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Determination of immunoreactive gonadotropin-releasing hormone in serum and urine by on-line immunoaffinity capillary electrophoresis coupled to mass spectrometry☆

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Abstract

The need for urgent diagnoses has propelled the development of automated analyses that can be performed in a short time at reasonable cost. One such method is immunoaffinity capillary electrophoresis. This emerging hybrid technology employs two powerful techniques coupled on-line for the direct and rapid determination of analytes present in biological fluids. The first technique, immunoaffinity, is used for the selective extraction of a molecule present in a complex matrix, utilizing a microscale-format chamber affinity device. An analyte (affinity target) present in serum or urine is captured by an immobilized molecular recognition antibody molecule (affinity ligand) bound to a solid support constituent (glass beads or an appropriate porous structure) of a microchamber affinity device. The second technique, capillary electrophoresis, is used for the high-resolution analytical separation of the purified and concentrated affinity target material after elution from the microchamber affinity device. In this work, immunoaffinity capillary electrophoresis was developed for the identification and characterization of a single constituent of a complex matrix. Immunoreactive gonadotropin-releasing hormone was determined in serum and urine specimens derived from a normal individual and from a patient suffering from benign prostatic hyperplasia. Furthermore, the on-line immuno-separation system was coupled in tandem to mass spectrometry to obtain molecular mass information of the affinity isolated and CE separated neuropeptide. This hybrid immuno-analytical technology is simple, rapid, selective and sensitive. In addition, an attempt was also made to characterize other urinary constituents by CE–MS that may lead to marker activity in the urine of the diseased subject. The hyphenation of analytical techniques has proved valuable in enhancing their individual features. The future of bioanalysis using miniaturized affinity systems is discussed in this paper. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Gonadotropin-releasing hormone; Immunoaffinity capillary electrophoresis; On-line preconcentration CE

1. Introduction

Miniaturized chemical and physical processes utilizing microscale devices are being incorporated into the design of modern analytical instruments. Devices that can process and analyze nano-, pico- and even attoliter quantities of samples and reagents are now being used in a variety of areas [1–7]. An increasing number of applications are in progress in many clinical laboratories employing capillary electrophoresis (CE) and microchip (MC) technology

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[1–21]. Significant potential benefits will impact the future of medical diagnosis. Although the advantages of microscale technology are unique and impressive, sensitivity and selectivity of detection are relatively weak points [22–33]. In particular, in many instances the limited availability of biological fluids significantly hinders the analysis of various relevant biochemical compounds.

To overcome some of these problems, several modes of capillary electrophoresis [34–42] and a few instrumental and chemical strategies ([21,43–50 and Table 1) have been described for increasing concentration, improving detectability and achieving better identification and characterization of physiological and pathophysiological markers, particularly for markers found at subnanomolar amounts in complex matrices such as biological fluids, cells and tissue specimens [8,16,19,21,51–61]. One interesting emerging and versatile preconcentration technique is on-line immunoaffinity capillary electrophoresis (IA–CE) [8,16,19,21,51–61]. A microchamber affinity device, located near the inlet of the separation capillary, contains an immobilized antibody bound to the surface of a porous structure or directly to the walls of a portion of the capillary. The antibody selectively captures and concentrates the compound(s) of interest (present in a simple or complex matrix) without sample pretreatment. Furthermore, it protects the isolated compound from any possible degradation that might occur when present in a complex matrix, presumably by some kind of steric protective mechanism or other unknown mechanism(s). The purified and concentrated analyte(s) is then separated by capillary electrophoresis after desorption from the microchamber affinity device.

IA–CE has previously been demonstrated for the determination of analytes in biological fluids [8,16,19,21,51–61] as well as for the in situ determination of neuropeptides present in tissue biopsy [60]. In this latter report, four monovalent Fab antibody fragments were immobilized to a portion of the capillary to specifically capture four neuropeptides found in tissue biopsy samples. The selected neuropeptides were then eluted from the microchamber affinity area of the capillary and resolved by capillary electrophoresis at the picogram level of concentration. The method was rapid, simple, specific, sensitive and did not require pretreatment of the crude homogenate to selectively isolate and quantify specific neuropeptides present in a tissue biopsy sample.

Presently, the coupling of CE to mass spectrometry is generating growing interest, because the acquisition of mass information facilitates the identification and characterization of the analytes of interest [54,62–65]. In this report, we have coupled on-line immunoaffinity capillary electrophoresis method to mass spectrometry (IA–CE–MS) for the determination and characterization of the peptide gonadotropin-releasing hormone (GnRH), in serum and urine. The addition of an immunoaffinity principle to the CE–MS technology permitted a detectability of GnRH to at least 1 ng/ml level of concentration using UV and MS detection. GnRH, previously termed luteinizing hormone-releasing hormone (LHRH), is an important decapeptide, classi-

Table 1
Systematic approaches for determination of analytes in biological fluids by capillary electrophoresis

