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Interfacing microbore and capillary liquid chromatography to continuous-flow fast atom bombardment mass spectrometry for the analysis of glycopeptides

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ABSTRACT

This work describes a system to interface either microbore or packed capillary gradient liquid chromatography (LC) to fast atom bombardment mass spectrometry (FAB-MS). The interface incorporates on-line ultraviolet detection and post-column matrix addition to enable independent optimization of both LC and FAB-MS. The glycopeptide antibiotic teicoplanin was chosen as a model system because this group of compounds places severe demands on the chromatographic separation and is difficult to analyze by FAB-MS. For both microbore and capillary LC, high-quality mass spectra of the major components in teicoplanin were obtained; however, the increased sensitivity of the capillary system allowed spectra to be obtained at low picomole concentrations. The sensitivity and ease of use make capillary LC the preferred system for use in LC–FAB-MS.

INTRODUCTION

The use of fast atom bombardment (FAB) as an ionization technique to obtain the mass spectra of polar and ionic compounds, such as peptides and oligosaccharides, has become universally accepted in the short time since its discovery in 1981 by Barber et al. [1]. Unfortunately, the incorporation of mass spectrometric (MS) detection for analyzing mixtures of these compounds by liquid chromatography (LC) is much less routine. One of the most promising interfaces for performing LC–MS is continuous-flow FAB. In this method, effluent containing a low percentage of FAB matrix is supplied to the

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FAB probe at low microliter flow-rates. Several approaches to this technique have been reported by Caprioli et al. [2,3] and Ashcroft et al. [4]. The constraint of low flow-rates (2–5 μl/min) and the need for a non-volatile FAB matrix, such as glycerol, have presented problems in the routine application of LC to continuous-flow FAB-MS. To date, most investigators have used effluent splitting along with isocratic LC systems to meet the constraints of constant low flow-rates. Applications of this technique using microbore and capillary LC for the analysis of peptides and oligosaccharides have been recently reported [5–8]. In all of the reports cited above, glycerol has been added to the mobile phase at concentrations of 5–15% to allow compatibility with the FAB ionization process. This imposes severe limitations on the LC in terms of flow-rate, pressure and separation. The advantage to adding the FAB matrix post-column is that it allows independent optimization of the conditions for LC and FAB-MS similar to the approach taken for post-column addition of buffers in thermospray LC–MS.

A recent report by Moseley et al. [9] describes the post-column addition of FAB matrix using a coaxial sheath with use of open tubular and packed fused-silica LC columns (50 μm I.D.). The coaxial design for post-column addition is especially applicable to separation techniques with flow-rates in the low nl/min range such as is encountered with open tubular LC and capillary zone electrophoresis [10].

Our work describes the design and application of very low dead volume, dynamic tees for LC effluent flow-rates in the low μl/min range to allow effluent splitting and/or the post-column addition of matrix for LC–FAB-MS. The LC system described is capable of gradient elution on either microbore (1.0 mm I.D.) or packed fused-silica capillary (320 μm I.D.) columns.

EXPERIMENTAL

Microbore high-performance liquid chromatography (HPLC)

The need to perform gradient separations in micro LC with post-column addition of matrix was met by using a Waters 600-MS LC system (Waters Chromatography Division of Millipore, Milford, MA, U.S.A.). A linear gradient was performed from an initial mobile phase of water–acetonitrile (85:15, v/v) containing 0.1% trifluoroacetic acid (TFA) to a final mobile phase of water–acetonitrile (50:50, v/v) containing 0.1% TFA in 40 min. To achieve a flow-rate of 75 μl/min on the microbore column (Spheri S5, ODS2, Applied Biosystems, 25 cm × 1.0 mm I.D.), the pump flow-rate was run at 1.5 ml/min and the effluent was split by a tee prior to the injector using a dynamic balance column (5-μm Spherisorb ODS, 25 cm × 4.6 mm I.D.). The overall schematic of the gradient microbore LC system is shown in Fig. 1. A Rheodyne Model 7413 injection valve with a 0.5-μl internal injection loop was used for sample injection. The end of the microbore column was connected directly to the flow

