Matrix-Assisted Laser Desorption Mass Spectrometry of Biotransformation Products of Dynorphin A in Vitro

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The utility of matrix-assisted laser desorption mass spectrometry for characterizing products of in vitro processing of synthetic dynorphin A (Dyn A) peptides in biologic matrices is described. A series of laser desorption matrices were tested for their response to Dyn A (1-6), Dyn A (1-7), Dyn A (1-8), Dyn A (1-9), Dyn A (1-10), Dyn A (1-13), Dyn A (2-17), and Dyn A (1-17). α-Cyano-4-hydroxycinnamic acid was chosen as a suitable matrix for subsequent studies. Mass spectra of dynorphin peptides indicated a good signal-to-noise response down to (1) 10 fmole of Dyn A (1-10) amide standard in aqueous acidic solution and (2) a concentration of 10^{-7} M for seven dynorphin peptides spiked into human plasma. Two examples of the mass spectrometric analysis of the products of in vitro processing are presented: Dyn A (1-13) and Dyn A (1-17) in human blood. The presence and identity of processed peptides can be simply inferred from the molecular masses provided by the mass spectrometric measurement without extensive sample purification. A comparison of matrix-assisted laser desorption mass spectrometry is made with high-performance liquid chromatography. *(J Am Soc Mass Spectrom 1994, 5, 10–16)*

A number of studies using mass spectrometric techniques have been reported for characterizing and measuring neuropeptide levels in human and animal tissues and fluids [1–5]. For example, fast-atom bombardment mass spectrometry, coupled with extensive multidimensional reversed-phase high-performance liquid chromatography (HPLC), has been used in the analysis of endogenous met-enkephalin [1], β-endorphin [2], dynorphin (Dyn) A (1-6), and Dyn B (7-13) [3] in human pituitary and met-enkephalin in human cerebrospinal fluid [4]. Recently, electrospray mass spectrometry was used to determine endogenous met-enkephalin and β-endorphin levels extracted from human pituitary gland [5].

Matrix-assisted laser desorption mass spectrometry [6–8] is also a powerful analytical method for analyzing peptides and proteins. Among the strengths of the technique are its high sensitivity [9, 10]; its ability to examine, simultaneously and without extensive chromatographic purification, many peptide and protein components in a complex mixture [11]; and its facility for making such measurements on mixtures containing large quantities of nonproteinaceous impurities (e.g., salts) [11].

We report here the development of a matrix-assisted laser desorption mass spectrometry technique to characterize processing in body tissues and fluids of exogenously added or administered neuropeptides. In this study, two examples of the mass spectrometric analysis of the products of in vitro processing are presented: Dyn A (1-13) [12] and Dyn A (1-17) in human blood. The presence and identity of processed peptides can be readily inferred from the mass spectrometric measurement. A comparison of the present technique is made with standard HPLC analysis.

Materials and Methods

Dynorphin Peptides

The dynorphin peptide standards Dyn A (1-6), Dyn A (1-7), Dyn A (1-8), Dyn A (1-9), Dyn A (1-10), Dyn A (1-10) amide, Dyn A (1-13), Dyn A (2-17), and Dyn A (1-17) were purchased from Peninsula Laboratories (Belmont, CA). Some of these peptide standards [Dyn A (1-9), Dyn A (1-13), and Dyn A (1-17)] were also obtained from Multiple Peptide Systems (San Diego, CA) and made available by the National Institute on Drug Abuse (NIDA). Dyn A (1-13), used in the human blood processing study, was generously donated by Neurobiological Technologies Inc. (Richmond, CA). This sample of Dyn A (1-13) contained mannitol as an excipient prepared for human use. The amino acid sequence of Dyn A (1-17) is given in Figure 1. HPLC-grade acetonitrile was purchased from Burdick & Jackson (Muskegon, MI) and trifluoroacetic acid (TFA) from Fisher Scientific (Fair Lawn, NJ).

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1044-0305/94/$7.00

Received June 2, 1993
Revised August 23, 1993
Accepted August 27, 1993

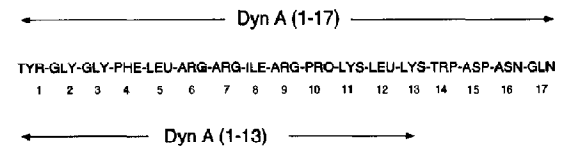

Figure 1. The amino acid sequence of dynorphin A (1-17).