·	Utilization of various modes of capillary electrophoresis
·	UV profile of separated underivatized and derivatized analytes at various wavelengths
·	UV spectrum of separated analytes
·	Mass spectra of analytes
·	Spiking the sample with a known standard and comparing the profile to unknown analyte
·	Fluorescence detection of separated tagged analytes
·	Application of off-line and/or on-line preconcentration methods using low and high selective immobilized ligands to enhance detectability
·	A combination of all methods described above, including hyphenation to immunological and/or spectroscopic techniques

fied as a neuropeptide, having several unique physiological features. GnRH is synthesized in the brain, although some recent evidence indicates that GnRH is present also in the ovary and testis [66–68]. This hormone is clinically important and is a target for control of its biological activity through GnRH agonists, antagonists and analog peptide molecules [69]. Agonists to the peptide have been widely used as effective agents in endocrine therapy for prostate cancer. Antagonists to the peptide have also been used for prostate cancer, benign prostate hypotrophy and in vitro fertilization studies [66–74]. Given the short plasma half-life of GnRH (3–6 min) [66], as well as the anatomical inaccessibility of the hypophysioportal circulation, it is generally not possible to sample GnRH in a meaningful fashion.

It has been documented that establishing the diagnosis of patients with lower urinary tract symptoms is complicated. Many tests have been developed for prostate cancer and benign prostatic hyperplasia, but the importance of these tests in the diagnostic process is still not well understood [75,76]. IA–CE–MS could be used as an additional test to gain new information for obtaining an accurate diagnosis of a common affliction of older men. IA–CE–MS is a simple, rapid, selective and sensitive method for the determination of GnRH in serum and urine. Furthermore, an attempt was made to identify other urinary components directly by CE–MS (without a preconcentration step), searching for a lead in the identification of a marker in the urine of a patient suffering from benign prostatic hyperplasia. Although a few laboratories have reported the use of on-line preconcentration capillary electrophoresis coupled to MS, they have utilized a microchamber device containing C-18 immobilized to a solid support in order to capture nonspecifically a few analytes [77–93]. We have focused on the use of a highly selective molecular recognition antibody to capture one antigen or hapten of interest. The determination of GnRH in biological fluids by IA–CE–MS may provide more specific and promising results for the diagnosis of benign prostatic hyperplasia.

In this study, monovalent FAb antibody fragments (instead of whole antibodies) were used to facilitate an increase in the binding surface area, as well as to increase the number of integral binding sites without causing steric interference. To the best of my knowledge, this is the first time that immobilized antibody fragments to a solid support structure have been used for on-line immuno-affinity preconcentration CE coupled to MS.

Although IA–CE–MS was used in this study for the determination of the neuropeptide GnRH in serum and urine, it may also become useful for the determination of other diagnostic markers found in minute concentrations in biological fluids, cells and tissue specimens. Furthermore, the technique may find application in environmental science, the food industry, forensic science, the pharmaceutical industry, the biotechnology industry and the Human Proteomic Project.

2. Materials and methods

2.1. Chemicals

All chemicals were of the highest grade available. Organic solvents were purchased from AlliedSignal, Burdick & Jackson, Muskegon, MI, USA. Sodium phosphate monobasic and sodium hydroxide acid were obtained from Fisher Scientific, Fair Lawn, NJ, USA. Sodium tetraborate (Borax), boric acid, formic acid, ammonium bicarbonate, ammonium acetate, creatinine, urea, malonic acid, (*R*)-(−)-3-hydroxybutyric acid (sodium salt), magnesium chloride, ethylene glycol, Nonidet P-40, leupeptin, 3-aminopropyltriethoxysilane, glycine and bovine serum albumin were purchased from Sigma Chemical Co., St. Louis, MO, USA. Glacial acetic acid and hydrochloric acid were obtained from Mallinckrodt, Paris, KY, USA. Human gonadotropin-releasing hormone (luteinizing hormone-releasing hormone) was purchased from Peninsula Laboratories, Belmont, CA, USA and Sigma Chemical Co. Bare capillary columns were purchased from Polymicro Technologies, Phoenix, AZ, USA. Underivatized controlled pore glass (CPG) beads (3000 A pore size, 200–400 mesh, irregularly shaped) was obtained from CPG Inc., Fairfield, NJ, USA. Deionized water was purified with a Milli-Q-Plus Ultra-Pure water system from Millipore Corporation, Bedford, MA, USA. Filters to remove particulate matter (0.20 μm) were purchased from Gelman Sciences, Ann Arbor, MI,

USA. A Protein A affinity column was obtained from Amersham Pharmacia Biotech, Piscataway, NJ, USA. Keyhole limpet hemocyanin subunits, sulfosuccinimidyl 4-(-maleimidomethyl) cyclohexane-1-carboxylate (SSMCC), an ImmunoPure $F(Ab)'_2$ preparation kit and Cleland's reagent were purchased from Pierce, Rockford, IL, USA.

2.2. Methods

2.2.1. Preparation of serum samples

Serum samples from healthy and pathophysiological individuals were obtained as morning specimens. Blood was collected in untreated tubes containing no chemicals to allow blood clotting and the formation of serum. After a few minutes, the tube was centrifuged and the serum was separated. The serum was then divided into two aliquots. One aliquot was immediately filtered. To the other was added a cocktail of protease inhibitors [94], including leupeptin at 1 m*M* [60], before filtration. Both serum aliquots were subjected to a filtration process utilizing 0.20 μm porosity filters to remove particulate matter. Two filters were used in tandem to filter the serum specimens. One was made of Nylon and the other of PTFE.

