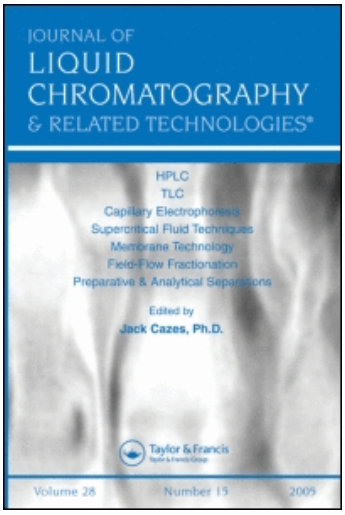

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Simple Free Amino Acid Separation by Reversed-Phase Ion-Pair Liquid Chromatography Using Column Switching Technique

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SIMPLE FREE AMINO ACID SEPARATION BY REVERSED-PHASE ION-PAIR LIQUID CHROMATOGRAPHY USING COLUMN SWITCHING TECHNIQUE

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ABSTRACT

Reversed-phase ion-pair liquid chromatography with copper (II) ions (RP-IPC-Cu) was applied to develop a high speed separation of free amino acids. Dynamic gradient elution in RP-IPC-Cu could not achieve this purpose due to base line shift, therefore a column switching technique was used with a combination of different alkyl-bonded silica gel columns under isocratic elution. The flexibility of eluent components makes it easy to realize a rapid separation of a mixture of targeted amino acids.

INTRODUCTION

Many workers have attempted to reduce the separation time of free amino acids, and present amino acid analyzers using ion-exchange resin as the packing require only 30 minutes for a separation of 18 common amino acids. Reversed-phase liquid chromatography, which is widely used, has flexibility and dynamic separation power due to the high number of

theoretical plates of columns and to complexed eluent components. The separation mechanism of reversed-phase ion-pair liquid chromatography (RP-IPC) is like a combination of ion-exchange and reversed-phase liquid chromatographies, and is suitable for separation of ionizable compounds like catechol amines [1], apomorphines [2], neostigmine [3], pyrimidines [4], choline [5], alkaloids [6], prostaglandines [7], theophylline [8], pilocarpine [9], peptides [10], levamisole [11], penicillamine [12], riboxamide [13] and salbutamol [14]. The separation of free amino acids by RP-IPC has also been described [15-19]. The separation however required a very long time, and many polar acidic and small amino acids were not well separated. First a gradient elution was applied to improve the separation and analysis time, but the appearance of system peaks did not permit a complete separation of free amino acids. A column switching technique effective in liquid chromatography [20-24] was therefore applied to improve the separation and analysis time.

EXPERIMENTAL

The liquid chromatograph consisted of an ERC-3310 degasser from ERMA Inc.(Tokyo, Japan); two HPLC pumps Model 576, and spectro detectors Model 502T from Gasukuro Kogyo Inc. (Tokyo, Japan); a spectro multi channel photodiode array detector MCPD-3500 from Otsuka electronics (Osaka, Japan); and an injector and a four-way valve, model 7125 and 7040 respectively, from Rheodyne. An octadecyl-bonded silica gel column (Inertsil ODS-2), an octyl-bonded silica gel column

(Inertsil C8 and 300 C8) and a propyl-bonded silica gel column (Inertsil C3) were obtained from Gasukuro Kogyo Inc.. These columns were 250, 150, 100 and 50 mm length x 4.6 mm i.d., and thermostated at 45 °C. The eluent was prepared by dissolving appropriate amounts of a given hexane-, heptane- and octanesulphonate together with copper (II) acetate in an acetate buffer. All alkylsulphonates were purchased from Gasukuro Kogyo Inc. Amino acids were purchased from Ajinomoto (Tokyo, Japan).

RESULTS AND DISCUSSION

It was impossible to separate polar acidic and small amino acids due to their very weak retention in a liquid chromatographic condition as reported by Grushka et al.[19, 25]. Therefore the effect of a counter ion and of its concentration which have very important role in RP-IPC [26, 27] were reexamined in RP-IPC-Cu. The retention of amino acids in RP-IPC-Cu became longer with increasing alkyl-chain length of alkylsulphonate as in RP-IPC [17, 28, 29]. Heptanesulphonate was a suitable counter ion for the separation of polar acidic and small amino acids, and it stabilized the retention time of amino acids with a concentration of 6.4 mM or more heptanesulphonate in 0.01 M sodium acetate buffer (pH 5.6) containing 0.4 mM copper acetate.

Under these conditions, the retention of hydrophobic and basic amino acids was too long on an octadecyl-bonded silica gel, and therefore the selectivity of alkyl-bonded silica gel

was examined with trimethyl-, octyl- and octadecyl-bonded silica gels for better separation of free amino acids in RP-IPC-Cu. The retention of amino acids was longer on more hydrophobic phases as usually expected, however there was no significant difference between octyl- and octadecyl-bonded silica gels made from the same silica gel as was found in RP-IPC [17].

