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# Simple interface for microbore LC and electrospray ionization mass spectrometry and analysis of melphalan–alkylation sites in metallothionein

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**Abstract**: A microbore high pressure liquid chromatograph has been interfaced to a Vestec electrospray ionization source retrofitted to a Hewlett–Packard quadrupole mass spectrometer. The chief features include the absence of a splitter, the use of a second, ballast column to provide a stable flow rate across the gradient, and an in-line UV detector. The system was evaluated for analysis of peptide mixtures and applied to identification of drug-modified peptides released by tryptic digestion of drug-alkylated metallothionein.

**Keywords**: *Microbore HPLC; electrospray ionization mass spectrometry; peptide mapping; melphalan; metallothionein.*

## Introduction

Since its introduction 10 years ago [1] electrospray ionization mass spectrometry (ESIMS) has become widely used for the analysis of both low and high molecular weight compounds [2–4]. One of its most important features is its compatability with high pressure liquid chromatography. Recently a few manufacturers have offered integrated LC–ESIMS systems, however, most laboratories using LC–MS with electrospray have had to take responsibility for interfacing their own systems. The majority of the papers published to date report interfaces with ionspray sources, a technique closely related to electrospray [4–7]. However, separation techniques have been interfaced to several kinds of electrospray sources as well [3, 6–12]. One such source is that provided by Vestec Corp. (currently PerSeptive Biosystems/Vestec Products), which differs from those of other manufacturers by the use of a heated block for declustering ions in the spray [13, 14]. This thermally assisted source has been retrofitted to low voltage mass spectrometers from a variety of manufacturers [10–19]. It has been interfaced to accept samples from size-exclusion chromatography [18], capillary electrophoresis [10, 11], and nanoscale capillary liquid chromatography [10–12].

This report describes an interface developed to introduce the effluent from a microbore LC directly into the Vestec electrospray source, evaluation, and optimization of the performance of the interfaced system for gradient separation and molecular weight determination of peptides alkylated by the chemotherapeutic agent melphalan.

## Experimental

### *Materials and methods*

Synthetic peptides (bradykinin, Met-Lys-bradykinin, buccalin, momany peptide and substance P fragment (4–11) were used as purchased from the Sigma Chemical Co. (St Louis, MO, USA); neurotensin was purchased from Bachem Bioscience Inc. (Philadelphia, PA, USA). LC grade methanol and acetonitrile were purchased from J.T. Baker Inc. (Phillipsburg, NJ, USA). LC grade acetic acid was purchased from Fisher Scientific Co. (Fair Lawn, NJ, USA). Rabbit liver metallothionein 2 [15] (the Sigma Chemical Co.) was incubated with melphalan (the Sigma Chemical Co.) for 30 min in 0.1 M potassium phosphate buffer at pH 7.4 and purified by LC. The alkylated

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protein was cleaved using a 1:200 ratio of trypsin–protein, incubated at pH 8.0 for 10 min.

*Chromatography*

An ISCO (Lincoln, NE) μLC-10 chromatograph equipped with two 100 DM syringe pumps was used. For all separations, Synchrom (Lafayette, IN, USA) C18 (1.0 × 250 mm and 2.1 × 250 mm, particle size 6.5 μm) columns were employed. For solvent gradients A was 0.25% acetic acid in water and B was 0.25% acetic acid in acetonitrile. UV detection was at 206 nm. A typical flow rate through the working column was 25 μl $min^{-1}$.

*Mass spectrometry*

A Hewlett–Packard (Palo Alto, CA, USA) quadrupole 5988A mass spectrometer equipped with a Vestec (Houston, TX, USA) thermally assisted electrospray ion source and Phrasor (Duarte, CA, USA) high energy ion multiplier was used. All mass spectral data were collected and processed by a Vector Two data system from Technivent Corp. (St Louis, MO, USA).

