Modified Solvent System for Reversed-Phase Liquid Chromatography/Mass Spectrometry

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Liquid chromatographic influence on changing the solvent system from the non-volatile buffer containing inorganic salts or phosphates to some volatile buffers suitable for the direct coupled liquid chromatography/mass spectrometry was investigated by means of the reversed-phase high-performance liquid chromatography. On a separation of ten methylxanthines including methyluric acids, almost the same chromatograms of the mixture were obtained among all buffers examined. Similar modifications on the solvent system were tried to separate a mixture of nine non-steroidal anti-inflammatory drugs and anthranilic acid. In an application of the modified buffer to the FRIT-FAB liquid chromatography/mass spectrometry, informative negative ion mass spectra of acetyl-coenzyme A and structurally related compounds could be obtained by using diethanolamine-acetic acid buffer. Diethanolamine, its acetate in the buffer, acts effectively as a matrix for the FRIT-FAB liquid chromatography/mass spectrometry.

1. Introduction

Today, liquid chromatography/mass spectrometry (LC/MS) has become one of the most powerful tools in the field of analytical chemistry through an appearance of commercially available LC/MS instruments with the direct coupled interfaces such as thermospray (TSP)¹⁾, direct liquid introduction²⁾, and direct coupling of micro

(or semi-micro) liquid chromatography with fast atom bombardment (FAB) mass spectrometry using a stainless steel frit (FRIT-FAB)³⁾. Wide utility of the LC/MS technique has been expected for the analysis of polar, thermally labile or high-molecular-weight compounds in the mixed medium, especially in the biomedical and biological fields. However, some limitations and difficulties still remain on the practical applica-

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tion at a choice of the solvent system which may affect fully on the chromatographic and mass spectrometric performance.

As is well known, buffers containing non-volatile inorganic salts or phosphates are frequently used as the eluant on the reversed-phase liquid chromatography (LC) that has gained great popularity in the conventional LC analysis. The non-volatile buffers allow satisfactory efforts for the liquid chromatographic separations of many types of compounds, but can not be applied to the direct coupled LC/MS without any damage of the instrument. To cope with this problem, it needs to modify the solvent system from the non-volatile buffer to the volatile one suitable for the LC/MS analysis. Among several parameters which affect the LC/MS performance, the choice of the buffer is a fundamental item because the compounds affect not only the chromatographic separation but also the spectral characteristics and sensitivity of the analyte. In principle, the choice of the solvent system must be considered according to the differences of the ionization process, its mode, and the ion detection mode on each LC/MS instrument together with properties of the analyte itself. Some papers⁴⁻⁶⁾ related to the selection of the solvent systems for the LC/MS analysis have been presented; however, these reports are limited in the study of optimization or ionization mechanism on the TSP LC/MS.

Our first interest in this study is to see the chromatographic influence on changing the solvent system from a non-volatile buffer to volatile ones which are expected to be used for the LC/MS analysis. For this purpose, methylxanthines including methyluric

acids, non-steroidal anti-inflammatory drugs, and acetyl-coenzyme A (acetyl-CoA) and structurally related compounds in each mixture were examined for their separations by means of the conventional LC analysis. In addition, one of the modified buffers was applied to the FRIT-FAB LC/MS measurement of acetyl-CoA and related compounds.

2. Experimental

Chemicals and Reagents

Paraxanthine, 1-methylxanthine, 3-methylxanthine, 7-methylxanthine, 1, 3, 7-trimethyluric acid, 1, 7-dimethyluric acid, and 7-methyluric acid were purchased from Fluka AG. Naproxen, indomethacin, flufenamic acid, ibuprofen, mefenamic acid, acetyl-coenzyme A, and adenosine were purchased from Sigma Chemical Co. Caffeine, theobromine, theophylline, anthranilic acid, monoethanolamine, and diethanolamine were obtained from Nakarai Chemicals, LTD. Following chemicals were: sulindac (Kyorin Pharmaceutical Co.), alclofenac (Chugai Pharmaceutical Co.), diclofenac (Ciba-Geigy), coenzyme A (Kohjin Co.), and adenosinediphosphate (Boehringer Mannheim). Flunixin was supplied by courtesy of Equine Research Institute, Japan Racing Association. Monoethanolamine and diethanolamine were re-distilled under the reduced pressure after drying for about two weeks with sodium hydroxide. Other chemicals and reagents, analytical grade, were obtained from commercial sources.

