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Biomedical applications of high-performance liquid chromatography–mass spectrometry with continuous-flow fast atom bombardment

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ABSTRACT

This report describes the application of high-performance liquid chromatography combined with continuous-flow fast atom bombardment mass spectrometry to analytical problems in the biomedical laboratory. Applications include the compound-specific detection of diagnostic acylcarnitines in human urine, the separation and analysis of acyl-coenzyme A thioesters, and qualitative studies on complex mixtures of modified peptides (dansyl and dinitrophenyl derivatives). For each of these applications standard analytical columns (3.9 mm I.D.) and 1 ml/min flow-rates were employed with post-column stream splitting (1:100) before mass spectrometry. Various mobile phase compositions and solvent gradients were employed. The addition of 1–5% glycerol to the mobile phase was shown to have little effect on the chromatography. For all compounds studied (acylcarnitines, acyl-coenzyme A thioesters, and derivatized peptides) molecular weight information was obtained and sufficient sensitivity was achieved to allow unambiguous identification of trace components in complex mixtures.

INTRODUCTION

The continuous-flow fast atom bombardment (CF-FAB) interface has significantly increased the scope and application of combined high-performance liquid chromatography–mass spectrometry (HPLC–MS). Since its introduction [1,2], HPLC–CF-FAB-MS has been applied to the compound-specific analysis of peptides [3–8], sugars [9], nucleotides [10], acylcarnitines [11,12], and acyl-coenzyme A compounds [13], to name a few examples. Although limited to comparatively low flow-rates (5–10 μl/min) and requiring the presence of a suitable FAB matrix (*e.g.* glycerol), CF-FAB has sensitivity advantages for polar and surface active

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compounds and has a high tolerance for a wide variation of chromatographic conditions including non-volatile mobile phase additives and solvent gradients. It is also possible to interface CF-FAB with different HPLC system types with different standard analytical (3.9 mm I.D.; ml/min flow-rate), microbore (1 mm I.D.; 50 μl/min flow-rate), packed fused-silica (0.32 mm I.D.; 5 μl/min flow-rate), and open-tubular fused-silica columns (10 μm I.D.; 50 nl/min flow-rate).

This report describes the application of HPLC–CF-FAB-MS to biomedical analytical problems for which thermospray (TSP) ionization HPLC–MS has had limited success. These include the compound-specific detection of acylcarnitines, acyl-coenzyme A thioesters, and chemically modified peptides (dansyl and dinitrophenyl derivatives). Acylcarnitines and acyl-coenzyme A compounds are metabolites associated with the catabolic pathways for fats and branched-chain amino acids. The accumulation of specific acylcarnitines in urine, blood, and tissue has been associated with specific enzyme deficiencies in these catabolic pathways. The accumulated acylcarnitine which is excreted is derived from a corresponding acyl-coenzyme A metabolic intermediate of identical acyl group structure. An example is given of a detailed analysis of acylcarnitines in the urine of a patient affected with medium-chain acyl-coenzyme A dehydrogenase (MCAD) deficiency [11,12]. The analysis of acyl-coenzyme A thioesters by HPLC–CF-FAB-MS with an application to enzymology has been described previously in this journal [13]. Additional data are provided here regarding comparison with HPLC–TSP-MS analysis, and retention of chromatographic integrity in HPLC–CF-FAB-MS.

In order to make peptides and amino acids amenable to HPLC–UV or HPLC-fluorescence analysis the N-terminus of the molecule may be reacted with either 2,4-dinitrofluorobenzene or dansyl chloride to form the corresponding dinitrophenyl or dansyl derivative. Certain applications in enzymology biotechnology require that such derivatization reactions be performed on complex mixtures of peptides. An example is here described in which HPLC–CF-FAB-MS was employed to characterize mixtures of both peptide derivatives.

EXPERIMENTAL

Reagents and materials

Methanol and acetonitrile utilized for chromatography were high-purity HPLC grade (J. T. Baker, Phillipsburg, NJ, U.S.A.) or equivalent. Aqueous portions of mobile phases were prepared from deionized, distilled water, HPLC-grade ammonium acetate (Fisher Scientific, Fair Lawn, NJ, U.S.A.), and high-purity trifluoroacetic acid (Sigma, St. Louis, MO, U.S.A.; for example). All mobile phase components were filtered through 0.45-μm Nylon 66 membranes (Alltech Assoc., Deerfield, IL, U.S.A.) and continuously sparged with helium throughout all HPLC–MS experiments. Acylcarnitine standards were either synthesized in this laboratory or purchased commercially and characterized as previ-

ously described [12]. Urine samples were prepared for HPLC–CF-FAB-MS analysis by one of the extraction and clean-up procedures also previously described [12]. Acyl-coenzyme A thioesters were obtained from Sigma [13]. Mixtures of unknown dansylated and dinitrophenyl derivatized peptides were obtained by solid-phase peptide synthesis.