## Results and Discussion

Flow rate has been one of the major considerations in interfacing LC to ionspray and electrospray ionization sources. The Vestec source used in this study had been used with flow rates up to 80 μl $min^{-1}$ [14] and performed optimally between 20 and 30 μl $min^{-1}$ with the peptide mixtures studied here. This range is readily compatible with the eluent from microbore columns, but requires post-column splitting if conventional packed columns are used. Transfer of the entire sample into the ionization source was preferable because the overall sensitivity of the system would be maximized. In our experiments a flow rate as low as 25 μl $min^{-1}$ was used to separate analytes in the microbore column and to deliver them into the electroscopy. To achieve a stable flow rate at that level, both LC pumps were operated at higher flow rates and the solvent was split after mixing and before the sample was injected (Fig. 1). Use of a second C18 column of a larger diameter as a ballast column allows a constant splitting ratio to be maintained as the solvent gradient changes. This cannot be achieved by using a piece of silica capillary as ballast, because the back pressure of the working column and that of the ballast capillary change in different ways across the gradient due to differences in their coefficients of viscosity.

Typically the mobile phases that go into the solvent gradient in a reversed-phase LC separation consist of water and acetonitrile containing small amounts (usually 0.1%) of trifluoroacetic acid. It was determined in this

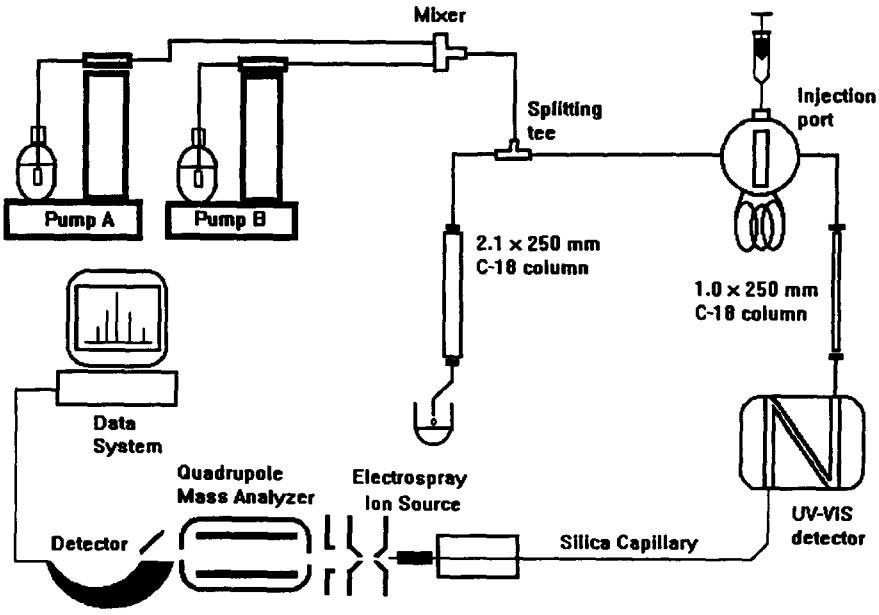


**Figure 1**
Schematic diagram of the LC/ESIMS interface.

study that the use of acetic acid in place of trifluoroacetic acid increases the sensitivity of this electrospray source for the production of cations or anions [19] by at least a factor of 10.

Chromatographic separation was maintained with gradients of mobile phases consisting of 0.25% acetic acid in water and 0.25% acetic acid in acetonitrile.

The overall performance, chromatographic fidelity and limits of detection were tested with a mixture of synthetic peptides. The sample injected was 2 μl of a solution of 0.25% acetic acid in deionized water containing 10 μM bradykinin, Met-Lys-bradykinin and neurotensin, and 50 μM buccalin, momany peptide and substance P fragment (4–11). Figure 2(a) shows the chromatographic separation recorded by a UV detector in line between the column and the electrospray source, and Fig. 2(b) shows it reconstructed from the summed ion currents in mass spectra repetitively scanned from 200 to 1400 amu. Although some peak broadening occurs in the transfer with these samples, generally the chromatographic fidelity is retained as the eluent flows into the ESI source.