2.2.2. Preparation of urine samples

Urine samples from healthy and pathophysiological individuals were collected as morning clean-catch urine specimens. There was no processing of collected urine other than a filtration step, to remove cells and particulate matter. The urine was divided into two aliquots. One aliquot was immediately filtered. To the other was added a cocktail of inhibitors [94] including leupeptin at 1 m*M* [60], before filtration. Two filters were used in tandem to filter the urine specimens. One was made of Nylon and the other made of PTFE. Both were 0.20 μm in porous diameter.

2.2.3. Preparation of antibodies

Polyclonal antibodies directed against commercially available gonadotropin-releasing hormone coupled covalently to albumin or keyhole limpet hemocyanin (KLH) subunits were raised in rabbits similar to a method described elsewhere [94]. Albumin was further purified to remove trace amounts of peptidases in order to avoid cleavage of GnRH. The purification of the antibodies from rabbit antisera was carried out by HPLC using Protein A affinity chromatography as previously described [95].

2.2.4. Preparation of monovalent FAb fragments

Purified antibodies were subjected to partial enzymatic digestion to generate $F(Ab)'_2$ antibody fragments using the ImmunoPure $F(Ab)'_2$ preparation kit according to the manufacturer's instructions. The formed divalent $F(Ab)'_2$ antibody fragments were then reduced to monovalent FAb antibody fragments, by incubation with equal volumes of 200 m*M* Cleland's reagent for 30 min at 37°C [52,60]. A minor purification of the final product (FAb peptide) was achieved by gel filtration chromatography.

2.2.5. Coupling of FAb fragments to glass beads

Controlled-porous glass beads, previously utilized to link antibodies directed against methamphetamine [51], were used to bind monovalent FAb fragments [52,60]. The irregularly shaped beads were incubated at 90°C for 60 min in the presence of 10% aqueous 3-aminopropyltriethoxysilane. This treatment was repeated four times. The beads were then incubated at 90°C for 60 min with 10 m*M* hydrochloric acid. The beads were washed extensively with distilled/deionized water before preparing the maleimide-activated surface. The beads were then incubated at 30°C for 60 min with a buffer solution containing 50 m*M* sodium borate, pH 7.6 and 1 mg/ml SSMCC. The beads were finally washed extensively with 50 m*M* sodium borate buffer, pH 7.6 and then incubated overnight at 4°C with approximately 500 μg/ml of FAb peptide in 50 m*M* sodium borate buffer, pH 7.6.

2.2.6. Fabrication of an analyte concentrator

The fabrication of an analyte concentrator was carried out as a minor modification of a method previously described [21,51]. A piece of fused-silica capillary (150 μm×10 mm) was filled with irregularly shaped glass beads of controlled porosity (CPG) containing covalently attached FAb fragments. The beads were held inside the column by two porous frit structures (Fig. 1). This piece of capillary of approximately 10 mm in length was then fused to two longer capillary columns (75 μm) using a Teflon sleeve and glued with an epoxy resin. The entire

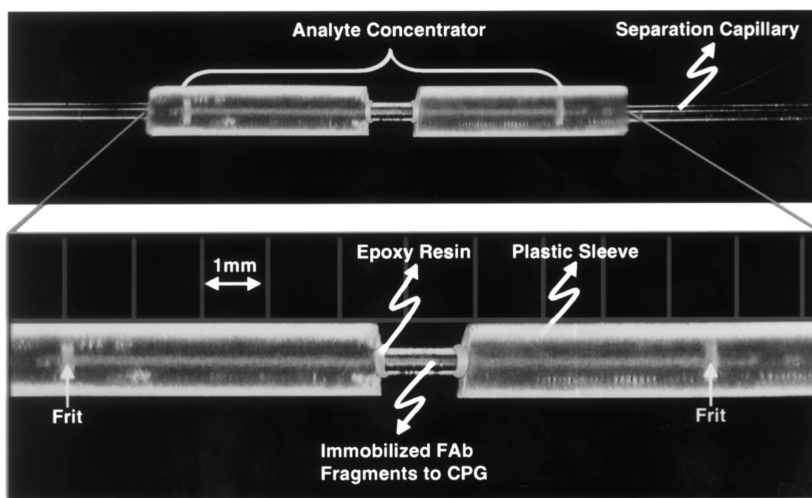

Fig. 1. Microphotograph of an analyte concentrator fabricated with FAb antibody fragments immobilized to controlled-pore glass silica. The irregularly shaped beads were housed between two frit structures. The analyte concentrator device was connected to two separation capillaries by a Teflon sleeve. The plastic connector was glued to the separation capillaries by an epoxy resin. The entire fabrication process was monitored by an stereo microscope.

process of fabrication of the analyte concentrator was monitored under a stereo microscope. The capillary containing the analyte concentrator was prepared to fit into a commercial cartridge–cassette device that held the capillary in a coiled configured form (Fig. 2).

