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Accurate mass measurement using multiple sprayer nano-electrospray mass spectrometry combined with nano-scale high-performance liquid chromatography on a magnetic sector instrument

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Abstract

A new technique for accurate mass measurement utilizing multiple sprayer nano-electrospray ionization mass spectrometry (nano-ESI-MS) combined with nano-scale high-performance liquid chromatography (nano-HPLC) on a magnetic sector instrument is described. Both metal-coated glass capillaries and fused-silica capillaries were used as nano-ESI sprayers. A metal-coated glass capillary was used for the introduction of the Ref. compound solution, and a metal-coated fused-silica capillary was used for connection to the nano-HPLC column. By shifting each sprayer's position relative to the sampling orifice, spectra were obtained of both the sample components as eluted from the column and reference compounds. Several standard compounds were examined and satisfactory accurate masses were obtained. Problems arising from differences in ionization efficiency between the sample and reference compounds were not observed.

Keywords: Mass measurement; Magnetic sector instrument

1. Introduction

Electrospray ionization mass spectrometry (ESI-MS) is a powerful technique for characterization of macrobiomolecules [1–3]. When combined with separation by high-performance liquid chromatography (HPLC), ESI-MS is commonly used for characterization of small organic compounds such as peptides [4,5], metabolites [6], and metal complexes [7,8]. Standard ESI sources typically operate at a flow-rate of 1–200 μl/min and use a nebulizing gas to assist in droplet formation. However, when the amount of sample is limited, it is advantageous to use a minimal volume of sample solution introduced at a much lower flow-rate. Recently, such nano-scale electrospray ion sources (nano-ESI) have been developed, and several applications have been reported [9–14]. The nano-ESI source produces a stable ion signal at a flow-rate of 10–100 nl/min, leading to smaller size droplets compared to the standard ESI sources. The flow-rate of nano-ESI depends on the inner diameter of the sprayer tip and/or the viscosity of the sample solutions. For example, using 1 μl of sample solution, it is possible to perform analysis for a period of up to 30 min, which allows extensive MS–MS investigation to determine the amino acid

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peptide sequence. Furthermore, nano-scale HPLC (nano-HPLC) with ESI-MS detection may be applied to the separation analysis of a limited amount of sample mixtures.

Accurate mass measurement [15] is a very useful technique to obtain information on elemental compositions of unknown compounds. However, it is usually necessary to use reference compounds for calibration. When performing an accurate mass measurement using a sector instrument equipped with an ESI source and a conventional HPLC, the reference compound solution is introduced to the source by postcolumn addition. The eluted sample solution and the reference compound solution are mixed in a low volume static mixer. In the case of using a nano-HPLC, however, using the postcolumn addition technique is difficult. If it is used, the sample components separated in the nano-column are diffused in the static mixer, because the flow-rate of mobile phase is extremely low (<500 nl/min). Also if the sample compound and reference compound are mixed together and introduced into the nano-ESI capillaries, in some cases, the reference compound can suppress ionization of the sample, or vice versa. Also in some cases, a signal from the reference compound overlaps signals from a sample compound of the same mass.

In a previous paper [16], we reported accurate mass measurements by reference compound infusion during direct sample introduction using a nano-ESI-MS system and the multiple sprayer nano-ESI technique. Because the sample solution and the reference solution are introduced into different nano-ESI capillaries, the difference of ionization efficiency between sample compound and reference compound may be ignored.

In this paper, we investigate the accurate mass measurement of several standard compounds by using a multiple sprayer nano-ESI source combined with a nano-HPLC on a magnetic sector mass spectrometer. To the author's knowledge, the application of multiple sprayer electrospray ionization mass spectrometry combined with a nano-scale HPLC on a magnetic sector instrument has not yet been reported. By using this system, the sample compounds and the reference compounds are introduced to a nano-ESI source from different capillaries by shifting each sprayer's position relative to the sampling orifice. Since the samples are introduced in separate capillaries, differences of ionization efficiency may be ignored and accurate mass measurements are easily obtained. The performance was demonstrated by using a mixture of peptides and other small organic compounds.

