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High-performance liquid chromatography–time-of-flight mass spectrometry and its application to peptide analyses

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ABSTRACT

High-performance liquid chromatography (HPLC) has been successfully interfaced on-line with liquid secondary-ion time-of-flight mass spectrometry, utilizing a continuous-flow interface. Time-of-flight mass spectrometry (TOF-MS) is a low-resolution, high-mass-range technique, compatible with extremely rapid data acquisition rates. Thus a TOF-MS system is extremely well suited for coupling with HPLC. This paper describes the interface used to couple the HPLC and TOF-MS as well as the basic operating principles of such a system. Using both standard and packed-capillary reversed-phase HPLC columns, the HPLC–TOF-MS system has been successfully used to separate and detect peptides, providing molecular weight information for the peptide analytes. Experimental data, including chromatograms (UV, reconstructed ion and selected ion) and mass spectra, are presented to demonstrate the ability of the HPLC–liquid secondary-ion TOF-MS system to resolve chromatographically analytes as well as to resolve mass spectrometrically analytes which are unresolved on the chromatographic column.

INTRODUCTION

The past decade has witnessed a vast increase in the number of techniques used to interface high-performance liquid chromatography (HPLC) with mass spectrometry (MS). To date, some of the more common interface techniques have included moving belt [1], thermospray [2], continuous-flow fast-atom bombardment (FAB) [3] and particle beam [4]. In recent years, there has been an increased interest in developing interfaced HPLC–MS techniques which are suited for use with large and somewhat fragile macromolecules of biological interest. HPLC–continuous-flow FAB-MS has proven to be quite amenable to such applications, in large part due to its high-

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mass-range capabilities (approximately 10 000 daltons) and the gentle ionization process, which preserves molecular information. Unfortunately, in order to achieve the high-mass capabilities, continuous-flow FAB is typically performed with a magnetic sector mass spectrometer which generally operates at relatively slow scan rates (s/decade) over the mass range being monitored. Such slow scan rates may result in "missing" a chromatographic peak or skewing the mass spectral information obtained within an eluted chromatographic peak. Substitution of a quadrupole mass spectrometer for the magnetic-sector instrument results in vastly improved scan rates (perhaps 1 s/2–3 decades). The use of the electrospray interface with a quadrupole instrument has proven to be very successful in the HPLC–MS analyses of biopolymers [5,6]. Unfortunately, quadrupole instruments have somewhat limited upper mass ranges, typically 2000 to 3000 daltons. The ideal solution to this dilemma would be to utilize a gentle ionization technique such as FAB in conjunction with a mass spectrometer possessing both a high mass range and a rapid scan rate.

One possible means of approaching these goals is to interface a liquid chromatograph with a time-of-flight (TOF) mass spectrometer. A TOF instrument has a mass range at least as large as that of a magnetic sector instrument. Recently, a TOF instrument was used to acquire the mass spectra of proteins with molecular weights in excess of 200 000 daltons [7]. Additionally, a TOF instrument has scanning speeds which easily surpass those obtained on a quadrupole system [8,9].

Our laboratories have been involved in the development and application of techniques which interface liquid chromatography with TOF-MS. The present paper describes the principles and use of HPLC-liquid secondary-ion time-of-flight mass spectrometry (LSI-TOF-MS) and its application to the HPLC–MS analyses of peptide mixtures. Examples in which both standard and capillary HPLC are employed are presented to demonstrate the capabilities of the interfaced system.

EXPERIMENTAL

The LSI-TOF-MS instrument was constructed in-house and has been described previously [8–10]. Briefly, an ion gun provides a pulsed primary ion beam of 5 keV Xe^+, which are directed at the glycerol–analyte target exiting the tip of the continuous-flow probe interface. The pulsed beam of primary ions sputters secondary analyte ions from the glycerol target. The primary ion pulse width is long enough (approximately 10 μs) to produce a high yield of the secondary analyte ions. After a brief delay, these secondary analyte ions are then extracted from the source region by application of voltage pulses to the source backing plate, draw-out and accelerating grids. The delay time and draw-out pulses provide time, energy and spatial focusing of the analyte ions. The analyte ions are then accelerated to 3 keV and enter the field-free drift tube for mass separation prior to striking a dual channelplate detector at the end of the flight tube. The resulting detector signal is then amplified and directed into the analog input of a high-speed data acquisition system. The high-speed data acquisition system, including the associated software package, was also constructed in-house and is controlled by a 80386-based PC system. Operating principles of the data acquisition and processing system have been discussed in detail elsewhere [10] and will not be repeated in the present paper.