Apparatus

Shimadzu LC-4A equipped with SPD-2A variable wavelength ultraviolet spectrometer and Chromatopac C-R3A

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(Shimadzu) were used for LC analyses. The chromatographic separations were effected by using 150 x 4.6 mm i.d. Cosmosil 5C₁₈ column with 50 x 4.6 mm i.d. Cosmosil 10C₈ pre-column (Nakarai Chemicals LTD).

A JMS-DX 303 double-focusing mass spectrometer with JMA-DA 5000 data system (JEOL) was used. A Familic-100 semi-micro liquid chromatograph (JASCO) with a Rheodyne 7125 injector and a UVIDEX-100 variable wavelength ultraviolet spectrometer (JASCO) was directly connected to the mass spectrometer through a FRIT-FAB LC-MS interface (JEOL) for LC/MS measurements. The block diagram of the system is shown in Fig. 1. A stainless steel frit was connected with a fused silica capillary tube (60 μ m i.d., 1 m length). A semi-micro column, SFPK-ODS 5S25 (250 x 1.7 mm i.d., JEOL) was used at a flow rate of 150 μ l/min and one portion (1/30 volume) of the eluent was introduced into the ion source through a splitter. Other conditions were as follows: ion source temperature 50°C, xenon beam energy 4 keV, accelerating

voltage 3 kV, scan range m/z 50 to 1000, and scan speed 3 sec.

3. Results and Discussion

3.1 LC Separation of Methylxanthines and Methyluric acids

It was tried to separate a mixture of ten methylxanthines including methyluric acids, which are known as urinary metabolites of caffeine, according to the LC conditions reported by Scott et al.⁷⁾ Separation of the compounds was carried out on a reversed-phase column (Cosmosil 5C₁₈) under a gradient elution. The solvent system consists of eluant A being 1% tetrahydrofuran in 10 mM sodium acetate-acetic acid (pH 4.8) and eluant B being 15% acetonitrile-1% tetrahydrofuran in 10 mM sodium acetate-acetic acid (pH 4.8). Eluant A was pumped for the first 5 min and mixed in eluant B at rates of 5%, 10% and 2% increase per min for 5 min each step. Other conditions were: flow rate at 1.5 ml/min, wavelength at 280 nm, and oven temperature at 35°C. Figure 2a shows a chromatogram of the mixture obtained

Fig. 1. Block diagram of the FRIT-FAB LC/MS system. 1: LC pump, 2: line filter, 3: injector, 4: column, 5: splitter, 6: syringe, 7: waste reservoir, 8: connection pipe, 9: glass flange, 10: FRIT-FAB probe, 11: target, 12: ion gun, 13: ion source.

Fig. 2. Separation of methylxanthines and methyluric acids. Step gradient elution using following solvent systems.

(a): 1% tetrahydrofuran in 10 mM sodium acetate-acetic acid (pH 4.8) and 15% acetonitrile-1% tetrahydrofuran in 10 mM sodium acetate-acetic acid (pH 4.8); (b): 1% tetrahydrofuran in 10 mM monoethanolamine-acetic acid (pH 4.8) and 15% acetonitrile-1% tetrahydrofuran in 10 mM monoethanolamine-acetic acid (pH 4.8).

The chromatographic conditions are as described in text.

Peaks are: 1, 7-methyluric acid; 2, 7-methylxanthine; 3, 3-methylxanthine; 4, 1-methylxanthine; 5, theobromine; 6, 1, 7-dimethyluric acid; 7, paraxanthine; 8, theophylline; 9, 1, 3, 7-trimethyluric acid; 10, caffeine.

Fig. 3. Retention data of methylxanthines and methyluric acids on various buffer systems. The chromatographic conditions are as described in text. Compound numbers are: 1, 7-methyluric acid; 2, 7-methylxanthine; 3, 3-methylxanthine; 4, 1-methylxanthine; 5, theobromine; 6, 1, 7-dimethyluric acid; 7, paraxanthine; 8, theophylline; 9, 1, 3, 7-trimethyluric acid; 10, caffeine.

under these conditions. A complete separation was performed as described in the reference paper⁷⁾.