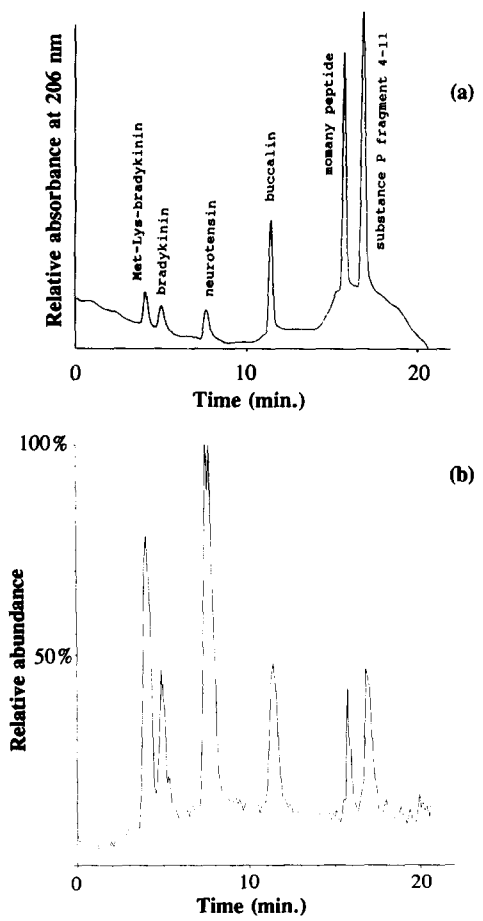


**Figure 2**
Chromatograms of the test mixture of peptides: (a) UV chromatogram (at 206 nm); (b) total ion current chromatogram. The gradient was 5–25% B in 15 min and 25–45% B in 7.5 min. Full scans were acquired across the range 200–1400 *m/z*.

Limits of detection were evaluated using reconstructed ion chromatograms comprising the $MH^+$ signal for each peptide. In this case, 10 pmol of each peptide could be detected. At that level only two of the peptides were detected by UV absorbance at 206 nm. It is worth mentioning that the first three compounds of the test mixture are rather hydrophilic, and they elute when the organic solvent is between 5 and 15% in the gradient. Nonetheless, the thermally assisted electrospray source provided stable and intensive peptide signals with the predominantly aqueous solvent. The interfaced system was then applied to the analysis of a mixture of peptides produced by tryptic digestion in a study of the conjugation of the anticancer agent melphalan by the ubiquitous cytosolic protein metallothionein [20]. Using electrospray ionization the products of an *in vitro* incubation were all found to comprise 1:1 molar adducts. A closer look at the sites of reaction was provided by analysis of peptides produced by digestion with trypsin.

On-line LCMS is a powerful analytical tool for rapid characterization of chemically modified peptides from a protein whose sequence and molecular weight are already known. In this case 2 μl of the 50 μM tryptic digest was injected into the LCMS system and full-scan mass spectra were acquired repetitively through the mass range 200–1200. Chromatograms detected by UV absorbance at 206 nm and reconstructed from total ion current are shown in Fig. 3. When this chromatographic peptide map was compared with the map from a trypsin digestion of unmodified metallothionein, several new peaks were recognized.

