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AUTOMATED DRY FRACTION COLLECTION FOR MICROBORE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

A method is presented for the collection of dry fractions from a microbore high-performance liquid chromatographic column. These fractions are electrosprayed onto a foil strip that is being moved past the spray in steps. These solid deposits are in a form which is compatible with solid-phase secondary-ion mass spectrometry, in particular the time-of-flight instrument that has been developed in our laboratory. Because the type of ionization used in static secondary-ion spectrometry is essentially non-destructive, the non-volatile eluent is available for any other analytical method after mass analysis. The chromatogram of a mixture of peptides was re-constructed from the mass spectra of fractions collected in this way. This chromatogram is shown and its features are examined.

INTRODUCTION

Since the publication of results obtained using microbore high-performance liquid chromatography (HPLC) columns by Scott and Kucera[1,2], research and development on this type of column has been steadily increasing. Over the past few years, equipment manufactured specifically for use with microbore columns has been appearing on the market. The availability of this equipment has allowed the use of microbore columns in laboratories that were previously unwilling to use microbore columns, due to the specialized skills required to pack their own columns and modify pumps and detectors for use with low flow-rates.

Microbore HPLC has established itself as a suitable technique for analytical work, particularly in the case of trace analysis[3,4]. Trace analysis involves the screening of unknown mixtures using the retention times of observed peaks with reference to known standards. The other major application of HPLC is the separation of complex mixtures into fractions that can then be assayed for biological or chemical activity or used as a stage in a purification process. This latter application has yet to exploit the advantages of microbore HPLC. The difficulty in using microbore columns for this purpose is the extremely small volumes of liquid that must be collected from a microbore column to take advantage of the column's resolution; volumes on the order of 1–10 μl. The problem of the collection and storage of such small fractions has yet to be addressed by commercial HPLC manufacturers.

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Such small fraction volumes result from the reduction in liquid flow volumes made possible by the reduction of the inside diameter (I.D.) of the column. A small I.D. allows the maintainence of linear flow velocities similar to those in conventional columns, with a reduction of approximately two orders of magnitude in solvent volume used. This feature of microbore columns has also attracted researchers interested in coupling HPLC to mass spectrometry (LC–MS)[5,6]. When the eluent of an HPLC column is introduced into the ion source of a mass spectrometer, volatile solvents must be removed either before or during the introduction into the vacuum system. The reduction in volume flow made possible by microbore HPLC decreases the difficulty of the removal of volatile solvents from the eluent, thus reducing a major problem facing all LC–MS interfaces.

Recently there has been a great deal of interest in developing LC–MS interfaces. One approach that has received a great deal of attention is the "on-line" interface. An on-line interface is one that analyses the eluent of an HPLC column in real time, working as an outlet detector. The methods that currently show the most promise (and an example of each) are direct liquid introduction (DLI)[7], MAGIC[8], moving belt[9], electrospray[10], fast atom bombardment (FAB) moving belt[11], FAB direct introduction[12] and thermospray[13].

Another approach to LC–MC is the "off-line" interface. In an off-line interface the eluent from an HPLC column is stored and then prepared (usually by the removal of solvent) before it is introduced into the mass spectrometer. This method seems the most compatible with particle-induced desorption (PID) MS techniques and several such interfaces have been developed. Examples of these interfaces are given in refs. 14 and 15.

Our research into developing an off-line LC–MS interface for our time-of-flight (TOF) secondary ion mass spectrometer led us to the construction of a system for the automated collection of dry fractions from a microbore HPLC column. This collection was achieved by spraying the eluent of a microbore column directly onto a metal foil substrate, using a method of spray deposition known as electrospray[16] (not to be confused with electrospray ionization[10]). This type of spray removes the solvent from a sprayed solution by rapid evaporation at room temperature and pressure. The foil onto which the eluent is deposited is moved past the spray in a discontinuous fashion, pausing on one spot for a given period of time, and then rapidly moving to another adjacent position on the foil. Thus, the non-volatile portion of the eluent is deposited onto the strip in a series of fractions (1-mm diameter spots). This strip, bearing the actual chromatogram, is then in a form compatible with secondary ion mass spectrometry (SIMS). By inserting the foil strip into the mass spectrometer and moving the collected spots past the primary ion beam, it is possible to obtain mass spectra of each individual fraction in a time-ordered fashion. Furthermore, the method of ionization that we use only affects the surface of a sample (typically to a depth of tens of Ångstroms) and not the bulk; submicrogram samples are typically not degraded by analysis. Therefore, collected fractions are available for assays or other analytical techniques after mass analysis, as are fractions collected using normal HPLC proceedures. The method of collection also allows the convenient storage of many fractions in a small physical volume, with a typical density of 150 fractions on a strip of foil 25 cm long and 1.5 mm wide.