2. Experimental

2.1. Chemicals

Reserpine (M_r: 608) and polyethylene glycol 600 (PEG-600) were obtained from Wako (Osaka, Japan). Bradykinin (M_r: 1059), gramicidin-S (M_r: 1040) and leucine enkephalin (M_r: 555) were obtained from Sigma (St. Louis, MO, USA) and were used without further purification. Compounds were mixed and introduced to the low dead-volume injector in the nano-HPLC system. PEG-600 was used as the reference compound. The purity of each chemical was higher than 97%.

A 200-fmol amount of bradykinin, 1 pmol of leuenkephalin, 500 fmol of reserpine and 10 pmol of gramicidin-S were dissolved in 1 μl of pure water. The injection volume was 100 nl. PEG-600 was dissolved at a concentration of 50 ng/μl in methanol.

2.2. Nano-HPLC ESI-MS

The multiple sprayer nano-electrospray ionization mass spectrometry (nano-ESI-MS) was carried out using a Jeol JMS-700 reverse-geometry double focusing mass spectrometer equipped with a nano-ESI source. A nano-HPLC system was constructed using two conventional HPLC pumps (Shimazu 10ADvp, Japan), a solvent splitter with static mixer (LC Packings Acurate™, San Francisco, CA, USA), a low dead-volume injector (LC Packings) and a nano-scale C_{18} column (LC Packings). The HPLC flow-rate was 100 μl/min, and was split into 400 nl/min for the column effluent with the solvent splitter. The calibrator was used to adjust the flow-rate of the nano-column. A 150×0.075 mm column was used with a linear gradient from 100% A (99.9% H_2O with 0.1% formic acid) to 100% B (99.9% acetonitrile with 0.1% formic acid) over 5.0 min. A

300×0.02 mm fused-silica capillary was used to connect the column and the nano-ESI capillary.

A schematic diagram of the multiple sprayer nano-ESI source combined with a nano-HPLC is shown in Fig. 1.

Up to three capillaries can be connected to the probe. The spacing between capillaries is ~2.5 mm. The PEG-600 reference solution is injected into one capillary. The column end can be connected to the remaining two capillaries. If each capillary is connected to the end of a different column and the sprayer's position rapidly shifted, the results of two samples can be obtained in a single measurement. In the present experiment, a single column end was connected to one capillary.

Nano-ESI capillaries, made from glass and coated with gold, were obtained from Protana (Odense M, Denmark) and New Objective (Woburn, MA, USA). The capillaries purchased from New Objective were used for connection with the column end, and were used without modification. The capillary tips purchased from Protana were cut 1–2 mm from the end using a special tool (fabricated from a pair of scissors modified with a file) before measurement. The PEG-600 solution was injected into the Protana capillary. Spectra of sample components and PEG-600 were obtained by vertically shifting the sprayer's position relative to the sampling orifice during the measurement. The mass calibration was carried out with the PEG-600 spectrum, and this result was applied to the sample components spectra. Then accurate masses of sample components separated by a nano-column can be obtained in one measurement

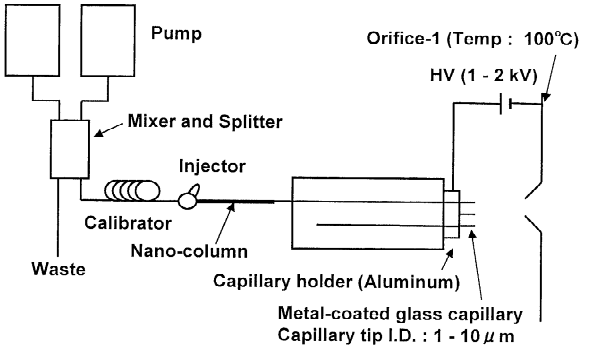


Fig. 1. Schematic diagram of the multiple sprayer nano-ESI source combined with nano-HPLC.