2.2.7. Separation of analytes by CE-UV

A capillary electrophoresis instrument [HP ^{3D}CE, Agilent Technologies, Inc. (formerly Hewlett-Packard Company), Wilmington, DE] equipped with a diode array detector was used for the analysis. A CE ChemStation (Agilent Technologies), using software

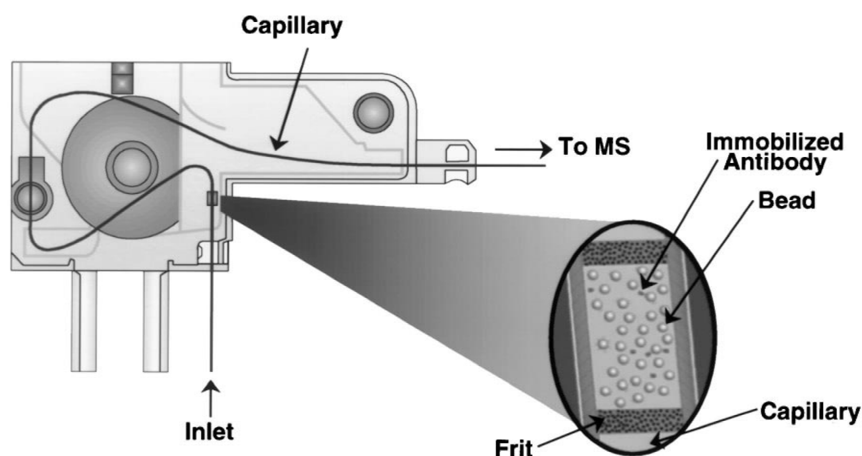

Fig. 2. Schematic representation of a commercial cartridge–cassette containing a fused-silica capillary with an analyte concentrator positioned at approximately 10 cm from the inlet of the capillary.

version Rev.A.06.01-403, was used for instrument control, data acquisition and data handling. Separation was performed in fused-silica capillaries. For experiments that did not use on-line preconcentration, bare capillary columns were 75 μm I.D. and 80 cm in length. A metallic alignment interface containing an appropriate optical slit was matched to the corresponding capillary I.D. used. All experiments were carried out in normal or cathodic mode (anode at the inlet and cathode at the outlet). During sample analysis, a constant voltage (25 kV) was applied and the capillary was thermostated at 22°C. Samples were kept at ambient temperature in the autosampler and introduced into the capillary by applying positive pressure of 30 mbar for 20 s (approximately 60 nl) [46]. UV detection was carried out at 195, 230, 260 and 292 nm with a bandwidth of 8, 10, 16 and 20, respectively. Bare capillary (without the analyte concentrator device) was regenerated by rinsing the capillary with 1.0 *M* NaOH, followed by water and separation buffer. The separation buffer consisted of 80 m*M* sodium tetraborate buffer, pH 9.1, containing 1% (v/v) acetonitrile. When not in use, the capillary was washed with acetonitrile and water and then stored in air at 25°C.

2.2.8. Separation of analytes by CE–MS

Mass spectra data were generated using a HP ^{3D}CE coupled to a Agilent Technologies model 1100 MSD single quadrupole electrospray ionization mass spectrometer. Instrument control, data acquisition and data processing were performed using ChemStation software version Rev.A.06.03-509 (Agilent Technologies). Separation was performed using a 75 μm I.D. bare fused-silica capillary column with a column length of 120 cm. A plastic alignment interface containing an appropriate optical slit was matched to the corresponding capillary I.D. used. All experiments were carried out in normal or cathodic mode (anode at the inlet and cathode at the outlet). During sample analysis, a constant voltage (22 kV) was applied and the capillary was thermostated at 22°C. Samples were kept at ambient temperature in the autosampler and introduced into the capillary by applying positive pressure of 30 mbar for 35 s (approximately 70 nl) [46] when using CE–MS. When using IA–CE–MS, samples were applied at 930 mbar for 9 min (approximately 30 μl) [46]. UV detection was carried out at 195, 230, 260 and 292 nm with a bandwidth of 8, 10, 16 and 20, respectively. When the instrument was used for CE–MS, the bare capillary without the analyte concentrator device, was regenerated by rinsing the capillary with 1.0 *M* ammonium hydroxide, followed by water and separation buffer. When the instrument was used for IA–CE–MS, the capillary containing the microchamber affinity device, was generated by rinsing the capillary with 1% Nonidet P-40, followed by water and then extensively with the separation buffer. The separation buffer consisted of 60 m*M* ammonium bicarbonate buffer, pH 8.0, containing 1% (v/v) acetonitrile. When not in use, the capillary was stored in air at 4°C.

For electrospray ionization operation, the sheath liquid consisted of a 20 m*M* acetic acid solution in 50% methanol flowing at a speed of 0.500 ml/min. A positive polarity mode was employed. The scan mass range utilized for the analysis of small-molecular-mass urine constituents (analysis without on-line preconcentration step) was from 50 to 600 amu, at 1.04 s/cycle. The scan mass range utilized for the analysis of gonadotropin-releasing hormone (analysis with on-line preconcentration step) was from 50 to 1250 amu and from 950 to 1250 amu, at 1.04 s/cycle. Ion source temperature was 150°C at a nebulizer pressure of 10 p.s.i. (68.9 kPa). The peakwidth was set at 0.10 min and the capillary voltage was 4000 V. The slope sensitivity was 10, the ion width 5 m/z and the abundance cut-off 1000.

At the end of each day, the interface chamber was cleaned with a soft paper impregnated with a solution of 80% (v/v) water, 18% methanol and 2% glacial acetic acid.

