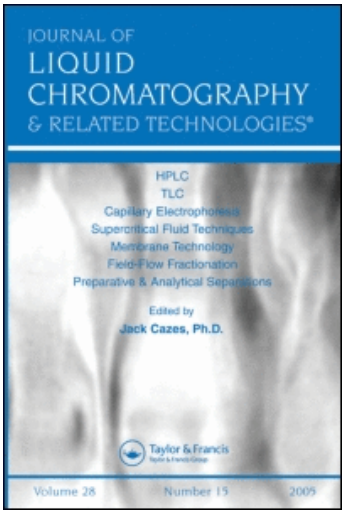

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

A Review of Combined Liquid Chromatography and Mass Spectrometry

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To cite this Article Desiderio, Dominic M. and Fridland, Genevieve H.(1984) 'A Review of Combined Liquid Chromatography and Mass Spectrometry', Journal of Liquid Chromatography & Related Technologies, 7: 10, 317 — 351
To link to this Article: DOI: 10.1080/01483918408073941
URL: http://dx.doi.org/10.1080/01483918408073941

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(S-2), 317-351 (1984)

A REVIEW OF COMBINED LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY

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ABSTRACT

The area of research involving the on-line combination of mass spectrometry and liquid chromatography has recently experienced a period of intense growth. A dependable LC-MS combination is eagerly awaited for by many researchers. The salient features of the combination aspect of LC-MS are analyzed in this review. LC-MS may operate in either the off-line or the on-line mode. The off-line mode is illustrated with the analytical measurement of biologically important peptides.

I. THE ON-LINE COMBINATION OF LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY

A. INTRODUCTION

The on-line instrumental combination of high performance liquid chromatography (HPLC) and mass spectrometry (MS) is a natural combination that effectively couples the separation power of HPLC with MS, which is capable of achieving high levels of sensitivity and molecular specificity. The driving force for scientists to develop an on-line combination

 0148-3919/84/07S2-0317$3.50/0

of HPLC and MS is the need to decrease the number of experimental steps and manual interventions required to manipulate small amounts of precious endogenous biological materials, especially peptides. However, the coupling of these two very dissimilar instrumental methods is difficult to achieve and indeed, it is the authors' opinion that the rate of development of achieving an efficient and dependable on-line coupling for LC-MS is relatively slow, compared to the corresponding initial development of the combination of gas chromatography with mass spectrometry (1). Nonetheless, investigators are creatively approaching this interface problem and it is simply a matter of time until this combination technique will be in the hands of many investigators.

The literature pertaining to on-line LC-MS has been reviewed extensively (2-4).

B. BASIC CONSIDERATIONS

In principle, there are two different interfacing methods to achieve the on-line combination of HPLC with MS. On one hand, a direct liquid introduction (DLI) interface performs exactly what the name implies, that is, the liquid emerging from the HPLC column is introduced directly into the mass spectrometer. The solvent is removed by some appropriate process such as nebulization, thermospray, etc. On the other hand, the effluent from the HPLC column may be deposited upon a moving belt, which transports a chromatographic fraction from the external, high pressure region of the HPLC unit, through an interface, and into the high vacuum portion of the mass spectrometer where ionization is effected. These two separate interfacing techniques, DLI and moving belt, will be discussed in greater detail in the next section.

There are five operational parameters which must be thoughtfully analyzed and experimentally optimized for any particular type of on-line HPLC-MS development and

experimentation. These parameters include the use of a normal HPLC column versus a microbore column (5,6); the use of either a high voltage magnetic instrument or a low voltage quadrupole mass spectrometer; use of either the split or splitless mode for the LC-MS interface following the HPLC column; the use of either volatile or nonvolatile buffers for HPLC separations; and the selection of the ionization process, which plays a significant role in that either electron ionization (EI) or chemical ionization (CI) may be used to ionize the HPLC fraction, as opposed to a surface method such as secondary ion mass spectrometry (SIMS) or fast atom bombardment (FAB) mass spectrometry.

C. DIRECT LIQUID INTRODUCTION INTERFACE

One of the first sets of experiments to study the DLI of solutions into a mass spectrometer involved the use of CI mass spectrometry (7-11). The CI source for LC-MS followed the work with LC-MS in the EI mode. Chemical ionization LC-MS was developed to such a point whereby polypeptide sequencing was possible. For determining the amino acid sequence of a peptide, the authors felt that the information from a CI mass spectrum appeared to be at least as valuable as that information derived from EI. Direct solution introduction has the further advantage that the resulting direct CI mass spectrum allows sequencing of less volatile samples such as underivatized pentapeptides. In this study, the zwitterionic character of the peptide is eliminated by acetylation of the free amino group and esterification of the free carboxyl groups. The LC solvents used included acetonitrile and water. A capillary splitter interface is used in front of the HPLC UV detector and continuously introduces five to ten microliters of the LC effluent per minute into a CI mass spectrometer. Because only about one or two percent of

the peptide sample enters the mass spectrometer, the sensitivity of the method is decreased by a factor of 100. Mass spectral scans are cycled continuously during LC elution, and two or three corresponding mass spectra were obtained per HPLC peak. The HPLC solvent serves readily as the CI ionizing reagent although other reagents can be added concurrently to alter that type of ionization.

The critical instrumental parameters necessary for combining LC with a MS have been optimized. In one case, discussion centered on whether or not the solvent should be removed in the interface (12). It is stated that the basic philosophy in the development of the first LC-MS systems included:

1. introduction of the total HPLC column effluent into the separator;
2. selected removal of the chromatographic carrier;
3. vaporization of the neutral analyte;
4. ionization and mass analysis.

These initial developments led to transport systems which are based on the use of a moving belt or wire for conveying the solution, then the solute, through these four stages. In the cases of ionization, it was soon realized that preformed ions already existing in the solution could be directly vaporized and analyzed when enough energy is supplied to the liquid solution. Several methods are available for ionization and include electrospraying, electrohydrodynamic (EHD) ionization, field desorption (FD), thermospray ionization, and FAB mass spectrometry.

When interfacing a chromatographic column to a mass spectrometer, it is important to realize that the chromatographic column performs both as a separation and as a dilution unit (13). The problems involved with interfacing an HPLC unit to a mass spectrometer stem from the relative incompatibility between the HPLC effluent solution and the low pressure inside the source of the mass spectrometer (13). These two instrumental features make the coupling of the two instruments more difficult compared to the interfacing of a gas chromatograph to a mass spectrometer.

It is important to consider the main constraints which are introduced by the chromatographic process as well as to couple those constraints with the minimum requirements that the interface must possess. These considerations will accurately describe the degree of competitiveness that this interface would possess and will suggest compromises which are acceptable from the chromatographic point of view.

When combining an HPLC unit with a mass spectrometer, it is important to realize that, as this process occurs spontaneously (irreversibly) from a thermodynamic point of view, the decrease in entropy which arises from the chromatographic separarion is more than compensated for by the increase in entropy deriving from the dilution in the mobile phase. Whichever type of LC-MS interface that is selected for the on-line coupling, the use of a non-volatile HPLC buffer seems prone to considerable difficulties. Furthermore, although gradient HPLC elution is much talked about in LC research circles, that form of separation is relatively rarely used.