Matrix-assisted Laser Desorption Mass Spectrometry

The matrix-assisted laser desorption mass spectrometric measurements were carried out with a linear time-of-flight mass spectrometer and laser desorption ion source constructed at the Rockefeller University. Details of the instrument and the data acquisition/analysis methods have been described previously [9, 11–13]. The peptide samples were prepared for mass spectrometric analysis by mixing them with concentrated solutions of a variety of different matrix compounds: sinapinic acid, ferulic acid, caffeic acid, α-cyano-4-hydroxycinnamic acid, and α-cyano-3-hydroxycinnamic acid (Aldrich, Milwaukee, WI); 2,5-dihydroxybenzoic acid (Sigma Chemical Co., St. Louis, MO); and 3-indoleacrylic acid (Fluka AG, Buchs, Switzerland). The matrix compounds were dissolved at a saturation concentration of 5 to 10 g/L in 33% acetonitrile and 67% of 0.1% aqueous TFA (v/v). Adjustments of the organic/aqueous ratio were sometimes needed to maximize the matrix concentration. The samples and standards were also dissolved in solutions of similar organic/aqueous ratio. Typically, 2 μL of matrix solution and 2 μL of peptide sample solution were mixed, and about 0.5 μL of the mixture was applied to the mass spectrometer sample probe tip, which is 2 mm in diameter. The liquid was dried with a stream of cool forced air. The probe tip was inserted into the mass spectrometer through a sample lock, and the spectrum acquired after a few minutes of vacuum pumping to allow the pressure to reach approximately 10^{-7} torr and to thoroughly remove residual solvents from the sample. The UV laser wavelength was set at 355 nm. The laser pulse duration was approximately 10 ns, and the power density deposited on the sample probe was approximately 10^6 watts/cm^2. The individual spectra from 100 laser shots were averaged to obtain a mass spectrum exhibiting good statistics. With the laser operating at a repetition rate of 2.5 pulses/s, the acquisition of a spectrum was completed in 40 s.

Separation Methods Used Prior to Matrix-assisted Laser Desorption Mass Spectrometry

The separation methods used to remove potentially interfering contaminants in human plasma included Sep-pak mini-extraction cartridges and membrane filters with 3000 or 5000 Da molecular weight (Mw) cut-off. The solid phase C_{18} Sep-pak extraction columns (Waters-Millipore, Milford, MA) were activated by a wash with acetonitrile followed by 0.1% TFA in H_2O. Typically, 1 mL of plasma sample was diluted in 5 to 10 mL of 0.1% TFA, and the resulting mixture was applied to the activated C_{18} minicolumn. A vacuum was applied to initiate and sustain the flow. The columns were washed with 0.1% TFA in H_2O three times and partially dried for a minute by drawing air through with the application of vacuum. The peptides were eluted by acetonitrile (40–80%) in 0.1% TFA. The eluate was placed in a speed vacuum concentrator (Savant Instruments, Farmingdale, NY) and the volume reduced until a 10 to 20 μL residue of the eluate remained. The resulting peptide solution was combined with the matrix solution for mass spectrometric analysis.

The membrane filters were made of regenerated cellulose with 3000 Da Mw cut-off (Centricon 3 microconcentrators, Amicon-Grace, Beverly, MA) or 5000 Da Mw cut-off (Ultrafree-Cl filters, Millipore, Milford, MA). The low molecular weight peptides of interest were separated from the high molecular weight proteins in plasma by centrifugation (3000–4000 g) at low temperature (4 °C). Prior to use, the filters were washed sequentially with 2 mL each of deionized H_2O, 0.1 M NaOH, warm H_2O (~ 70 °C), and 0.1% TFA to remove contaminating chemicals that often reduce the laser desorption mass spectrometric response. Typically, 0.3 mL of plasma sample was diluted with 1.7 mL of 0.1 to 0.5% TFA in H_2O. The diluted mixture was pipetted into a microconcentrator containing a membrane filter and centrifuged (4 °C) for 2 h. The filtrate was placed in a speed vacuum concentrator until its volume was reduced to 10 to 20 μL for subsequent mass spectrometric analysis.