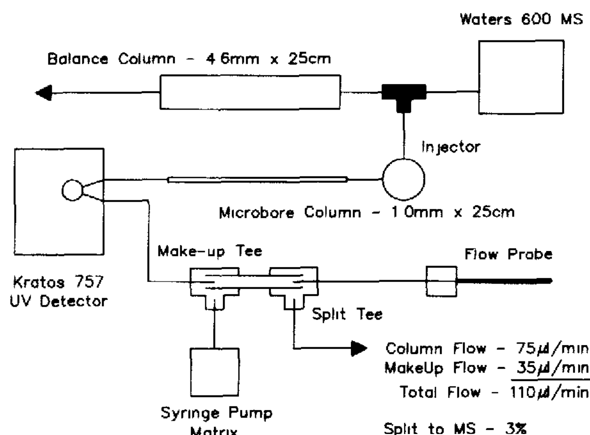

Fig. 1. Schematic of microbore LC system.

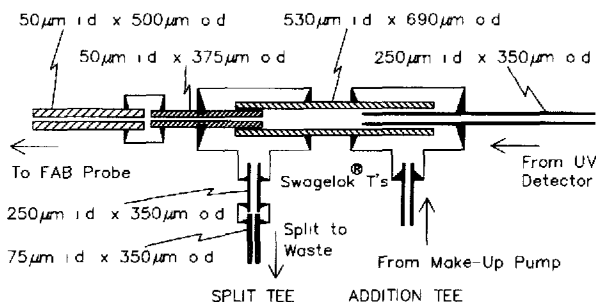

Fig. 2. Details of microbore LC addition and split tees.

cell (0.5 μl) in a Kratos 757 absorbance detector (ABI, Ramsey, NJ, U.S.A.) operated at 254 nm. Connections from the outlet of the flow cell were made with fused-silica tubing (Polymicro Technologies, Phoenix, AZ, U.S.A.). The tubing was then connected to an addition tee where 35 μl/min of a glycerol–methanol (50:50, v/v) mixture was added to the column effluent to give a total flow-rate of 110 μl/min. This glycerol–methanol mixture was delivered by a Waters prototype syringe pump (stepped syringe, 30 nl per pulse) controlled by a Hewlett Packard 3311A function generator. The combined flow was then dynamically split so that approximately 3 μl/min of effluent entered the fused-silica transfer tubing (500 μm O.D.$\times$50 μm I.D.) which carried the column effluent and FAB matrix to the tip of the continuous-flow FAB probe over a distance of approximately 1 m. The remainder was directed to waste. The details of the addition and split tees are shown in Fig. 2. The use of fused-silica tubing of various inside and outside diameters allowed the construction of coaxial, post-column addition and split tees that had low dead volume and inertness.

Packed capillary high-performance liquid chromatography

The gradient system used for the packed capillary LC involved the use of the same equipment as described for the microbore system with some modification. The Waters 600-MS LC system was used along with the same balance column to produce an effective flow-rate of 2 μl/min through the capillary column. A linear gradient was used from an initial mobile phase of water–acetonitrile (85:15, v/v) containing 0.1% TFA to a final mobile phase of water–acetonitrile (50:50, v/v) containing 0.1% TFA in 50 min.