The continuous-flow probe which is actually used to interface the liquid

chromatograph to the mass spectrometer is a modified version of a previously reported probe, constructed in our laboratory [11]. The modifications primarily consist of employing a metal solder seal around the fused-silica capillary at the outlet tip of the probe. The use of a metal seal provides better electrical conductivity to reduce charge build-up on the probe tip and better thermal conductivity, which provides more uniform evaporation of liquid from the probe tip, resulting in more stable pressures within the mass spectrometer.

To prevent freezing and associated blockage of the probe tip due to mobile phase evaporation in the high vacuum of the mass spectrometer, heat was applied to the tip of the flow probe. Electrical current was passed through a length of copper wire, wrapped around the probe tip, providing resistive heating of the wire. To monitor the temperature of the probe tip, a thermocouple was placed within the metal solder seal at the probe tip. The current passing through the heating wire was adjusted to maintain a tip temperature in the range of 40 to 60 °C, a typical value being 50°C. Variation of the tip temperature over this range did not appear to affect significantly the quality of the mass spectra obtained.

The flow-rate of the liquid actually passing into the mass spectrometer was set at 1 μl/min. When the LSI-TOF-MS system was interfaced to a conventional HPLC system, operating at a mobile phase flow-rate of 1 ml/min, a 1:1000 split of the mobile phase was required after the UV detector and prior to the mass spectrometer. The split was accomplished by placing 1 m of 60 μm I.D. fused-silica capillary (SGE, Austin, TX, U.S.A.) between the UV detector and the flow-probe tip. The inlet end of the capillary was simply inserted (not sealed) into the liquid flow path at the exit of the UV detector. The vacuum of the mass spectrometer drew liquid into the capillary, while the length and I.D. of the capillary provided sufficient flow resistance to regulate the liquid flow into the mass spectrometer to 1 μl/min. The remaining 0.999 ml/min was collected as waste at the outlet of the UV detector.

When interfacing a capillary HPLC system to the LSI-TOF-MS system, the mobile phase flow-rate was fixed at 1 μl/min and the chromatographic system was mechanically connected to the inlet of the flow probe with a 60-μm I.D. fused-silica capillary. The mobile phase was entirely transferred into the mass spectrometer, eliminating the need for a splitter.

The conventional HPLC system was a Beckman 114M binary solvent system (Berkeley, CA, U.S.A.), fitted with a Rheodyne 7125 injection valve with a 50-μl sample loop (Cotati, CA, U.S.A.). The variable-wavelength UV detector was a Kratos Spectraflow 757 (Ramsey, NJ, U.S.A.), set at 280 nm.

The capillary HPLC system was a μLC-500 pump and a μLC-10 UV detector, having a 30-nl flow cell and 1 mm optical path-length (Isco, Lincoln, NE, U.S.A.), fitted with a Model CI4W micro-injection valve with a 200-nl internal sample loop (Valco Instruments, Houston, TX, U.S.A.).

Columns

The conventional HPLC column was a base-deactivated 5 μm C_{18} cartridge column, 83 mm × 4.6 mm (Perkin-Elmer, Norwalk, CT, U.S.A.). The fused-silica capillary column was 20 cm × 200 μm I.D. Fused-silica capillary tubing was obtained from Polymicro Technologies (Phoenix, AZ, U.S.A.) and cut to length. The column blank was prepared as described by Shelly *et al.* [12]. However, EPO-TEK 377 epoxy

(Epoxy Technology, Billerica, MA, U.S.A.) was substituted for the originally described formulation due to its acid resistance in organic solutions. Additionally, 2 μm pore size ultra-high-molecular-weight polyethylene frit material (Upchurch Scientific, Oak Harbor, WA, U.S.A.) was substituted for the porous PTFE frit material originally described. The column blank was slurry-packed in-house with 5-μm base-deactivated C_{18} material (Supelco, Bellefonte, PA, U.S.A.).

Reagents and chemicals

All biochemicals were obtained from Sigma Chemicals (St. Louis, MO, U.S.A.). HPLC grade solvents were obtained from Burdick and Jackson (Muskegon, MI, U.S.A.). All other chemicals were of reagent-grade purity.