HPLC Methods Used for Comparison with Matrix-assisted Laser Desorption Mass Spectrometry

Chromatographic analysis was performed using a SMART microbore HPLC system (Pharmacia LKB Biotechnology, Piscataway, NJ) fitted with a C_2/C_{18} reverse phase column (3.2 mm × 3.0 cm) with a UV detector (254 nm). The mobile phase was made up of two components: component A consisting of 0.1% TFA in H_2O and component B consisting of 80% acetonitrile in 20% of 0.1% TFA. The flow rate was 300 μL/min. The separation method used for this comparison consisted of an initial aqueous mobile phase (0% B for 6 min and 0–10% B in 5 min), followed by a gradient with a low rate of change (10–20% B in 24 min). After the gradient separation, the column was washed with 100% B for 10 min. The standard mixture of eight dynorphin peptides (10 μL, 10^{-5} M) was injected into the SMART system, and fractions containing all the chromatographic peaks were collected for subsequent mass spectrometric analysis.

Results and Discussion

Matrix Selection for Dynorphin Peptide Analysis

To obtain an optimal matrix-assisted laser desorption mass spectrum for a given application, it is necessary to select an appropriate matrix [6, 7, 10, 14]. A mixture of dynorphin standards—Dyn A (1-6), Dyn A (1-7), Dyn A (1-8), Dyn A (1-9), Dyn A (1-10), Dyn A (1-13), Dyn A (2-17), and Dyn A (1-17)—was used to determine the response of the mass spectrometer to a selection of commonly used matrices (Figure 2). The amount of each peptide in the matrix solution applied to the probe of the mass spectrometer was 0.4 to 0.5 pmole. The spectra obtained from α-cyano-4-hydroxycinnamic (Figure 2a) and 3-indoleacrylic acids (Figure 2b) indicated that both are effective matrices for the dynorphin peptides. However, α-cyano-4-hydroxycinnamic acid offered a slightly stronger and more stable response than 3-indoleacrylic acid with a lower background below m/z 1000. Caffeic acid was also an effective matrix for these peptides (Figure 2c). However, it proved difficult to produce spectra uniformly from all positions on the probe tip because only selected areas yielded intense spectra. A significant amount of effort was required to obtain the spectrum shown in Figure 2c. Ferulic acid was a less effective matrix for these peptides, producing a relatively weaker response (Figure 2d). Although sinapinic acid generated quite strong spectra (Figure 2e) for peptides of larger masses, Dyn A (2-17) and Dyn A (1-17), the signal quality was reduced for the lower mass peptides. α-Cyano-3-hydroxycinnamic acid and 2,5-dihydroxybenzoic acid produced fairly good spectra of all the peptides, but again it proved necessary to search the probe tip for an area that yielded an intense spectrum.

These responses for the dynorphin peptides in the different matrices tested are in general agreement with previous findings by this and other groups [10–18]. α-Cyano-4-hydroxycinnamic acid has recently been demonstrated to be an excellent matrix for a variety of biopolymers [18]. However, one practical complication was the frequent formation of intense adduct species of copper with the peptides, occurring as a result of the presence of trace amounts of adventitious copper impurities of unknown origin [18]. In the present application, copper adduction is not a serious problem because the resulting adduct peaks in the spectrum are well resolved for the peptides of interest. Thus, α-cyano-4-hydroxycinnamic acid was chosen as the matrix for all subsequent analyses. We have found that this matrix also tolerates a large molar excess of involatile inorganic contaminants (e.g., salts) over the peptides of interest. For example, a mixture of the eight dynorphin standards in α-cyano-4-hydroxycinnamic acid, in which a 100,000 molar excess of phosphate buffer was present, produced a mass spectrum indistinguishable from the spectrum taken without added buffer (Figure 2a).