The overall schematic of the capillary LC system is shown in Fig. 3. The capillary column was fabricated in-house using 320 μm, I.D. fused-silica tubing. The column bed support was an in situ porous ceramic frit described by Cortes et al. [11]. The columns were slurry-packed with 5-μm Spherisorb ODS packing using a procedure similar to that described by Gluckman et al. [12]. The capillary column was directly connected to the Rheodyne internal loop (0.5 μl) injector. The end of the capillary column was connected to a tee shown in detail in Fig. 4. A solution of glycerol–methanol (50:50, v/v) was then added coaxially at a flow-rate of 1 μl/min by the syringe pump described above. The end of the capillary column was positioned approximately 1 mm from the fused-silica transfer tubing (1 m × 500 μm O.D. × 50 μm I.D.). At a point 3 cm beyond the addition tee, a 1-cm segment of the polyimide coating of the transfer line

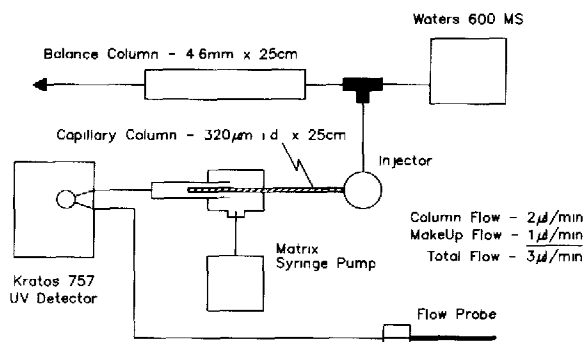

Fig. 3. Schematic of packed capillary LC system.

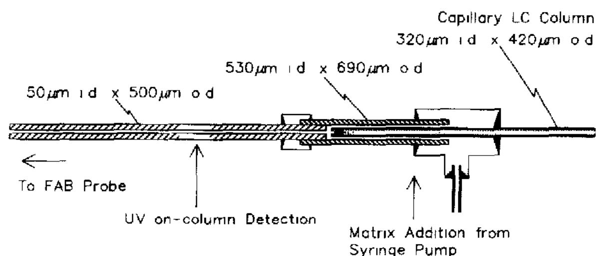

Fig. 4. Details of post-column addition tee for packed capillary LC.

was burned away to give a clear quartz tube which was positioned in a fabricated holder in the Kratos 757 absorbance detector to allow for "on-line" detection.

Mass spectrometry

A VG ZAB2-SE double-focusing mass spectrometer (VG Analytical, Manchester, U.K.) equipped with a VG continuous-flow FAB ion source and probe were used in these studies. Ionization was achieved with the use of a cesium ion gun operated at 25–30 kV, which, at the normal 8-kV acceleration voltage of the instrument, gave an effective particle energy of 17–22 keV. The indicated source temperature was 35–40°C. Except where noted, the mass spectrometer was operated at a resolution of 2500, and a scan rate of 30 per decade was used to cover a mass range of m/z 2300–1400. The resulting scan cycle time was about 10 s. Samples were dissolved in the initial mobile phases prior to injection.

Teicoplanin used in these studies was obtained from the Merrell Dow Research Institute (Cincinnati, OH, U.S.A.).

RESULTS AND DISCUSSION

Teicoplanin is a glycopeptide antibiotic obtained as a fermentation product from *Actinoplanes teicomyceticus* and is a mixture of multiple components. The six major components of teicoplanin are designated according to their relative retention order on a reversed-phase HPLC column [13]. Each of these compounds are analogues of the earliest eluting component A3, which represents the core glycopeptide. The five later eluting components (group A2) consist of the core glycopeptide with an additional N-acyl-D-glucosamine to which different acyl aliphatic chains are attached as shown in Fig. 5 [14].

Teicoplanin was chosen as a model system to evaluate the LC–FAB-MS system for two reasons. First, the separation of both the major and minor components of teicoplanin requires a high degree of resolution by LC, and to maintain this resolution through the entire LC–FAB-MS system would be a rigorous test of extra-column effects. Secondly, the ability to identify the individual components of teicoplanin from their FAB mass spectra at realistic concentrations is difficult. Therefore, the overall instrument response to teicoplanin is a more meaningful evaluation of LC–MS performance than the use of compounds ideally suited to FAB.