As a modification of the solvent system, four volatile buffers including ammonium formate-formic acid, ammonium acetate-acetic acid, ammonium oxalate-oxalic acid and monoethanolamine-acetic acid were examined for separation of the mixed compounds instead of using sodium acetate-acetic acid buffer. Other conditions were as quite the same as the original condition described above. The analytical data are summarized in Fig. 3 and a typical chromatogram obtained from monoethanolamine-acetic acid buffer is shown in Fig. 2b. As can be seen in these figures, almost the same separation of the mixed compounds were obtained among all buffers examined. These results suggest that the simple modification of the solvent system from the non-volatile buffer to the volatile ones may be possible only by exchanging components of the buffer. The ammonium acetate buffer has been used as the most popular eluant for the TSP LC/MS. Further, we found that the monoethanolamine-acetic acid buffer is useful for the FRIT-FAB LC/MS measurement, and it will be discussed below.

3.2 LC Separation of Non-Steroidal Anti-Inflammatory Drugs

A similar modification of the solvent system was attempted to separate a mixture of nine non-steroidal anti-inflammatory drugs and anthranilic acid. A separation of the compounds based on a method by Battista et al.⁸⁾ was achieved on a solvent programming under the following conditions. Eluant A was acetonitrile/0.05 M

sodium acetate-acetic acid, pH 4.5 (45:55) and eluant B was acetonitrile only. Eluant B was mixed with eluant A to make a linear running gradient, 0 to 30% B/A, at a rate of 2% increase per min. Other conditions were: flow rate at 1.0 ml/min, wavelength at 262 nm, and oven temperature at 35°C. A chromatogram of the mixture obtained under these conditions is shown in Fig. 4a.

Three modified buffers, ammonium formate-formic acid, ammonium acetate-acetic acid and monoethanolamine-acetic acid, were examined in place of the original non-volatile buffer at the same pH and the same concentration as described above. In these experiments, acetate buffers gave similar chromatographic separations with the original buffer (Fig. 4b), whereas the buffer consisted of ammonium formate-formic acid exhibited remarkable tailing on each peak of flunixin, flufenamic acid and mefenamic acid (Fig. 4c). A similar phenomenon was also observed on the chromatogram for the same compounds when monoethanolamine-formic acid buffer was used. Ammonium formate-acetic acid buffer gave peaks with less tailing. From these results, it is clear that the tailing on the peaks of some compounds having a N-phenylanthranilic acid skeleton in their structures is caused by the presence of free or dissociated formic acid in the buffer.

3.3 LC Separation of Acetyl-CoA and Related Compounds

A mixture of acetyl-CoA, CoA, adenosine-diphosphate and adenosine was separated based on a method by Halvorsen et al.⁹⁾. The chromatographic separation of the compounds was carried out on a reversed-phase column using 20 mM potas-

Fig. 4. Separation of non-steroidal anti-inflammatory drugs. Linear gradient elution using following solvent systems.

(a): acetonitrile/0.05 M sodium acetate-acetic acid, pH 4.5 (45:55) and acetonitrile; (b): acetonitrile/0.05 M monoethanolamine-acetic acid, pH 4.5 (45:55) and acetonitrile; (c): acetonitrile/0.05 M ammonium formate-formic acid, pH 4.5(45:55) and acetonitrile. The chromatographic conditions are as described in text. Peaks are: 1, anthranilic acid; 2, sulindac; 3, alclofenac; 4, flunixin; 5, naproxen; 6, diclofenac; 7, indomethacin; 8, flufenamic acid; 9, ibuprofen; 10, mefenamic acid.

sium phosphate buffer (pH 6.0)-methanol (91: 9) as an eluant at a flow rate of 1.0 ml/min, detection wavelenght of 254 nm. and oven temperature of 35°C. Under these conditions, all compounds in the mixture were successfully separated as shown in Fig. 5a. Adenosine-diphosphate appeared as a peak with small tailing on the chromatogram.

To modify the solvent system, 20 mM monoethanolamine-acethic acid buffer (pH 6. 0) was used instead of the potassium phosphate buffer for the LC separation of the mixed compounds under the conditions as described above. Under the modified conditions, three compounds except for adenosine were eluted faster than those at the original condition (Fig. 5b). On the chromatogram, adenosine-diphosphate was hardly retained on the column and appeared as a tailing

peak. By changing the pH value of the buffer and the mixing ratio between the buffer and methanol, we could get a better separation on acetyl-CoA, CoA and adenosine, but could not improve the tailing on the peak of adenosine-diphosphate (Fig. 5c).