Further, the pH effect was measured on octyl- and octadecyl-bonded silica gels for improvement of their separation. The retention of polar acidic and small amino acids was measured on a long octadecyl-bonded silica gel column 250 mm x 4.6 mm i.d. packed with Inertsil ODS-2, and that of hydrophobic and basic amino acids was measured on a short octyl-bonded silica gel column 100 mm x 4.6 mm i.d. packed with Inertsil 300 C8 in 0.01 M sodium acetate buffer (pH 4.5 - 5.4), containing 0.4 mM copper acetate, 6.4 mM heptanesulphonate and 3 v/v% methanol. The retention of amino acids increased at higher pH, except for aspartic acid, glutamic acid and histidine.

The spectra of amino acids in RP-IPC-Cu were measured by a photodiode array detector to obtain an optimal instrumentation conditions. When the column was not well equilibrated, the retention of amino acids was not long enough for their separation, and their maximum absorption wavelength was not constant. When the column was well equilibrated, the retention time of amino acids and their spectra became constant as shown in Figure 1.

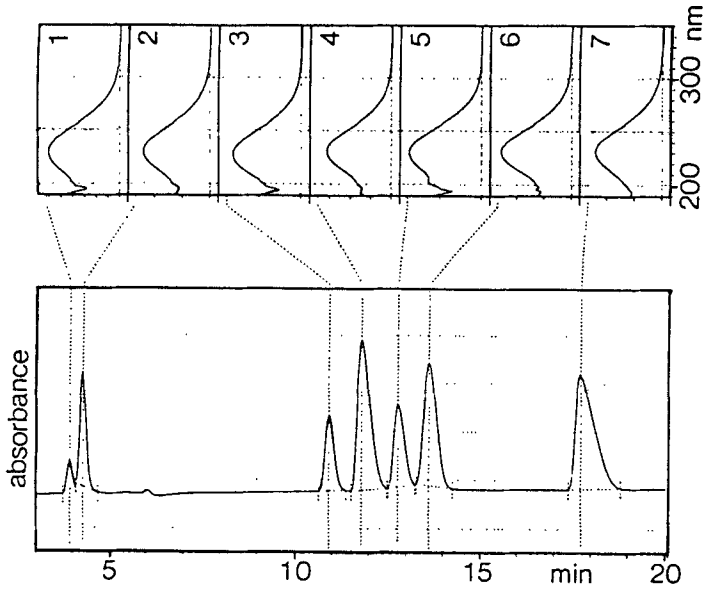

FIGURE 1 The chromatogram and spectra of amino acids obtained with a well equilibrated column. Column: Inertsil ODS-2 (250mm x 4.6mmi.d.). Eluent: 0.01M Sodium acetate buffer (pH 5.6) containing 0.4mM Copper acetate and 1.2mM Sodium octanesulphonate . Detector: MCPD 3500. 1: Aspartic acid (Asp) 2: Glutamic acid (Glu) 3: Glycine (Gly) 4: Serine (Ser) 5: Asparagine (Asn) 6: Alanine (Ala) 7: Threonine (Thr)

A high speed separation of amino acids was first attempted by using a gradient elution based on the above results, however the appearance of system peaks [30, 31] and high base line shift frustrated their separation.

Column switching, which was one method of using different types of columns for a separation of complex mixtures, was therefore applied to achieve the desired separation in minimum analysis time. As seen in Figure 2, the effluent from the first column (A) was either passed to a

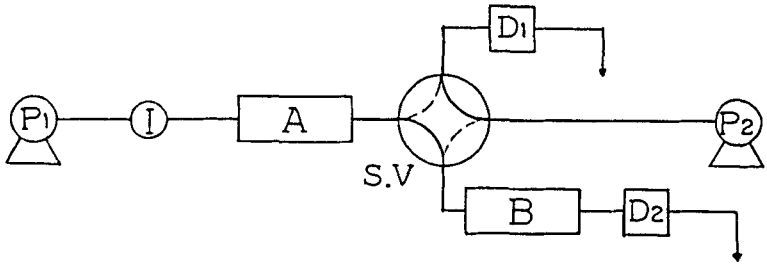

FIGURE 2 Flow diagram of column switching system. P1, P2: Pump. I: Injector. A, B: Column. D1, D2: UV detector. S.V: Switching Valve.

second column (B) or a detector (D1) through a switching valve (S.V.). The timing of the column switching was carefully planned so that only the chromatographic zone containing unseparated peaks was transferred to column B. The residual peaks on column A and the unseparated peaks transferred to column B from A, were further separated isocratically by the same eluent pumped from pumps P1 and P2 respectively, and both effluents were separately monitored by two detectors D1 and D2. A short column, 50 mm x 4.6 mm i.d. packed with Inertsil C3, was selected as column A, and a long column, 250 mm x 4.6 mm i.d. packed with Inertsil ODS-2, was selected as column B. The eluent was 0.01 M sodium acetate buffer, pH 5.0, containing 0.4 mM copper acetate, 6.4 mM heptanesulphonate and 3 v/v% methanol. As seen in Figure 3, 17 amino acids were separated within 25 minutes.