Our initial evaluation of continuous-flow FAB using approaches outlined in the literature suggested areas for possible improvement including both the use of gradient elution LC and post-column addition of the FAB matrix. Our first attempt at gradient elution LC involved the use of microbore columns. The reason for this approach was the commercial availability of the column and the higher flow-rates (50–100 μl/min) which would make it easier to run gra-

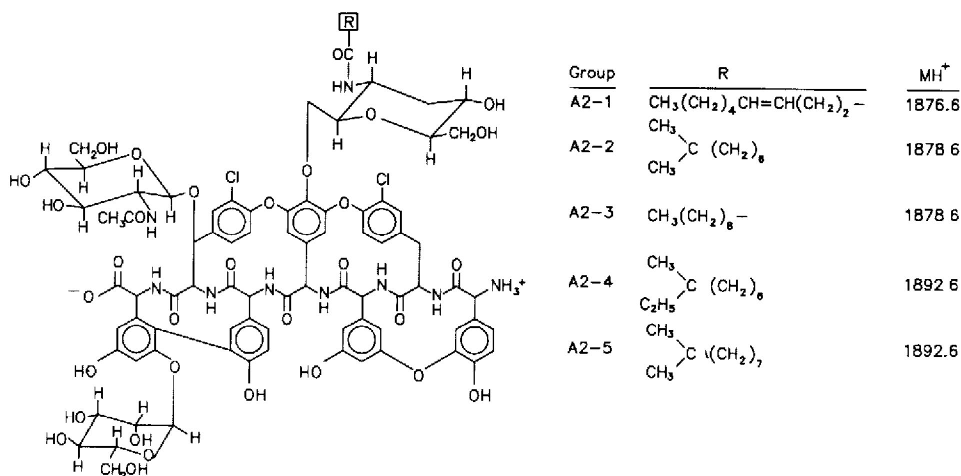

Fig. 5. Structures of the teicoplanin A2 group.

dients, and, in general, to perform post-column matrix addition and UV detection. One of the essential features of this system was the use of a standard analytical column (25 cm × 4.6 mm I.D.) as a dynamic, parallel restrictor to allow gradient formation at flow-rates compatible with the gradient HPLC pump (1.5 ml/min). A split in the flow between the microbore column and the dynamic restrictor column was then achieved based on the ratio of their cross-sectional areas. This resulted in a flow-rate of about 75 μl/min through the microbore column to which a matrix solution with a flow-rate of 35 μl/min was added post-column. The combined flow-rate of 110 μl/min then had to be split as the pumping capacity of the MS system was limited to only 3 μl/min. This resulted in a transfer efficiency of only about 3%. In spite of the low transfer efficiency, the results obtained with this system for teicoplanin were encouraging. The chromatograms obtained from the total ion current (TIC) of MS and the UV detection are shown in Fig. 6. The comparison of these two chromatograms shows a small loss in resolution in the TIC; but good overall chromatographic integrity. The FAB mass spectra of the major A2 components of teicoplanin were of interpretable quality as shown by the mass spectrum of the A2-2 component presented in Fig. 7. Unfortunately, this sensitivity was insufficient for the detection of the A2 components of teicoplanin when lower concentrations of the total mixture were injected onto the microbore column. On average, the A2-2 is the major component and accounts for 40% of the total teicoplanin content. Even with an injection of 28 μg of teicoplanin, the spectrum presented in Fig. 7 represents only a maximum of 180 pmol of A2-2 reaching the mass spectrometer after the split interface.