3. Results and discussion

3.1. Capillary electrophoresis determination of immunoreactive gonadotropin-releasing hormone in serum specimens

In general, examination of blood reflects not only the overall metabolism of the tissues, but also provides the most accessible method for the sampling of bodily fluids. The direct analysis of serum constituents by CE is not simple, since albumin and

immunoglobulins are present in relatively large quantities. It becomes quite a challenge to separate and detect low-molecular-mass serum components [8–21] in the presence of biopolymers. Albumin and immunoglobulins, as well as other macromolecules present in serum such as hemoglobin, lipoproteins, fibrinogen and transferrin, contribute to the masking of low-molecular-mass substances, hindering their CE separation and detection [8–21].

In addition, other technical aspects must be taken into consideration when using CE for the analysis of serum constituents. For example, the limited amounts in which important markers may be present in serum (e.g., subnanomolar quantities) may require their detection by methods other than UV (e.g., fluorescence, mass spectrometry, etc.) or the use of a preconcentration step prior to CE separation. Furthermore, when analyzing peptides by CE, special conditions must be implemented in the processing of samples in order to obtain reproducible quantification of the analyte of interest in serum specimens. This is largely due to the varied degree of the stability of peptides. Although a large amount of peptides are stable in biological fluids, a significant number can be degraded easily. Proteolysis is usually one of the common culprits affecting peptides. The half-life of some known peptides is less than 5 min [66,96,97]. Thus, a rapid method of analysis may facilitate their quantification.

To overcome these limitations, several modes of CE [34–42] and a few instrumental and chemical strategies [21,43–50 and Table 1] have been reported for the analysis of serum or plasma constituents, including ions, drugs, metabolites and biopolymers. The determination of GnRH and various analogues has been accomplished by analytical and immunological techniques [66,67,98–104]. In this study, a method utilizing an on-line preconcentration immuno-affinity CE coupled to MS was utilized to determine the levels of gonadotropin-releasing hormone in serum obtained from normal and disease subjects in the absence and presence of protease inhibitors.

A typical example of the high specificity of an antibody for the monitoring of serum GnRH is demonstrated in Fig. 3. Serum sample obtained from a patient with benign prostatic hyperplasia (Fig. 3A) was treated with protease inhibitors, followed by

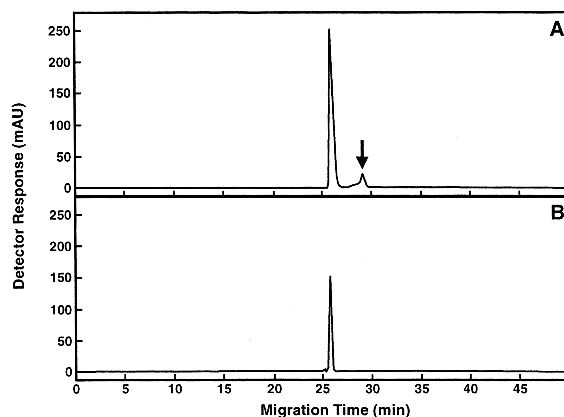

Fig. 3. Capillary electrophoresis profile of GnRH determined by IA–CE with UV detection. (**A**) The main observed peak represents the GnRH peptide extracted from serum derived from a patient with benign prostatic hyperplasia. The minor peak illustrated by the arrow represents an unidentified serum component, possibly related to GnRH. (**B**). GnRH peptide extracted from a serum from a healthy individual. Capillary electrophoresis was performed utilizing 60 m*M* ammonium bicarbonate buffer, pH 8.0, containing 1% acetonitrile. The electropherograms were monitored at 195 nm.

mixing and filtration. An aliquot of approximately 30 μl (930 mBar for 9 min) [46] of each serum sample was introduced by positive pressure into individual fused-silica separation capillaries. The complete fabricated capillaries (75 μm×120 cm) contained an analyte concentrator microchamber device (150 μm×10 mm) positioned 10 cm from the inlet section of the capillary. The affinity capillary column was then adapted to a commercial cartridge–cassette (Agilent Technologies, Inc.), as depicted in Fig. 2. The peptide hormone present in the serum and introduced into the capillary, was selectively captured by highly selective immobilized antibody fragments. The antibody fragments were covalently bound to an adsorbent porous structure (irregularly shaped controlled-pore glass beads) that formed the core of a microchamber device.

In this study, monovalent FAb fragments (derived from polyclonal antibodies) were used for immobilization to the surface of the beads. The antibodies were directed against human GnRH. It is noteworthy to mention that the random orientation of a coupled affinity ligand to a solid support structure has been documented to be a significant factor contributing to

the optimum performance of an affinity adsorbent [105]. Maintaining conformational integrity of the binding site upon immobilization is crucial to produce an active immunosorbent, in particular when using antibodies. One way to contribute to a better orientation and to increase the binding surface area of immobilized antibodies is to employ antibody fragments. These fragments will increase the number of integral binding sites without causing steric interference. The utilization of a microchamber affinity device containing immobilized FAb fragments should enable the selective capture of a larger number of antigens and haptens present in complex matrices.