The chromatographer is always surprised by the low ionization yield (E) of the mass spectrometer. The factor E represents the number of ions collected on the mass spectrometer detector per number of molecules which is introduced into the ion source. It is important to review briefly the various sources of those losses (13):

1. To detect a signal at a given mass, and to calculate the location of the signal maximum, that is, the corresponding molecular weight, one needs approximately 100 ions at the detector entrance slit; practically all ions reaching this slit are detected.

2. The object and image slits in most mass spectrometers are of a rectangular cross-section. When a mass spectrometer is scanned, the product of these two (presumably identical) slits is a triangle. We require 200 ions to enter the analyzer. Losses in the analyzer itself are assumed to be negligible.

3. The extraction yield of ions from the source to the

analyzer across ion optics (ion-focusing) is about 10%. Therefore, we need 10 x 200, or 2,000 ions in the source.

4. For identification purposes, we need to obtain a complete scanned mass spectrum and the previous figure (2,000 ions) must be applied to those ions which account only for small peaks in the mass spectrum. Peaks that are 10% of the base peak must be detectable. Therefore, at least 2×10^{4} molecular ions must be formed during the time when the corresponding mass is scanned.

5. The ionization yields vary widely with both the ionization method used and for the particular compound being analyzed. While that efficiency may be nearly one for electron capture of haloaromatics, it may, on the other hand, be as low as 10^{-4}. If these two values are averaged to 10^{-3}, then 2×10^{7} molecules should be present during the scan.

6. The time to scan one mass in a spectrum is about one msec, and the introduction of sample molecules into the source must therefore proceed at a speed of approximately 2×10^{10} molecules per second.

The maximum concentration C_M of the Gaussian band of a solute of retention volume V_r and efficiency N is:

$$C_M = m\, N^{1/2} / V_r\, (2\pi)^{1/2} \qquad \text{eq. 1}$$

where m is the sample mass. If the column capacity is k' and the liquid cross-section of the column is s, then:

$$C_M = (m/s)\, (N^{1/2}) / [L\, (1 + k')\, (2\pi)^{1/2}] \qquad \text{eq. 2}$$

where L and u are the column length and the solvent velocity, respectively. The mass flow-rate of sample into the mass spectrometer source is then the product $C_M F$, where F (= Su) is the solvent flow-rate. With a splitting ratio r, the mass flow-rate of sample into the source is:

$$dm / dt = C_M Fr = (m \cdot u \cdot r \cdot N^{\frac{1}{2}}) / [L \cdot (1 + k') \cdot (2\pi)^{\frac{1}{2}}] \quad \text{eq. 3}$$

Comparing equation 3 with the condition (6) above;

$$N' \cdot (m \cdot u \cdot r \cdot N^{\frac{1}{2}}) / [M \cdot L \cdot (1 + k') \cdot (2\pi)^{\frac{1}{2}}] = 2 \times 10^{10} \quad \text{eq. 4}$$

where M is the molecular weight of the solute and N' is Avogadro's number. With L = 15 cm, N = 1.5×10^4 plates, u = 0.05 cm per second, r = 1, M = 500, and k' = 1, then m = 2×10^{-10} = 0.2 nanograms = 200 picograms. This level of sensitivity is in general agreement with the specifications of modern mass spectrometer instruments which give the detection limit of 100 picograms of methyl stearate (M=298, m = 120 picograms). The latter specifications may not have been calculated with the rather favorable chromatographic conditions selected above: narrow peaks with small retention give large maximum concentration. The detection limit of a chromatographic detector is defined as the mass of a compound that generates a signal which is twice that of the noise.

On the other hand, if the mass spectrometer operates not in the scanning mode but rather as a true selected ion monitor, then a smaller amount of sample is necessary: with a one second time-constant, 1000 times less, or around one picogram would suffice for detection. The sensitivity values are similar for either a magnetic instrument or for a quadrupole. The only possibility of improving these detection limits in a significant fashion is whenever a very efficient ionization technique is utilized; a fact that explains why haloaromatics can be determined at the femotomole level with negative ions.

C.1. Controlled Desolvation Chamber

A high speed DLI interface is described (14). At the exit of the HPLC probe, a heated preevaporation chamber is provided, where the molecules have a low speed in a high

pressure region. Following that region is a short transition zone from which the molecules enter a high speed-low pressure region before entering the ion source. This latter region is the controlled desolvation chamber. The maximum velocity in the transition zone is sonic velocity.

A new desolvation chamber for droplet-focusing or Townsend discharge ionization is described as an interface for DLI into a mass spectrometer. This new desolvation chamber contains a conically-shaped volume and derives from considerations that utilize a slightly different concept because preliminary experiments show that the liquid droplets are highly positively-charged during nebulization, and that physical contacts with the walls of the desolvation chamber should in general be avoided. A positive potential on the walls therefore can be used to repel the droplets. On the other hand, by adoption of the appropriate conical geometry, droplets could either be focused along the drift tube axis or rather converge to an appropriate location. Other considerations of LC-MS interfacing have been published by this research group (15-17).

C.2. Thermospray

Thermospray is defined as the complete or partial vaporization of a liquid stream by heating it as it flows through a capillary (18). Thermospray has recently been demonstrated to be a versatile interface for combined LC-MS, where the heat is supplied electrically, and controlled by an electronic feedback system to maintain a constant level of vaporization. The optimal temperature for thermospray is a function of the solvent composition, flow rate, capillary dimensions and the sample to be analyzed. Thermospray interfaces were first heated by lasers, then by oxy-hydrogen torches, and most recently by cartridge heaters.

One of the first papers in this work describes a new soft ionization technique for the MS of complex molecules (19).

Briefly, the effluent from an HPLC column enters the vaporizer via a steel capillary tube which is partially immersed in a copper cylinder which is heated to about 1000°C by four small oxy-hydrogen flames. As a result of rapid heating, a jet of vapor and aerosol is produced near the exit of the stainless steel tube. The jet is further heated as it passes through the channel in the copper. It then undergoes an adiabatic expansion, and a portion passes through a skimmer to the ion source where the beam impinges on a nickel-plated copper probe which is electrically heated to about 250°C. While the ion source of the mass spectrometer is equipped with an electron gun for producing ions for normal CI operation, in the work described in this manuscript, that gun is turned off. When the charged particle undergoes a high energy impact with the heated probe, it is wholly or partially vaporized, and some of the resulting molecules are ionized. Spectra can be obtained for dinucleosides (CpG;ApU) and the pentapeptide leucine enkephalin (TyrGlyGlyPheLeu). Background ionization of the solvent is low and this fact, coupled with the reduced amount of fragmentation that is generally observed with this type of ion source, lead to the surprising result that the detection limit for the protonated molecular ion, $(M+H)^+$, of most of the nonvolatile substances investigated is substantially lower with the electron beam turned off!