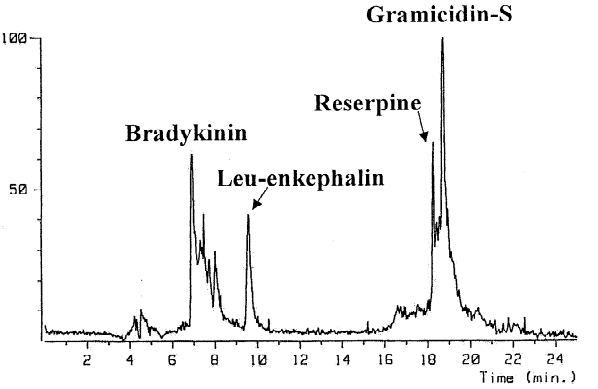


Fig. 2. Total ion chromatogram of the mixture.

by using this system. Further action of the probe will be described later.

No nebulizer gas or drying gas was employed. Needle voltage was 1.5–2.0 kV, orifice voltage was 0 V, ring lens voltage was 50 V, orifice temperature was 80 °C, and ion accelerating voltage was 5.0 kV. The low-resolution spectra were obtained by scanning the magnetic sector voltage. The low-resolution spectra were recorded by respective scanning from m/z 50 to 1500 every 1 s. The high-resolution spectra were obtained by scanning the electric sector (E) and the accelerating voltage (V) linked so that E/V was constant. The high-resolution spectra were

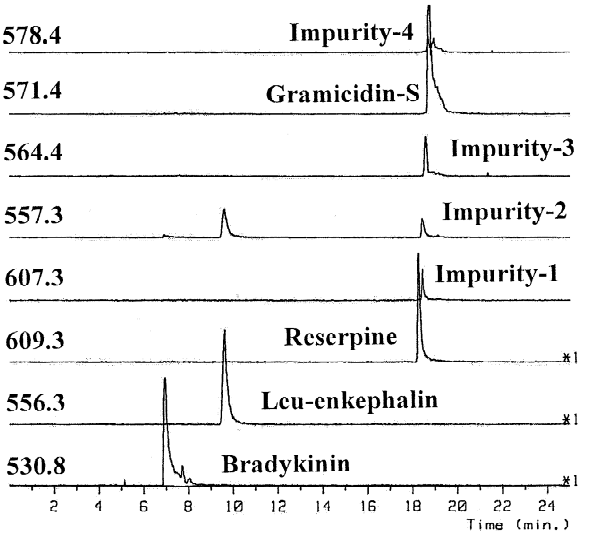


Fig. 3. Mass chromatograms of the components in the mixture.

recorded by respective scanning from m/z 520 to 620 every 1 s. Mass resolution was 1000 and 3000 (10%-valley definition). Accurate mass determination is also possible by using a time-of-flight (TOF) instrument. However, the resolving power of sector instrument is much higher than that of the TOF instrument because the resolution of the TOF instrument is defined on the full-width-at-half-maximum (FWHM).

3. Results and discussion

The total ion chromatogram (TIC), mass chromatograms and mass spectra of mixture sample at low resolution ($R=1000$) are shown in Figs. 2–4. In this measurement, the sprayer position relative to the sampling orifice (original position) was fixed which the eluted sample components spectra from nano-column were observed. The spectra and mass chromatograms of all standard sample components and impurities were observed. Several scans were accumulated in each case to obtain a clear spectrum. In Fig. 3, the mass chromatograms of m/z 531.3, 556.3, 609.3 and 571.4 correspond to the $[M+2H]^{2+}$ ion of bradykinin, $[M+H]^{+}$ ion of leu-enkephalin, $[M+H]^{+}$ ion of reserpine and $[M+2H]^{2+}$ ion of gramicidin-S, respectively. The mass chromatograms of m/z 607.3, 557.3, 564.4 and 578.4 are probably impurities of the standard compounds. The m/z 607.3 ion in Fig. 4c probably corresponds to $[M+H]^{+}$ of an unsaturated molecular component of reserpine. The m/z 557.3, 564.4 and 578.4 ions in Fig. 4d presumably correspond to $[M+2H]^{2+}$ ions