HPLC–CF-FAB-MS system

The basic design of the HPLC–CF-FAB-MS system has been described in an earlier report [13]. It includes a Waters 600-MS low-pressure gradient solvent delivery system (Waters Chromatography Division, Milford, MA, U.S.A), a Rheodyne 7125 injector (Cotati, CA, U.S.A.), analytical HPLC column, and post-column stream splitting into the CF-FAB probe. Acylcarnitines were separated with a Waters μBondapak C_{18} column (150 mm × 3.9 mm I.D.), modified peptides with two Waters Delta Pak C_{18} columns (150 mm × 3.9 I.D.) connected in series, and acyl-coenzyme A thioesters on either a Waters Nova-Pak C_{18} column (150 mm × 3.9 I.D.) or a 250 mm × 1 mm I.D. microbore column (Keystone Scientific Hypersil BDS, Bellefonte, PA, U.S.A.). In all cases, the particle size was 5 μm. System modifications and conditions for microbore HPLC–CF-FAB-MS were as previously described [13]. For peptide analysis, a Waters UV detector (Model 481) was placed after the HPLC column to monitor component elution (222 nm for dansyl derivatives; 375 nm for dinitrophenyl derivatives).

Mobile phase conditions have been reported previously for the analysis of acylcarnitines [11,12] and acyl-coenzyme A thioesters [13]. For elution of dansylated peptides, two solvent systems were prepared as follows. A: water containing 0.1% trifluoroacetic acid and 1% glycerol; B: 60% acetonitrile plus 40% water containing 0.1% trifluoroacetic acid and 1% glycerol. A solvent gradient was employed which progressed from 35% A plus 65% B to 10% A plus 90% B in 30 min with a 10-min final hold time. Dinitrophenyl-derivatized peptides were analyzed with the same mobile phase system employing a gradient from 45% B to 75% B in 90 min. Flow-rates for all HPLC–CF-FAB-MS analyses (except for microbore acyl-coenzyme A thioester elution; see ref. 13) were 1 ml/min.

The MS system consisted of either a VG Trio-2 quadrupole or Trio-3 triple quadrupole instrument (VG Masslab, Altrincham, U.K.) which incorporated VG Masslab dynamic-FAB probes as previously described [11–13]. Derivatized peptide analyses were also performed on a VG 70S double-focusing instrument (VG Analytical, Manchester, U.K.) which incorporated a similar probe. The FAB sources of all three instruments incorporated saddle-field ion guns (Ion Tech, Teddington, U.K.) which generated 5–6 keV xenon atom beams at 1–2 mA current. Stable conditions in each instrument were obtained as previously described [11–13] at source temperatures of 50°C. Scan conditions for various experiments were unique to the individual experiment and are detailed in later sections.

TSP HPLC–MS system

Acyl-coenzyme A thioester standard mixtures were analyzed using the same HPLC system (Waters Nova-Pak column; glycerol removed from the mobile phase) [13] interfaced to the VG Trio-3 triple quadrupole mass spectrometer via a VG Masslab TSP probe. The probe incorporates a resistively heated capillary vaporizer. TSP conditions (including vaporizer temperature, repeller electrode voltage, and ion source tune) were optimized using ammonium acetate–water–acetonitrile solvent cluster ions. In order to obtain good TSP spectra for all acyl-coenzyme A compounds over the solvent gradient, several runs were made at different capillary temperatures. In each run, the mass spectrometer was scanned from m/z 100 to 900 in 1 s with a 0.1 s delay. The TSP source temperature for all experiments was 280°C.

