ($r^2=0.9968$) and the detection limit was 55 fmol (S/N 3). By using this SRM chromatogram of the SA (Fig. 5), we examined the content of SA present in the cytosol fraction of P-815 cells, which were pretreated with 2.5 mmol/l of the acid for 0.5 h, and followed by washing by PBS according to the procedure described in Fig. 1.

On the other hand, compared the UV-HPLC analysis with the ESI-LC/MS/MS in operation time, the former required about 45 min while the latter took only about 8 min. Furthermore, the detection limits of SA in the former vs. the latter were 1 pmol (S/N 3) and 55 fmol, respectively. These results showed that the LC/MS/MS method was more rapid and sensitive than the HPLC-UV method. Under ESI conditions, slight ionization suppression was observed and the retention time of SA was slightly shorter than the standard one. This may have been caused by the cytosol fraction in what some call the matrix effect and may depend on the number of P-815 cells. However, although the retention time was changed, it was possible to detect SA by the SRM method. By using the SRM method under the conditions described above, the average of SA level recovered from the cytosol of P-815 cells, which were preloaded with SA (2.5 mmol/l) for 0.5 h, was 160 fmol/5×10^6 cells (n=3). On the basis of antibacterial activity of SA, it is assumed that the acid would be incorporated into yeast and fungi. However, as long as we know, there is no published date dealing with the incorporation of the acid in those organisms. In addition, compared with microorganism, the metabolism of SA has not been examined at all in mammalian tissues and cells. Therefore, the value mentioned above may be the first preliminary data suggesting the incorporation of SA in mammalian cells. However, it surely needs to determine the cellular localization of SA in the mammalian cells.

There have been several papers concerning designs to improve the specificity or sensitivity of SA determination. For example, Cartwright et al. studied the derivatisation of carboxylic acid groups for enhancement of sensitivity, and Negri et al. studied the single ion monitoring (SIM) technique for determination of SA in urine. However, the derivatisation reaction procedure requires extra time and produces unwanted side products. Also, the SIM technique is less selective and sensitive than SRM. This method could be applicable for the determination of trace amounts of SA in the mammalian tissues and cells.

**Conclusion**

In this study, we established a highly sensitive and specific analysis of SA via LC-negative ion ESI-MS/MS (SRM method) using a CH$_3$CN–H$_2$O system as an eluent of HPLC. This eluent system is effective for detecting acidic compounds in the negative ion mode, because it retains the anion form in the eluents of neutral pH. This is the first demonstration of a non-acidic and non-buffer eluent being used for highly sensitive analysis of SA. In addition, the method using our non-acidic and non-buffer eluent system is very simple and swift for the detection of SA in the homogenate of mastocytoma P-815 cells; the detection limit was 55 fmol and good linearity was obtained up to 500 fmol. This SRM method may be applicable to determine the localization and metabolic effect of SA in mammalian tissues and cells.

**Acknowledgments** The authors would like to thank Ms. N. Kato (Waters Co.) for her interesting suggestions and technical support.

**References**

2) (U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition.) FDA List of Food Additives that are Generally Recognized as Safe (GRAS) U.S. GPO, Washington, DC (2005).

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**Fig. 5. SRM Chromatogram of Cytosol Fraction from P-815 Treated with SA**

Eluent: CH$_3$CN–H$_2$O (40 : 60); flow rate: 0.4 ml/min.