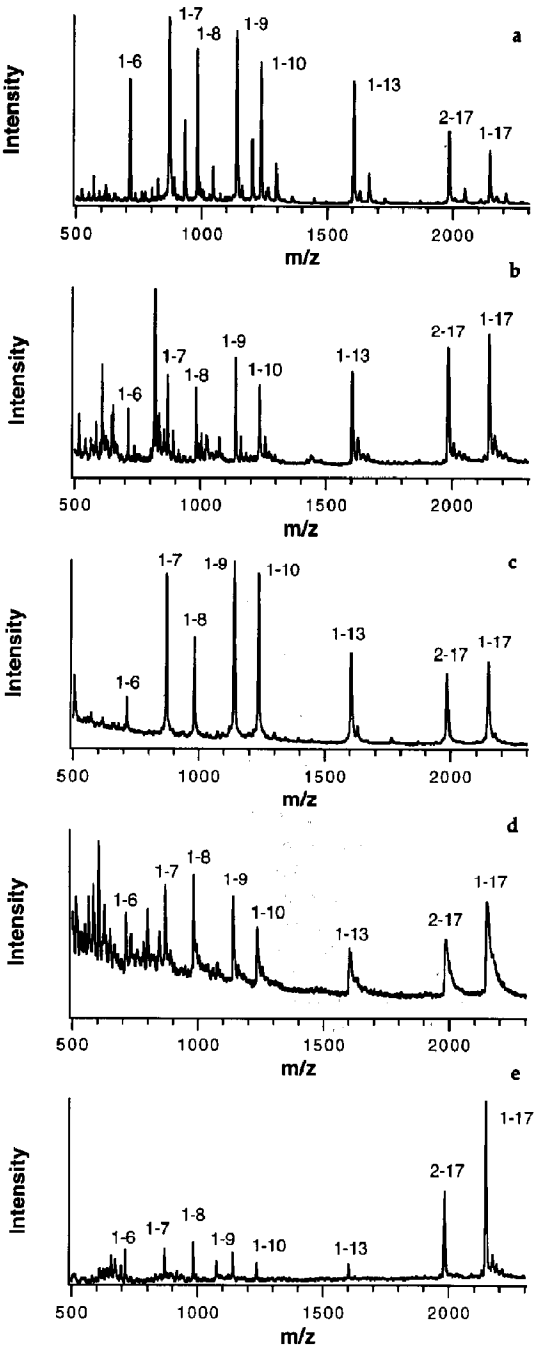

Figure 2. Matrix-assisted laser desorption mass spectrum of eight dynorphin standards in various matrices. **(a)** α-Cyano-4-hydroxycinnamic acid, **(b)** 3-indoleacrylic acid, **(c)** caffeic acid, **(d)** ferulic acid, and **(e)** sinapinic acid. The peak corresponding to Dyn A (1-6) is labeled 1-6. The other peaks are labeled using the same nomenclature.

Sensitivity for Detecting Dynorphin Peptides

Dilution studies using dynorphin standards showed that laser desorption mass spectrometry is a very sensitive method for detecting these peptides. A constant amount of angiotensin III (80 fmole) was used as an internal standard. The peak intensity ratio of Dyn A (1-10) amide to angiotensin III was plotted as a function of the amount of Dyn A (1-10) amide on the probe tip, as shown in Figure 3. At the lowest level investigated, a total amount of 10 fmole of Dyn A (1-10) amide applied to the 2-mm diameter probe tip yielded a protonated molecular ion peak having a signal-to-noise ratio of 27:1.

Effect of the Biologic Matrix on the Mass Spectrometric Detection of Dynorphins

The results obtained from the dynorphin standards encouraged us to examine the matrix-assisted laser desorption mass spectrometric response to dynorphin peptides spiked into plasma. The human plasma (New York Blood Center) used in these experiments was treated by chemical agents, which would be expected to deactivate most plasma enzymes. The plasma samples spiked with dynorphin standards were subjected to various extraction schemes to establish a method for retaining the maximum amount of the dynorphins of interest while removing as many of the interfering proteins, peptides, and other contaminating compounds as possible. Two techniques emerged as the most satisfactory. Plasma samples with the added dynorphin peptides were diluted with a fivefold to tenfold excess volume of acidic aqueous solution (0.1–0.5% TFA in deionized water). In the first technique, the diluted mixture was subjected to C_{18} Sep-pak column extraction, whereas in the second technique, separation was achieved by filtration through membranes with 3000 or 5000 Da Mw cut-off. Filtration using the membrane filters was found to be a straightforward and efficient first step for removing plasma proteins. Although the C_{18} Sep-pak column was similarly effective in removing plasma proteins and salts, the recovery of peptides from the Sep-pak columns was reduced rapidly as the dynorphin concentration was lowered toward 10^{-6} M.