After introduction of the serum into the capillary by positive pressure for a long period of time to permit maximum interaction of GnRH with the immobilized antibodies present in the microchamber, the column was washed extensively, also by positive pressure, with 60 m*M* ammonium bicarbonate buffer, pH 8.0. The bound and enriched GnRH was desorbed from the column by introducing an aliquot or plug (40 mBar for 35 s or approximately 90 nl) of a solution containing 0.3 *M* glycine-HCl buffer, pH 2.5. The sample was then separated by capillary zone electrophoresis (UV detection) coupled to mass spectrometry (with electrospray ionization interface). The concentration limit of detection for gonadotropin-releasing hormone in the serum, utilizing IA–CE with UV detection, was 1 ng/ml as determined by comparing the peak areas of various dilutions of a standard solution of the peptide hormone (data not shown), starting with the most diluted concentration first. It was also possible to lower the CLOD to pg/ml by labeling the peptide hormone with a chromophore substance (unpublished information), as previously reported by Phillips [60]. If the frit structure was well positioned within the microchamber affinity device and the plugging system was optimized, the system could be reused for at least 10 times.

Similar experiments were carried out with a control serum sample, as depicted in Fig. 3B. The major difference between the two electropherograms shown in Fig. 3A and B is the concentration of the peptide hormone in the serum. The results are clearly demonstrated when comparing peak areas of GnRH immunoextracted from serum derived from a normal subject and diseased subject. The area of the peak corresponding to the electropherogram of the diseased sample is approximately 3.5 fold larger than the area of the peak of the electropherogram from the control serum. Furthermore, as shown in Fig. 3A, a second minor peak was observed by UV detection in the eluted material (as indicated by the arrow), but this peak was not observed in the control serum (Fig. 3B). Currently, the chemical nature of this peak and the degree of the selectivity of the antibody are being investigated.

Quantitative data obtained from serum samples not containing protease inhibitors were reduced and the degree of degradation ranged from approximately 3–20% (based on peak areas), depending on the time of the exposure to the protease(s) present in the serum (Table 2). Although the half-life for GnRH has been reported to be 3–6 min, as represented by in vivo data [66], our in vitro data demonstrate that the proteolytic degradation of the neuropeptide is generated for a much longer time. This seems to be acceptable since the disappearance of a drug from body circulation is affected by other factors or conditions other than blood peptidases (for example, by influence of the liver, lung, kidney and muscle). One interesting observation has been reported for the in vitro degradation of GnRH by peptidases present in serum derived from several species. The half-life of GnRH varied from 3 h (guinea pig) to 9.8 h (human) [106].

In previous work [16,19,21], a desorption solution was used to remove the bound antigen (or hapten), 50 m*M* sodium tetraborate buffer pH 7.6 or 8.3, containing 2 *M* magnesium chloride and 25% highly purified ethylene glycol. Unfortunately, when other ethylene glycol reagents (acquired from different

Table 2
Effect of endogenous proteases on the in vitro degradation of serum GnRH

Incubation time at 37°C (min)	Peak Area (arbitrary units)	Percentage of control
0	5700	100.0
8	5540	97.2
15	5320	93.3
35	5016	88.0
60	4821	84.6
90	4602	80.7

vendors) were tested, high absorbance values were obtained in the area corresponding to the migration of the desorption plug. Furthermore, there was some interaction between the various ethylene glycols and the walls of the capillary, altering subsequent analyte migrations. Apparently, the use of highly purified reagents in the elution buffer is crucial for the generation of high-quality data. Further studies of optimization conditions for the chemical constituents of the plug utilized for desorption of bound antigens or haptens from their correspondent immobilized antibody or antibody fragments are under way in our laboratory.

Fig. 4B shows the total ion–current electropherogram (TICE) of the separated serum component(s) desorbed from the microchamber affinity device. The electrospray ionization method is well suited for CE–MS interfacing since it produces ions directly from liquid solutions at atmospheric pressure. A typical successful application has been reported for the analysis of peptides. Nevertheless, considerations for interfacing generally derive from limitations on buffer composition for direct electrospray ionization and the desire to position the interface as close as possible to the analytical capillary terminus, thus avoiding lengthly transfer lines. In this study, the commercial interface system utilized to couple CE to MS was simple and allowed good data reproducibility every time a new capillary was used (if experimental conditions were maintained as close to the ones used with the previous capillary). However, the mass spectrometry data generated for GnRH were obtained several minutes later than the data obtained by UV analysis. This migration delay was due to the extra path that the analyte has to migrate from the UV detection window to the mass spectrometer (see Fig. 2). The proper alignment of UV response (Fig. 4A) and mass data (Fig. 4B) was generated by a software package provided with the instrument. No mass spectrometry data were obtained for the second peak observed by UV, as depicted by an arrow in Figs. 3A or 4A, indicating either low ionization for the unknown molecule or a concentration too low to be detected. The spectrum corresponding to the electropherographic peak presents a single-charge state distribution: m/z 1183.5 (Fig. 5), that yield a molecular mass value of 1182.5 Da for the purified serum GnRH, corresponding to the mass value reported in the literature [107,108]. The same molecular mass value (1182.5 Da) was obtained for all GnRH peptides, immunoaffinity isolated from other samples studied utilizing IA–CE–MS (data not shown). Apparently, the only form