RESULTS AND DISCUSSION

HPLC–CF-FAB-MS analysis of urinary acylcarnitines

Acylcarnitines (Fig. 1) are quaternary ammonium compounds which lack a sensitive chromophore and are thus not amenable to analysis by HPLC–UV techniques. TSP [14] and CF-FAB [11,12] have been successfully applied. TSP spectra were obtained from individual acylcarnitines separated isocratically on an analytical (3.9 mm I.D.) HPLC column [14]. Although molecular ions were observed in all cases, significant fragmentation also occurred which was thought to result from a combination of thermolytic and unimolecular cleavage processes. This combination coupled with the dependence of acylcarnitine spectra on TSP temperature, mobile phase, and instrument tune conditions can result in a lack of reproducibility in acylcarnitine TSP spectra.

Previous studies with CF-FAB accomplished in this laboratory [11,12] employed packed fused-silica columns (0.32 mm I.D.) operating at approximately 10 μl/min flow-rates with no post-column splitting. Although possessing good chromatographic efficiency, these columns have limitations of capacity and are rather fragile. In this study, acylcarnitine analysis was performed by CF-FAB using an analytical HPLC column (3.9 mm I.D.) with post-column stream splitting (1:100). The chromatograms shown in Fig. 2 were derived from the CF-FAB analysis of a series of standard acylcarnitines (A) and acylcarnitines from the urine of a patient with a suspected defect of fatty acid catabolism (B). Each chromatogram is a computer-reconstructed extracted ion current profile of acyl-

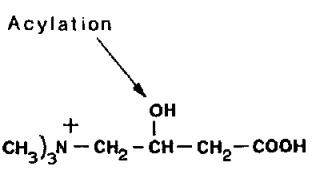

Fig. 1. Structure of acylcarnitines.

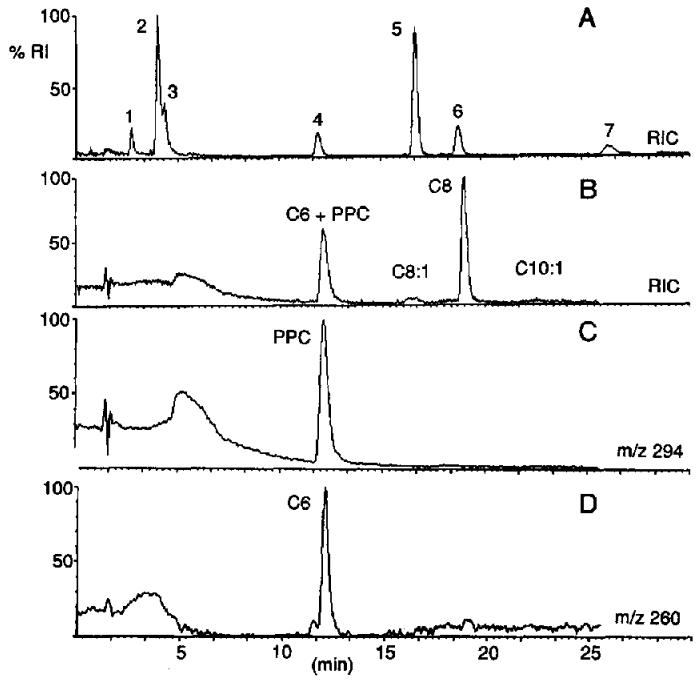

Fig. 2. HPLC–CF-FAB-MS analysis of acylcarnitines on the VG Trio-2 quadrupole mass spectrometer. (A) Summed reconstructed ion current profile (RIC) of acylcarnitine standards $[M+H]^+$; peaks: 1 = propionyl; 2 = isobutyryl; 3 = butyryl; 4 = hexanoyl; 5 = valproyl; 6 = octanoyl; 7 = decanoyl; (B) summed RIC $[M+H]^+$ of acylcarnitines from the urine of a patient with suspected metabolic disease; (C) extracted ion current profile (*m/z* 294) for the $[M+H]^+$ ion of phenylpropionylcarnitine (PPC); (D) extracted ion current profile (*m/z* 260) for the $[M+H]^+$ ion of hexanoylcarnitine. Conditions: Waters μBondapak C_{18} (150 mm × 3.9 mm I.D.); solvent gradient: from 90% solvent A (0.05 *M* ammonium acetate containing 2% glycerol) plus 10% solvent B (methanol containing 2% glycerol) to 40% solvent A plus 60% solvent B in 15 min; 1 ml/min flow-rate; mass spectrometer scan 100–650 in 1 s (0.1-s delay).

carnitine molecular $[M+H]^+$ ions from each sample. The FAB spectra of acylcarnitines are dominated by $[M+H]^+$ ions as shown in Fig. 3, which depicts the averaged and background-subtracted spectra from the major peaks in the patient sample.