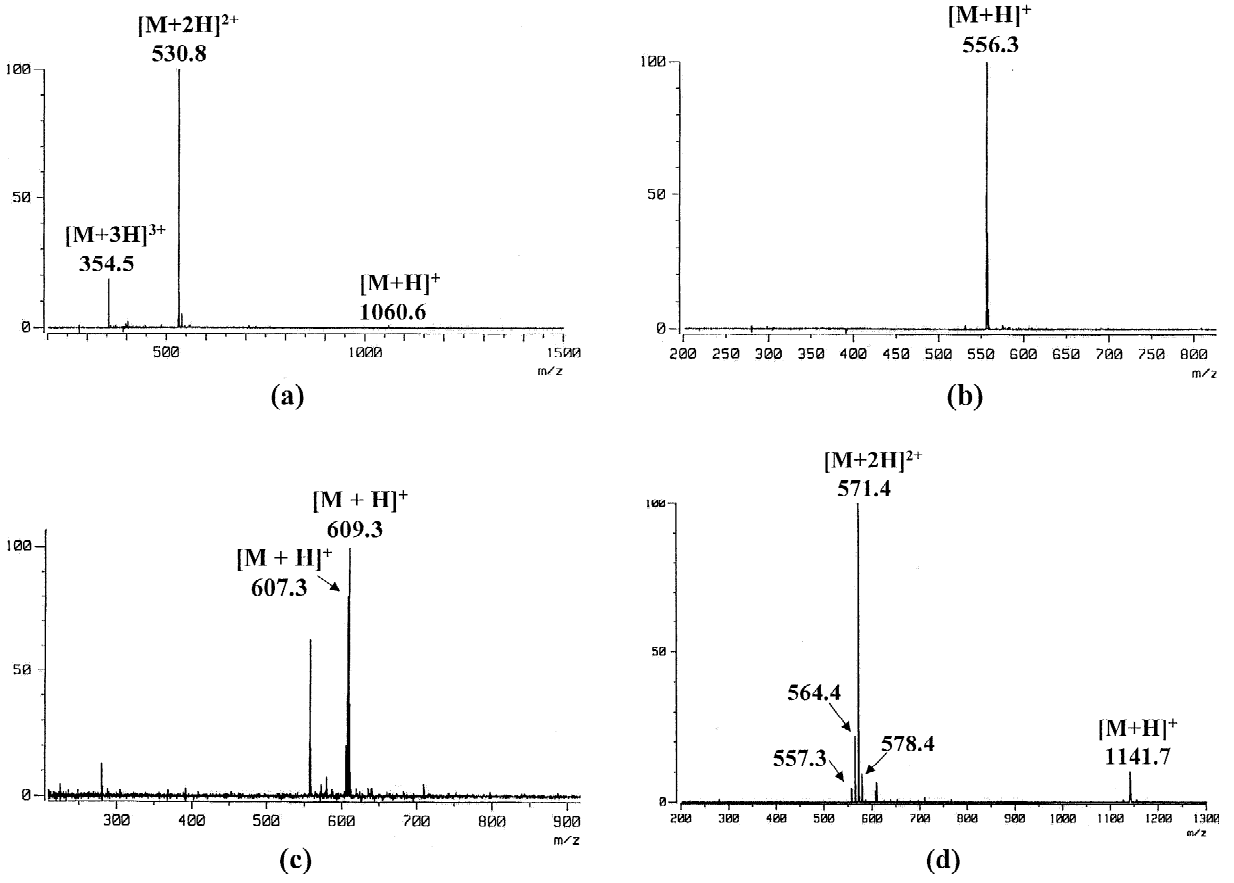


Fig. 4. Low-resolution mass spectra of (a) bradykinin, (b) leu-enkephalin, (c) reserpine and (d) gramicidin-S.

of analogues of gramicidin-S due to alkyl chain variation in the chemical structure. The differences of these components presumably correspond to CH_2 units. The chemical structure of gramicidin-S is shown in Fig. 5 [17].