Mobile phase

For conventional HPLC separations, the mobile phase was prepared by pre-mixing a solution of acetonitrile–water (25:75, v/v). This solution was then made 5% (v/v) in glycerol and 0.1% (v/v) in trifluoroacetic acid. The mobile phase flow-rate was 1 ml/min.

For capillary HPLC separations, the mobile phase was prepared by pre-mixing a solution of acetonitrile water (28:72, v/v). This solution was then made 10% (v/v) in glycerol and 0.1% (v/v) in trifluoroacetic acid. The mobile phase flow-rate was set at 1 μl/min.

All mobile phases were degassed by helium sparging prior to use.

RESULTS AND DISCUSSION

TOF-MS

Time-of-flight mass spectrometry involves measuring the time required for an ion to travel from the ion source region of the mass spectrometer to its detector. Ions are produced in "packets", then accelerated out of the source region and into a field-free drift tube, approximately 1 m in length. Our instrument has a 65-cm flight tube. All ions receive equal kinetic energy of approximately 3 keV during acceleration out of the ion source. Therefore, ions will separate into groups according to their velocity (and hence mass) as they travel the length of the flight tube. The mass-to-charge ratio of an ion is determined by its flight time. Since their kinetic energy is equal, the lighter low-mass ions transit the flight tube faster than heavier high-mass ions do. Eqn. 1, which describes the velocity of the ions in the flight tube, shows that the velocity (v) is inversely proportional to the square root of the mass of the ion (m).

$$v = \left[\frac{2zeV}{m}\right]^{1/2} \tag{1}$$

In practice, the velocities of the ions are not measured. Instead, the time required to transit the flight tube, or time of flight (TOF), is what is actually measured. Eqn. 2 shows the TOF as directly proportional to the square root of the mass of the ion (m), where L is the length of the flight tube, z is the charge on the ion, e is the charge of an electron and V is the accelerating potential. The mass spectrometer is calibrated by fitting the TOF for ions of known masses to eqn. 2.

$$\mathrm{TOF} = \left[\frac{m}{2zeV}\right]^{1/2} L \tag{2}$$

The concept of interfacing HPLC with a TOF-MS system is quite attractive, due to several characteristics of a TOF-MS instrument. A TOF-MS system has extremely rapid scan rates (up to 10^8 decade/s) thus enabling many scans to be acquired across even the narrowest of chromatographic peaks. In principle, no mass-dependent voltage or field is scanned in a TOF-MS instrument. Rather, masses are resolved in a time domain and recorded sequentially by a transient recorder. Obtaining such a large number of scans across the chromatographic peak permits signal averaging to be used as a means of improving the signal-to-noise ratio relative to that of a single or very small number of scans. Additionally, the wide mass range capability of a TOF-MS system is very well matched to the detection of macromolecular biomolecules such as peptides and proteins. Another important consideration is the fact that in TOF-MS all ions from each ionization event may be detected. This permits full-scan and selected-ion data to be acquired at the same time. This is in contrast to scanning magnetic-sector or quadrupole instruments, which are essentially mass filters, permitting only ions of a specific mass to reach the detector.

The use of secondary-ion mass spectrometry (SIMS), which might also be thought of as fast-ion bombardment (FIB), as the ionization mode in our instrument produces little fragmentation of labile molecules, resulting in the ability to obtain molecular weight information. The information obtained by SIMS ionization is very similar to that obtained with FAB ionization. Although our instrument utilizes an ion gun for SIMS ionization, an atom gun could, in principle, be substituted to yield FAB spectra utilizing TOF mass resolution.

Conventional HPLC LSI-TOF-MS

The UV and reconstructed ion chromatograms for a chromatographically resolved two-component peptide mixture are shown in Fig. 1. The excellent match of peak shapes and resolution in the two chromatograms indicates that the flow probe interface does not introduce any substantial band broadening, nor does it exhibit any substantial memory effect. A memory effect, due to the slow ionization and removal of analyte from the probe tip, would result in significant peak tailing and loss of chromatographic resolution. The 5-min time offset between the UV and ion chromatograms is due to the delay volume between the exit of the UV detector and the tip of the flow probe interface. The difference in peak height ratios observed in the two chromatograms indicates that the peptides have different molar absorbtivities at 280 nm, while their ionization efficiency is approximately equal.