The separation of acylcarnitine standards (propionyl- through decanoylcarnitine) is clearly adequate for the purpose of diagnostic confirmation. Note that two isomeric pairs (butyryl- and isobutyryl-; valproyl- and octanoyl-) were resolved. This is significant since only one of each pair (butyryl- and octanoyl-) is considered to be diagnostic of metabolic disease. Valproic acid is, in fact, a drug often given to children with seizure disorders which necessitates adequate identification procedures for the diagnostic metabolite. This particular child had been dosed with phenylpropionic acid which requires the MCAD enzyme for breakdown. The resulting phenylpropionylcarnitine (PPC) (*m/z* 294), a newly reported

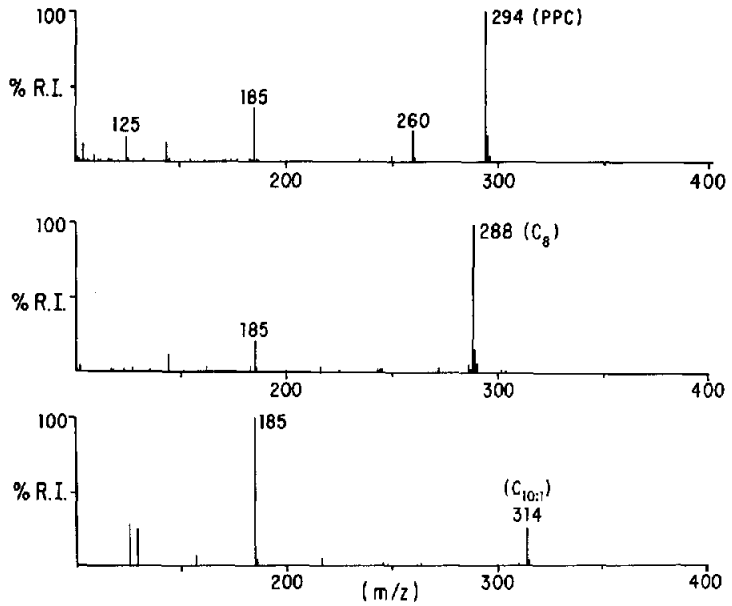

Fig. 3. Averaged and background-subtracted spectra of acylcarnitines from the major peaks in Fig. 2B: PPC = phenylpropionyl carnitine; C_8 = octanoylcarnitine; $C_{10:1}$ = decenoylcarnitine.

metabolite of phenylpropionic acid, is clearly distinguished from coeluting hexanoylcarnitine (*m/z* 260) by use of extracted ion current profiles (Fig. 2C and D). The identification of PPC was confirmed by mass spectral and elution time comparison with an authentic standard. The identification of appropriate diagnostic metabolites enables a presumptive diagnosis of MCAD deficiency.

The stability, sensitivity, and instrument tune of the CF-FAB system were not adversely affected by the steep solvent gradient required to elute diagnostic acylcarnitines. This tolerance of solvent gradients enables the separation and detection of acetylcarnitine (C_2 acyl group) through stearoylcarnitine (C_{18} acyl group)

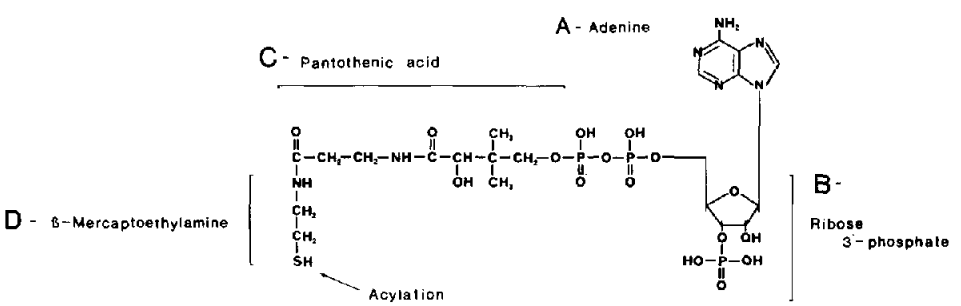

Fig. 4. Structures of acyl-CoA compounds.

in a single analysis. Detection limits for octanoyl- and propionylcarnitine may be estimated from a signal-to-noise ratio of better than 10:1 for 0.4 nmol of each compound injected, which corresponds to about 4 pmol reaching the CF-FAB probe tip in this system configuration.