The accurate masses of these eight components were measured using multiple sprayer nano-ESI combined with a nano-HPLC system. In this experiment, PEG-600 solution was injected to the Protana capillary, and was introduced to the nano-ESI source using the infusion mode by shifting the sprayer's position. TIC for the accurate mass measurement of the mixture of sample and PEG-600 is shown in Fig. 6. In Fig. 6, the peaks of PEG-600 were observed at four different retention times of 1, 5, 13.7 and 21 min. At these times, the sprayer's position relative to the sampling orifice was vertically shifted from the original position to the other position to obtain the PEG-600 spectrum. Several spectra of PEG-600 were observed, and then the sprayer's position was returned to the original position. Therefore, spectra

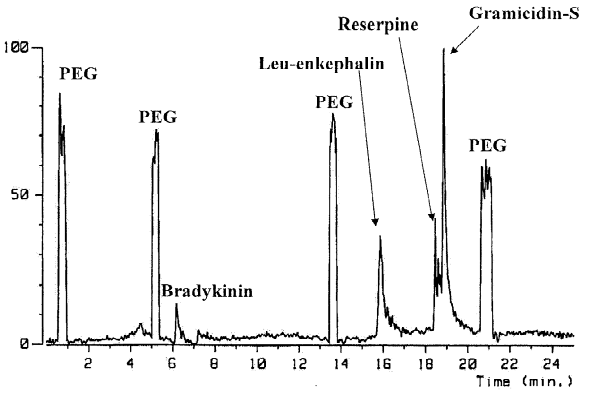
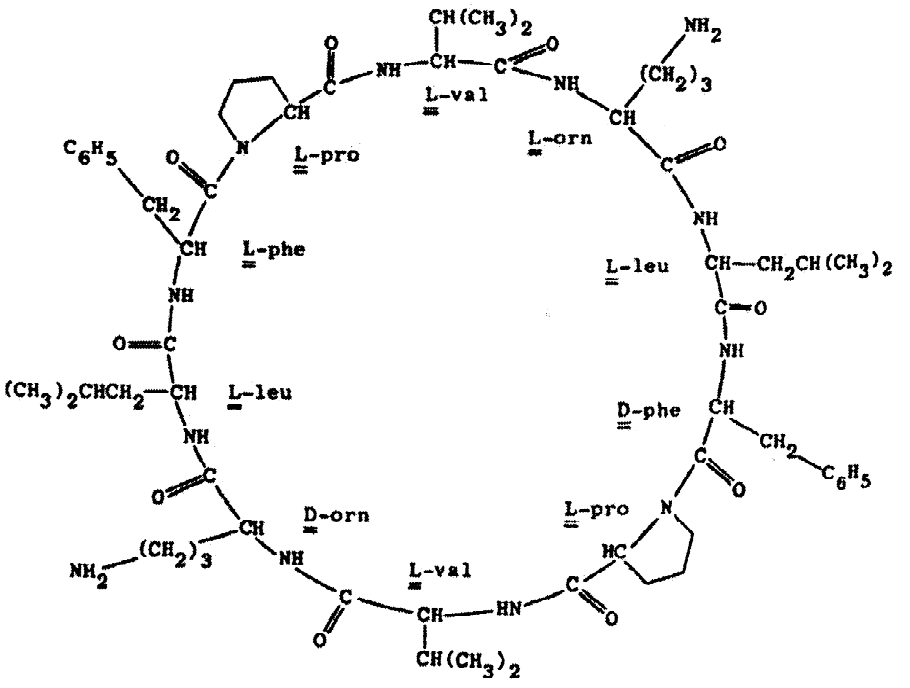


Fig. 6. Total ion chromatogram of the mixture and PEG-600.

of the sample components eluted from the column and PEG-600 were observed separately.

Fig. 7 shows PEG-600 spectrum and sample components spectra at resolution of 3000. Magnified peak profiles of the $[M+H]^+$ ion or the $[M+2H]^{2+}$ ion with its isotopes, for each components, are also



MW : 1140.7

Fig. 5. Chemical structure of gramicidin-S.

