Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
Journal of Pharmaceutical and Biomedical Analysis 41 (2006) 707–713
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

On-line sample enrichment system coupled to electrospray ionization time-of-flight mass spectrometry (ESI–TOF–MS)

Masahiko Okamoto*, Kazuko Yamashita, Kiyoshi Nakai

Environmental Health Science Laboratory, Sumitomo Chemical Co. Ltd., 3-1-98 Kasugade-naka, Konohana-ku, Osaka 554-0022, Japan

Received 11 October 2005; received in revised form 23 January 2006; accepted 28 January 2006
Available online 7 March 2006

Abstract

In order to enhance the sensitivity of LC–MS, a novel on-line sample enrichment system was integrated by inserting a column-switching device containing a peak parking loop after conventional LC column and UV detector. The system consists of conventional LC, micro-LC, a parking loop, and a micro-precolumn, all connected through one eight-port switching valve. An analyte peak of interest detected on the conventional LC is stored in the parking loop with an appropriate amount of added water. The analyte is then directly transferred to the micro-precolumn to be concentrated, and then eluted from the precolumn with linear gradient and separated by the micro-LC column. The sensitivity of LC–MS can be increased by a factor of up to a 100. By combining the micro-LC column with ESI–TOF–MS, some structural information including accurate molecular weight were obtained from analyte samples in the pmol range.
© 2006 Elsevier B.V. All rights reserved.

Keywords: Micro-LC; ESI–TOF–MS; Valve-switching; Accurate mass measurement; On-line concentration

1. Introduction

A comprehensive study has been undertaken to isolate and characterize impurities, degradation products, and metabolites by spectroscopic and spectrometric techniques in the fields of the agricultural and pharmaceutical products. Stringent new purity requirements from the regulatory authorities necessitate that an impurity profile study be carried out for any final product to identify and characterize all unknown impurities present at levels even below 0.05% of the UV peak area using LC analysis [1].

The need to employ ancillary techniques to confirm the identity of peaks eluted from a column was recognized early in the development of chromatography. It was soon found that the process of collecting and transferring a sample of the column eluent containing a solute peak to an appropriate spectrometer was tedious, clumsy, and often inefficient. Furthermore, it is possible for the impurities to be degraded during sample extraction and purification [2]. As a result, the concept of connecting the chromatograph directly to an appropriate spectrometer was developed to eliminate the manual solute transfer. It appears that the mass spectrometer has emerged as the most useful ancillary technique to LC for structural elucidation and solute identification. As an interface for coupling LC with MS, ESI is common and widely used at present; however, it is often difficult to accurately measure a spectrum peak smaller than 0.1%, which is the identification level required by the ICH guideline [2].

It is well known that increased mass sensitivity due to miniaturization of the column is useful in trace analysis and is especially favorable in the analysis of precious samples [3]. However, valve injection spoils most of the samples and thus is not preferred in the analysis of valuable samples, such as components present in blood serum. The precolumn concentration method is an effective solution to this problem. In this method, adequate volume of sample solution is passed through the micro-precolumn prior to the chromatographic run and all the concentrated solutes in the precolumn are then subjected to chromatographic separation [4]. Solutes of interest can be effectively concentrated in the precolumn by selecting the proper packing material and altering the properties of the matrix solution. In addition, the precolumn concentration method overcomes the drawback of the low concentration sensitivity inherent in micro-LC. For example, for a solute concentration of 1 ppb, 1 ng of the solute is separated for each 1 mL of the sample solution

* Corresponding author. Present address: Organic Research Laboratory, Sumitomo Chemical Co. Ltd., 3-1-98 Kasugade-naka, Konohana-ku, Osaka 554-0022, Japan. Tel.: +81 6 6466 5172; fax: +81 6 6466 5459.
E-mail address: okamotom@sc.sumitomo-chem.co.jp (M. Okamoto).

0731-7085/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2006.01.052

passed through the precolumn, and recovery is perfect. On the other hand, time-of-flight mass spectrometry (TOF–MS) is recommended for micro- or nano-LC column separations, since features of the equipment enable it to cope with the large amounts of data usually generated during such fast, high-resolution separations. Advanced digital electronics, which ensure fast and accurate data handling over a more or less “unlimited mass range”, make mass spectrometers the instruments of choice to sensitively cope with low flow rate, high-speed analyses, and often very narrow bandwidths without risking a loss of valuable information. In addition to the wide mass range, the high-speed data acquisition rate makes the TOF–MS especially useful and attractive among micro-separation detection techniques [5].

