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The efficient use of microcolumns in chromatographic systems*

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Abstract: Microcolumn techniques involving pre-column concentration and post-column derivatization make good use of micro-scale high-performance liquid chromatography. The efficient use of microcolumns is illustrated by some applications to the analysis of amino acids, bile acids and catecholamines. The potential of new detector techniques based on the multichannel photodiode array detector, and on immobilized enzyme post-column reaction systems with fluorimetric detection, is also discussed.

Keywords: *Micro HPLC; pre-column concentration; post-column derivatization; multi-channel photodiode array detection; biomedical applications.*

Introduction

Miniaturization of high-performance liquid chromatography (HPLC) has yielded several advantages over conventional systems: low consumption of mobile and stationary phases; increased mass sensitivity; the possibility of employing expensive or exotic phases; ease of coupling with mass spectrometry; high-speed separability at low flow-rate; and rapid heat transfer in the column. In order to achieve these advantages, microcolumn techniques and the ancillary technology have been developed or improved.

The concentration of solutes in the column effluent is higher in micro HPLC than in conventional systems and this can result in increased mass sensitivity with concentration-sensitive detectors. UV-visible spectrophotometers and fluorimeters can be adapted for detection in micro HPLC by replacing the conventional flow cell with a miniaturized version. Electrochemical detectors and multichannel photodiode array UV-visible detectors can also be employed in micro HPLC by appropriate design of the flow cell.

Increased mass sensitivity is especially desirable in the analysis of biomedical samples, where the amount of material available is sometimes restricted. In such cases, the syringe-less injection technique is preferred. The micro pre-column concentration technique [1] has been shown to afford a useful syringe-less injection method, thus

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making the best use of increased mass sensitivity of micro HPLC. This technique permits the analysis of components which are present in dilute solution.

As for the separation column in micro HPLC, column materials exert a profound effect on column efficiency. Fused-silica tubing is efficient in micro HPLC with respect to the inertness of its surface, mechanical strength and ease of handling, compared with PTFE, Pyrex glass and stainless-steel tubing [2]. Furthermore, fused-silica tubing can be used as connecting material of small dead-volume or as a flow cell.

This paper describes some chromatographic systems for micro HPLC and their application in biomedical analysis.

Experimental

The apparatus was generally constructed as previously described, using a micro-pump, a micro valve injector ML-422 (0.02 μl; Japan Spectroscopic Co., Tokyo, Japan), a micro pre-column, a separation column and a detector [1–4]. The gradient separation was carried out with home-made gradient equipment comprising a small-volume mixing vessel and a magnetic stirrer [3]. A micro pre-column comprised PTFE tubing packed with commercially available materials, which should be carefully selected with reference to the nature both of solutes and of the sample matrix solution. The UV spectrophotometers, fluorimeter, multichannel photodiode array detector and electrochemical detectors employed in this paper were as previously described [3–9]. The flow cell of each detector was designed and constructed in the authors' laboratory.

Results and Discussion

Liquid chromatography has been employed by many researchers for the analysis of amino acids. Derivatization with 5-dimethylaminonaphthalene sulphonyl (Dns or Dansyl) chloride is known to enable the sensitive HPLC analysis of amino acids. This can also be achieved by derivatization with *o*-phthalaldehyde, phenylisothiocyanate, fluorescamine or 2,4-dinitrofluorobenzene. Since the concentration of solutes in the column effluent increases with decreasing column dimensions, it is possible to increase the mass sensitivity by using a small-bore column and an appropriate detection system. Both isocratic and gradient separations of Dns-amino acids have been investigated in the reversed-phase mode [4]. Figure 1 shows the gradient separation of a standard mixture of Dns-amino acids on 3-μm ODS-Hypersil. At little as *ca* 40 pmol of each Dns-amino acid can be assayed with UV detection at 222 nm; the detection limit for a signal-to-noise-ratio of 2 was less than 1 pmol.

The separation of amino acids in 1.3-nl soya sauce is demonstrated in Fig. 2 [4]. The pH of the sample solution was adjusted to 9.7 and Dansyl-derivatization was carried out at 38°C. The samples in Figs 1 and 2 were loaded with the micro valve injector.