Both positive and negative ion CI mass spectra were obtained for nonvolatile biologic samples in the one to ten nanogram range for full mass scans, and subnanogram quantities with selected ion monitoring (SIM) (19). It has been noted that vaporization is possible without pyrolysis because the sample spends a very short time in the high temperature region in the thermospray LC-MS interface. During that transit time, these samples are protected from overheating by the solvent. The liquid entering the hot region is heated from ambient temperature to the vaporization point in a few nsec., and the

vapor is expelled from the heat region within a msec. or less after it is formed. Experiments show that the detection limit (signal-to-noise-ratio of two) for arginine is 500 picograms and, for methionine, 2.5 nanograms. If the SIM measurement mode were used for only the intensity of $(M+H)^+$, the detection limits were predicted to be about one order of magnitude more sensitive. In this study, phosphate buffers cannot be used, but ammonium formate and ammonium acetate are used extensively with essentially no difficulties. The major limitation to the operation of the mass spectrometer is that the solvent vapor is also the CI reagent. This dual role of the same substance can cause difficulty in the simultaneous optimization of both the HPLC separation and the mass spectrometric detection.

As mentioned above, cartridge heaters have been used to replace the oxy-hydrogen torches in a thermospray interface readily adapted to quadrupole mass spectrometers (20). HPLC effluents are thermosprayed directly into the ion source, and the excess vapor is pumped away by the added mechanical pump which is directly coupled to the ion source through a port opposite the electrically-heated themospray vaporizer. When used with mobile phases containing a significant concentration of ions in solution (ca. 10^{-4} to 1M), no external ionizing source is required to achieve the detection of many nonvolatile solutes at the subnanogram level. The mass spectrum of the tridecapeptide renin substrate was shown and represents the largest molecule which has been successfully detected by using thermospray ionization. The $(M+H)^+$ of the renin substrate is 1,758 mass units.

A review of all the ionization techniques currently available for mass spectrometry for nonvolatile molecules has been published (21). A common feature of all of the ionization methods appears to be the direct production of ions from a condensed phase without formation of a neutral gas-phase molecule as an intermediate. An attempt is made to present a

unified ionization model which at least qualitatively accounts for the results obtained by the various techniques. A laser desorption mass spectrometer was interfaced to an HPLC unit using a moving stainless steel belt, where samples are sprayed online onto the belt under partial vacuum through a thermospray vaporizer. Laser desorption occurred with a laser. Two modes of operation are presented for the thermosprayer, one with (closed) and one without (open) the transfer line. Using an electrospray method for sample deposition, comparisons were made amongst the electrospray, closed, and open modes. It was assumed that the electrospray method has a 100% efficiency to transfer the sample; closed correlates to 40% and the open mode to 20% efficiency of covering the belt. A major limitation of the present moving belt system was found to be that the HPLC separations must be accomplished in less then nine minutes in order to prevent sample components from overlapping each other on that belt.

Triply and quadruply-charged molecules of nonderivatized glucagon (molecular weight equals 3,483 a.m.u.) are observed (22). In another experiment, a procedure is presented for peptide sequencing by utilizing an immobilized exopeptidase column which is directly coupled to a thermospray mass spectrometer (22). Amino acid sequence determination starting at the C-terminus is effected in this manner. This experimentation parallels that found previously for an immobilized enzyme (23). The underivatized peptide solutions are injected through a column containing immobilized carboxypeptidase Y and the amino acids were released, starting from the C-terminus of the peptide chain, and directly transported by a continuously flowing aqueous buffer into a thermospray mass spectrometer where the $(M+H)^+$ of each amino acid is determined (22). Temperature of enzymolysis is a factor because, at 22°, only two amino acids are found, whereas at 42°, five amino acids are determined from angiotensin.

C.3 Microbore LC-MS

The effluent from a microbore HPLC which operates at a flow rate of eight microliters min^{-1}, is introduced into a quadrupole mass spectrometer ion source operating in the CI mode (24). This paper studied evaporation of liquids from a capillary into a vacuum, where a jet was observed inside a glass envelope. The jet appeared to travel straight in air. However, when a vacuum was applied, that jet started to bend. This phenomenon is explained by considering the fact that both large and small droplets are formed and that the small droplets evaporate more rapidly than the larger droplets. If the small droplets are predominantly formed on one side of the jet, owing to the irregular shape of the orifice, the evaporating vapors from the small droplets push the large droplets away from the axis of the jet when a vacuum is applied.

Instrumental and analytical advantages have been taken of microbore HPLC coupled through a DLI interface to a CI mass spectrometer (25). The analytical capabilities of a microbore HPLC which is interfaced to an unchanged quadrupole mass spectrometer demonstrate continuous monitoring of the total HPLC effluent. Full scan CI mass spectra of drugs are obtained in the range of one to five nanograms. A microbore HPLC flow rate of eight microliters per minute is utilized and SIM provides a 20 picogram detection limit of a tranquilizer.

Conventional HPLC instruments generally operate at flow rates of 0.5 to two milliliters per minute. It is not practical to introduce the total HPLC effluent through a DLI interface. However, microbore packed columns operate at eluant flow rates of two to forty microliters per minute and are ideal for DLI. Because the entire effluent from a microbore HPLC can be directed into the ion source for mass spectral analysis, nearly 100-fold increases in sensitivity may be realized (26). Conventional HPLC systems yield peak volumes of 0.5-2.0 ml, where that range depends upon the flow rate and column efficiency. On the other

hand, utilizing microbore HPLC, peak volumes are found in the range of 10 to 30 microliters. The smaller volume in the latter case provides a much more concentrated solution passing through the detector, with a concomitant increase in the detection limits (27).

C.4. Liquid Chromatography-Mass Spectrometry-Mass Spectrometry (LC-MS-MS)

In another study determining sulfa drugs in biologic fluids, it is noted that one limitation results from the DLI technique of LC-MS because the mass spectra are very simple and they lack sufficient specificity for elucidation. Futhermore, it is always possible that co-eluting compounds will appear superimposed in the mass spectrum, a process which renders the interpretation rather difficult. The novel technique of combined liquid chromatography-mass spectrometry-mass spectrometry (LC-MS-MS) offers the analytical capability of also providing collision activated dissociation (CAD) mass spectra of the characteristic LC-MS ions for each of the components of interest (28). In this study, the potential of the more open atmospheric pressure ionization (API) source design is utilized in combination with HPLC and mass spectrometry in a triple stage quadrupole (TSQ) instrument. Nitrogen is used as the collision gas with an effective target thickness of approximately 2×10^{14} molecules centimeter^{-2}. This study was prompted by the extreme paucity of fragmentation that occurs in the positive CI LC-MS mode.

C.5. Liquid Chromatography-Negative Chemical Ionization Mass Spectrometry

In another study utilizing HPLC, negative ion CI mass spectrometry was begun because of the need to analyze

mixtures of explosives (29). Post-explosion analysis of residues is noted to be very relevant in criminalistic bombing. A simplified construction of a new micro LC-MS probe is presented, where the volume of the HPLC microcell is calculated to be 1.2 microliter, and which maintains the one centimeter pathlength of the conventional cell, where the latter has a 15 microliter volume (30).

A desolvation chamber of new design sprays the HPLC effluent into droplets through a pinhole diaphragm (31). One of the most effective ways of controlling the desolvation of the solute droplets is to use a heated zone, usually called a desolvation chamber, in which the droplets acquire a high speed before entering the CI source. Three different desolvation chambers were studied and include the standard Hewlett-Packard, extended, and solvent-stripping models. A review of the micro LC-MS methodology used in drug analysis and metabolism studies has been published (32) and the quantitative analysis of betamethasone in equine plasma and urine by DLI micro-LC-MS is presented (33).