A novel on-line preparation system for LC–ESI–TOF–MS with a column-switching LC system was therefore developed for analyzing impurities present at levels even below 0.05% of the UV peak area using LC analysis. The system consists of conventional LC, micro-LC, a parking loop, and a micro-precolumn, which are all connected through one eight-port switching valve. An analyte peak of interest detected on the conventional LC is stored in the parking loop with an appropriate amount of added water. The analyte in solution is then directly transferred to the micro-LC precolumn for concentration, and then eluted from the precolumn with linear gradient and separated by the micro-LC column. The micro-LC column in the final step allows ESI–TOF–MS to increase sensitivity by several 100-fold over that of conventional LC/MS, and some structural information including accurate molecular weight were obtained from an amount of analyte in the pmol range.

In this paper, details of the system and applications to pharmaceutical products are presented.

2. Experimental

2.1. Reagents and materials

HPLC-grade acetonitrile and distilled water were obtained from Nacalai Tesque (Kyoto, Japan). Bradykinin, warfarin, flurbiprofen, chloropheniramine maleate, and propranolol were purchased from Wako Pure Chemical Industries (Osaka, Japan). Several 2,2,6,6-tetraalkylpiperidinyl derivatives were used as the calibrants for accurate mass measurement [6].

Other reagents were of guaranteed reagent grade and were obtained from Nacalai Tesque (Kyoto, Japan). All reagents were used without any further treatment, unless otherwise noted. Water used in all experiments was deionized and purified by a Milli-Q purification system from Millipore (Bedford, MA, USA).

The stationary phase of the conventional column employed in this work for the separation of the component of interest was SUMIPAX ODS A-212 (particle diameter, 5 μm; i.d., 6 mm; length, 150 mm), while Develosil C30-UG beads (particle diameter, 30 μm; Nomura Chemical, Seto, Japan) were packed into polyether ether ketone (PEEK) tubing (i.d., 0.3 mm; length, 35 mm) that was used as the micro-LC precolumn (referred to as the precolumn). PEEK tubing (i.d., 0.3 mm; length, 150 mm) packed with Develosil SR C18 beads (particle diameter, 5 μm; GL Sciences, Tokyo, Japan) was used as the separation micro-LC column (referred to as the micro-LC column). PEEK tubing (i.d., 0.75 mm; length, 5000 mm) was also used as a parking loop.

2.2. Chromatographic equipment

A schematic diagram of the instrument setup is shown in Fig. 1. The system consists of three LC instruments: a conventional LC column (Cl), a precolumn (C2), a micro-LC column (C3), and a parking loop, which are connected through a Valco eight-port switching valve (Cheminert C2, product no. C2-1008).

In the conventional LC column section of the apparatus, an LC pump (P1) (LC-6A; Shimadzu, Kyoto, Japan), which was controlled by a gradient controller (SCL-6B; Shimadzu, Kyoto, Japan), delivered the mobile phase (M1). The effluent was monitored with a variable-wavelength UV detector (D1) (SPD-6A; Shimadzu, Kyoto, Japan). Injection was carried out manually with a six-port injection valve (7125; Reodyne, Berkeley, CA, USA) equipped with a stainless-steel sample loop (10 μL).

In the precolumn section, another pump (P2) (LC-6A; Shimadzu, Kyoto, Japan) delivered the mobile phase (normally water) into a parking loop for adsorption of the compounds of interest on the precolumn.

In the micro-LC section, an LC pump (P3) (LC-6000; Hitachi, Tokyo, Japan) delivered the mobile phase (M2) with a flow rate of 5 μL/min for elution of the compounds of interest from the precolumn. Before being passed to the LC–MS interface, the compounds of interest were re-chromatographed on the micro-LC, monitoring with a UV detector (D2) (Spectra 200; Spectra Physics, Mountain View, CA, USA) with a micro-cell (cell volume, 0.6 μL). The introduction of the micro-LC to ESI–TOF–MS was performed with a pneumatic splitter (MS-PNS; JEOL, Tokyo, Japan) in order to reduce the flow rate from 500 to 6 μL/min. LC gradient program used for this study for both conventional and micro-LC is shown in Table 1.

2.3. Column-switching procedure

The valve-switching procedure was as follows.

In the first stage, the three columns were run independently. The sample solution was injected at the injector and eluted from the conventional LC column (C1), peaks being detected at the

Table 1
LC gradient program used for this study

Time (min)	Mobile phase A	Mobile phase B
0.0	90	10
2.0	90	10
3.0	50	50
15.0	10	90
25.0	10	90

Mobile phase was delivered at a flow rate of 1 mL/min in the case of conventional LC, and at 5–6 μL/min for the micro-LC.