In most cases of real sample analysis, sample pre-treatment plays an important role, viz. concentration of analytes and/or elimination of interfering compounds. The pre-column concentration method is a convenient pre-treatment stage in micro HPLC [1]. The validity of the micro pre-column concentration method is shown in Figs 3 and 4, where bile acids in a buffer solution are employed as test solutes and off-line pre-column concentration is adopted. Figures 3 and 4 illustrate the linear relationship of peak height with concentration and sample volume, respectively. The micro pre-column consisted of PTFE tubing (10 × 0.2 mm i.d.) packed with Develosil ODS-15/30 (15–30 μm; Nomura

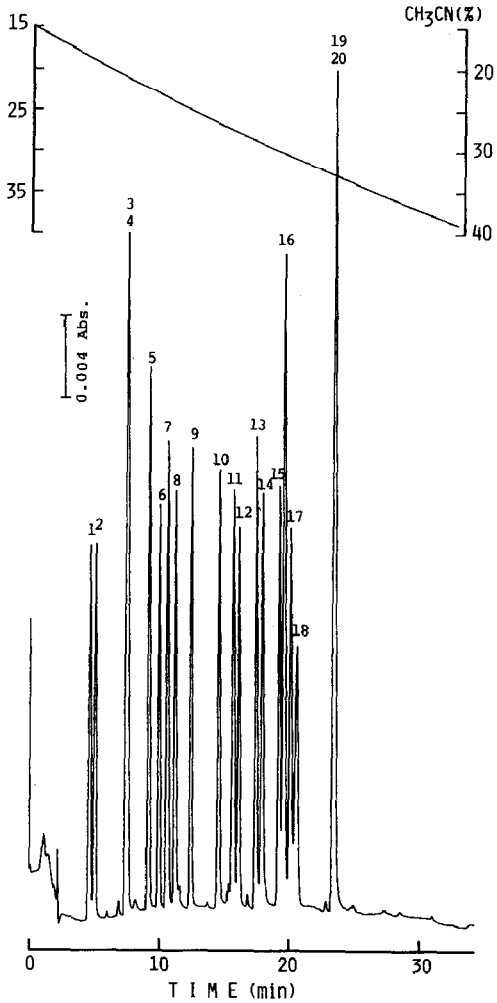

Figure 1
Separation of Dns-amino acids. Column: ODS-Hypersil (3 μm), 100 × 0.34 mm i.d. Mobile phase:acetonitrile–0.13 M ammonium acetate, gradient profile as indicated. Flow rate: 4.2 μl/min. Wavelength of UV detection: 222 nm. Key to Dns-amino acids in sample: 1, Asp; 2, Glu; 3, Hyp; 4, Asn; 5, Ser; 6, Thr; 7, Gly; 8, Ala; 9, Pro; 10, Val; 11, *N*val; 12, Met; 13, Ile; 14, Leu; 15, *N*leu; 16, Trp; 17, Phe; 18, NH_2; 19, di-Cys; 20, Cys. Reproduced from [4] with the permission of Elsevier Science Publishers.

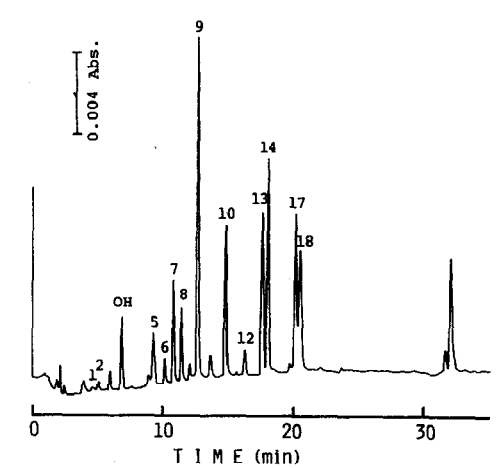

Figure 2
Separation of amino acids in soya sauce (1.3 nl). Operating conditions and key as in Fig. 1; OH = dansylic acid. Reproduced from [4] with the permission of Elsevier Science Publishers.