Acyl-coenzyme A thioesters

The analysis of acyl-coenzyme A thioesters (acyl-CoA compounds, Fig. 4) of various acyl group chain-length (C_2–C_{18}) and functionality (–COOH, –OH, –C=C–) by HPLC–CF-FAB-MS has already been described [13]. Two chromatographic systems, one utilizing an analytical HPLC column (3.9 mm I.D.) with 1 ml/min flow-rate and the other a microbore column (1 mm I.D.) operating at approximately 50 μl/min, were employed. Since acyl-CoA compounds are complex molecules of diverse functionality it was considered possible that chromatographic integrity would be compromised by the elution and spreading of mobile phase over the conical probe tip which precedes the volatilization–ionization process. Fig. 5 indicates, however, that this was not the case, since octanoyl-CoA

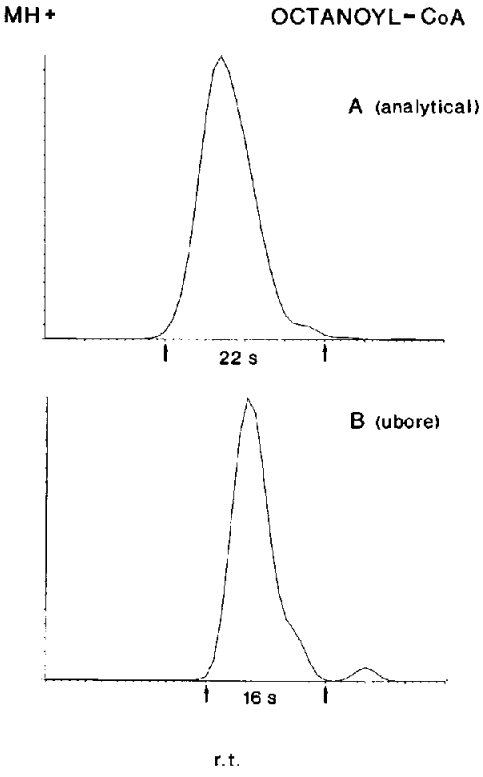

Fig. 5. Extracted ion current profiles $[M+H]^+$ (m/z 894) from the HPLC–CF-FAB-MS analysis of octanoyl-CoA on an analytical column (about 30 nmol injected onto a Waters Nova Pak C_{18}; 3.9 mm I.D.) and on a microbore column (about 0.5 nmol injected onto a Hypersil BDS; 1 mm I.D.) (see ref. 13).

produced symmetrical peaks from both CF-FAB systems. The microbore system produced slightly narrower peaks than the analytical column and allowed five to ten times greater sensitivity owing to the lower post-column split ratio (1:5 *versus* 1:100).

The CF-FAB spectra of acyl-CoA compounds exhibit molecular weight information in the form of protonated molecular ions $[M+H]^+$ as well as structurally useful fragmentation. This was not the case with TSP ionization. Fig. 6 compares the TSP (A) and CF-FAB (B) mass spectra of octanyl-CoA. Note the absence of molecular weight information in the TSP spectrum, probably due to thermolytic decomposition in the TSP capillary tube.

Derivatized peptides

The objective of these experiments was to characterize complex mixtures of dansylated (DNS) pentapeptides and dinitrophenyl (DNP)-derivatized heptapeptides for which HPLC conditions had been previously established. In the case of DNS-peptides TSP ionization was unsuccessful. These derivatives were also relatively poor FAB substrates. The signal-to-noise ratio in single-ion monitoring