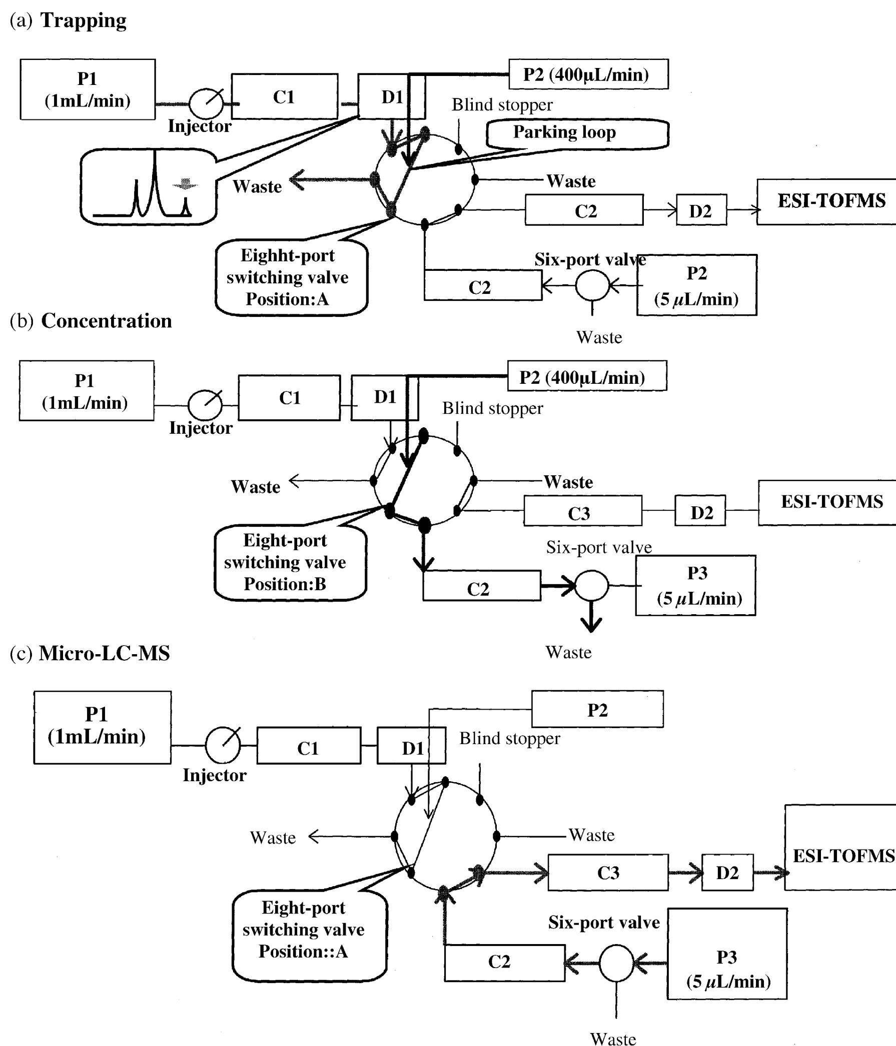

Fig. 1. Schematic diagram of the instrument setup of the on-line sample enrichment system.

detector (D1). When a peak was detected at the detector (D1), the valve was switched so the flow pattern changed to that shown in Fig. 1 (configuration (a): trapping). The valve was adjusted so that the target peak containing eluate flowed into the parking loop connecting the two, where it was stored (configuration (b): concentration). When the detector (D1) showed that sample material was no longer leaving the column, the valve was returned to its original position (configuration (a)).

In the second stage, the compounds in the parking loop were pumped out and sent to the precolumn (C2), while simultaneously being eluted with water in order to improve the adsorption of the compounds of interest on the precolumn (C2). When the flow rate of water was 2.0 mL/min, the dilution ratio of four was attained. The compounds were adsorbed on the precolumn (C2) and the mobile phase was exchanged with the mobile phase (M2). In the third stage, the adsorbed compounds were eluted from the precolumn (C2) with the mobile phase forced by the pump (P3), sent to the micro-LC column (C3) for the re-chromatography, and introduced into the ESI–TOF–MS system. After the completion of these three stages, the procedure was repeated for other samples.

2.4. Mass spectrometer

ESI–TOF mass spectrometric analysis was performed using a time-of-flight mass spectrometer (Mariner; Perseptives Biosystems, Framingham, MA, USA) equipped with an atmospheric pressure ionization (API) interface, throughout the studies.