An analysis of free amino acids in an artificially-flavored health drink on the market was carried out, and a

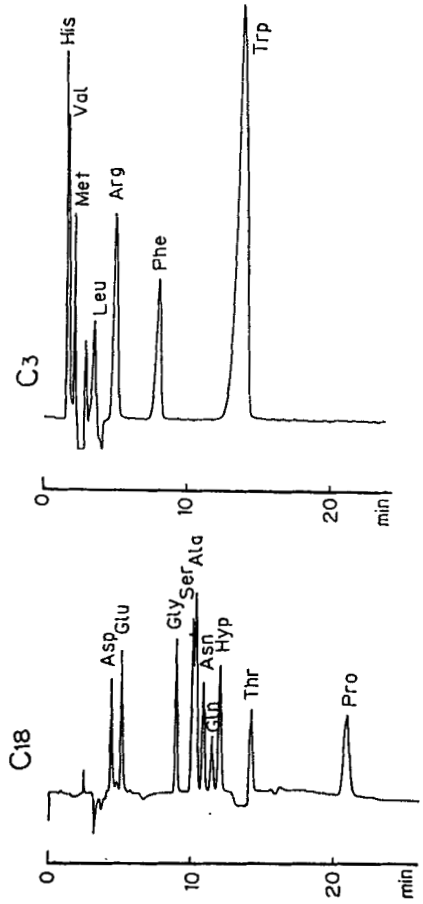

FIGURE 3 Chromatogram of standard amino acids using a column switching technique.
First column: Inertsil C3 (50mm x 4.6mmi.d.). Second column: Inertsil ODS-2 (250mm x 4.6mmi.d.). Eluent: 0.01M Sodium acetate buffer (pH 5.0) containing 0.4mM Copper acetate, 6.4mM Sodium heptanesulphonate and 3 v/v% Methanol. Column Temp.: 45°C. Flow rate: 1.0mL/min. Detector: UV 230nm.

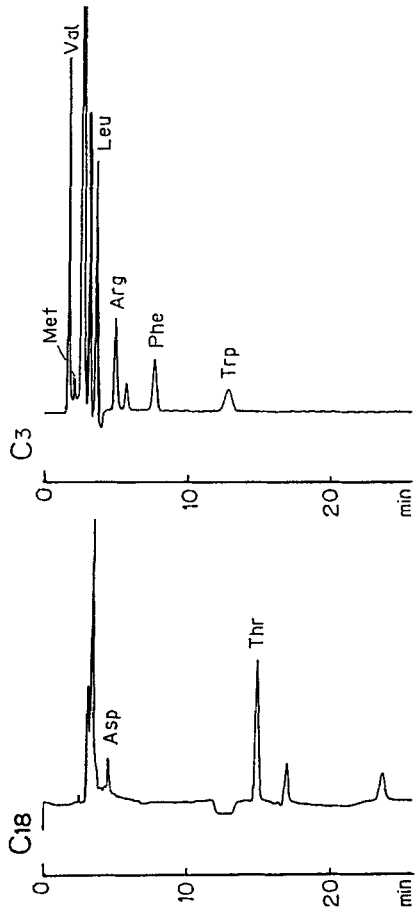

FIGURE 4 Chromatogram of amino acids in a drink using a column switching technique.
Experimental condition: see Figure 3.

chromatogram is shown in Figure 4. All amino acids that might be contained in the drink were separated by the above system.

The sensitivity and linearity of this amino acid analysis system were measured on an Inertsil ODS-2 column, 150 mm x 4.6 mm i.d. in an eluent of 0.01 M sodium acetate buffer, pH 4.5, containing 0.4 mM copper acetate, 6.4 mM heptanesulphonate and 22 v/v% methanol. In this system, several ng (S/N=2) of alanine, proline, threonine, methionine and isoleucine could be detected, and the calibration curves were linear over a concentration of 3 order range from 10 ng to 10 μg.

This method has the advantage not only of time saving, but also of improving the separation. The flexibility of eluent and the easy set-up of the system can be useful to analyze a limited number of free amino acids within a short time. Further improvement of the sensitivity and separation is required to apply this system in clinical analysis.

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