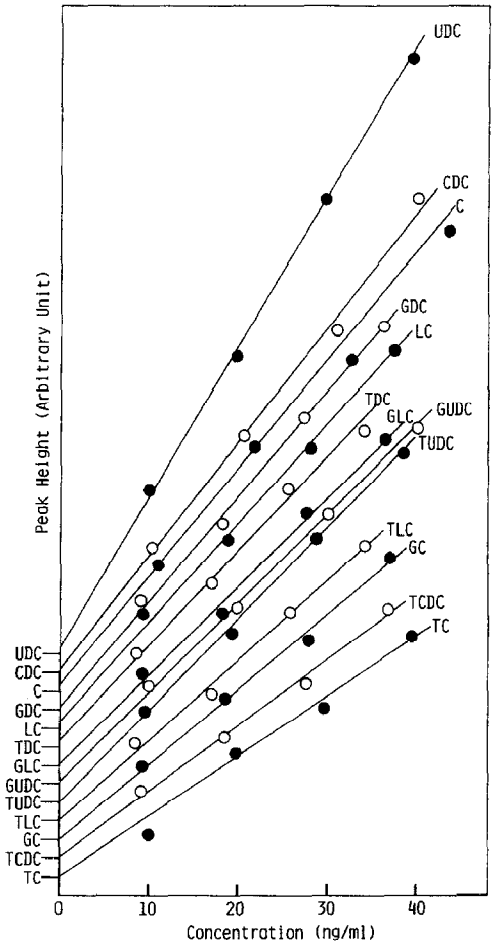

Figure 3
Micro pre-column concentration method [1] with post-column derivatization and fluorimetric detection [6, 10]: relationship between peak height and concentration. Column: silica ODS SC-01 (5 μm), 200 × 0.26 mm i.d. Guard column: silica ODS SC-01, 5 × 0.20 mm i.d. Pre-column: 15 to 30-μm, Develosil ODS-15/30, 10 × 0.20 mm i.d. Post-column derivatization with 3α-hydroxysteroid dehydrogenase, immobilized on glass beads in fused-silica tubing (20 × 0.34 mm i.d.). Mobile phase: acetonitrile–phosphate buffer containing 1.8 mM NAD [6, 10]. Flow rate: 2.1 μl/min. Sample volume: 1 ml. Key to samples: UDC, ursodeoxycholic acid; CDC, chenodeoxycholic acid; C, cholic acid; GDC, glycodeoxycholic acid; LC, lithocholic acid; TDC, taurodeoxycholic acid; GLC, glycolithocholic acid: GUDC, glycoursodeoxycholic acid; TUDC, tauroursodeoxycholic acid; TLC, taurolithocholic acid; GC, glycocholic acid; TCDC, taurochenodeoxycholic acid; TC, taurocholic acid. Fluorimetric detection at λ_{ex} = 365 nm; λ_f = 470 nm [6, 10].

Chemical Company, Seto-shi, Japan). Post-column derivatization detection was employed, as described below.

HPLC analysis of bile acids using a 3α-hydroxysteroid dehydrogenase (HSD) post-column derivatization appears to be very promising [10] compared to other methods, with respect to resolution, sensitivity and quantitation. The 3α-hydroxy group in each bile acid is oxidized to a keto group by the enzyme reaction, while β-nicotinamide-adenine dinucleotide (NAD) is reduced to NADH, which is detected fluorimetrically. The authors adopted a pre-mixing system, in which NAD is pre-mixed with the mobile phase, so that consequently a single pump is employed [5]. The pH of the mobile phase and the concentration of NAD greatly affected the sensitivity of detection. The post-column reactor consisted of fused-silica tubing (20 × 0.34 mm i.d.) packed with HSD-immobilized controlled-pore glass beads (200–400 mesh).

Detection sensitivity was increased to such an extent that the amount of sample required could be decreased to 0.1 ml of serum. Figure 5 shows the separation of bile acids in the serum of a patient with alcoholic cirrhosis [6]. The upper tracing was obtained without a post-column, in which fluorescent compounds other than NADH were detected. The serum was diluted ten times in 10 mM phosphate solution (pH 7 to 8) and 1 ml of the solution forced into the pre-column.

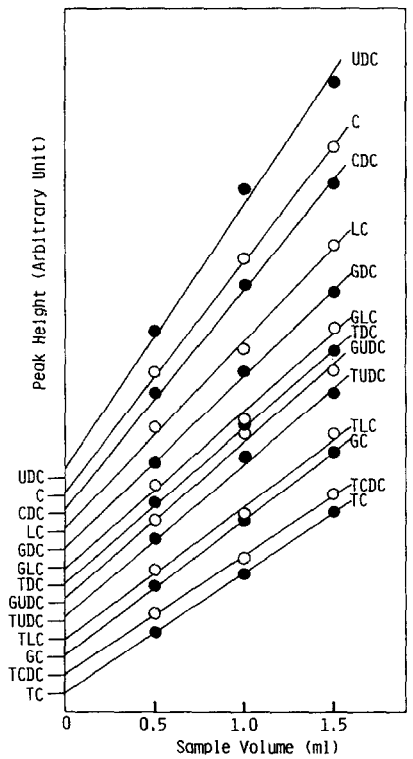

Figure 4
Micro pre-column concentration method [1] with post-column derivatization and fluorimetric detection [6, 10]: relationship between peak height and sample volume. Operating conditions as in Fig. 3. Sample concentration: 17–22 ng/ml.