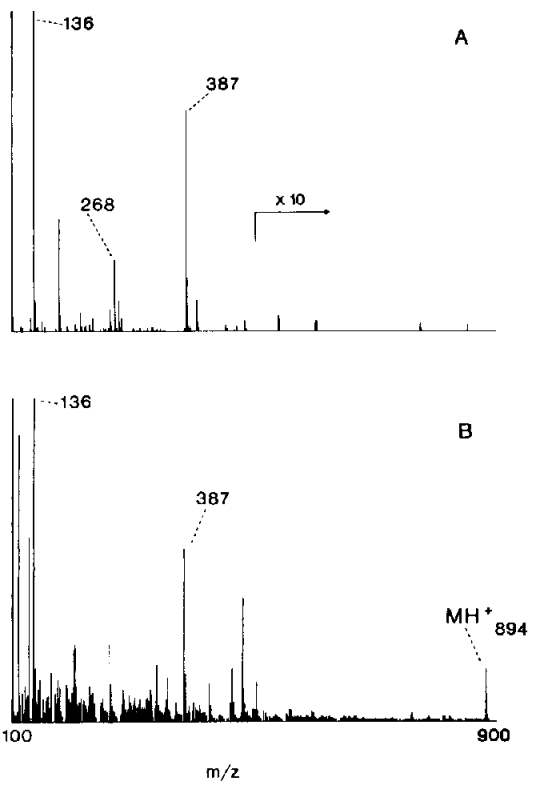

Fig. 6. TSP (A) and CF-FAB (B) mass spectra of octanoyl-CoA.

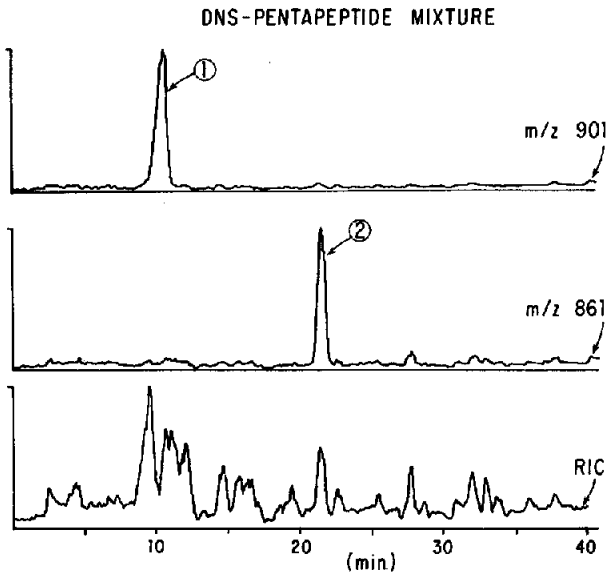

Fig. 7. HPLC–CF-FAB analysis of a mixture of dansylated pentapeptides including the summed reconstructed ion chromatogram (RIC) and selected-ion chromatograms of two individual components.

mode at the $[M+H]^+$ ion of a standard (DNS-Ile-Ile-Trp-Val-Asn, 10 nmol injected) was only about 10:1. Nevertheless, using selected-ion monitoring on the Trio-2 instrument, a complete characterization of the molecular components of the pentapeptide mixture was obtained. The ions monitored were the $[M+H]^+$ ions for the expected peptides in the mixture. Their elution positions were further

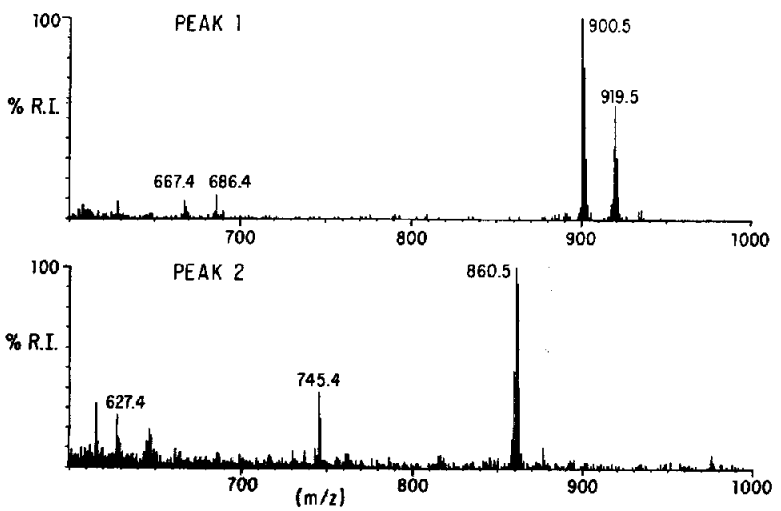

Fig. 8. Mass spectra derived from the HPLC—CF-FAB analysis of dansylated pentapeptides on the VG 70S instrument. Components correspond to peaks 1 and 2 in Fig. 7.