A new version of the thermospray LC-MS interface is described, where this version differs from the previously described interfaces in that it is a dual purpose probe-type interface which is introduced conveniently into the mass spectrometer via a standard direct insertion probe inlet (34). The device is a dual purpose LC-MS interface and it can provide conventional DLI LC-MS, or the copper vaporizer may be heated electrically to produce thermospray ionization. Detection limits are currently about 100 nanograms in the thermospray mode for nonvolatile, labile compounds using two mm i.d. microbore LC columns and a flow rate of about 150 microliters per minute.

C.6. Ultrasonic Interface

An ultrasonic spraying device was constructed to overcome difficulties in spraying aqueous solvents into the vacuum

system of a mass spectrometer. The ultrasonic vibration is achieved by means of a magnetoconstriction in the nickel inlet tube (35).

C.7. Segmented Wire Interface

It has been noted that the DLI technique has intrinsically simple implementation and that the moving belt interface has significant advantages in several parameters including solute concentration- a process that introduces a greater proportion of the sample into the mass spectrometer ion source (35,36). This group approached the interface problem by preconcentrating a liquid stream and introducing the effluent into the MS by means of DLI. The viewpoint is that the advantages of both ordinary DLI and the moving belt technique would be combined. The described interface device concentrates a liquid stream by allowing it to flow down a resistance-heated stationary wire having three successively-decreasing diameters of 0.8 millimeters, 0.6 millimeters and lastly, 0.3 millimeters, where each section has a length of 15, 7.5, and 1.5 centimeters, respectively. When a drop becomes too large, it flows either down the wire or along the outer surface of the DLI probe, and is lost. A light-emitting diode and photocell are fitted at the gap and sense the size of the drop. Electronic feedback from the photocell controls the current through the wire to hold the drop size constant. The instrumental parameters are met only with the wire that was not of homogeneous construction, and the three-segment wire design described above was arrived at empirically. A description is provided where the performance of the concentrator wire is tested in an effort to determine the maximun flow which could be accomodated and still maintain a 95% evaporation of the solvent, corresponding to a 230-fold increase in sample concentration.

C.8. Nebulization

The design of a DLI interface is described which uses a jet of helium gas to aid in the nebulization of the HPLC effluent and sample into the MS source (37). Interfaces used in coupling HPLC with MS fall into two basic categories: transport interfaces and DLI interfaces. A number of systems have been used in the DLI approach for introducing the LC effluent into the MS source and include formation of liquid jets through either viscous flow capillary or 1.5 micron diaphragms and vacuum nebulization techniques.

C.9. Supercritical Fluid Injection

Direct supercritical fluid injection interfaces have been designed for use with mass spectrometry (38). Direct fluid injection (DFI) mass spectrometry utilizes supercritical fluids for solvation and transfer of materials to a CI mass spectrometer source. Supercritical carbon dioxide with isobutane as the CI reagent gas has been used and DFI MS/MS is also illustrated for major ions in the isobutane CI mass spectrum of T2 toxin. More polar compounds may be analyzed using supercritical ammonia. This alternative LC-MS approach uses a supercritical fluid or "dense gas" for efficient transfer of material to the gas phase in the CI source. The DFI method allows mass spectra to be obtained for essentially any compound which is soluble in the supercritical fluid and hence allows a rapid qualitative evaluation of fluid phase solubility.

At high pressures and above the critical temperature, the resulting fluid or dense gas attains a density approaching that of a liquid which has relatively strong internal molecular interactions and therefore assumes some of the properties of a liquid. In describing the DFI process, the supercritical fluid exits from a 50 to 500 atmosphere pressure region through a

hole into another pressure region which contains a Mach disk, where initial clusters have a diameter of approximately 30 angstroms. The shock waves resulting after the Mach disk then transfer into a molecular spray which enters a CI region of a mass spectrometer at a pressure of approximately one torr. The Mach disk is characterized by two phemomena - the destruction of the highly-directed jet and the collisional energy transfer resulting in a redistribution of the directed kinetic energy of the jet among the various translational, vibrational, and rotational modes of the molecule.

The use of capillary column supercritical fluid chromatography (SFC)- MS can obviate the difficulities associated with previous interfaces and allows a simple interface readily adapted to existing GC-MS systems (39). The combination of SFC with mass spectrometry offers the following potential advantages relative to GC-MS or LC-MS methods:

1. high molecular weight, polymeric, polyfunctional, and thermally labile compounds can be separated, as well as more volatile species;

2. capillary SFC columns can provide greatly enhanced chromatographic efficiency relative to HPLC due to solute diffusivities which are about 100 times greater in the supercritical fluid than in the corresponding liquid phase and similar to the gas phase;

3. soluting power of the mobile phase can be readily controlled with pressure programming. Mixed mobile phases, gradient, and temperature programming are also feasible;

4. SFC using a capillary column provides low mobile phase flow rates which, when coupled with high mobile phase volatility, allows optimal interfacing of SFC and mass spectrometry.

Optimal mobile phase flow rates of five to 80 microliters per minute (supercritical fluid), depending on column diameter and pressure, may be obtained in this combination instrument. The SFC mobile phases used up to now in this work are isobutane and normal pentane. Initial evaluations of

the capillary SFC-MS interface demonstrate it to be mechanically simple and reliable. Polyaromatic hydrocarbon studies indicate that a detection limit of approximately one picogram is achieved.

C.10. Examples of LC-MS Analysis of Compound Types

Glucuronides are characterized using a thermospray LC-MS interface (40). Ten nanograms of the glucuronide injected onto a column suffice for characterization by a scanned mass spectrum. Unlike many of the other currently available LC-MS interfaces, the thermospray ionization interface allows 100% of an aqueous effluent to enter the mass spectrometer at flow rates up to 1.5 ml per minute.

Nanogram amounts of the peptides leucine enkephalin, methionine enkephalin (TyrGlyGlyPheMet), and alpha-amanitin are obtained by direct LC-MS (41). Both $(M+H)^+$ and $(M-H)^-$ ions are obtained in the positive and negative ion modes, respectively.

D. MOVING BELT INTERFACES

D.1. Introduction

The addition of a modified segmented flow extractor between an HPLC and a mass spectrometer permits the direct coupling of an HPLC operated in the reversed phase (RP) mode to a mass spectrometer, without compromising the operational characteristics of either instrument (42). Ion-pairing techniques were studied and demonstrate compatibility with on-line LC-MS (43).

Several approaches have been explored for the direct LC-MS combination:

1. A moving wire/belt system that transports the LC effluent through a series of vacuum locks for the solvent to be

evaporated. The sample is then introduced into the ion source for analysis by either EI or CI.

2. A 1% split of the LC effluent into the mass spectrometer, where the LC solvent is used as the CI reagent gas.

3. A vacuum nebulizing interface to introduce total effluent from a microbore HPLC into the ion source.

4. The system that converts the LC effluent into a molecular beam by forcing it through a nozzle restriction, followed by flash evaporation using a laser beam or sonic radiation, and then mass spectrometric analysis in either the EI or CI mode.