A spectrum of a mixture of seven dynorphins spiked into plasma is shown in Figure 4. The initial concentration of each dynorphin in the human plasma was 10^{-7} M in a total plasma volume of 200 μL. This sample was prepared by diluting 1 μL of a standard solution of seven peptides (20 μM) into 200 μL of plasma. The spiked plasma was diluted with 2 mL of 0.1% TFA, and the resulting solution was filtered through a 3000 Da Mw cut-off membrane filter. The filtrate was placed in a speed vacuum concentrator until its volume was reduced to about 10 μL for mass spectrometric analysis. Only one-fortieth of this total sample (0.5 pmole of each peptide) was finally subjected to mass spectrometric analysis. All of the spiked dynorphin peptides are readily identified in Figure 4, although the signal intensities are weaker than those of the standards in acetonitrile/0.1% TFA (Figure 2a). This reduction in response likely arises from losses that occurred during the filtration and from interference of residual contaminants. A large quantity of salt passes through the membrane filter. Therefore, a rapid wash (1 s) of the probe tip was needed to remove these salts using cold (4 °C) aqueous acidic solution (in which the matrix is relatively insoluble). The same sample preparation method was used in the subsequent analysis of dynorphin processing in human blood.

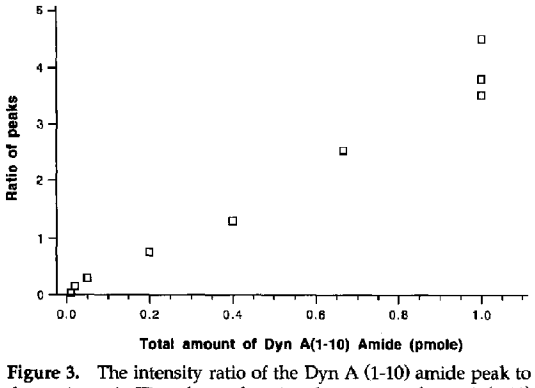

Figure 3. The intensity ratio of the Dyn A (1-10) amide peak to the angiotensin III peak as a function the amount of Dyn A (1-10) amide on the probe tip. The amount of angiotensin III was kept constant at 80 fmole. The reproducibility of the ratio determination is indicated by three replicate measurements obtained from 1 pmole of Dyn A (1-10) amide.

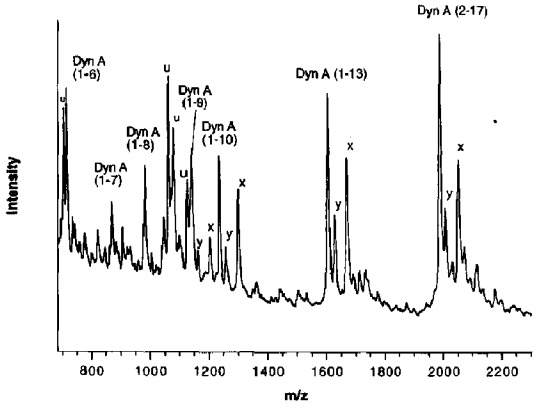

Figure 4. Matrix-assisted laser desorption mass spectrum of seven dynorphin standards spiked into human plasma at a concentration of 10^{-7} M for each peptide (see text). Sodium adducts are indicated as y, copper adducts are indicated as x, and the unidentified peaks are marked as u.

In Vitro Processing of Dyn A (1-13) and Dyn A (1-17) in Human Blood

In preparation for proposed dynorphin peptide pharmacokinetic studies in humans, it is essential to ascertain (1) whether dynorphin maintains its integrity after intravenous administration and (2) whether any further processing of dynorphin peptides occurs in fresh blood (without or with addition of enzyme inhibitors) after it is drawn from a person. As an initial step to address both of these questions, studies were performed to determine the in vitro processing of Dyn A (1-13) and Dyn A (1-17) in freshly drawn human blood.

A relatively high concentration (10^{-4} M, 150 μL) of Dyn A (1-13) was immediately added (delay time < 30 s) into freshly drawn blood (1.5 mL) taken from normal healthy human volunteers after the blood was collected in a sterilized 10-mL VACUTAINER tube (Becton Dickinson, Rutherford, NJ). To obtain plasma, the spiked blood was promptly centrifuged at 4 °C for 5 min at 2500 g. In the same manner as described in part c for the sample shown in Figure 4, the plasma samples were diluted with 0.1% TFA, filtered through 3000 Da Mw cut-off membrane filter, and the filtrate concentrated before mass spectrometric analysis.