For accurate mass measurement, a calibrant introduction system designed as shown in Fig. 2 was used. The valve for the calibrant solution was connected just before the interface in order to conduct accurate mass measurement. By switching the valve, the calibrant solution was injected just after the eluent from the micro-LC column was detected as a peak in the UV detector.

3. Results and discussion

3.1. Signal enhancement of the samples with precolumn

The enrichment efficiency of the precolumn was tested using authentic bradykinin as the sample. Separation of bradykinin is shown in Fig. 3. Acetonitrile–water (1:99) containing 0.1% trifluoroacetic acid solution was used as the eluent and detec-

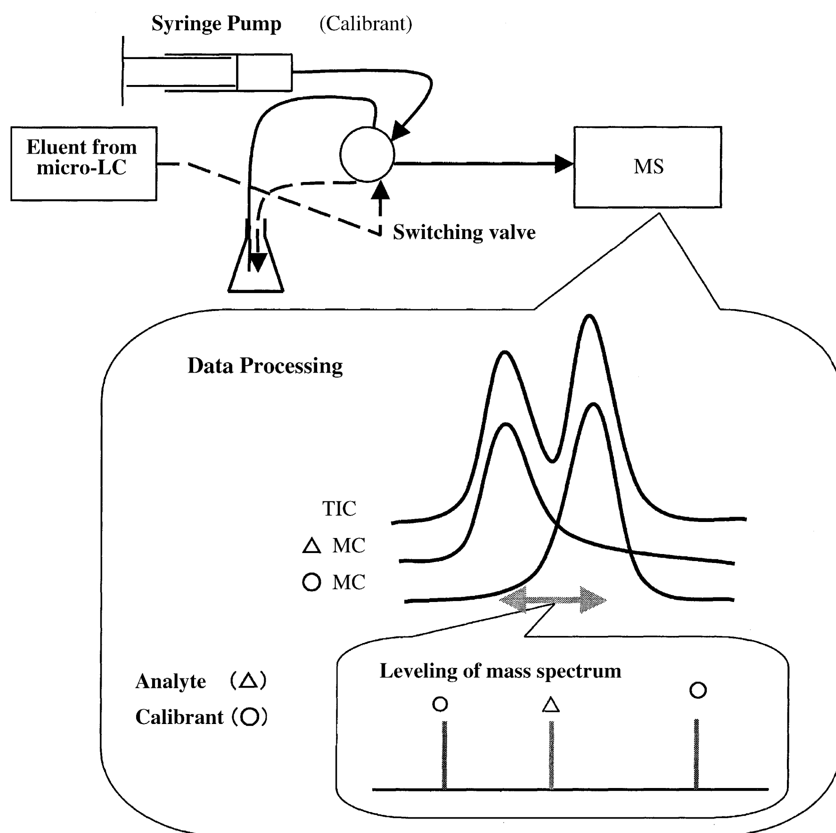

Fig. 2. Diagram of the calibrant introduction system for accurate mass measurement. The calibrant was introduced to MS by switching the flow after introduction of the eluent from the micro-LC. Data processing was conducted to choose the range within which the ion intensity from both calibrant (△) and analyte (○) corresponded to the same level, and to average them.

tion was performed at 220 nm with a UV absorbance detector (D1). Fig. 3a was obtained when a 50 pmol (1 μL) sample of bradykinin was injected into the column, while Fig. 3b was obtained when the sample was concentrated on-line into the precolumn after injection. It is interesting to note that the peak area was increased by ca. 100-fold, and the peak shape estimated by theoretical plate (N) was improved from 12,600 to 25,600, as can be seen by comparison of Fig. 3a and b. In addition, the peak height was increased by 16 times. Since the enrichment of the eluent was achieved using the precolumn, the signal-to-noise ratio (S/N) of the mass spectrum of bradykinin could be considerably improved, as shown in Fig. 3c and d.

3.2. Enrichment efficiency and recovery

The enrichment efficiency of the precolumns was tested using flurbiprofen ($\log P = 4.2$) and warfarin ($\log P = 2.7$) as model substances in order to scrutinize the effect of the hydrophobicity to the enrichment efficiency and recovery. Twenty picomoles (10 μL) of each of the samples (the total sample size was 20 pmol) was loaded into the conventional LC column. The relationship between the eluent passing through the enrichment column on recoveries and the dilution ratio of the eluent stored in the parking loop was scrutinized. The experiments were repeated after replacing the packed C18 precolumns with a packed C30 precolumn. This C30 precolumn has almost the same hydrophobicity as Develosil C18 beads, and its carbon contents correspond to that of Develosil C18 beads. The relative recoveries and dilution ratios are summarized in Table 2. By increasing the water ratio, larger recoveries were obtained, as shown in Table 2. When the eluent in the parking loop was diluted with water to a dilution ratio of four, satisfactory recoveries from 43.7 to 67.0% were obtained. Even at this dilution ratio, no precipitates were observed. Thus, a dilution ratio of four for water added to the parking loop was adopted, unless otherwise noted.