5. Direct evaporation of the total effluent into the mass spectrometer ion source followed by CI under API conditions.

6. A silicone membrane enrichment device which removes the solvent and permits preferential entry of the solute into the mass spectrometer.

The segmented-flow LC-MS interface desalts the organic phase with high efficiency. This fact is demonstrated by analyzing sodium ions and phosphate ions at the 0.3 ppm and 10 ppm levels, respectively.

In a further development of this type of LC-MS interface, a specially designed nebulizer was constructed for deposition of the effluent from the HPLC column onto a moving belt (44). Another aerosol liquid deposition device was described (45). Measurements of the solvent transfer efficiency to the belt were performed by first spraying a peak from the HPLC column onto the belt, and then comparing that peak area with the area obtained by depositing the same mass of sample onto the belt with a syringe, where the syringe was assumed to provide 100% efficiency in the transfer of the solvent onto the belt. A belt interface can be thought of as consisting of four basic steps, each of which may potentially effect chromatographic performance:

1. transfer or deposition step;
2. evaporation of the solvent remaining on the belt with an infrared heater;

3. passing of the solvent into the vacuum lock system;
4. desorption of the sample from the belt into the ion source.

The influence that the deposition step can have on the performance of the LC-MS interface is critical when comparing the conventional method of flowing the effluent onto the belt in a continuous stream versus spray deposition. Dispersion of the LC effluent into a fine mist can provide an efficient evaporation step. It is found that the dimensions of the orifice through which the liquid flows must be minimized to prevent liquid from accumulating on the glass tip, and also to allow formation of the smallest possible droplets. By using a 60° angle between the spray tip and belt, droplet formation on the belt behind the tip is minimized. Attention to these critical experimental steps is of primary importance in order to obtain good chromatographic fidelity including peak shape, variance, area, and reproducibility.

D.2. Peptide Studies

Using a quadrupole mass spectrometer outfitted with a moving belt interface, N-acetyl-N,O,S-permethylated oligopeptides were analyzed (46,47). Isobutane CI yields good intensity $(M+H)^+$ and N- and C-terminal ions. In addition, C-methylated peptides are separated by LC.

Permethylated peptides and peptide mixtures have been studied employing normal phase chromatography and a moving belt LC-MS interface (48). Eighteen different peptides, ranging in size from di- to heptapeptides, were studied and it was found that on-line LC-MS ammonia CI spectra produced complete or almost complete amino acid sequence determination information.

D.3. Analysis-of-Variance of System Components

Extra-column band-spreading on an HPLC-MS moving belt interface was analyzed by a numerical evaluation of the system variance (49). Spraying effects may be evaluated by considering the increase of the variance (the second moment of mass) of the chromatographic band. Variances are additive when contributions are independent.

D.4. Review of LC-MS Transport Devices

HPLC interfaces with transport devices were reviewed (50) and a comparison is made of moving belt interfaces for LC-MS (51). In the interfacing of the HPLC to a mass spectrometer, three fundamental problems must be overcome:

1. how to make the mass spectrometer, which can handle 20 cc per minute of gas if configured for CI, compatible with solvent flow rates of the order of one cc per minute which result in gas volumes in the range of 150 to 1200 cc per minute, depending on the solvent used;

2. introduction of the solute into the mass spectrometer so that mass spectral information can be obtained and the solute does not undergo thermal decomposition;

3. coupling of the HPLC with the mass spectrometer so that there can be no loss of the chromatographic performance.

It is noted that a further problem which occurs is that, with aqueous solvent systems containing more than 50% water, beading of the solvent on the belt causes pressure fluctuations in the ion source resulting in poor mass spectral data. Although microbore HPLC was initially utilized with the moving belt system, it has had little subsequent use.

A table in this review (50) collects the applications and corresponding references of HPLC using transport type

interfaces and includes the following compound types: aflatoxins, Amaryllidaceae alkaloids, antibiotics, aromatic acids, bile acids and their conjugates, carbamate pesticides, chinchona alkaloids, chlorinated phenols in urine, coal liquification products, dinitrophenyl hydrazones, drugs, effluent analysis, ergot alkaloids, glycosides, herbicides, lipids, liquid crystals, natural coumarins, nucleosides, peptides, pesticides, petroporphyrins, polychlorinated biphenyls and their metabolites, polynuclear aromatics, rotenoids, steroids, sugars, triglycerides, and waxes.

D.5. Ribbon Storage Device

A novel ribbon storage interface is described (52, 53). The distance between the mass spectrometer and the HPLC unit approximates five feet. This LC-MS interface is designed for use with SIMS and with conventional EI. The LC-MS interface includes a 120 cm region at atmospheric pressure. Aerosol deposition of the HPLC effluent allows the complete evaporation of the mobile phase before the first vacuum slit, and a 320 cm total length allows the storage of chromatographically-separated materials. Ten picograms of amino acids have been detected. The long ribbon also allows temporary storage of five to 60 minutes of HPLC separations on the ribbon for subsequent reanalysis by SIMS or EI.

A new method for ribbon cleaning using vapor deposition of a thin layer of silver is also described (52,53) which reduces background from contaminants and residues on the ribbon and is superior to heaters or solvent baths for ribbon cleaning. The liquid is deposited on the ribbon surface by an aerosol liquid deposition device. The heart of the interface is a ribbon (0.63 cm wide, 0.0087 cm thick, and 320 cm long) spot-welded to perform a continuous loop. Ribbons of high-purity nickel, molybdenum, and platinum are found to

have acceptable mechanical properties; most of the work is done with high-purity nickel.

E. IONIZATION METHODS FOR LC-MS

Ionization methods available for LC-MS are reviewed (54). Nine different methods are described and include desorption chemical ionization (DCI), laser desorption (LD), field desorption (FD), electrohydrodynamic ionization (EHI), ^{252}Californium plasma desorption (PD), SIMS, FAB, API and thermospray ionization technique.

F. CONCLUSIONS

At the present time, no one of the on-line interface devices described above has taken charge of the field to become universally useful in laboratories around the world. Each one of the proponents of the different on-line techniques can describe the advantages and disadvantages of each system. It is clear from the data and experience published in the literature that more developmental time is needed. That hesitation of employing the commercially available HPLC-MS interfaces notwithstanding, it is quite clear that this type of on-line LC-MS interface, once it has been appropriately developed and utilized, will significantly increase the use of mass spectrometry as the detector for HPLC for the measurement of endogenous compounds.

II. OFF-LINE COMBINATION OF LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY: EXAMPLES OF PEPTIDE MEASUREMENTS.

A. INTRODUCTION

While the first section reviewed the on-line combination of LC-MS, this section will focus on the off-line combination of

LC and MS, where a compound eluting at a known retention time is manually collected and is measured by MS techniques. Extensive analytical measurements have been made of endogenous peptides extracted from biological sources.