Figure 5a shows the mass spectrum of the peptides extracted from plasma separated from spiked blood immediately after Dyn A (1-13) was added (0 min waiting time). The processed products were identified, from the molecular weights of the peaks, as Dyn A (1-6), Dyn A (2-11) or (4-12), Dyn A (3-12), Dyn A (2-12), and Dyn A (1-12). The masses were determined with an accuracy of better than ± 0.4 Da, so that all but one of the peptides could be identified unambiguously. The calculated molecular weight difference between Dyn A (2-11) and Dyn A (4-12) is only 0.9 u. Because the observed mass of one product fell between the two calculated molecular weights, there is uncertainty as to the identity of this product. The observed rapid cleavage of the amino terminal tyrosine and the carboxyl terminal lysine of Dyn A (1-13) has been reported previously in studies of processing of Dyn A (1-13) in rat brain in vitro [19, 20].

The results of a similar study of in vitro processing of Dyn A (1-17) in human blood is shown in Figure 5b. Dyn A (1-17) (10^{-4} M, 150 μL) was immediately added to freshly drawn human blood (1.5 mL) to yield a final concentration of 10^{-5} M, whereupon the plasma was separated promptly (0 min waiting time). In Figure 5b, the compound giving rise to the predominant peak is unprocessed Dyn A (1-17), whereas the major products of processing are identified as Dyn A (9-17), Dyn A (8-17), Dyn A (7-17), Dyn A (1-6), and Dyn A (2-17). The mass spectrum of a control sample of human blood with no added dynorphin A peptides is given in Figure 5c. No peaks were observed in this control spectrum that corresponded to any processed products from Dyn A. Further study is being conducted to examine the details of the differential processing of Dyn A (1-13) and Dyn A (1-17) in human blood and to study the appearance and disappearance of metabolites as a function of time (Chou et al., manuscript in preparation).

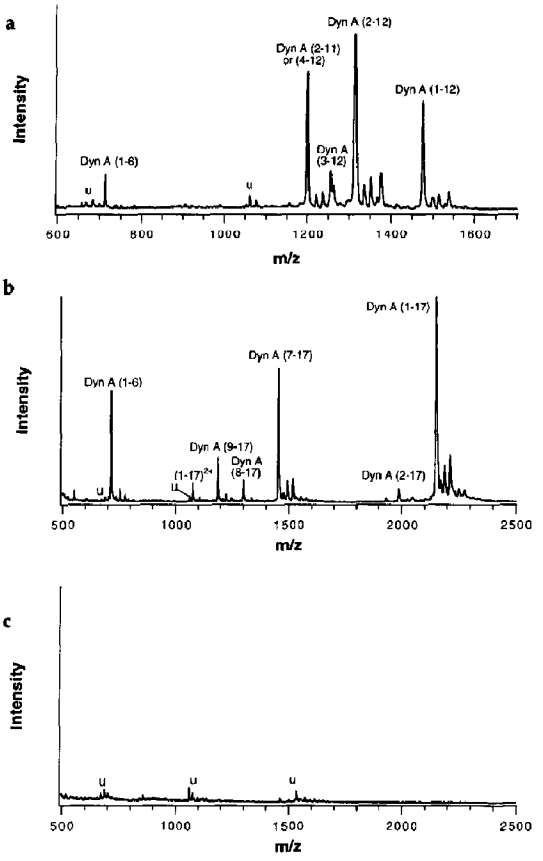

Figure 5. Matrix-assisted laser desorption mass spectra of dynorphin peptides after in vitro processing in human blood. The starting peptides and the time periods that the spiked blood was kept on ice were (a) Dyn A (1-13), 0 min; and (b) Dyn A (1-17), 0 min. The small peaks on the high mass side of the major peaks arise from sodium, potassium, and copper adduct species, respectively [28]. The unidentified peaks are marked as u. (c) The mass spectrum obtained from a sample of human blood with no added Dyn A peptide.

Contrasting Matrix-assisted Laser Desorption Mass Spectrometry with HPLC Analysis of Dynorphin Peptide Mixtures

HPLC is a widely used technique for the separation and quantitation of neuropeptides [21]. However, HPLC alone cannot always determine the identity of a single peptide or a mixture of peptides in a given chromatographic peak, even when all of the peptides possibly present are available to be used as standards.