EXPERIMENTAL

Chemicals

The organic solvents used, methanol and 2-propanol, were both purchased from Caledon Labs. (Georgetown, Ontario, Canada) and were distilled-in-glass grade. The trifluoroacetic acid (TFA) was Aldrich (Milwaukee, WI, U.S.A.) 99% grade and the triethylamine (TEA) was Fisher (Fairlawn, NJ, U.S.A.) reagent grade. TFA and TEA were redistilled in our laboratory to remove dissolved solids. The amino acids and peptides were purchased from Sigma (St. Louis, MO, U.S.A.). The water used was Fisher W-2 grade. All of the solvents were selected from a range of brands tested on the basis of a minimum amount of residue on evaporation. In particular, solvents containing "large" amounts of alkali metal ions (more than 1 mg/l) were rejected because of the strong influence these cations have on SIMS spectra.

The buffers referred to below were made from 1% (v/v) stock solutions of TEA and TFA in water. A volume of the TFA stock was titrated with TEA until the desired pH was obtained. The pH measurement was made by placing drops of the solution onto "colorpHast" indicator stick obtained from MCB Reagents (Gibbstown, NJ, U.S.A.). Indicator paper was used rather than a pH meter because the pH electrodes available to our laboratory produced an unacceptably high amount of ionic contamination. The indicator paper was used by pipeting a small amount of the buffer onto a strip of paper when a pH measurement was necessary.

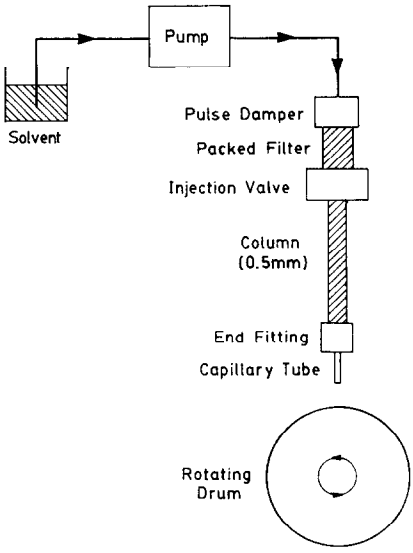

Fig. 1. A block diagram of the HPLC system.

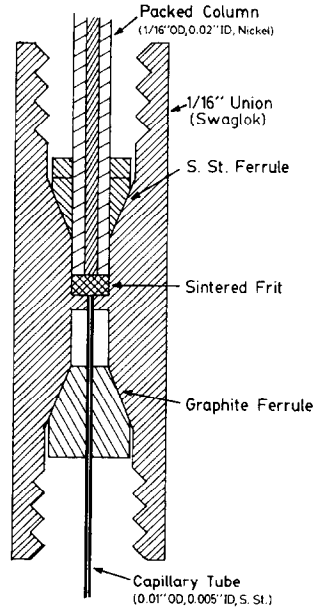

Fig. 2. End-fitting of the microbore column attaching the spray capillary.

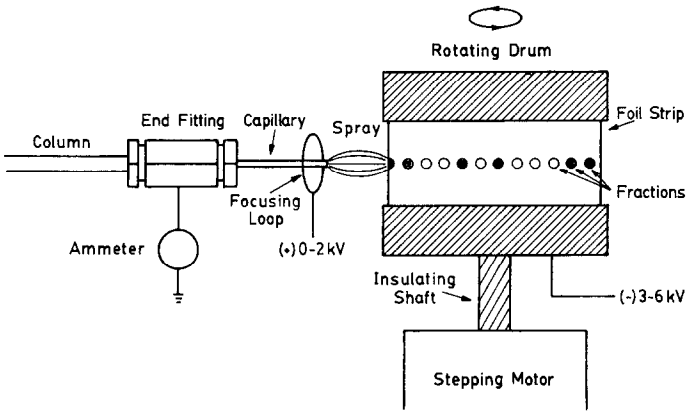

Fig. 3. The electrospray deposition apparatus.

HPLC apparatus

The columns used were packed in our laboratory using a method already described[17]. The columns were 1.6 mm O.D., 0.5 mm I.D. stainless-steel blanks (thoroughly washed to remove contaminants) packed with RSil 10 μm C_3 or C_{18} reversed-phase irregular silica, obtained from Alltech Assoc. (Deerfield, IL, U.S.A.).