In this section, advantage is taken of the fast and facile production of $(M+H)^+$ ions of biologically important peptides by means of FAB mass spectrometry (55). Doubly-charged molecular ions of insulin have been observed in some instruments (56) and singly-charged ions of the proinsulin molecule in other instruments (57). However, because of the relatively high background produced by the matrix needed for FAB mass spectrometry, the resolution of this analytical system must be increased in one of two ways (58). On one hand, the mass resolution can be increased up to one part in ca. 20,000 to effectively isolate the accurate mass of $(M+H)^+$. However, especially in the case of peptides, it is known that even the $(M+H)^+$ of a peptide lacks sufficient molecular specificity for unambiguous quantification. On the other hand, structural resolution can be significantly increased by taking advantage of linked-field scanning MS techniques (59), especially in the B/E mode, where one selected product ion is selected from a precursor ion. In both cases, fragmentation can either be increased, or created, by the use of CAD processes.

Internal standards (60, 61) can be efficiently produced for measurement of peptides by incorporating a stable isotope such as ^{18}O into the peptide molecule (62, 63).

B. CONSTRUCTION OF CALIBRATION CURVE

The method of analysis employing the FAB-CAD-B/E-B'/E'-SIM-microcomputer measurement mode must demonstrate a linear response over the range of concentrations of endogenous peptides. The use of both the stable

isotope-incorporated peptide internal standard and this novel analytical measurement mode are important to demonstrate linearity and to overcome biologic matrix effects. Primary data from one of the accelerating voltage alternation experiments for leucine enkephalin versus ^{18}O leucine enkephalin were used to monitor the peak at mass 336 in the former case and 340 in the latter case. This selected pair of ions corresponds to the tripeptide sequence -GFL which derives from the CAD-B/E analysis mode (64). This amino acid sequence-determining ion is a product ion arising only from the $(M+H)^+$ ion of leucine enkephalin- a most significant experimental fact that rigidly maintains the molecular specificity of the analytical measurement.

The microcomputer interface is used to accept a large number of analog signals to optimize ion statistics (65). Integrated areas are calculated and the ratio of the known amounts of the ^{16}O and the ^{18}O species are plotted. The calibration curves for both the methionine enkephalin (ME) and leucine enkephalin (LE) experiments show that both curves intercept at, or very near, the origin and have correlation coefficients near unity. The statistical parameters for the two best-fit straight lines are, for ME: $y = 1.09x + 0.06$, $r^2 = 0.999$ and for LE: $y = 0.69x$, $r^2 = 0.995$.

C. HYPOTHALAMUS TISSUE EXTRACTS

The RP-HPLC chromatogram for canine hypothalamus tissue demonstrates that, at the UV wavelength being monitored (200 nm), the RP-HPLC chromatogram of a hypothalamic peptide-rich fraction is relatively clean of UV-absorbing material (66, 67). It is quite important to remember that, of course, a wealth of biologically active radioreceptorassayable, bioassayable, and/or radioimmunoassayable material may, and generally does, coelute on this chromatogram at the indicated retention times

and usually at other retention times (68, 69). However, it is experimentally observed that, at a UV detection sensitivity level of 0.1 AUFS, microgram amounts of peptides are detected (70) whereas radioreceptor assay (RRA), radioimmunoassay (RIA), and bioassay (BA) are capable of detecting ng and pg amounts, but with no structural information attached to those measurements.

While experimental experience indicates that the RP-HPLC resolution of the two enkephalin peaks may be increased by alterations of several experimental parameters including recycling and/or change of the buffer and/or organic modifier, flow rate, temperature, etc., it must be remembered that in the novel mode of off-line LC-MS analysis, the detector is not limited to only UV absorption, but rather, utilizes a unique amino acid sequence-determining ion which arises from a peptide eluting at one selected retention time. In this type of measurement mode, virtually all chromatographic and chemical background noise disappears. The plot of leucine enkephalin and the ^{18}O leucine enkephalin internal standard $(M+H)^+$ ratios for this hypothalamus data shows that the intersection of the signal and noise levels corresponds to 170 ng leucine enkephalin g^{-1} hypothalamus tissue (71).

D. THALAMUS TISSUE EXTRACTS

^{18}O-incorporated internal standards are used to determine endogenous amounts of enkephalin in canine thalamus tissue (72). The straight line intersects the abcissa at values corresponding to 62 ng LE and 125 ng ME g^{-1} thalamus, respectively.

E. PITUITARY

A number of canine pituitaries (70) is neuroanatomically separated into the anterior (1.9 g. total wet weight) and

posterior (0.44 g total wet weight) portions. The tissue is homogenized in acetic acid (1 M) and divided into three equal samples. In the anterior pituitary, 70 ng of LE and 2,950 ng of ME g^{-1} are found while 2 ng LE and 3760 ng ME g^{-1} are measured in the posterior pituitary extracts.

F. CAUDATE NUCLEUS

FDMS methods have been used to measure the endogenous amount of enkephalin in canine nucleus tissue extracts (71). The amount of endogenous leucine enkephalin in the canine caudate nucleus tissue extract is 1,500 ng leucine enkephalin g^{-1} tissue.

G. TOOTH PULP

Tooth pulp tissue is collected from four animals and pooled (four teeth from each animal; total = 16). Canine tooth pulp RP-HPLC chromatograms have been published (74). The endogenous amount of ME for pooled tooth pulp tissue is 3 micrograms g^{-1} tooth pulp tissue.

H. ELECTROSTIMULATED TOOTH PULP

In a study of nociceptive processes, levels of enkephalins are determined in canine tooth pulps which had been electrostimulated in vivo and compared to levels of these peptides in tooth pulps of control animals. The current working hypothesis underlying this type of physiologic study is that three peptidergic pathways (endorphinergic, dynorphinergic, and enkephalinergic) are available to a cell to maintain a dynamic homeostatic relationship and to deal with noxious stimuli. The three peptidergic pathways are composed of large

precursor peptides, the opioid oligopeptide, and metabolites. Noxious stimuli are hypothesized to activate the peptidergic pathways, and individual opioid peptides may have decreased concentrations following stimulation. Electrostimulation significantly decreases by 20% the amount of endogenous ME.

A general overall trend noted is that the two opioid pentapeptides ME and LE are altered upon electrostimulation (75,76). Electrostimulation is performed to elucidate those molecular mechanisms operating during a physiologically stressful situation. These preliminary data indicate that the three peptidergic pathways (dynorphinergic, endorphinergic, enkephalingeric) may be mobilized in the following sequence:

Large precursor ⟶ intermediate precursor(s) ⟶ pentapeptide(s) ⟶ inactive metabolites (77).

On one hand, there may be a naturally-occurring pool of pentapeptides which is electrostimulated towards metabolism or, on the other hand, the entire metabolic scheme noted above may be stimulated to produce a lowered endogenous amount of each constituent peptide. Other human and in vivo dynamic studies are needed to resolve that question.

I. CEREBROSPINAL FLUID

LE (44 ng ml^{-1}) was measured in canine CSF (71). This type of measurement is important in current clinical studies aimed towards the elucidation of molecular mechanisms involved in pain.

J. CONCLUSIONS

Several significant conclusions are derived from the experiments utilizing the off-line LC-MS measurement modes which are reported in this section.

A fast and facile method of tissue sample acquisition and procurement from the canine animal model is described (78).