The LSI-TOF-MS spectra obtained for each component of this chromatographically resolved mixture are shown in Fig. 2. The spectra in this paper are the raw spectra. They have not been converted into the familiar "stick" spectra. The protonated molecular ion is the predominant species observed in each spectrum, with no evidence of any significant fragmentation of the molecular ion. The ions in the spectra labeled as "G_xH^+" are protonated oligomers of the glycerol matrix, and are commonly observed. These ions may be used for mass calibration of the TOF instrument.

Although the previous example presents the type of data obtainable with the

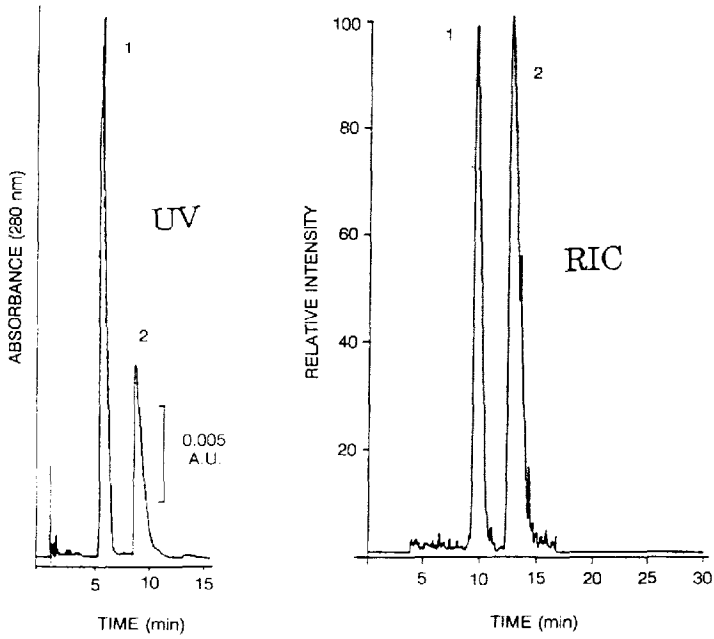

Fig. 1. Chromatograms of a chromatographically resolved two-component peptide mixture obtained by conventional HPLC–LSI-TOF-MS. (Left) UV chromatogram; (right) reconstructed ion chromatogram. 50 nmol of each peptide injected on-column and detected by UV; 50 pmol of each component transferred to the mass spectrometer. Peaks: 1 = Tyr-Gly-Gly-Phe-Met (mol.wt. 574); 2 = Tyr-Met-Gly-Phe-Pro-NH_2 (mol.wt. 613). (Reprinted with permission from ref. 10. © 1990 American Chemical Society.)

HPLC–LSI-TOF-MS instrument, it does not provide an example of some of the more powerful aspects of a combined HPLC–MS system. A more illustrative example of that is given in Fig. 3. This figure shows a chromatogram of a four-component peptide mixture, where components 3 and 4 are not chromatographically resolved. However, by manipulation of the mass spectral data, the selected-ion chromatograms for the protonated molecular ion region for each of the two coeluting peptides may be generated. Thus, even though the two peptides are not chromatographically resolved, they may be mass spectrometrically resolved.

To verify that the two coeluting peptides can be mass-spectrometrically resolved, the mass spectra of the individual components as well as that of the peak containing the coeluting peptides must be examined. In Fig. 4 the LSI-TOF-MS spectrum of pure peptide 3 is presented, as well as the spectrum for the peak containing coeluting peptides 3 and 4. As is clearly demonstrated, the mass spectrometer easily resolves the individual peptides within the merged chromatographic peak. Thus, the selected-ion chromatograms in Fig. 3 are specific for each peptide.

Capillary HPLC–LSI-TOF-MS

The previous examples were for a conventional HPLC instrument interfaced to the LSI-TOF-MS instrument via a 1:1000 flow splitter. Although the performance of the system and quality of the data are satisfactory, one must realize that 99.9% of the