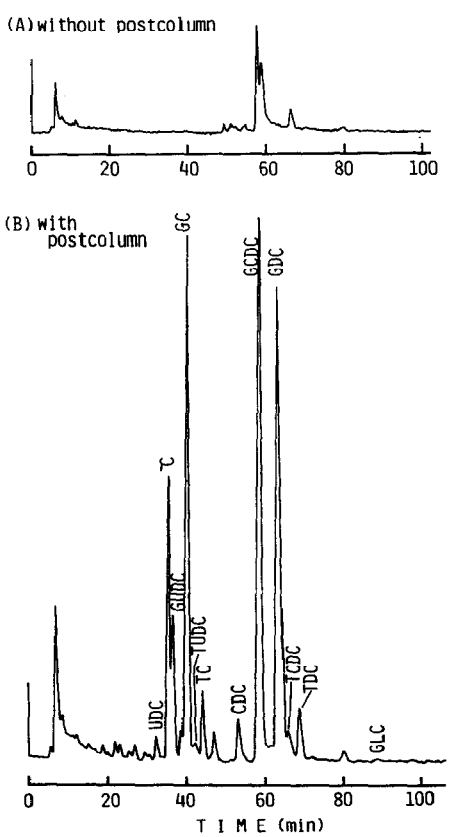

Figure 5
Gradient separation of bile acids in the serum of a patient with alcoholic cirrhosis. Operating conditions as in Fig. 3. Sample: 0.1 ml of serum from a patient with alcoholic cirrhosis. Key as in Fig. 4, plus GCDC, glycochenodeoxycholic acid. Reproduced from [6] with the permission of Elsevier Science Publishers.

Typical chromatograms of catecholamines in human serum from healthy individuals obtained using the micro HPLC system with on-line pre-column concentration and parallel-opposed dual electrochemical detection are shown in Fig. 6 [7]. By recording the cathodic current, interfering compounds could be eliminated. The twin-electrode thin-layer electrolytic cell in parallel-opposed configuration was designed for a dual electrochemical detector. The thin-layer cavity was constructed of two fluorocarbon resin blocks separated by a PTFE sheet 50-μm thick and 2-mm wide. Two working electrodes were made with glassy carbon plates 1-cm long and 2-cm wide and supported in each block. The silver/silver chloride reference electrode and the platinum counter electrode were assembled *in situ*. This detection system afforded powerful and selective detection of reversible and/or quasi-reversible species from irreversible species. The system can provide an enhancement in sensitivity by recycling oxidation and by re-reduction between the anode and cathode at low mobile phase flow rates.

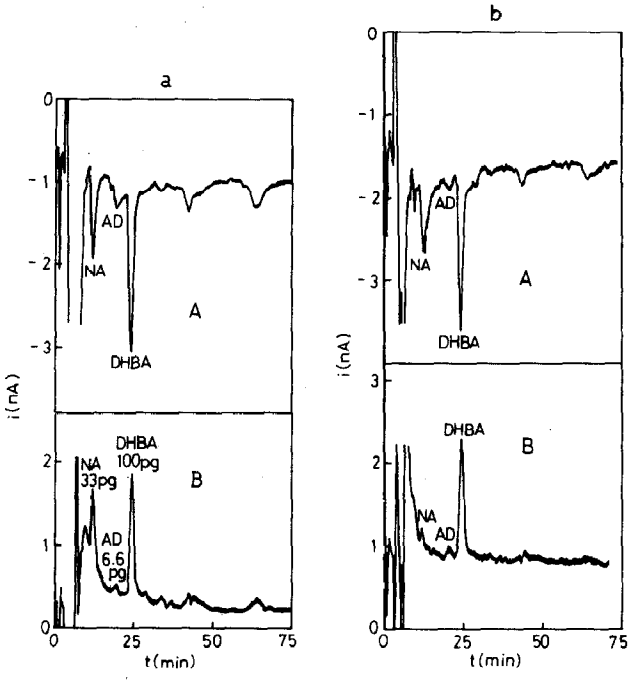

Figure 6
Typical chromatograms of catecholamines in human serum from healthy individuals by internal standard addition. (A) Anodic response; (B) cathodic response. Sample: 200 μl of human serum spiked with 100 pg of 3,4-dihydroxybenzylamine hydrobromide (DHBA). Column: Yanapak ODS-T (10 μm), 150 × 0.5 mm i.d. Pre-column: LiChrosorb Alox T (30 μm), 20 × 0.5 mm i.d. Mobile phase: Britton–Robinson buffer (pH 1.8) containing 2 mM sodium 1-heptanesulphonate, 0.1 mM EDTA and 50 mM sodium perchlorate. Flow rate: 8.3 μl/min. Electrode potentials (vs Ag/AgCl): anode +0.60 V; cathode +0.20 V. Sections a and b represent two different individuals. Key to solutes: NA, noradrenaline; AD, adrenaline. Reproduced from [7] with the permission of Elsevier Science Publishers.