Table 2
Influence of precolumn characteristics on the dilution ratio[a] and recoveries (%)[b]

Analyte	Precolumn	Dilution ratio		
		1.2	1.5	4
Warfarin	A	4.6	15.8	
	B		37.9	43.7
Flurbiprofen	B		10.3	
	C		11.5	67.0

Twenty picomoles (10 μL) of the samples (the total sample size was 20 pmol) were loaded into the system.
[a] Dilution ratio is defined as the flow rate of conventional LC (mL/min) + volume of water added (mL/min).
[b] Recovery was calculated from the following equation: recovery (%) = (peak area obtained with the micro-LC column after passing through the system)/(peak area obtained by direct injection into the micro-LC column) × 100.

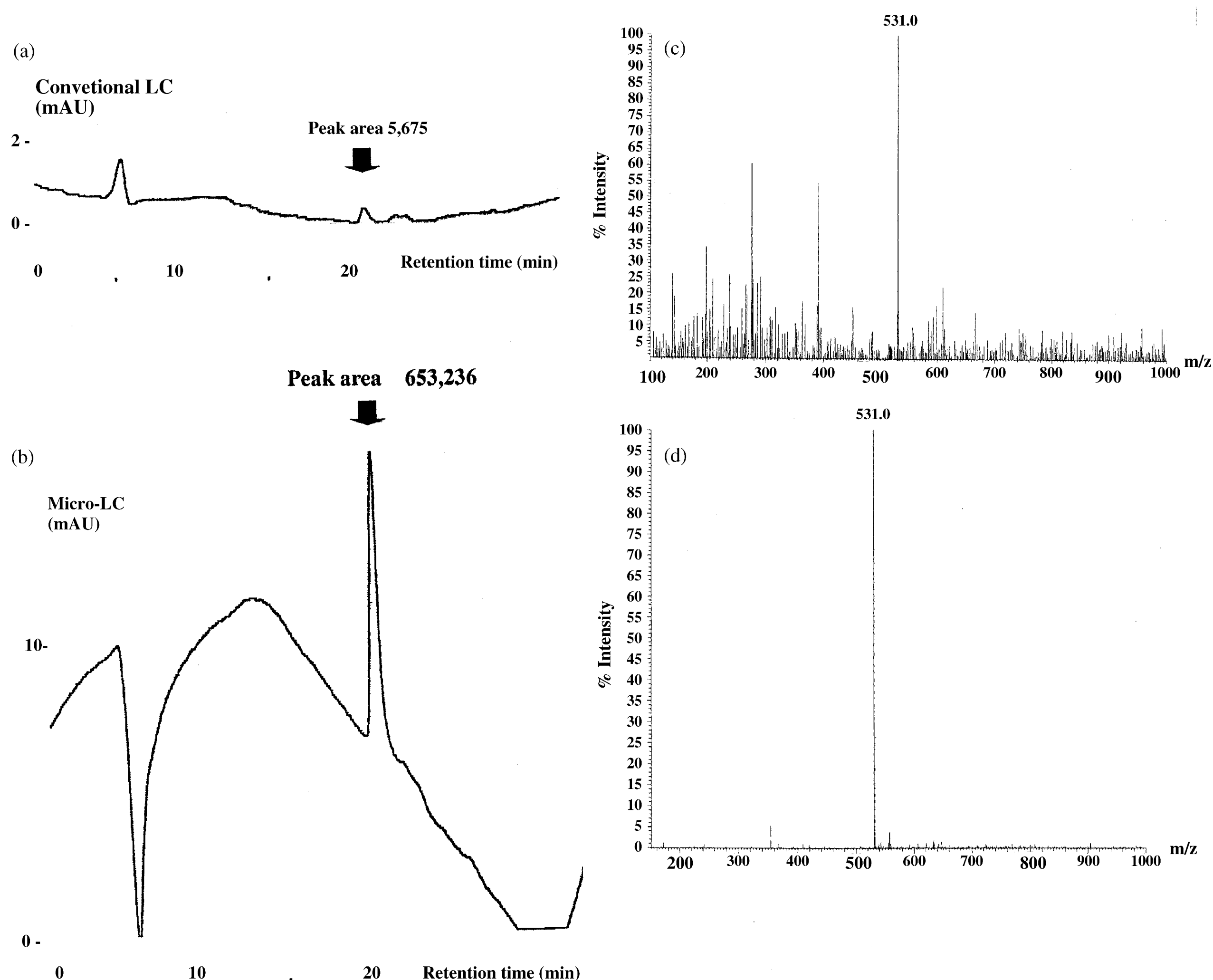

Fig. 3. Enrichment efficiency of the precolumn concentration. Conventional LC conditions: column, L-column ODS (particle dia., 5 μm; i.d., 4.6 mm; length, 150 mm). Mobile phase: 0.1% TFA-H_2O (mobile phase A)/0.08% TFA-acetonitrile (mobile phase B), flow rate: 1 mL/min. The LC gradient program used for the study is shown in Table 1. Micro-LC conditions: column, Develosil SR (particle dia., 5 μm; i.d., 0.3 mm; length, 150 mm). Mobile phase: 0.1% TFA-H_2O (mobile phase A)/0.08% TFA-acetonitrile (mobile phase B), flow rate: 5 μL/min. The micro-LC gradient program used for the study is shown in Table 1: (a) conventional LC (Cl) chromatogram of bradykinin without enrichment (50 pmol injected); (b) micro-LC (C3) chromatogram of bradykinin with precolumn (C2) concentration (50 pmol injected); (c) LC-ESI-MS spectrum of bradykinin from conventional LC without enrichment; (d) LC-ESI-MS spectrum of bradykinin from micro-LC with precolumn concentration.

3.3. Effects of the precolumn packing material particle size