Because the sequence of rabbit liver metallothionein 2 was known, it was possible to predict the molecular weight of every peptide produced by trypsin's specific cleavage at arginine and lysine. It was also possible to predict the molecular weight of every peptide potentially conjugated to melphalan. Ion chromatograms such as the two shown in Fig. 4 were reconstructed from the large set of scanned spectra and used to search for alkyl-

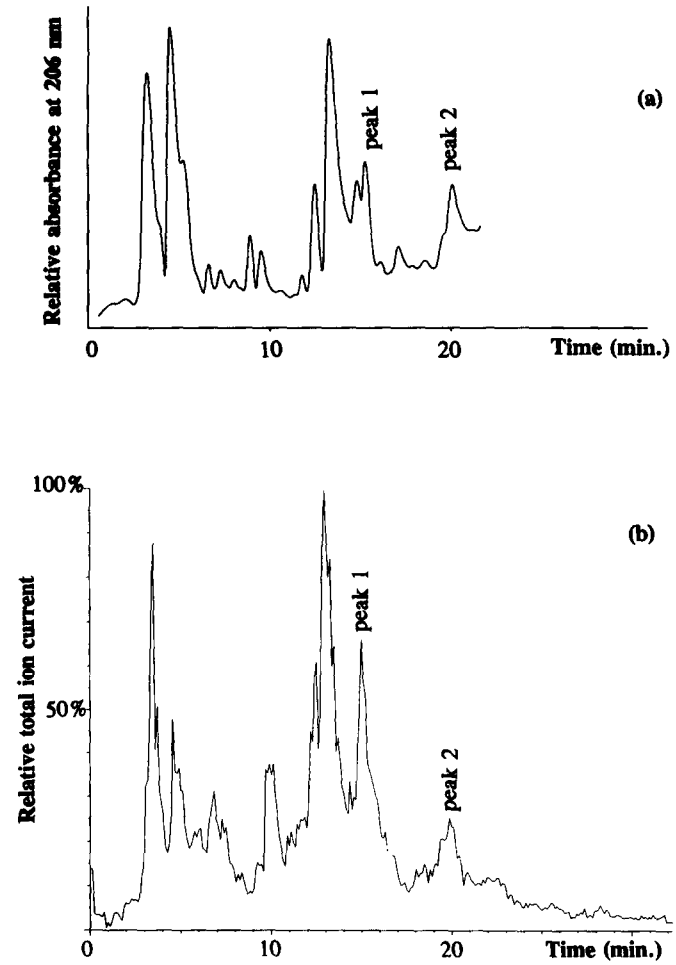


**Figure 3**
Map of the tryptic digest of a melphalan-treated metallothionein: (a) UV chromatogram (at 206 nm); (b) total ion current chromatogram.

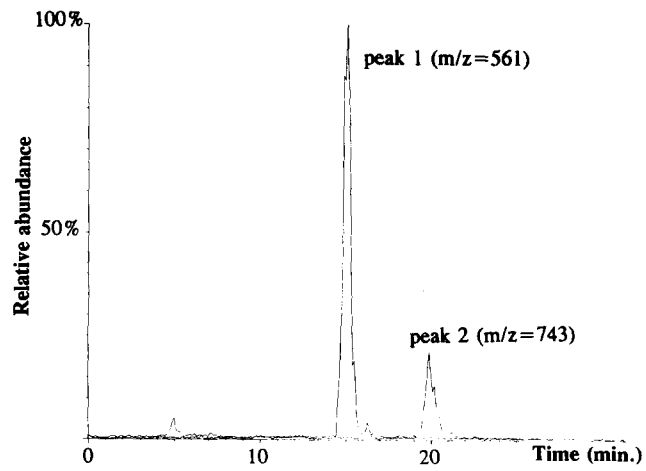


**Figure 4**
Reconstructed ion chromatograms of doubly protonated molecular ions predicted for two melphalan–alkylated tryptic peptides.