The use of multichannel photodiode array detectors has improved the qualitative capability of HPLC, which hitherto has been poor when using a conventional UV-visible spectrophotometer as a detector. Since it is capable of obtaining a large amount of spectral information in a single chromatographic run, multichannel detection yields many advantages over conventional single-wavelength detection [8, 9, 11]. These include improved identification capability, possibilities for checking the purity of chroma-

tographic peaks [11], rapid selection of an optimal detection wavelength, and economy in the use of solvents and samples.

The authors have developed a multichannel photodiode array UV-visible detector with a 50-nl flow cell for micro HPLC [8, 9]. The decrease in light intensity reaching the detector, constructed in reversed-optics configuration [11], was compensated by decreasing the number of optical parts such as lenses and mirrors, and by reducing the distance between the light source and the flow cell and between the flow cell and the diffraction grating. This detection system has been used for the analysis of real samples, as illustrated in Fig. 7, which shows a three-dimensional spectrochromatogram of the three components in a commercially available drug [9].

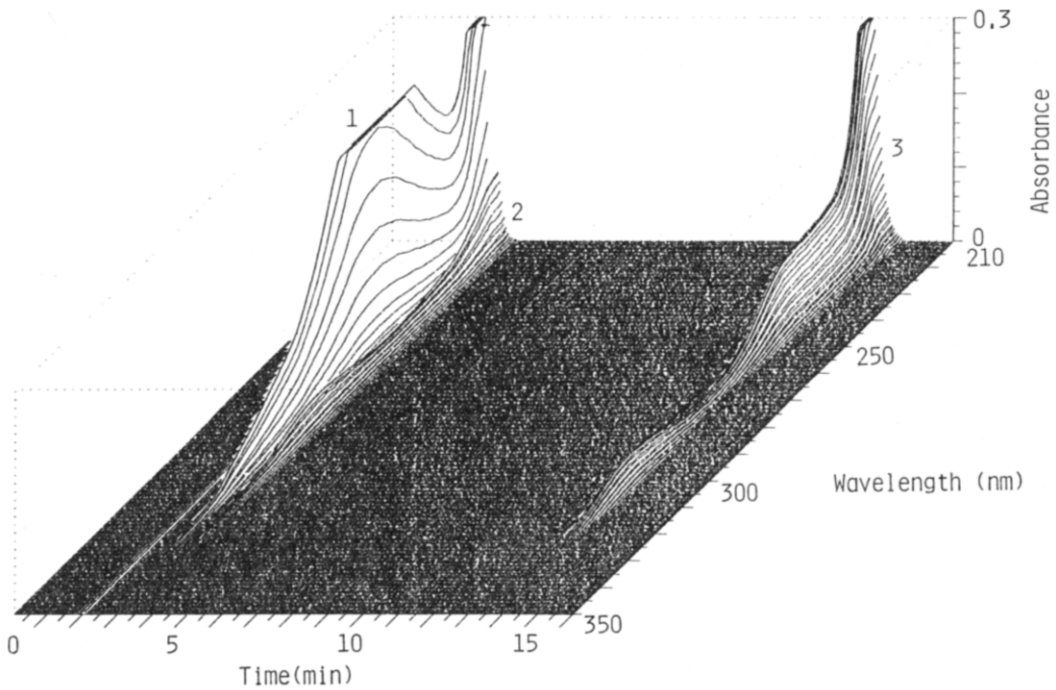

Figure 7
Three-dimensional spectrochromatogram of components in a commercially available drug. Column: ODS-Hypersil (3 μm) packed in fused-silica column, 100 × 0.34 mm i.d. Mobile phase: acetonitrile–methanol–60 mM potassium phosphate (pH 3.1) (14:6:80, v/v/v). Flow rate: 4.2 μl/min. Sampling time: 25 msec × 8, corresponding to ensemble averaging over 200 msec. Detector: micro gate photodiode array detector (Union Giken, Osaka, Japan) with MC-800 microcomputer and MC-920 digital plotter (Union Giken); flow cell dimensions were 1 × 0.25 mm i.d. (50 nl). The sample (19 nl) was a filtered methanolic extract of a tablet containing acetaminophen (1), caffeine (2) and ethenzamide (3). Reproduced from [10] with the permission of Dr Alfred Huethig Publishers.