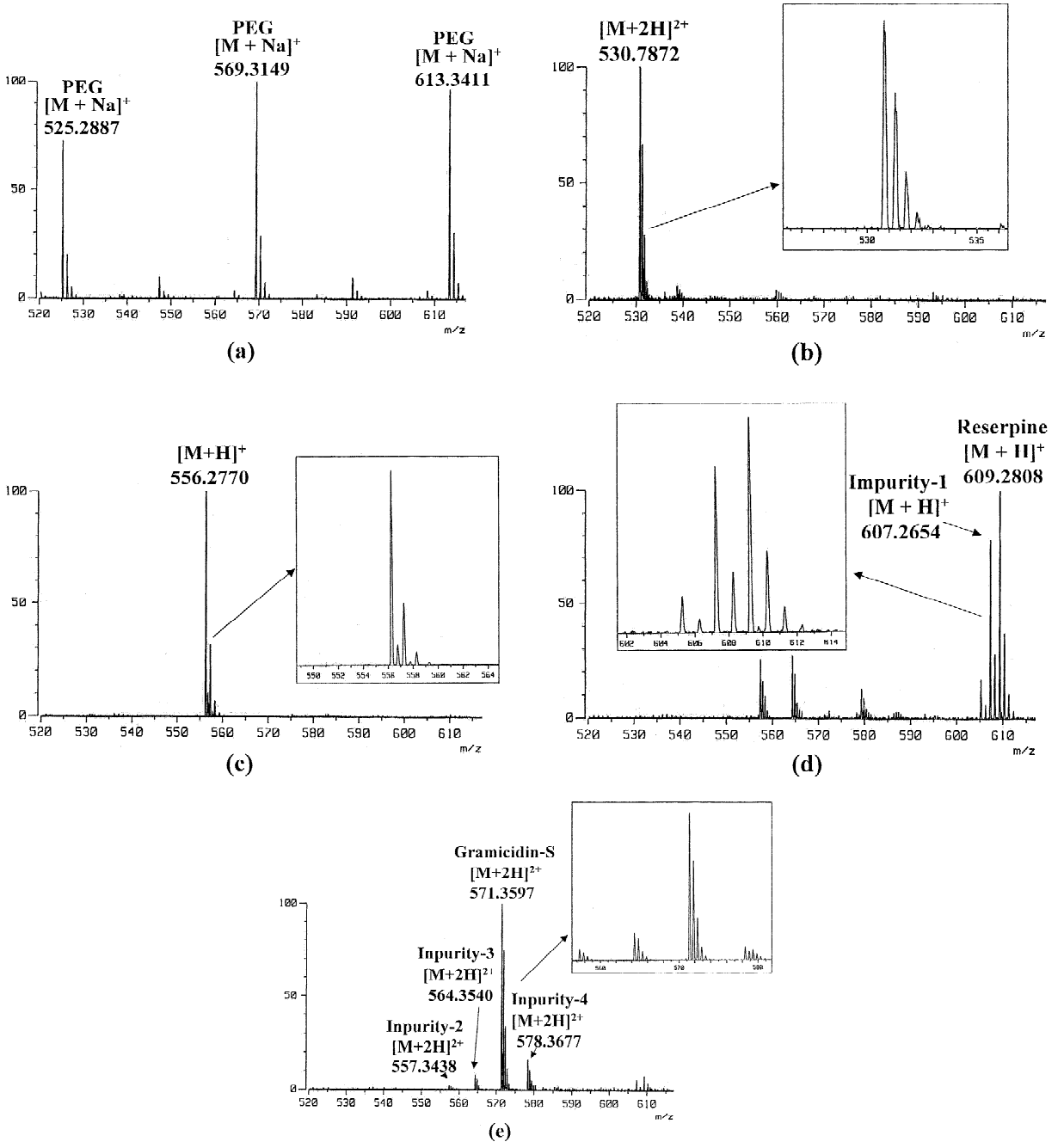


Fig. 7. High-resolution mass spectra of (a) PEG-600, (b) bradykinin, (c) leu-enkephalin, (d) reserpine and impurities, and (e) gramicidin-S and impurities.

shown in Fig. 7. The dynamic mass resolution during measurement was indeed observed to be ~3000. In Fig. 7c, the magnified peaks of the $[M+H]^+$ ion of leu-enkephakin seem to be a doubly charged ion. The doubly charged ion of a dimer probably overlapped with the $[M+H]^+$ ion of leu-enkephakin. Additional details of this ion will not be described in this paper.