Laser desorption mass spectrometry proves useful for identifying the molecular weights of putative individual components separated by HPLC, and thus removes some of the ambiguity inherent in HPLC. For example, Figure 6a shows an HPLC chromatogram of 10 μL of a dynorphin standard mixture (10^{-5} M in 0.1% aqueous TFA) that contained Dyn A (1-6), Dyn A (1-7), Dyn A (1-8), Dyn A (1-9), Dyn A (1-10), Dyn A (1-13), Dyn A (2-17), and Dyn A (1-17). The mixture was injected into the SMART system with a programmed method described in the Method section. Each chromatographic peak was fraction-collected from the microbore HPLC and concentrated for mass spectrometric analysis. Although six of the seven chromatographic peaks corresponded to individual dynorphin peptides (Figure 6a), the mass spectrum of the fraction corresponding to the seventh peak (peak A) clearly revealed the presence of two dynorphin peptides: Dyn A (1-8) and Dyn A (1-10) (Figure 6b). The two peptides coeluted, yielding a single chromatographic peak even in a gradient with a low rate of change (see Experimental section). In the absence of mass spectrometry, the identification of these two coeluting dynorphin peptides would be problematic.

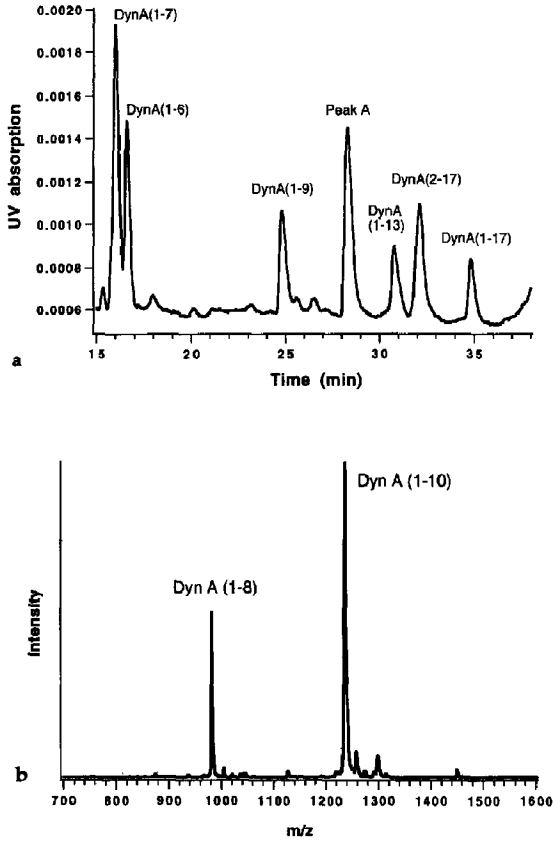

Figure 6. (a) A microbore high-performance liquid chromatogram of a mixture of dynorphin standards. The fractions of chromatographic peaks are collected and identified by laser desorption mass spectrometry. The indicated fraction A was collected for mass spectrometric analysis. (b) The mass spectrum of fraction A. Two peptides were found in the single chromatographic peak.

In addition, matrix-assisted laser desorption mass spectrometry was found to be highly effective despite the presence of a large amount of residual impurities in the samples (e.g., peptides, salts, and lipids). Because of this high tolerance for impurities, the sample purification/preparation steps required prior to mass spectrometric analysis are not more stringent than those required prior to HPLC. The sensitivity and speed of the laser desorption mass spectrometric analysis are comparable to or faster than standard microbore HPLC analysis.

HPLC has been developed for quantitation of some peptides under optimized conditions. By contrast, quantitative analysis using matrix-assisted laser desorption mass spectrometry has yet to be developed (work in progress). However, initial studies of the mass spectrometric response of Dyn A (1-10) amide standard as a function of the amount applied to the sample probe tip revealed an approximate linear relationship (Figure 3) in the 10 fmole to 1 pmole range, suggesting that such quantitative analysis may be feasible.

Conclusions

The mass spectra obtained from human plasma spiked with dynorphin peptides and from dynorphin processing studies in human blood demonstrate that matrix-assisted laser desorption mass spectrometry is a powerful analytical tool for studying the biotransformation of these neuropeptides. The technique allows simultaneous mass determination and identification of all significant peptide components in a mixture of biotransformation products. These measurements can be made rapidly from a variety of biologic materials with minimal requirement for purification of the sample.

Acknowledgments

We thank Thomas Gfroerer and Dr. Jeff Price of Neurobiological Technologies Inc., Richmond, CA, for generously supplying the dynorphin peptides for clinical use. This work is supported in part by NIH-NIDA Research Center grant I-P50-DA05130, Aaron Diamond Foundation (MJK and JZC); Aaron Diamond Postdoctoral Fellowship (JZC), NIH-NIDA Research Scientist Award, K05-DA00049 (MJK), and NIH RR00862; GM38274 (BTC).

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