This study demonstrates that rapid freezing of tissue is needed in a fashion similar to that described in the discovery of 19OH-PGE_2 compounds in human seminal fluid (79-81). Rapid freezing avoids, or at least minimizes, metabolic and chemical interconversions and also enhances the possibility of measuring only those endogenous target compounds and not artifacts or chemical/enzymic products. The need for an internal standard for an MS analytical measurement is demonstrated by previous workers (60, 61) to overcome the limitations imposed by the experimentally rather ill-defined, yet very real, biological matrix effects. Stable isotope-incorporated peptides are the most appropriate internal standards for measurement of endogenous peptides. Stable isotope-incorporated peptide internal standards also have hydrophobicity and MS behavioral characteristics similar to the endogenous peptides. An internal standard is added as soon as possible after tissue acquisition and before homogenization in the separation scheme as a means to accurately represent the endogenous amount of peptide, and also to provide sufficient time for equilibration (61) of the exogenous and endogenous peptides.

The most significant experimental parameter of any analytical measurement of a biological compound is the molecular specificity of that measurement; namely, is the compound one thinks is being measured the compound that is actually being measured? This concept of specificity is easy to state but experimentally rather difficult to prove unambiguously. One author calls this experimental phenomenon the "chromatographic uncertainty principle" (82). Many other assay methods are generally utilized because of their relative ease, low cost, high speed, high sensitivity, and putatively high molecular specificity. For example, chromatography, color reactions, enzymatic reactions, HPLC, BA, RRA, and RIA are some of the measurement procedures which are used in most laboratories around the world. However, one of the purposes of this review is to state unequivocally that the

molecular specificity of all of the above measurement methods is insufficient for unambiguous structural proof during an analytical measurement (78). Of course, the non-MS assay methods listed above will always be used, but investigators must at least be aware of and state the limitations of any statements made relating to structure. Only one measurement process, namely MS, offers unambiguous molecular specificity. On one hand, ability to produce the $(M+H)^+$ ion of a biologically important peptide is a signal advancement in the measurement of endogenous peptides. But even this parameter, $(M+H)^+$, while significantly increasing specificity, does not confer unambiguous molecular specificity to that measurement. The only analytical method currently available and which uses MS to provide maximum molecular specificity is to use an amino acid sequence-determining ion from the $(M+H)^+$ ion produced by FAB with either unimolecular or CAD processes, and then to collect by a linked-field scan only one unique amino acid sequence-determining ion. Furthermore, use of a stable isotope-incorporated internal standard, which is the same peptide as that being measured, additionally substantiates the molecular specificity of the analysis. In the study discussed above, the C-terminal tripeptide sequence ions -GFL from LE and -GFM from ME are selected for monitoring, and the analytic measurement of endogenous peptides is based upon those two ions and their corresponding ^{18}O-internal standards.

The detection sensitivity of the novel LC-MS process described above is quite encouraging for analytical measurement of endogenous peptides in most biological tissues and fluids. For example, enkephalin peptides in canine caudate nucleus tissue extracts are measured at the 200-400 ppb level. The current instrumental limitation corresponds to the 30 ppb level. It is significant to realize that several significant instrumental increases (10-100x) in the detection limits are forthcoming.

The peptide measurements include endogenous amounts of both ME and LE extracted from a variety of biological sources including hypothalamus, CSF, anterior and posterior pituitary, caudate nucleus, and tooth pulp (pooled and electrostimulated). It is important to undertake this type of analytical/physiological study within one laboratory to ensure quality control over all experimental manipulations which range from the live animal model through exsanguination, tissue procurement, homogenization, chromatography, MS, and data analysis. Inter-animal biologic variations are observed and it is possible to have one animal serve as it own control.

REFERENCES

1. J.T. Watson and K. Biemann, Anal. Chem., 36, 1135 (1964).

2. C.G. Edmonds, J.A. McCloskey and V.A. Edmonds, Biomed. Mass Spectrom. 10, 237 (1983).

3. P.J. Arpino, Trends Anal. Chem., 7, 154 (1982).

4. W.H. McFadden, J. Chromatogr. Sci., 19, 97 (1980).

5. C. Eckers, K.K. Cuddy and J.D. Henion, J.Liq. Chromatogr., 6, 2383 (1983).

6. C. Eckers, D.S. Skrabalak and J.D. Henion, Clin. Chem., 28, 1882 (1982).

7. B.G. Dawkins, P.J. Arpino and F.W. McLafferty, Biomed. Mass. Spectrom., 5, 1 (1978).

8. M.A. Baldwin and F.W. McLafferty, Org. Mass Spectrom., 7, 1111 (1973).

9. P.A. Arpino, B.G. Dawkins and F.W. McLafferty, J. Chromatogr. Sci., 12, 574 (1974).

10. F.W. McLafferty and B.G. Dawkins, Biochem. Soc.Trans. 3, 856 (1975).

11. B.G. Dawkins and F.W. McLafferty, GLC and HPLC Determination of Therapeutic Agents, Vol 1, Tsuji-Morozowich, (Eds), Marcel Dekker, 1978, p. 259.

12. P.J. Arpino and G. Guiochon, J. Chromatogr., 251, 153 (1982).

13. G. Guiochon and P.J. Arpino, J. Chromatogr., 271, 13 (1983).

14. M. Dedieu, C. Juin, P.J. Arpino, J.P. Bounine and G. Guiochon, J. Chromatogr., 251, 203 (1982).

15. P.J. Arpino, Liquid Chromatography Detectors, T.M. Vickrey (Ed.), Marcel Dekker, New York, 1983, p.243.

16. P.J. Arpino, J.P. Bounine and G. Guiochon, J. Chromatogr., 251, 203 (1982).

17. P.J. Arpino, J.P. Bounine, M. Dedieu and G. Guiochon, J. Chromatogr., 271, 43 (1983).

18. D. Pilosof, H.Y. Kim, D.F. Dyckes and M.L. Vestal, Anal. Chem., in press.

19. C.R. Blakley, J.C. Carmody and M.L. Vestal, Clin. Chem., 26, 1467 (1980).

20. C.R. Blakley and M.L. Vestal, Anal. Chem., 55, 447 (1983).

21. M.L. Vestal, Mass Spectrom. Rev., 2, 447 (1983).

22. D. Pilosof, H.Y. Kim, M.L. Vestal and D.F. Dyckes, Biomed. Mass Spectrom., in press.

23. H.E. May, F.S. Tanzer, G.H. Fridland, C. Wakelyn and D.M. Desiderio, J. Liq. Chromatogr., 5, 2135 (1982).

24. A.P. Bruins and B.F.H. Drenth, J. Chromatogr., 271, 71 (1983).

25. J.D. Henion and G.A. Maylin, Biomed. Mass Spectrom., 7, 115 (1980).

26. J.D. Henion, Adv. Mass Spectrom., 8, 1241 (1980).

27. J.D. Henion, J. Chromatogr. Sci., 19, 57 (1981).

28. J.D. Henion, B.A. Thomson and P.H. Dawson, Anal. Chem., 54, 451 (1982).

29. C.E. Parker, Y. Tondeur and J.R. Hass, J. Forens., Sci., 27, 495 (1982).

30. C. Eckers, D.S. Skrabalak and J. Henion, Clin. Chem., 28, 1882 (1982).

31. F.R. Sugnaux, D.S. Skrabalak and J.D. Henion, J. Chromatogr., 264, 357 (1983).

32. J. Henion, D. Skrabalak, E. Dewey and G. Maylin, Drug Metabol. Rev., 14, 961 (1983).

33. J. Henion and D.S. Skrabalak, Fifth International Symposium Equine Medication Control., G.H. Johnston and J.W. Martin (Eds), in press.