The high split required for the microbore system and the resulting limitation

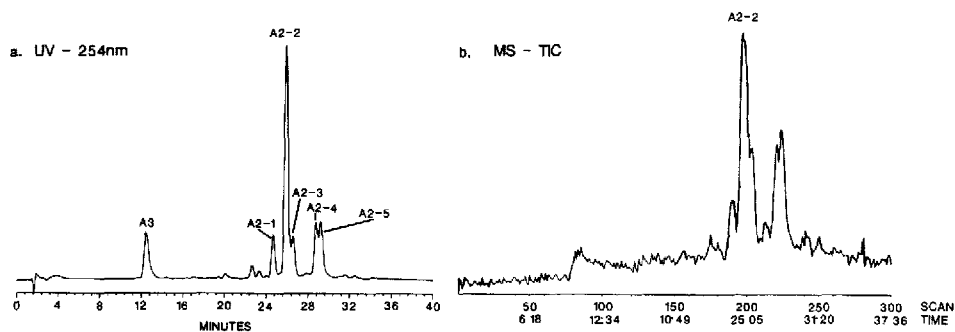

Fig. 6. Chromatograms of teicoplanin by microbore LC representing 28 μg (15 nmol) injected and 3% (440 pmol) split to MS. (a) UV (254 nm); (b) MS total ion current (TIC).

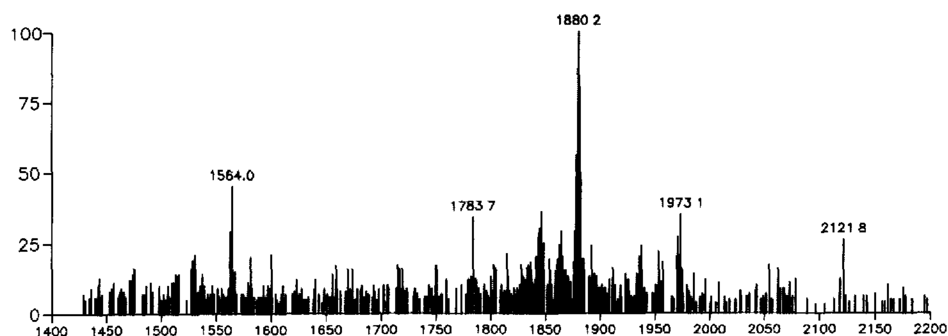

Fig. 7. Mass spectrum of teicoplanin A2-2 from the microbore LC system (180 pmol).

in sensitivity was a major consideration for designing a gradient capillary system for LC–FAB-MS. The same philosophy of gradient formation was used with the packed capillary column resulting in a flow-rate of about 2 μl/min. Matrix solution was added post-column at 1 μl/min and the entire effluent directed to the MS system. The TIC and UV chromatograms for a 2-μg injection of teicoplanin on this system are shown in Fig. 8. As shown, there is excellent resolution in the TIC chromatogram. In contrast to the microbore LC system, all the sample injected on the packed capillary column was transferred to the mass spectrometer. This resulted in mass spectra of high quality as indicated by the FAB mass spectrum for the A2-2 peak shown in Fig. 9 which represents 420 pmol. The mass spectra of other A2 components, which are present at lower concentrations, also exhibited good quality. For example the A2-1 component of teicoplanin is usually present at a level of approximately 10% of the total mixture. The mass spectrum of A2-1 from the same LC run shown in Fig. 10 represents 100 pmol of A2-1. Indeed, the mass spectra obtained using capillary LC–FAB-MS showed that the mass measurement and isotope distribution for a single scan were in close agreement with the theoret-

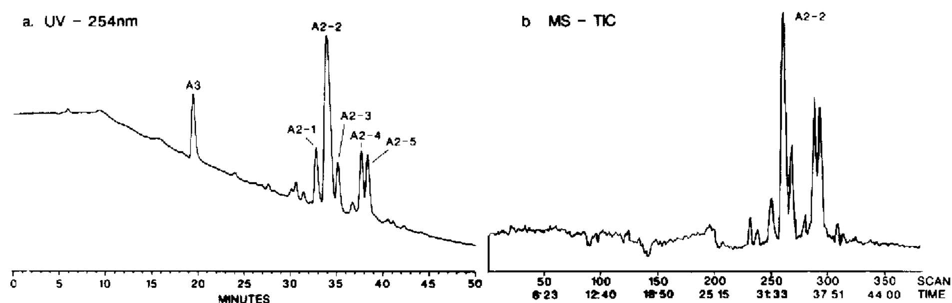

Fig. 8. Chromatograms of teicoplanin by packed capillary LC representing 2.0 μg (1 05 nmol) injected. (a) UV (254 nm); (b) MS total ion current (TIC).