Normally, PEG is ionized as $[M+H]^+$ ions by using an ESI source, but in some cases, PEG ions are also observed as Na^+, K^+ and/or NH_4^+ adducts [18]. In this case, $[M+Na]^+$ ions for PEG-600 were observed as shown in Fig. 7a. The mass calibration of the PEG-600 spectrum was carried out, and the result was applied to the sample spectra to calculate the accurate masses of the molecular ions of each component. The PEG-600 spectrum of the 5 min retention time peak was used to calculate the $[M+2H]^{2+}$ ion for bradykinin; the peak at 13.7 min was used to calculate the $[M+H]^+$ ion for leuenkephalin; and the peak at 21 min was used to calculate the $[M+H]^+$ ion for reserpine and $[M+2H]^{2+}$ ion for gramicidin-S. The results of the accurate mass measurement are shown in Table 1. The accurate masses of the sample components were observed with good accuracy, with errors of −2.7 mmu (2.5 ppm) for bradykinin, −0.1 mmu (0.2 ppm) for leuenkaphalin, −0.4 mmu (0.7 ppm) for reserpine and −2.2 mmu (1.9 ppm) for gramicidin-S. The accurate masses of leu-enkephalin and reserpine were calculated as [M+H], and those of bradykinin and gramicidin-S were calculated as [M+2H] which is twice the accurate masses of $[M+2H]^{2+}$ ions.

The accurate mass of the impurity-1 was 607.2654, which corresponds to the elemental composition of $C_{33}H_{39}O_9N_2$. This result suggests that the impurity corresponds to an unsaturated molecular component of reserpine, as described above. However, this result is inconsistent with the relationship of the retention time between reserpine and impurity-1. If impurity-1 has a structure which corresponds to an unsaturated molecular component of reserpine, the retention time of the impurity-1 should be shorter than reserpine. The structure of the impurity-1 could not be decided in this experiment.

The calculated elemental compositions of impurities 2, 3 and 4 were $C_{59}H_{92}O_{10}N_{12}$, $C_{58}H_{90}O_{12}N_{12}$ and $C_{61}H_{96}O_{10}N_{12}$, respectively. These results support the author's consideration, as described above. The concentrations of these impurities are expected very low, because the purity of gramicidin-S is ~97%. We can conclude that this system is useful for the accurate mass measurement of low-concentration impurities in complex mixture with nano-HPLC separation.

The mass accuracy obtained using this system was very good, with errors within 5 ppm of the known molecular masses of sample compounds. These results are nearly the same or much better than those obtained with TOF instruments [19].

4. Conclusion

Accurate mass measurement was straightforward using a multiple sprayer nano-ESI arrangement on a double-focusing magnetic sector instrument combined with nano-scale HPLC. We demonstrated that it is possible to measure the accurate masses of a small amount of mixture sample. Furthermore, it was

Table 1
Accurate mass measurement results for the components in the mixture

Compound name	Measured mass	Calculated mass	Composition	Error, mmu (ppm)
Bradykinin	1061.5743 ([M+2H])	1061.5770	$C_{50}H_{75}O_{11}N_{15}$	−2.7 (2.5)
Leu-enkephalin	556.2770 ([M+H])	556.2771	$C_{28}H_{38}O_7N_5$	−0.1 (0.2)
Reserpine	609.2808 ([M+H])	609.2812	$C_{33}H_{41}O_9N_2$	−0.4 (0.7)
Impurity-1	607.2654 ([M+H])	607.2656	$C_{33}H_{39}O_9N_2$	−0.2 (0.3)
Gramicidin-S	1142.7194 ([M+2H])	1142.7216	$C_{60}H_{94}O_{10}N_{12}$	−2.2 (1.9)
Impurity-2	1128.7080 ([M+2H])	1128.7060	$C_{59}H_{92}O_{10}N_{12}$	2.0 (1.7)
Impurity-3	1114.6876 ([M+2H])	1114.6903	$C_{58}H_{90}O_{10}N_{12}$	−2.7 (2.4)
Impurity-2	1156.7354 ([M+2H])	1156.7372	$C_{61}H_{96}O_{10}N_{12}$	−1.8 (1.6)

not necessary to consider the difference of ionization efficiency between sample compounds and the reference compound. The mass accuracy obtained using this system was very good, with errors within 5 ppm of the known molecular masses of the sample compounds. The elemental compositions of several impurities of standard compounds were also obtained with good accuracy. These results confirm that this system is useful for the accurate mass measurement of low-concentration impurities in complex mixture with nano-HPLC separation.

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