34. T. Covey and J. Henion, Anal. Chem., 55, 2275 (1983).

35. R.G. Christensen, H.S. Hertz, S. Meiselman and E. White, Anal. Chem., 53, 172 (1981).

36. E. White, H. S. Hertz and R.G. Christensen, US patent #4281246 (1981).

37. J.A. Apffel, U.A.Th. Brinkman and R. W. Frei, Anal. Chem., 55, 2280 (1983).

38. R.D. Smith and H.R. Udseth, Anal. Chem., 55, 2266 (1983).

39. R.D. Smith, W.D. Felix, J.C. Fjeldsted and M.L. Lee, Anal. Chem., 53, 1883 (1982).

40. D.J. Liberato, C.C. Fenselau, M.L. Vestal and A.L. Yergey, Anal. Chem., 55, 1741 (1983).

41. C.N. Kenyon, Biomed. Mass Spectrom., 10, 535 (1983).

42. B.L. Karger, D.P. Kirby, P. Vouros, R.L. Foltz and B. Hidy, Anal. Chem., 51, 2324 (1979).

43. D.P. Kirby, P. Vouros and B.L. Karger, Science, 209, 495 (1980).

44. M.J. Hayes, E.P. Lankmayer, P. Vouros, B.L. Karger and J.M. McGuire, Anal. Chem., 55, 1745 (1983).

45. R.D. Smith and A.L. Johnson, Anal. Chem., 53, 739 (1981).

46. T.J. Yu, H. Schwartz, R.W. Giese, B.L. Karger and P. Vouros, J. Chromatogr., 218, 519 (1981).

47. T.J. Yu, B.L. Karger and P. Vouros, Biomed. Mass Spectrom. 10, 633 (1983).

48. P. Roepstorff, M.A. McDowall, M.P.L. Games and D.E. Games, Int. J. Mass Spectrom. Ion Phys., 48, 197 (1983).

49. D.E. Games, M.J. Hewlins, S.A. Westwood and D.J. Morgan, J. Chromatogr., 250, 62 (1982).

50. N.J. Alcock, C.Eckers, D.E. Games, M.P.L. Games, M.S. Lant,

M.A. McDowall, M. Rossiter, R.W. Smith, S.A. Westwood and H.Y.Wong, J.Chromatogr., 251, 165 (1982).

51. D.E. Games, M.A. McDowall, K. Levsen, K.H. Shafer, P. Dobberstein and J.L. Gower, Biomed. Mass Spectrom., 11, 87 (1984).

52. R.D. Smith, J.E. Burger and A.L. Johnson, Anal. Chem., 53, 1603 (1981).

53. R.D. Smith and A.L. Johnson, Anal. Chem., 53, 1120 (1981).

54. N.M.M. Nibbering, J. Chromatogr., 251, 93 (1982).

55. M. Barber, R.S. Bordoli, G.V. Garner, D.B. Gordon, R.D. Sedgwick, L.W. Tetler and A.N. Tyler, Biochem. J., 197, 401 (1981).

56. D.M. Desiderio and I. Katakuse, Biomed. Mass Spectrom., (1984) in press.

57. M. Barber, R.S. Bordoli, G.J. Elliott, and N.J. Horoch, Biochem. Biophys. Res. Commun., 110, 753 (1983).

58. D.M. Desiderio, Adv. Chromatography Vol. 22, J.C. Giddings, E. Grushka, J. Cazes and P.R. Brown (Eds). Marcel Dekker, N.Y., 1983 p. 1.

59. K.R. Jennings and R.S. Mason, Tandem Mass Spectrometry, F.W. McLafferty (Ed.), Wiley, N.Y., 1983, p. 197.

60. B.J. Millard, Quantitative Mass Spectrometry, Heyden, London, 1978.

61. A.M. Lawson, C.K. Lim, W. Richmond, D.M. Samson, K.D.R. Setchell and A.C.S. Thomas, Current Developments in the Clinical Applications of HPLC, GC and MS, A.M. Lawson, C.K. Lim and W. Richmond, (Eds), Academic Press, London, 1980, p. 135.

62. D.M. Desiderio and M. Kai, Biomed. Mass Spectrom., 10, 471 (1983).

63. D.M. Desiderio and M. Kai, Int. J. Mass Spectrom. Ion Phys., 48, 261 (1983).

64. D.M. Desiderio, I. Katakuse and M. Kai, Biomed. Mass Spectrom., 10, 426 (1983).

65. D.M. Desiderio, J. Laughter, M.Kai and J. Trimble, J. Com. Enchanc. Spectros., in press.

66. D.M. Desiderio, J.Z. Sabbatini and J.L. Stein, Adv. Mass Spectrom., 8, 1298 (1980).

67. D.M. Desiderio, J.L. Stein, M.D. Cunningham and J.Z. Sabbatini, J. Chromatogr., 195, 369 (1980).

68. F.S. Tanzer, D.M. Desiderio, C. Wakelyn and J. Walker, J. Dent. Res., submitted.

69. D.M Desiderio, H. Onishi, H. Takeshita, F.S. Tanzer, C. Wakelyn, J. Walker and G. Fridland, J. Neurochem., submitted.

70. D.M. Desiderio and M.D. Cunningham, J. Liq. Chromatogr., 4, 721 (1981).

71. D.M. Desiderio, M. Kai, F.S. Tanzer, J. Trimble and C. Wakelyn, J. Chromatogr., in press.

72. D.M. Desiderio and M. Kai, Biomed. Mass Spectrom., 10, 471 (1983).

73. S. Yamada and D.M. Desiderio, Anal. Biochem., 127, 213 (1982).

74. F.S. Tanzer, D.M. Desiderio and S. Yamada, Peptides: Synthesis-Structure-Function, D.H. Rich and E. Gross (Eds), Pierce Chem. Co., Rockford, Ill., 1981, p. 761.

75. T. Kudo, H.-L. Chang, S. Maeda, Y. Uchida, J. Kakamae and R. Inoki, Life Sci., 33, 677 (1983).

76. T. Kudo, S. Maeda, J. Nakamae, H.-L. Chang, Y. Uchida and R. Inoki, Life Sci., 33, 681 (1983).

77. J. Hughes, Brit. Med. Bull., 39, 17 (1983).

78. D.M. Desiderio, Peptide Measurements by HPLC and MS, Elsevier, Amsterdam, in press.

79. H.H. Jonsson, B.S. Middleditch and D.M. Desiderio, Science, 187, 1093 (1975).

80. H.T. Jonsson, B.S. Middleditch, M.A. Schexnayder and D.M. Desiderio, J. Lip. Res., 17, 1 (1976).

81. D.L. Perry and D.M. Desiderio, Prostaglandins, 14, 745 (1977).

82. M.F. Delaney, LC Magazine, 2, 85 (1984).