The enrichment efficiency of the precolumn was tested using warfarin as the sample. A theoretical plot of peaks generally shows increasing height with decreasing size of packing materials such as silica gel, while the column head pressure increases. In addition, the flow rate of the mobile phase decreases with decreasing particle size of packing materials, if the column head pressure is constant. In view of these considerations, packing materials of 5 μm particle size are normally used. In the system described here, the flow rate of the precolumn packed with 5 μm silica gel was between 15 and 20 μL/min when the column head

Table 3
Influence of precolumn characteristics on the time required for concentration

Precolumn	Stationary phase	Dimension (mm)	Particle size (μm)	Relative surface area[a]	Concentration time (min)
A	C18	0.3 i.d. × 50	5	1	120
B	C18	0.5 i.d. × 50	5	2.8	60
C[b]	C30	0.3 i.d. × 35	20	0.6	3

The column head pressure was between 19.6 and 24.5 MPa during these experiments.
[a] The relative surface area was estimated using the surface area of A as 1.
[b] The capacity of the stationary phase is comparable with that of C18.

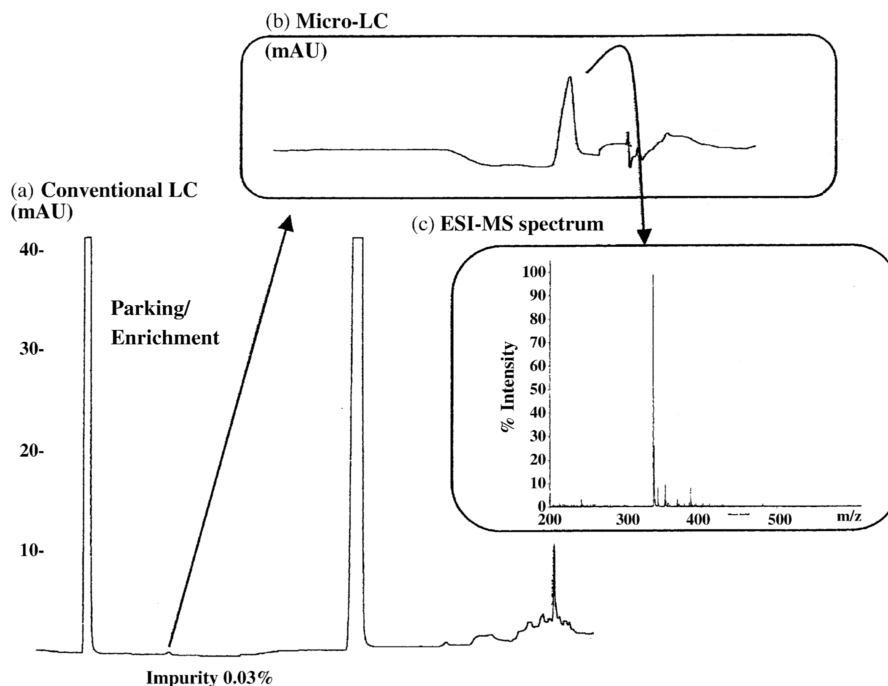

Fig. 4. LC–MS analysis of the impurity of a drug substance with precolumn concentration. Conventional LC conditions: column, Sumipax ODS A-212 (particle dia., 5 μm; i.d., 6.0 mm; length 150 mm). Mobile phase: 5 mM Na_2HPO_4 (mobile phase A)/acetonitrile (mobile phase B), flow rate: 1 mL/min. The LC gradient program used for the study is shown in Table 1. Micro-LC conditions: Develosil SR (particle dia., 5 μm; i.d., 0.3 mm; length 150 mm). Mobile phase: H_2O (mobile phase A)/acetonitrile (mobile phase B), flow rate: 5 μL/min. The LC gradient program used for the study is shown in Table 1.