A block diagram of the system used is shown in Fig. 1. The pump was an Eldex Laboratories (Menlo Park, CA, U.S.A.) Model A-30-S metering pump, which could be used at very low flow-rates. The pulse damper was 10 cm of 1.6 mm O.D., 1.3 mm I.D. stainless-steel tube with one end blocked with silver-enriched solder, and connected to the solvent line with a Valco 1/16 in. tee. The packed filter was 5 cm of 1.6 mm O.D., 1.3 mm I.D. stainless steel tubing pascked with 10 μm RSil C_{18} irregular silica. This filter was placed in the solvent delivery line to filter and precondition the solvent entering the main column. The injection valve was a Rheodyne Model 7410 valve with a 0.5-μl internal sample loop. The end-fitting which attached the column to the spray needle is shown in Fig. 2.

The electrospraying apparatus has been discussed in detail separately[17]. A diagram of the system is shown in Fig. 3.

Mass spectrometry

The mass spectrometer used was the Manitoba time-of-flight secondary-ion mass spectrometer[18,19]. The most important feature of this instrument for this application is its focussed primary ion beam, which has a diameter of *ca.* 1 mm at the target. This small primary beam allowed the analysis of the small spots (fractions) deposited on the foil substrate by the apparatus in Fig. 3 without overlap between fractions. To allow the introduction of a long foil strip into the ion source of the mass spectrometer, the sample-holding ladder was modified[17]. This modification permitted the accurate positioning of the foil inside of the machine.

RESULTS AND DISCUSSION

One of the important features of this technique is that it allows the coupling

of HPLC with PID–MS. PID is any mechanism in which the energy to perform the desorption and ionization is provided by an incident energetic particle, *e.g.* a keV/u particle (ion or atom), a photon or an MeV/u particle (^{252}Cf fission fragment or accelerated ion). For reasons not yet clear, PID produces intact pseudo-molecular ions for molecules that cannot be ionized using conventional electron impact ionization or chemical ionization methods. Peptides and oligonucleotides are examples of molecules that have been extensively examined using PID methods[20–22].

This type of ionization is particularly well suited to modern TOF mass spectrometers. These new TOF instruments are unlike those produced in the 1960s and early 1970s that used gas-phase ion sources and conventional ionization methods. The new instruments use solid-phase targets and can achieve mass resolutions of $m/\Delta m \geq 2000$[19]. These new machines also have very high sensitivity because (1) individual ions are counted instead of ion currents, and (2) the entire mass spectrum is collected simultaneously rather than scanned. The detection of organic materials present as a sub-monolayer on metal foils by SIMS is now quite common[23,24]. Therefore, samples eluting from the HPLC column at the picomole level should be sufficient to produce mass spectra. A demonstration of this low level sensitivity is shown by the mass spectrum in Fig. 4. This spectrum was obtained using the system described above, with 40 pmol of leucine enkephalin (LE, Tyr-Gly-Gly-Phe-Leu) injected onto the column and a mass spectrum taken of the fraction which contained the peptide. The mass spectrum shows two sodium adduct peaks [m/z = 578 $(M+Na)^+$ and m/z = 600 $(M+2Na-H)^+$] characteristic of LE. The peaks m/z = 120 and m/z = 136 are characeristic of Phe and Tyr present in the peptide sequence. These masses are typical of SIMS spectra of peptides[25]. The other peaks in the mass spectrum are due to contaminants in the HPLC solvents (see below for a discussion of m/z = 149), this assignment being made because these peaks occur in all fractions. These peaks are suppressed when larger amounts of sample are used. It should also be noted that at this low level of sample the structure of the peptide cannot be inferred from the fragmentation pattern; the fragment peaks seem to be suppressed. Currently, this level is the practical detection limit for the system, with the characteristic ion intensities dropping rapidly below 40 pmol. It is assumed that impurities in the solvents begin to play an important role in the suppression of ionization in the MS.