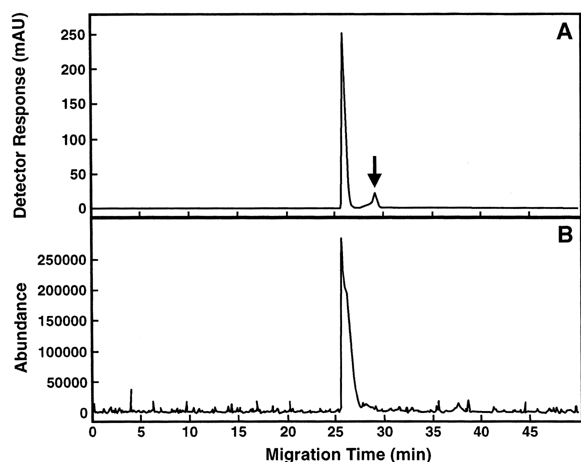

Fig. 4. Capillary electrophoresis mass spectrometry profile of GnRH present in serum derived from a patient with benign prostatic hyperplasia. (**A**) Electropherogram of the affinity-purified serum peptide, monitored at 195 nm. The arrow represents an unidentified component, possible related to GnRH. (**B**) Total ion–current electropherogram of the affinity-purified serum peptide. Capillary electrophoresis was performed utilizing 60 m*M* ammonium bicarbonate buffer, pH 8.0, containing 1% acetonitrile.

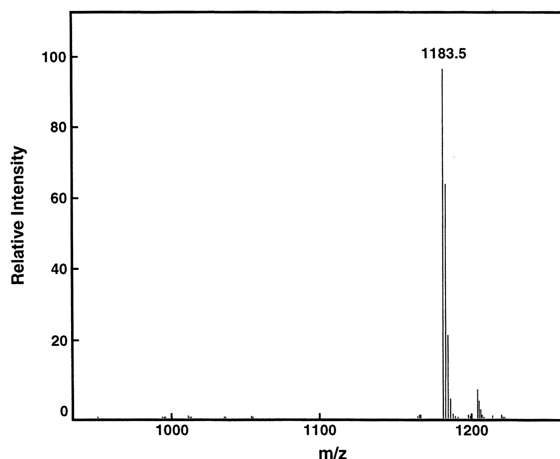

Fig. 5. Mass spectra data (shown as MH^+) of the affinity-purified gonadotropin-releasing hormone derived from the serum of a patient with benign prostatic hyperplasia (molecular mass, 1182.5 Da).

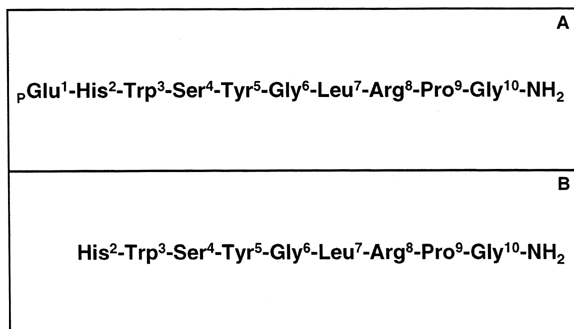

Fig. 6. Diagram of the amino acid composition of the GnRH decapeptide and nonapeptide. (**A**) The amino acid in position 1 of the decapeptide is pyroglutamic acid. The amino acid in position 10 is glycine amide. The intact decapeptide is found primarily in serum. (**B**) The nonapeptide lacks the pyroglutamic acid in position 1 and it is found primarily in urine.

of the peptide, immunoextracted from serum by the antibody generated in this laboratory, is the 1–10 decapeptide and not the 2–10 nonapeptide (Fig. 6) found primarily in urine specimens (see below). Other fragments of serum GnRH were not observed in this study, even when a scan mass range from 50 to 1250 amu was used. Pentapeptides 1–5 and 6–10 have been identified as GnRH degradation products in synaptosomes isolated from rat hypothalamus and cortex [109]. Currently, antibodies raised in animals other than rabbits are being tested in our laboratory, as well as different inhibition conditions for peptidases, with the hope to identify other GnRH degradation products in human serum and urine.

3.2. Capillary electrophoresis determination of immunoreactive gonadotropin-releasing hormone in urine specimens

Urinalysis is one of the most useful noninvasive procedures available to the physician as an indicator of health or disease, especially in the areas of metabolic and renal disorders. It is estimated that the number of cells in the complex human body is approximately on the order of billions or perhaps a few trillion. Thus, hundreds of thousands of analytes with diverse chemical nature, including drugs, metabolic intermediates, substances derived from the breakdown of biopolymers and even small amounts of large complex structures such as proteins, could be found in the urine of healthy individuals [110–112]. However, a large majority of urinary components are found present at very low concentrations and comprise a vast complexity of chemical entities, making separation and quantification very difficult. Although medical history books and reviews are full of fascinating information about the study of urine, only a few more than 1000 urinary components have been identified so far. Furthermore, despite the number of methodologies described in the literature, they all require laborious sample pretreatment [113–115]. Capillary electrophoresis can be developed into a rapid diagnostic tool to help critically ill patients who crucially need identification of the appropriate diagnostic marker to provide adequate therapy and avoid fatal consequences. Gonadotropin-releasing hormone has been quantified primarily in 24 h collected urine specimens for a variety of physiological and pathophysiological conditions [116]. The concentration values reported by radioimmunoassays ranged from approximately 3 to over 45 ng/24 h.