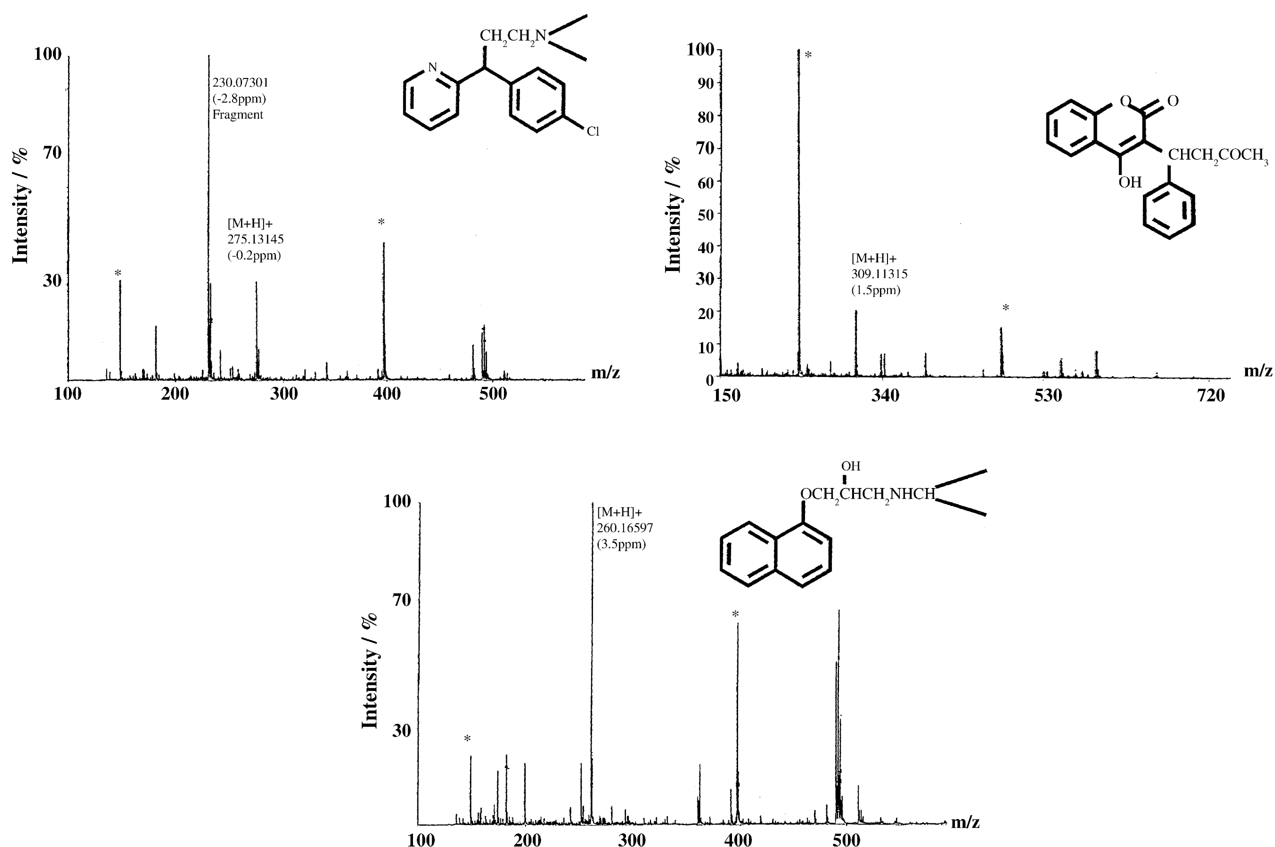

Fig. 5. Accurate mass measurement of drug substances, chloropheniramine (upper), warfarin (middle), and propranolol (lower) with precolumn concentration. The spectra were obtained using this system with 50 pmol of warfarin, 10 pmol of chloropheniramine, and 10 pmol of propranolol. (*) denotes the calibrant ions.

