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On-line desalting–mass spectrometry system for the structural determination of hydrophilic metabolites, using a column switching technique and a volatile ion-pairing reagent

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Abstract

A novel desalting method, using a column switching technique and a volatile ion-pairing reagent, pentadecafluorooctanoic acid, was developed. This system allows hydrophilic and cationic compounds in a nonvolatile buffer to be directly introduced into a mass spectrometer for structural elucidation. The desalting procedure consists of four steps: (1) the fractionation of a target compound from a separation column, (2) the removal of salts with pentadecafluorooctanoic acid on the trap column, (3) the desorption of the compound from the trap column, and (4) the re-equilibration of the trap column with a pentadecafluorooctanoic acid solution. In this procedure, we investigated the methods for optimizing the desalting and re-equilibration steps. Various amino acids, including branched chain amino acids, aromatic amino acids, basic amino acids and methionine, after separation with phosphate buffer on a cation-exchange column, were successively desalted by this method, and were observed as protonated ions by mass spectrometry. This desalting system could be useful for the structural elucidation of unknown hydrophilic compounds eluted by conventional high-performance liquid chromatography methods, such as ion-exchange chromatography, with mobile phases containing nonvolatile salts. As an example, we present the structural elucidation of unknown metabolites in bovine serum.

Keywords: Liquid chromatography–mass spectrometry; On-line desalting; Column switching; Volatile ion-pairing reagent; Hydrophilic metabolite

1. Introduction

High-performance liquid chromatography (HPLC) is a popular analytical method, because it is easy to learn and is not limited by the class of analytical targets, from small molecules to peptides. Therefore, this versatile method has been widely used for studies of pharmacokinetics, residual agricultural chemicals, metabolomics, proteomics and so on [1–4]. The separation is achieved by the fact that certain compounds have different retention rates with a particular column and mobile phase. Various kinds of column matrices with reversed-phase and ion-exchange properties are commercially available. An appropriate buffer is usually used to control the pH of mobile phase to achieve good separation and reproducible results. Among the kinds of buffers, phosphate buffer is one of the most versatile mobile phases for any kind of column. It is frequently used for HPLC analyses, because it has an excellent buffer action over a wide pH range and little UV absorption, and it generates good chromatographic peak shapes. Especially, in situations requiring separation of hydrophilic compounds like metabolites, the use of phosphate buffer with an ion-exchange column and UV detector is very effective.

Quantitative detection and chromatographic profiling of known compounds are based on the area/height count and retention time of each peak compared with its authentic standard. However, it is difficult to elucidate the structures of unknown compounds based on the HPLC. This is commonly accomplished by trial and error comparisons of the retention times with those of authentic standards. Recently, liquid chromatography-mass spectrometry (LC–MS), which can provide information on not only the retention time but also the mass and fragmentation pattern by MS/MS, has become a powerful tool for structural elucidation [5–7]. However, a problem in using MS is that nonvolatile buffers, such as phosphate buffer, cannot be introduced into a mass spectrometer, since the nonvolatile

Abbreviations: HPLC, high-performance liquid chromatography; LC–MS, liquid chromatography–mass spectrometry; PDFOA, pentadecafluorooctanoic acid; TDFHA, tridecafluoroheptanoic acid; Q-TOF, quadrupole time-of-flight; C30, triacontyl-bonded silica

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salts markedly degrade the performance of MS. Therefore, in order to achieve the assignment of unknown peaks from the various commonly used separation modes, the development of a new interface for desalting between HPLC and MS is necessary.

In this paper, we focused on the establishment of LC–MS analysis method for hydrophilic and cationic compounds, detected by a UV detector, using phosphate buffer. As a result, a novel on-line desalting–MS system, which can remove phosphate ions and inorganic cations from the eluate, was developed. For this purpose, a column switching technique was adopted, and pentadecafluorooctanoic acid (PDFOA) [8–12], a highly volatile ion-pairing reagent was used in the desalting process. The optimization of the desalting parameters was investigated, using cation-exchange chromatography as a model of separation with phosphate buffer. Amino acids were used as an example of standard compounds with hydrophilic and cationic properties. By this new desalting method, the protonated ions of amino acids, except for anionic and extremely hydrophilic amino acids, were easily observed. This system was also applied to an analysis of the metabolites in bovine serum, as an example of a biological sample. The peaks detected on a cation-exchange column with sodium phosphate buffer were determined by MS after the desalting procedure. This novel interface will become a powerful tool for the structural elucidation of unknown peaks by UV detection, using versatile phosphate buffer.