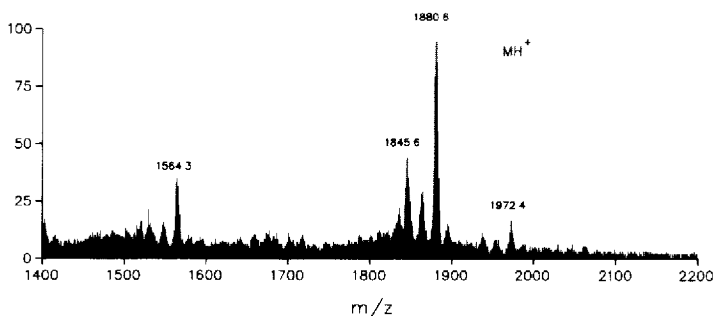

Fig. 9. Mass spectrum of teicoplanin A2-2 from the packed capillary LC system (420 pmol).

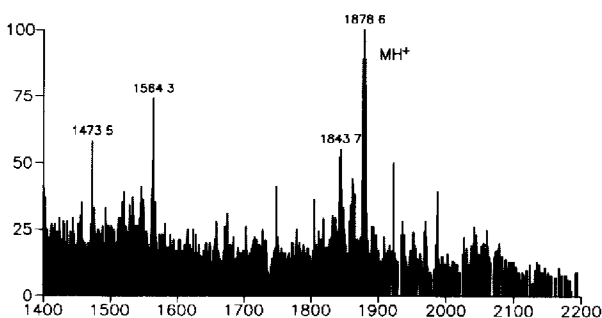

Fig. 10. Mass spectrum of teicoplanin A2-1 from the packed capillary LC system (100 pmol).

ical prediction. This is illustrated in Fig. 11 for the molecular ion region of the teicoplanin A2-2 component and for the molecular ion region of vancomycin, another member of the glycopeptide antibiotic family, each acquired at the 500-pmol level.

As a comparison to those results obtained with the glycopeptides teicoplanin and vancomycin, the sensitivity of the capillary LC–FAB-MS system was evaluated using a more ideal analyte, a synthetic decapeptide with a molecular weight of 1328 daltons. A sample of the decapeptide was chromatographed

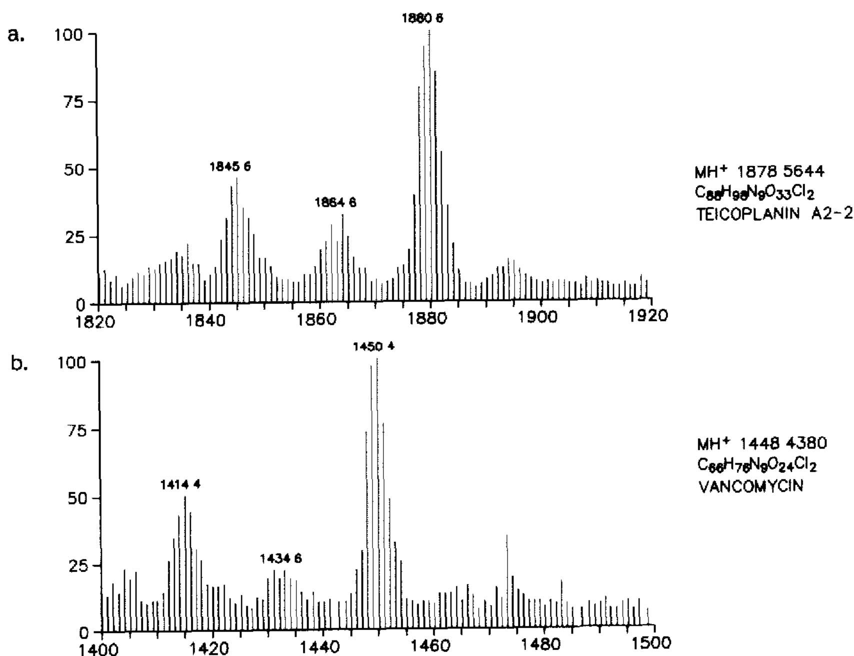

Fig. 11. Mass spectra of the molecular ion region of (a) teicoplanin and (b) vancomycin illustrating mass measurement and isotope distribution at 500 pmol.