High-speed chromatographic separation is generally required for routine work that involves diagnosis, resolution of unstable chemical species or the kinetic study of reaction processes [12]. Micro HPLC offers some advantages over conventional HPLC for very high-speed separation: a less restricted choice of flow rates; low consumption of the mobile phase; and rapid heat transfer in the column. However, it is important to pay serious attention to band broadening in the instrument.

The present authors have assembled instruments for fast micro HPLC [13] and demonstrated rapid separations, such as that illustrated in Fig. 8 for the separation of

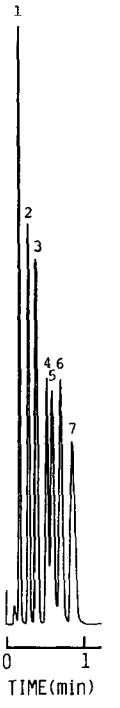

Figure 8
Rapid separation of catecholamines and interfering compounds. Column: ODS-Hypersil (3 μm), 50 × 0.34 mm i.d. Mobile phase:methanol–60 mM potassium phosphate (pH 3.0) (8:92, v/v) containing 0.4 mM sodium 1-octanesulphonate and 0.2 mM EDTA. Inlet pressure: 200 kg/cm^2. Flow-rate: 32 μl/min. Wavelength of UV detection: 220 nm. Key to samples (each 20 ng): 1, 3,4-dihydroxyphenyl glycol; 2, norepinephrine; 3, epinephrine; 4, 3,4-dihydroxybenzylamine; 5, normetanephrine; 6, 3,4-dihydroxyphenylacetic acid; 7, dopamine.

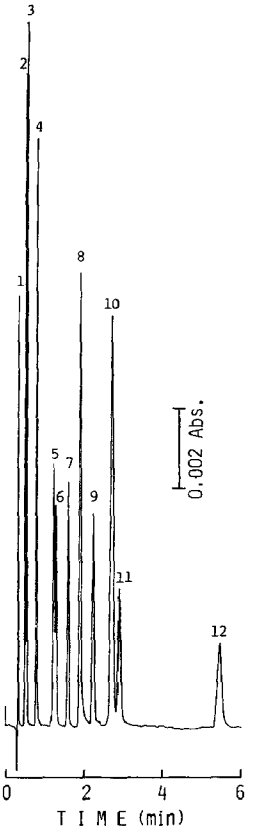

Figure 9
Supercritical fluid chromatographic separation of polynuclear aromatic hydrocarbons. Column: silica ODS SC-01, 150 × 0.34 mm i.d. Mobile phase: carbon dioxide. Inlet pressure: 150 kg/cm^2. Column temperature: 35°C. Wavelength of UV detection: 245 nm. Key to samples: 1, benzene; 2, naphthalene; 3, biphenyl; 4, fluorene; 5, phenanthrene; 6, anthracene; 7, *p*-terphenyl; 8, 9-phenylanthracene; 9, fluoranthene; 10, 1,3,5-triphenylbenzene; 11, pyrene; 12, chrysene. Reproduced from [14] with the permission of Elsevier Science Publishers.

catecholamines and interfering compounds by micro HPLC in one minute. It is evident that fast HPLC will contribute to a decrease in the time required for analytical separations.

The fast micro HPLC system has been shown to be applicable to supercritical fluid chromatography (SFC) using carbon dioxide as the mobile phase [14]. Figure 9 demonstrates the SFC separation of polynuclear aromatic hydrocarbons. In SFC, the separation can be performed faster than in HPLC, owing to the low viscosity and large diffusivity of the mobile phase. It should be possible to separate biomedical samples rapidly by SFC using a modified carbon dioxide mobile phase.

Conclusions

In this brief review of the contemporary applications of micro HPLC in biomedical analysis, it has been shown that these systems can be exploited with optimal efficiency when used in combination with 'state-of-the art' technology. Hybrid micro HPLC systems incorporating pre- and post-column technology, with rapid-scanning multi-channel detection, fluorimetric detection or electrochemical detection, can significantly extend the problem-solving capability of micro HPLC in the biomedical sciences.

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