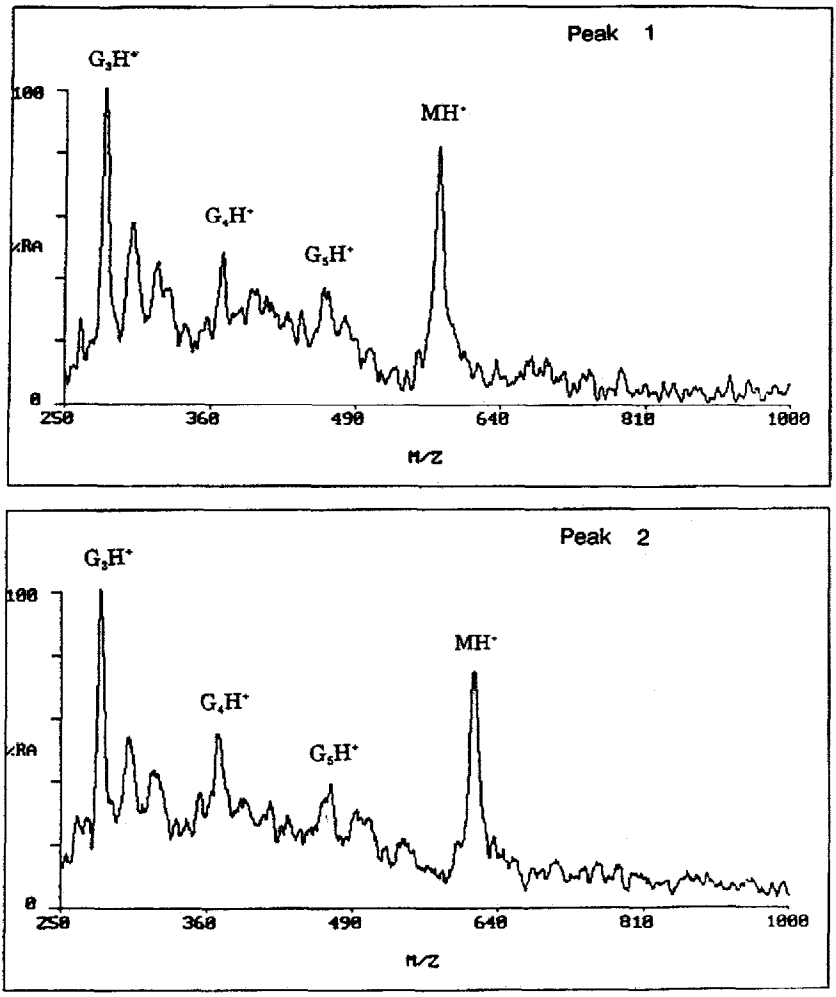

Fig. 2. HPLC–LSI-TOF-MS spectra for each of the peptides chromatographically resolved in Fig. 1.

sample injected into the HPLC column is directed to waste or a fraction collector at the exit of the UV detector. A much better method would be to utilize flow-rates low enough that a splitter is not required and the entire mobile phase and analyte load are transferred directly to the mass spectrometer. The low mobile phase flow-rates used in capillary HPLC is a primary advantage of interfacing a capillary HPLC system with the LSI-TOF-MS instrument.

The capillary HPLC–LSI-TOF-MS UV and reconstructed ion chromatograms for a two-component peptide mixture are presented in Fig. 5. As indicated by Fig. 5, the flow probe appears to be quite compatible with capillary columns, and has no adverse influence on the chromatographic resolution or peak shape.

The use of a capillary HPLC system does not affect the type of mass spectral information acquired. The LSI-TOF-MS spectrum of the first peak in the capillary