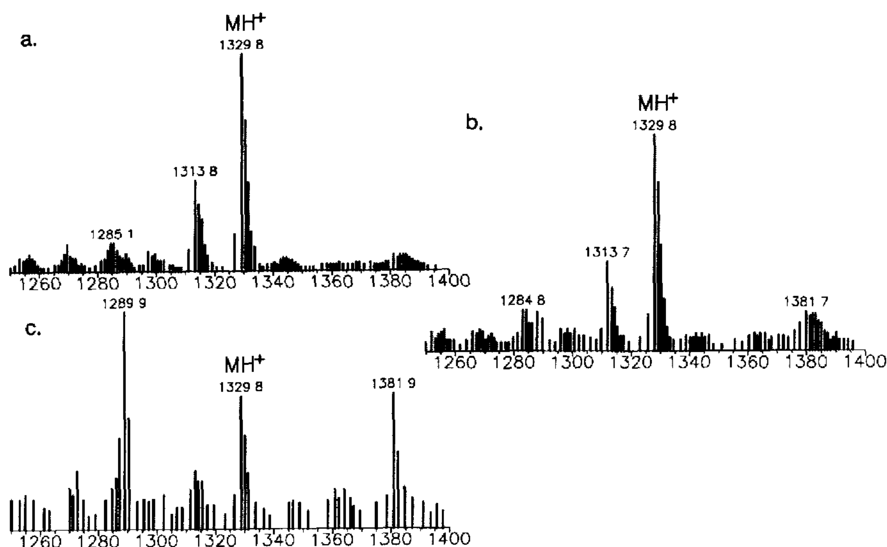

Fig. 12. Mass spectra of the molecular ion region of a synthetic decapeptide (succinyl-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-Glu-OH, MW 1328) showing full-scan sensitivity for (a) 100 pmol, (b) 10 pmol and (c) 1 pmol injected on-column.

isocratically with the final mobile phase composition noted in the Experimental section. The mass spectrometer was scanned at a rate of 10 s per decade over the mass range of 1800–500 daltons giving a cycle time of about 6 s. The results appear in Fig. 12. Under these conditions, an injection of 100 pmol gave an intense response for both the mass spectrum and the TIC. For 10 pmol the response was still well above background, but at 1 pmol the matrix ions became significant although the protonated molecular ion was still easily identified. The detectability of a compound is dependent on several variables including its molecular weight, the FAB matrix used and the accessibility of the compound to FAB ionization. In general, with this capillary LC–FAB-MS system, peptides with molecular weights in the range of 1000–2000 daltons are usually detected with 10–100 pmol injected on-column and often exhibit sequence-specific fragment ions in their mass spectra. In contrast, glycopeptides such as teicoplanin are detected at 50–500 pmol injected.

CONCLUSIONS

The increased sensitivity of the capillary LC–FAB-MS system is primarily due to the fact that all material injected onto the LC column reaches the mass spectrometer. In addition, the chromatographic resolution of the capillary system appears somewhat better than the microbore system due to the deletion of the extra column effects contributed from the post-column splitter section in the interface. These advantages outweigh the problems involved in fabricating the packed capillary LC columns and make this the preferred system for LC–FAB-MS. The use of gradient elution minimizes the effect of large volume injection (0.5 μl) and makes this a useful system for trace analysis with scanning detection limits in the low picomole range. The advantages of this approach to obtain FAB mass spectra in complex peptide mixtures is evident, especially those obtained from the enzymatic digestion and solid-phase peptide synthesis.

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