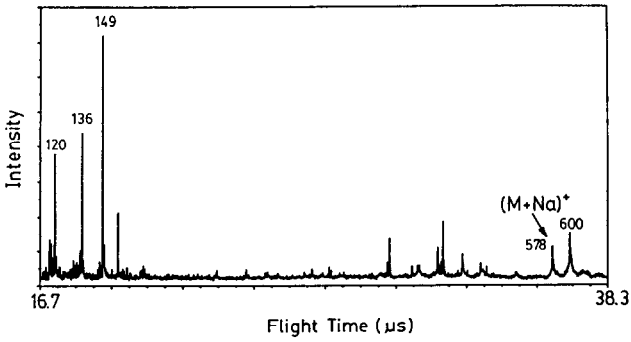

Fig. 4. Mass spectrum of a chromatographic fraction containing LE. Injection: LE and ME, 40 pmol of each. Chromatographic conditions: 2-propanol–water (60:40), 1 m*M* TFA–TEA buffer (pH 2.0), 25-cm C_3 column.

The subject of impurities in the mobile phases used in HPLC cannot be overstressed in any discussion of LC–MS coupling. In normal HPLC applications, the detector being used is much more selective than a mass spectrometer[26]. A UV detector will only detect compounds that absorb at the wavelength selected. Electrochemical detectors only detect changes in the electroactivity of the eluent. Fluorescence detectors specifically monitor the fluorescence of compounds of interest and cannot see other materials. This insensitivity to solvent impurities is not the case with a mass spectrometer. Any material introduced into the ion source of a PID mass spectrometer will produce some sort of ion signal, whether it be a molecular or fragment ion. Conventional mass spectrometers are also very sensitive to low level contaminants[27]. The range of reagents (other than the solvent which can be added to the mobile phase) is limited. Ion-pairing reagents and involatile buffers and salts, commonly used to enhance the resolution or change the retention time of certain compounds, all pose problems for LC–MS. These compounds can produce unacceptably high backgrounds in mass spectra or may suppress the yield of the ion of interest. These comments are particularly true for surfactants such as *p*-toluenesulfonic acid and/or long chain hydrocarbons or silicones, such as the greases commonly used in syringes. Plasticizers, such as phthalates, frequently appear in mass spectra due to their prevalence in laboratories and air-conditioned buildings in general[27]. The mass peak at $m/z = 149$ in Fig. 4 has been assigned to phthalate contamination of the mobile phase. The presence of this peak in a mass spectrum is a well known indication of phthalate contamination[27] and is very common in SIMS and FAB spectra.

With these considerations in mind, the criteria for selecting appropriate solvents and chromatographic conditions for LC–MS are clearly different than those used for normal HPLC. Solvents of "HPLC grade" are not, in our experience, of sufficiently high purity for this application. Although these solvents are UV transparent, they contain substantial amounts of material that, while not UV absorbent, show up in great abundance in mass spectra. A criterion that we have found useful is that the solvents used should have the lowest amount of dissolved solids possible. This is tested by electrospraying pure solvent onto clean foil and then performing a SIMS analysis of deposit. Buffers used should be "volatile", such as the TFA–TEA buffers used here. The term "volatile" for these organic buffers is a little deceptive, because they are solids at room temperature and pressure. If only small amounts of these buffers are used, however, they will evaporate in the spray process.

Inspection of the block diagram of the chromatographic system in Fig. 1 shows that no detector is being used at the outlet end of the HPLC. The absence of a detector is possible due to a property of electrosprayed deposits. The involatile material deposited on the foil surface is in the form of small lumps (0.1–1.0 μm in diameter[28]). These lumps are of just the right size to scatter visible light very efficiently. As little as 5 ng of material on the foil will produce a white spot on the shiny backing when viewed in oblique light. Thus, once fractions have been sprayed onto the foil, the fractions containing significant amounts of deposited material can be easily determined by visual inspection. The limit of 5 ng of material was not a limitation in the work so far done.

The chromatogram shown in Fig. 5 is a demonstration of the capabilities of our off-line microbore LC–MS system. A mixture containing Phe, Tyr-Gly-Gly-Phe,

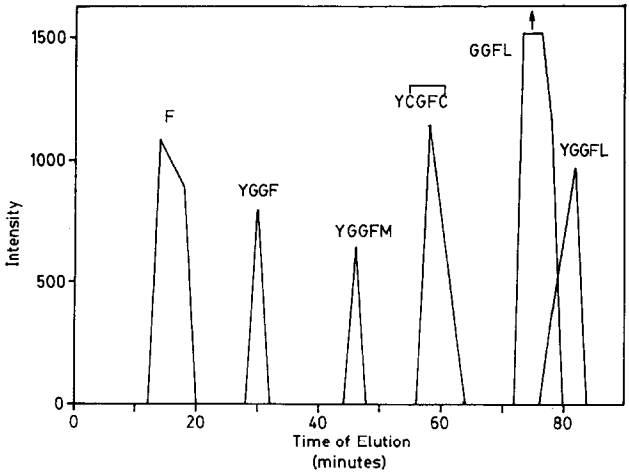

Fig. 5. Chromatogram of a mixture of peptides. Injection: mixture of 25 ng of each peptide. Chromatographic conditions: 2-propanol–water (70:30), 1 m*M* TFA–TEA buffer (pH 2.0), 25-cm C_3 column.