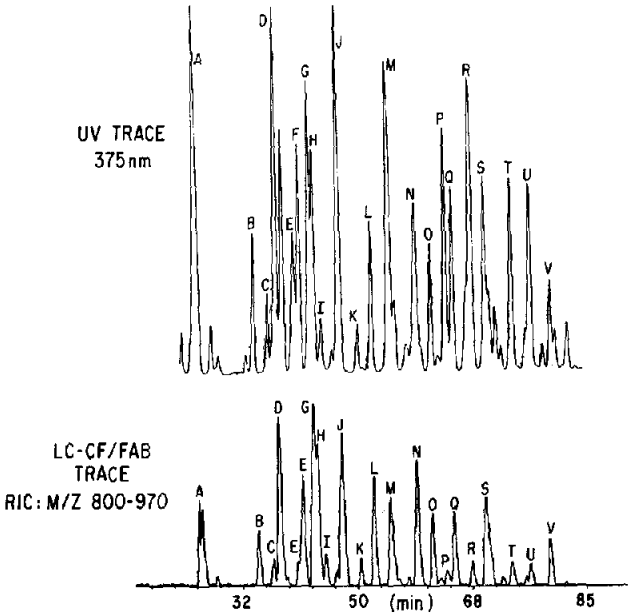

Fig. 9. HPLC–CF-FAB analysis of a mixture of dinitrophenyl-derivatized heptapeptides on the VG 70S instrument with on-line UV detection.

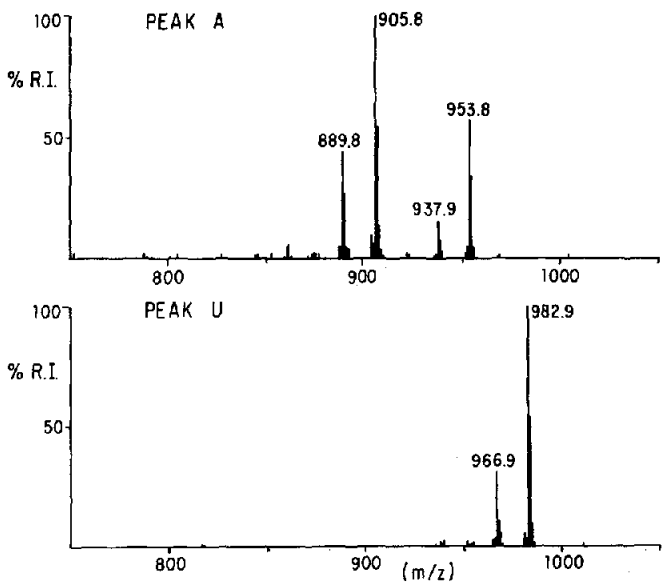

Fig. 10. Mass spectra taken from dinitrophenyl-derivatized heptapeptides corresponding to A and U in Fig. 9.

established by correlating on-line UV detection (222 nm) with $[M+H]^+$ selected-ion current profiles.

Two such profiles corresponding to $[M+H]^+$ ions in the mixture are shown in Fig. 7 relative to the summed ion current profile. Despite the relatively poor chromatography of this mixture, individual candidate $[M+H]^+$ ions are easily extracted. Their identities were confirmed in a second HPLC–CF-FAB-MS experiment on the more sensitive magnetic sector instrument (VG 70S) which permitted full-scan acquisition of mass spectra. The spectra shown in Fig. 8 correspond to components 1 and 2 (Fig. 7) in the mixture. They represent the average of about twenty scans collected during peak elution. Peak 1 obviously contains an unresolved second component (MW = 918.5).

A mixture of DNP-heptapeptides, each containing an arginine residue, was analyzed by HPLC–CF-FAB on the VG 70S with on-line UV detection (375 nm). Note the correlation between UV trace and total-ion chromatogram (Fig. 9) which enables molecular weight information to be obtained on all mixture components. The spectra were characterized by prominent $[M+H]^+$ and $[M+H-16]^+$ ions with little further fragmentation. The examples shown in Fig. 10 indicate the presence of two unresolved components in peak A and one component in peak U. The spectra resulted from averaging at least ten scans over each peak, with no background subtraction.

CONCLUSIONS

The examples shown here indicate the scope of HPLC–CF-FAB-MS for problem solving in biomedical research. The interface and chromatographic system were both stable and robust, tolerating a wide range of solvent composition without difficulty. Satisfactory results were achieved using standard HPLC equipment with a quadrupole mass spectrometer, although improvements in performance were achieved by using a microbore HPLC system or a magnetic sector mass spectrometer. The addition of 1–5% of glycerol in the mobile phase had no perceptible effect on chromatographic behavior. Much greater utilization of the technique is envisaged, especially in cases where TSP is inapplicable or of marginal utility.

ACKNOWLEDGEMENTS

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