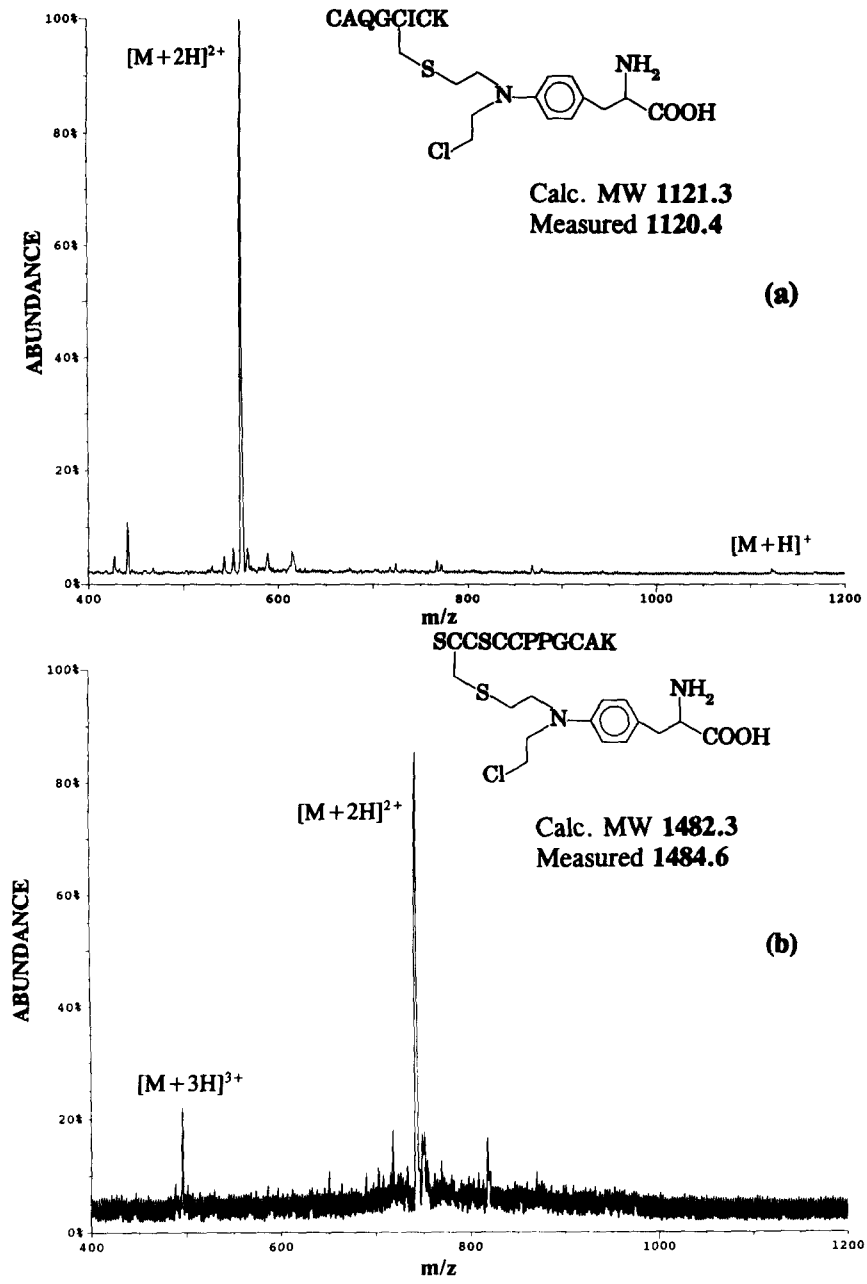


**Figure 5**
Mass spectra of two drug-modified peptides: (a) peak 1 in Fig. 3; (b) peak 2 in Fig. 3. Structures are taken from (20).

ated peptides. The two peaks identified from predicted ion chromatograms are identified in Fig. 3, and the corresponding full scan spectra are presented in Fig. 5. The molecular weights provided by this LCMS experiment identified two modified peptides and confirmed the displacement of one chloride ion from the conjugated melphalan. The site of alkylation within each tryptic peptide was established subsequently by tandem mass spectrometry experiments [20].

## Conclusions

The experiments reported here indicate that microbore LC can be readily interfaced in-house to a thermally assisted Vestec electrospray ionization source and provide satisfactory LCMS analyses of peptides and drug-alkylated peptides.

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