2. Experimental

2.1. Chemicals and reagents

HPLC grade methanol and sodium dihydrogen phosphate were purchased from Junsei Chemical (Tokyo, Japan). Phosphoric acid and formic acid were purchased from Kanto Kagaku (Tokyo, Japan). The purified PDFOA, which was re-crystallized from the LC–MS grade product, was a kind gift from Tokyo Kasei Kogyo (Tokyo, Japan). The tridecafluoroheptanoic acid (TDFHA) was also obtained from Tokyo Kasei Kogyo. Amino acids were purchased from Sigma (St. Louis, MO, USA). Distilled water was prepared with a Milli-Q system (Millipore, Bedford, MA, USA). Bovine serum was purchased from Thermo Trace (Melbourne, Australia).

2.2. Samples

A concentration of 0.3 mg/mL valine in distilled water was prepared for the method optimization. Another amino acid was also prepared individually to be at a concentration of 0.3 mg/mL. Glutamic acid, aspartic acid, and tyrosine were dissolved in 0.1% aqueous formic acid, respectively. The other amino acids were dissolved in distilled water.

A 100 μL aliquot of the bovine serum sample was diluted with 400 μL of acetonitrile and then was centrifuged for 15 min at 12,000 rpm by the Eppendorf centrifuge 5417R (Eppendorf Japan, Tokyo, Japan). A 5 μL aliquot of the supernatant was injected onto an analytical column.

2.3. Apparatus

The LC-integrated sample desalting system was composed of Co-Sense for LC-NMR (Shimadzu, Kyoto, Japan), equipped with a system controller (SCL-10Avp), four pumps (P1 and P3: LC-10ADvp, P2 and P4: LC-10ATvp), a UV detector (D: SPD-10Avp), an auto-sampler (SIL-10AF), a column oven (CTO-10ACvp), five sampling loops (L), and three switching 6-port valves (V1, V2 and V3: FCV-12AH). The five sampling loops were connected in parallel. The capacity of each loop could be varied, and the capacity used in this study was 1 mL.

A primesep 100 column (250 mm × 4.6 mm, 5 μm, SIELC Technologies, IL, USA), which is a reversed-phase column with embedded acidic ion-pairing groups, was used as the analytical column. A Develosil ERP20 column (30 mm × 4.0 mm, 20 μm, Nomura Chemicals, Aichi, Japan), in which the stationary phase was triacontyl-bonded silica (C30), was used as the trap column.

MS was performed on a quadrupole time-of-flight (Q-TOF) mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray interface. The instrument was operated in the positive ion mode. The capillary voltage was maintained at 3.0 kV. The voltages of the sample cone, the collision, and the multi channel plate detector were set to 10 V, 8.0 V, and 2200 V, respectively. The flow rates of the nebulizer gas and the cone gas were adjusted to 500 L/h and 50 L/h, respectively. The desolvation and the source temperature were 120 °C and 80 °C, respectively. The acquired mass range was set from 50 to 450 a.m.u. The acquisition duration per spectrum was set to 1.0 s, with a delay of 0.1 s. One-fifth of the effluent was introduced into the mass spectrometer.

2.4. Analytical chromatographic conditions

The temperature of the analytical column (Ca) was maintained at 40 °C. The UV detector was operated at a wavelength of 210 nm. Buffer A (2 mM sodium phosphate pH 2.0), and buffer B (100 mM sodium phosphate pH 1.5) were used as mobile phases. The flow rate was 1.0 mL/min throughout the analysis. After an 8 min isocratic run at 100% of buffer A, the ratio of buffer B was linearly increased to 65% for 35 min, to 100% of buffer B for 40 min, and then 100% of buffer B was maintained for 10 min. Column equilibration was carried out with 100% of buffer A for 15 min. A 10 μL aliquot of the sample solution was injected onto the column for analyses, using an auto-sampler (Fig. 1-I).

2.5. On-line desalting procedure

The on-line desalting system consisted of four steps. The valve-switching procedure and the flow path of each step are illustrated in Fig. 1.

2.5.1. Fractionation of the target compound

When the peak of a target compound was detected at the UV detector (D), the V1 valve was switched, as illustrated in Fig. 1-II, for the fractionation of the targeted compound in the 1 mL loop (L).