pressure was 24.5 MPa. In this case, it takes 2 h to concentrate 2 mL of the eluent (0.5 mL from the conventional LC column, and 1.5 mL from the water) on the precolumn. In addition, 60 min were needed to concentrate 2 mL of eluent even when the precolumn (i.d., 0.5 mm; length, 50 mm) was used as shown in Table 3. To shorten the analysis time and to minimize the pressure, packing material with larger, 20 μm particles was used in order to increase the flow rate. In the case of the precolumn packed with 20 μm of silica gel, a flow rate of ca. 400 μL/min was achieved with a column head pressure of 19.6 MPa. The time to concentrate 2 mL of the eluent was 3 min, as shown in Table 3. Therefore, we selected 20 μm particle dia. silica gel as the packing material for the precolumn (i.d., 0.3 mm; length, 35 mm) thereafter. The reduction of the concentration time on the precolumn is important when columns of different sizes, such as the separation column (4.6 mm × 150 mm) and the precolumn (0.3 mm × 35 mm), are connected on-line.

3.4. Application to pharmaceutical analysis

The on-line concentration system using a precolumn was applied to the analysis of impurities in drug substances. A 0.3 mm i.d. precolumn packed with C30 (20 μm) was used because it resulted in good recovery for analysis, as described above.

In this system, the compound of interest could be eluted with the mobile phase with a linear gradient of water–acetonitrile (total 30 min) in the final step so that desalting could be attained without incurring a decrease in the sensitivity of MS.

Fig. 4 illustrates the results of an impurity in a drug substance with a concentration of 0.03%. In this case, it was impossible to obtain the spectrum by normal LC–MS analysis, because phosphoric acid, a so-called non-volatile buffer, in the mobile phase was inhibited by ionization. However, a clear mass spectrum of the impurity could be obtained with this system, since the desalting was performed during the precolumn concentration process (Fig. 4c). This system is thus very powerful and offers high sensitivity.

Accurate mass measurement for the determination of elemental formulae can also be done with this system. Warfarin, chloropheniramine, and propranolol were analyzed with this system. The spectra of these compounds are shown in Fig. 5. The accuracies of observed masses were coincident with those of theoretical values within an error of 5 ppm.

4. Conclusion

This work has focused on the evaluation of our new on-line sample enrichment system coupled to LC–ESI–TOF–MS for characterizing impurities in raw drug substances. The technique could be applicable to the investigation of formulated drug substances, where impurities often appear as a result of reaction between the drug substances and the formulation compound, or to samples from degradation studies. These types of adduct can sometimes be unstable during sample extraction and purification. This area is of considerable practical importance in the pharmaceutical industry and is worthy of further study.

The application of the system presented here has led to efficient identification of the impurities without recourse to development of time-consuming isolation and purification procedures. Using conventional methods, the isolation would have required laborious preparative HPLC using large amounts of sample and the final isolated product would also have been prone to contamination from the solvent residues during preparation. The superior sensitivity of LC–MS conducted with the system presented, compared with normal LC–MS detection, has enabled the identification of these impurities using <1 μg of each substance. This work has demonstrated the applicability of these techniques in the analyses of the products of synthetic organic chemistry and is therefore potentially of great value in pharmaceutical analysis and is likely to be an important new approach for regulatory authorities. Additionally, the system presented here can be coupled to many types of mass spectrometers, for example, TOF, sector, quadrupole, ion trap, ion cyclotron resonance, and so on. By choosing the type of mass spectrometer, a variety of information such as accurate mass and MS spectra can be obtained in addition to the molecular weight. Further studies on the development of a fully automated on-line preparation system for LC–MS based on the system presented here are in progress.

Acknowledgement

The authors gratefully acknowledge Prof. Dr. Toyohide Takeuchi at Department of Chemistry, Faculty of Engineering, Gifu University, Japan for valuable discussion on the design of this system.

References

[1] International Conference on Harmonization (ICH), Document on Impurities in New Drug Substances.
[2] E. Gelpí, J. Mass Spectrom. 37 (2002) 241–253.
[3] N. Mistry, I.M. Ismail, M.S. Smith, J.K. Nicholson, J.C. Lindon, J. Pharm. Biomed. Anal. 16 (1997) 697–705.
[4] D. Ishii, K. Hibi, K. Asai, M. Nagaya, J. Chromatogr. 152 (1978) 341–348.
[5] I.M. Lazar, G. Naisbitt, M.L. Lee, Analyst 123 (1998) 1449–1454.
[6] K. Yamashita, M. Okamoto, K. Nakai, Anal. Sci. 19 (2003) 1191–1193.