3.4 FRIT-FAB LC/MS Measurement of Acetyl-CoA and Related Compounds

CoA and its acylated analogues are complex molecules containing numerous thermally-labile, polar groups. Therefore, informative mass spectra of the compounds could not be observed until the application of FAB mass spectrometry by Millington et al.^{10). In the FAB mass spectra, characteristic ions related to the molecular species were observed on both the positive and negative ion modes. A group of the same investigators¹¹⁾ have also applied various}

Fig. 5. Separation of acetyl-CoA and related compounds. Solvent systems: (a), 20mM potassium phosphate (pH 6.0)/methanol (91:9); (b), 20 mM monoethanolamine-acetic acid (pH 6.0)/methanol (91:9); (c), 20 mM monoethanolamine-acetic acid (pH 7.0)/methanol (95:5). The chromatographic conditions are as described in text. Peaks are: 1, adenosine-diphosphate; 2, CoA; 2', impurity of CoA; 3, adenosine; 4, acetyl-CoA; 4', impurity of acetyl-CoA.

acyl-CoA compounds to the TSP LC/MS measurement on a reversed-phase column using 0.1 M ammonium acetate-methanol as the solvent system. Although their TSP mass spectra in the positive ion mode exhibited some characteristic fragment ions containing the acyl group and the adenosine moiety as prominent ions, any $[M+1]^+$ ions or the cluster ions corresponding to the molecular ion species were not observed.

As one of the applications in this study, we tried to measure FAB mass spectra of acetyl-CoA and related compounds by means of the FRIT-FAB LC/MS. Separations of the compounds were performed on a reversed-phase semi-micro column using 100 mM diethanolamine-acetic acid buffer (pH 7.0)-methanol (85:15). The FAB mass spectra were detected on both the positive and negative ion modes. Figure 6 shows a typical chromatogram obtained by the selected ion monitoring on each $[M-1]^-$ ion of the compounds in the negative ion detection. On the chromatogram, all compounds in the mixture were completely separated. Figure 7 shows the FAB mass spectra of each compound in the negative ion detection. The $[M-1]^-$ ions, as prominent ions, and diagnostic fragment ions corresponding to the partial structures of each molecule were observed as shown in these figures. On the other hand, FAB mass spectra of the compounds in the positive ion detection gave relatively complex patterns containing adduct ions and lower sensitivity than those of the negative ion detection.

It is known that addition of glycerol or thioglycerol at suitable concentration, below 10% (*v/v*) in most cases, into the eluant is essential for the FRIT-FAB LC/MS

Fig. 6. Selected ion recordings of acetyl-CoA and related compounds by the FRIT-FAB LC/MS. Each $[M-1]^-$ ion of the compounds was detected on the negative ion mode. Column, SFPAC-ODS 5S25 (250 x 1.7 mm i. d.); eluant, 100 mM diethanolamine-acetic acid (pH 7.0)/methanol (85:15); flow rate, 150 μ l/min. A mixture of the compounds corresponding to 2.5 μ g each in aqueous solution was injected. Peaks are: 1, adenosine-diphosphate; 2, CoA; 3, adenosine; 4, acetyl-CoA.

measurement^{3,12,13}, because these chemicals act not only as a matrix for the conventional FAB ionization but also to disperse the eluent and concentrate the analyte on the surface of the frit. As described above, we could get satisfactory results on both the chromatographic separations and the FAB mass spectra of acetyl-CoA and related compounds in the FRIT-FAB LC/MS measurement by using diethanolamine-acetic acid buffer and the negative ion detection. This means that diethanolamine, its acetate, in the solvent system can be acceptable itself as a matrix in the FRIT-FAB LC/MS without adding any chemicals such as glycerol or thioglycerol. Moreover, it was ascertained that the buffer including monoethanolamine or triethanolamine had the similar effects in a course of this study. The details concerning the utility of the buffers with ethan-

ol amines for the FRIT-FAB LC/MS will be presented in the near future.

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Fig. 7. Negative FAB mass spectra of acetyl-CoA and related compounds by the FRIT-FAB LC/MS. Operating conditions are as in Fig. 6.

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Keywords

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