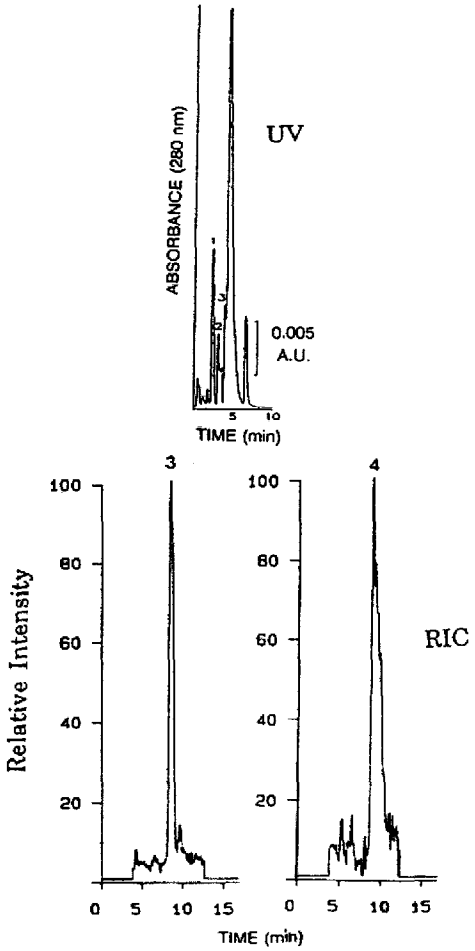

Fig. 3. UV and selected ion chromatograms of a four-component peptide mixture. Components 3 and 4 are not chromatographically resolved. They may be mass spectrometrically resolved by selected-ion chromatograms of the protonated molecular ion region for each peptide. Peaks: 1 = Tyr–Gly–Gly–Phe–Met (mol.wt. 574); 2 = Tyr–Met–Gly–Phe–Pro–NH_2 (mol.wt. 613); 3 = Arg–Tyr–Leu–Gly–Tyr–Leu (mol. wt. 784); 4 = Leu–Trp–Met (mol.wt. 448). (Reprinted with permission from ref. 10. © 1990 American Chemical Society.)

chromatogram of Fig. 5 is shown in Fig. 6. As was observed in the spectra acquired with the conventional HPLC–LSI-TOF-MS system, the capillary system yields spectra in which the predominant species is the protonated molecular ion of the peptide analyte. In addition, the mass spectral data obtained with the capillary system may be subjected to the same types of data manipulation and processing as that used on the conventional HPLC–LSI-TOF-MS system discussed earlier.

There are issues remaining to be resolved which have not been addressed in this paper. One of these is the relatively low mass-spectrometric resolution achieved by a TOF system, typically in the range of 100 to 500. This resolution is substantially less than that which may be achieved with a magnetic sector instrument. However, several

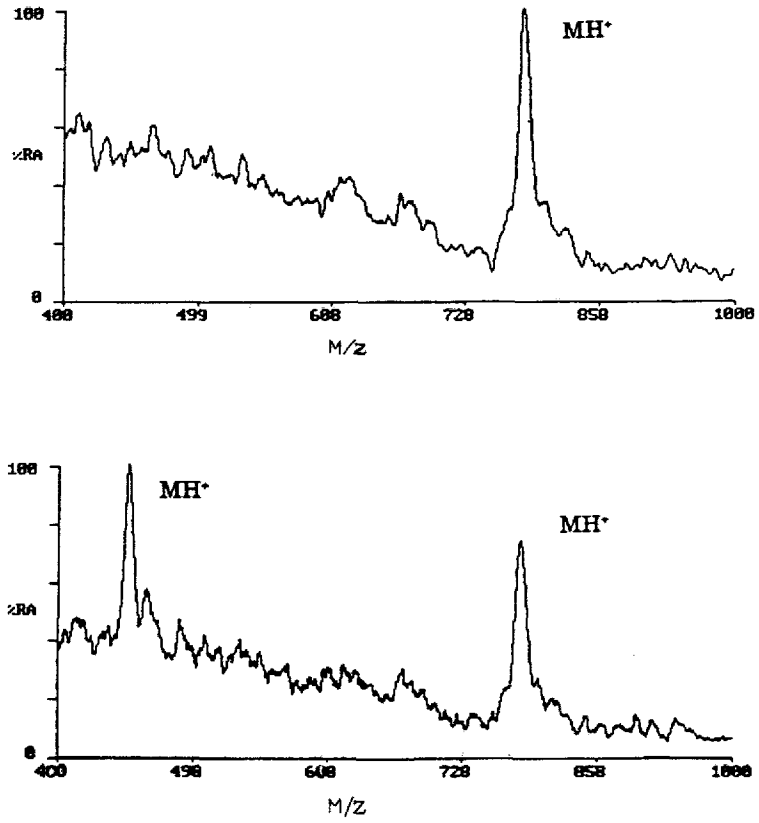

Fig. 4. HPLC–LSI-TOF-MS spectra for pure peptide 3 (top) and of the peak containing a mixture of peptides 3 and 4 (bottom). (See Fig. 3.)

laboratories are developing methods to increase the resolution obtainable with a TOF system to that obtainable with magnetic instruments [13,14].

Also, a system such as that described in the current paper is not yet commercially available. Our sytem is a prototype, built primarily in-house. Thus, many laboratories may not be able to gain immediate access to such a system.

Finally, the very rapid scanning rate over a wide mass range which can be achieved with TOF-MS generates huge amounts of data at extremely rapid rates. Not only must the data system be capable of acquiring data very rapidly, it must also be capable of quickly and efficiently storing the raw data. These requirements are beyond the abilities of most data acquisition systems, thus requiring the use of specialized or custom-built data processing systems.