methionine enkephalin (ME, Tyr-Gly-Gly-Phe-Met), Tyr-Cys-Gly-Phe-Cys, Gly-Gly-Phe-Leu and LE (each component at the 50 ng/μl level) was injected onto the column. The fraction collection time was 4 min per fraction. (Note: the apparently wide peak corresponding to Phe in the chromatogram was caused by the elution of Phe during a change of fractions.) The separated fractions deposited on the foil by the spray apparatus were then inserted into the mass spectrometer and a mass spectrum taken of each fraction. The spectra were then analysed individually to identify any molecular ions present. The quantity plotted on the ordinate of Fig. 4 is the normalized integrated intensity of the sodium adduct molecular ion $(M+Na)^+$ of a particular compound. This intensity was normalized to the primary ion beam current used to obtain the spectrum (typically 10 pC per spectrum). The intensities of each compound are plotted at the center point of the fraction elution time and points corresponding to the same compound are connected with a straight line.

The chromatogram shown is only a fraction of the information available from this technique. It is possible to sequence a short (10 or less units) peptide or oligonucleotide from the fragmentation pattern observed in its PID spectrum[29,30]. By interpreting the results from each of the individual mass spectra it should be possible to determine the structure of unknown molecules that appear in a chromatogram. This information is available without derivatization of the eluent molecules.

The chromatogram in Fig. 5 compares favorably with results obtained by other investigators, when analysing similar systems. A direct comparison between our system and a commercially available DLI interface can be made from the work of Kenyon[31]. Kenyon describes the separation and detection of ME and LE. The detection limits demonstrated were 500 ng of an individual peptide injected on column, using the mass spectrometer in the selected-ion monitoring mode. This limit compares with a detection limit of 25 ng of an individual peptide for our system. Smith *et al.*[32] investigated the use of a moving-belt type of interface for a quadrupole mass spec-

trometer equipped with a primary ion beam. Although chromatograms of peptide mixtures were not reported, the detection limits for arginine with no HPLC column in-line show the viability of the system. A moving-belt system that utilized electrospray as well as thermospray for eluent deposition and laser desorption ionization was reported by Hardin *et al.*[33]. Comparison of our results with Hardin's is difficult because there was no HPLC column in-line for his results and the lack of belt-cleaning apparatus in the interface limited the time span of a chromatogram to 9 min. A similar difficulty arises in a comparison with the work of Whitehouse *et al.*[10]. The reported sensitivity of the electrospray ionization method appears to be very high (50 amol for gramicidin S); however, no results obtained with an HPLC column in-line were reported. The use of electrospray ionization appears to hold great promise for LC–MS and is well suited for use with microbore columns.

Our method is not without difficulties. As is, the method is incompatible with gradient elution. The voltage conditions that allow a stable electrospray (see Fig. 3) are very dependent on the solvent mixture and flow-rate of the mobile phase. Changes in the water content of the mobile phase must be compensated for by changes in these voltages. The construction of the necessary feedback loops has not been attempted because pumps that produce accurate gradients at a flow-rate of less than 10 μl/min have yet to be produced. The problem of volatile buffers is also still under active consideration. As stated above, the organic buffers that are usually referred to as volatile are actually solid at room temperature and pressure. Therefore, to evaporate these buffers it is necessary to slightly heat the foil onto which the eluent is being sprayed. This evaporation is done using a 200-W heat lamp shining on the collection drum. The use of the heat lamp is only effective at low buffer concentrations, however; typically 1 m*M*. Other organic buffers are currently under examination to determine if they would be more suitable. The amount of time necessary to produce a chromatogram is also a current concern. The chromatogram in Fig. 5 took 90 min to obtain, while other chromatographic systems have been able to resolve enkephalin related peptides in less than 10 min[31]. This problem is mainly due to the very low flow-rate that must be used with the electrospray. A realistic upper limit for flow-rate in our system is 3 μl/min. This flow-rate is lower than optimum for even a 0.5 mm I.D. column, causing long analysis times and band broadening. Currently research is being carried out into the use of smaller bore columns.

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