Although these factors currently represent obstacles which must be overcome, they also represent, in combination with the current achievements, the numerous research opportunities available in the further development and refinement of HPLC–LSI-TOF-MS.

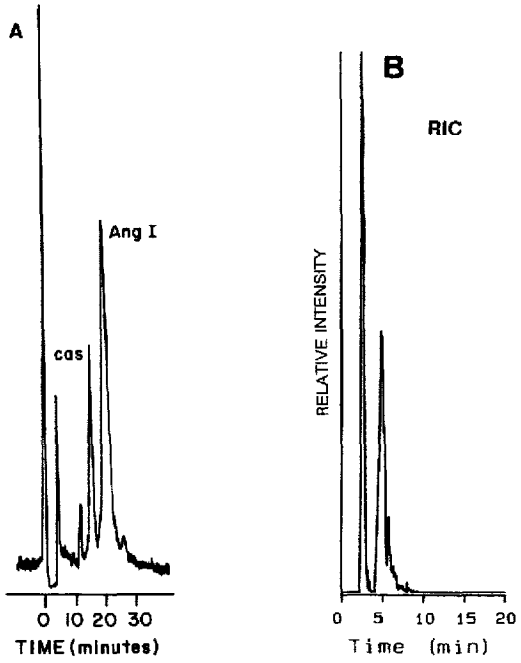

Fig. 5. Capillary HPLC–LSI-TOF-MS (A) UV and (B) reconstructed ion chromatograms of a two-component peptide mixture. 25 pmol α-casein [fragment 90–95] (mol.wt. 784, first peak) and 15 pmol human angiotensin I (mol.wt. 1296, second peak) were injected.

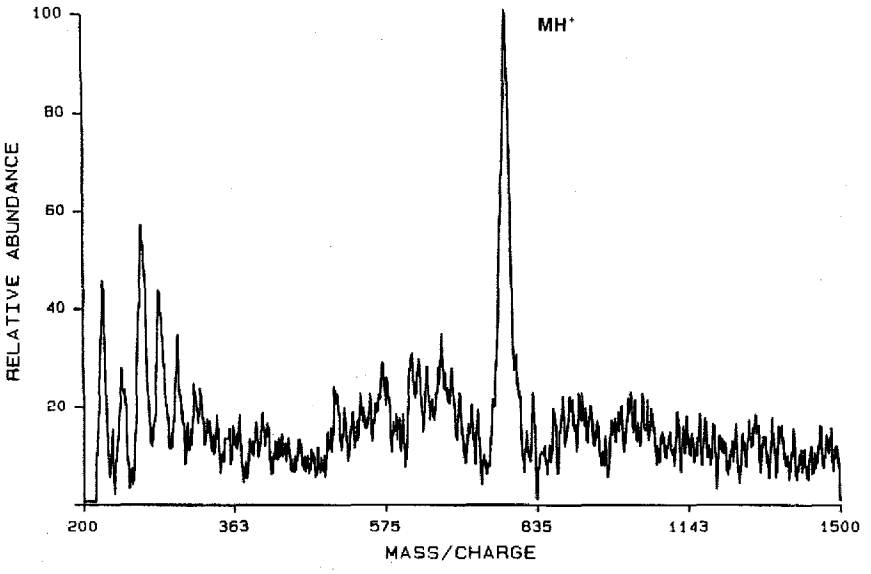

Fig. 6. HPLC–LSI-TOF-MS spectrum of the first peptide peak obtained from capillary HPLC–LSI-TOF-MS separation of a two-component peptide mixture shown in Fig. 5.

CONCLUSIONS

The preliminary data obtained and presented in this paper provide a strong argument for the further development of HPLC–LSI-TOF-MS systems. We have demonstrated that interfacing HPLC to LSI-TOF-MS is possible, and that such a system is quite versatile in that it may utilize either conventional or capillary HPLC. Even under the stringent dead-volume requirements of capillary HPLC, the interfaced system showed no signs of significant deterioration in the quality of the chromatographic separations obtainable. The sensitivity of the HPLC–LSI-TOF-MS instrument is approximately constant, whether operated in the conventional or capillary modes. For the peptides examined in this paper the sensitivity was in the low picomole range. As demonstrated by the examples presented, the HPLC–LSI-TOF-MS system produces spectra which exhibit predominantly protonated molecular ions with little fragmentation. Although the peptides used as examples all have molecular weights under 1000 daltons, the TOF-MS technique is applicable to much larger molecules.

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