In this study, we determined GnRH in urine specimens derived from normal and diseased individuals. The processing of the urine (in the absence and presence of inhibitors of proteolytic enzymes) and the use of the IA–CE–MS procedure to analyze GnRH were identical to that performed for serum samples. Immunoreactive gonadotropin-releasing hormone was detected in urine specimens derived from normal subjects (Fig. 7A) and treated with

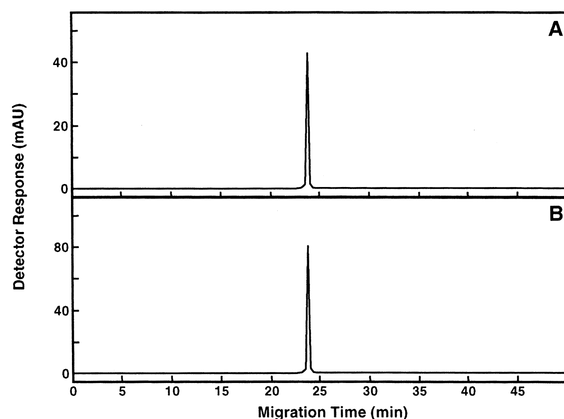

Fig. 7. Immunoaffinity capillary electrophoresis studies of urinary GnRH. (**A**) Electropherogram of GnRH extracted by immunoaffinity from a urine specimen derived from a normal subject. (**B**) Electropherogram of GnRH extracted by immunoaffinity from a urine specimen from a patient with benign prostatic hyperplasia.

protease inhibitors, at lower concentrations than those obtained for normal serum samples (Fig. 3B), utilizing the same experimental conditions. However, approximately double peak area values were detected in the urine derived from a patient suffering from benign prostatic hyperplasia (Fig. 7B), but lower than those found in the diseased serum (Fig. 3A). Similar data were observed when the urine specimens were not treated with protease inhibitors (data not shown) indicating not further proteolytic cleavage of GnRH in urine matrix. Interestingly, IA–CE–MS studies of urinary GnRH demonstrated a molecular mass value of 1071.3 Da for the immunoaffinity isolated urinary GnRH derived from the various urine specimens studied (data not shown). This experimental value apparently corresponds to that of the nonapeptide 2–10 instead of the decapeptide 1–10 (Fig. 6). No other fragments of urinary GnRH were observed in this study when using the antibody generated in our laboratory, even when a scan mass range from 50 to 1250 was used. Furthermore, some differences were observed when comparing migration times of urinary GnRH with serum GnRH. Migration time (average of three consecutive determinations) for serum GnRH was 25.3 min; migration time (average of three consecutive determinations) for urinary GnRh was 24.2 min. These experimental values correspond to those predicted by the principles of CE in which the nonapeptide (smaller analyte) migrated faster than the decapeptide (larger analyte), having both the same charge.

3.3. Capillary electrophoresis determination of urine constituents

An attempt was made to determine if other urinary constituents were elevated in the urine of patients suffering from benign prostatic hyperplasia. Thus, we subjected the urine specimens to CE and CE–MS studies. Samples were introduced directly into the capillary without an on–line preconcentration step. For capillary electrophoresis studies employing UV detection, 80 m*M* sodium tetraborate buffer, pH 9.1, containing 1% (v/v) acetonitrile was used as separation buffer.

Fig. 8 depicts an electropherographic profile of four urine specimens obtained from three apparently healthy representative adults (two females and one male) and from one male subject suffering from benign prostatic hyperplasia. All electropherograms showed at least 10 major peaks, as previously demonstrated [6]; four of these peaks are predominant when analyzed at 195 nm. Peak 1 represents (as confirmed below by CE–MS studies) primarily creatinine, with urea comigrating. Peak 2 is presently an unidentified substance. Peak 3 appears to be hydroxybutyric acid but other substances are not ruled out. Peak 4 corresponds to uric acid. Fig. 8A shows the CE profile of a urine specimen (male subject) containing a low amount of the peak 2 component. Fig. 8B represents a urine sample (female subject) with almost equal amounts of peaks 2, 3 and 4, but containing a significant amount of a peak identified as albumin (depicted by an arrow). Fig. 8C best represents a control urine (female subject) containing average normal values. Fig. 8D shows an increased area for peak 3 and the presence of an unidentified compound (arrow) in urine from a patient (male subject) suffering from benign prostatic hyperplasia.

Fig. 9 shows the UV spectrum profile of all four major peaks observed in Fig. 8A–D. The UV spectrum of Fig. 9A corresponds to that of a creatinine standard and is very similar to peak 1 (Fig. 8A–D). Urea does not absorb significantly in the UV [117]. The Fig. 9B profile corresponds to that of peak 2 in Fig. 8A–D and is yet to be identified. Fig. 9C corresponds to that of peak 3 in Fig. 8A–D and is probably hydroxybutyric acid. Fig. 9D corresponds to peak 4 in Fig. 8A–D and shows a characteristic profile of uric acid as demonstrated by the correspondent reference standard.

Fig. 10 shows a separation profile of the constituents of a urine specimen determined by CE–MS. Fig. 10A depicts the electropherographic profile of the separated urinary constituents using an aliquot of the same urine specimen utilized for generating the data shown on Fig. 8C. Fig. 10B shows the total ion–current electropherogram of the separated components. In the data presented in Fig. 8A–D, the separation profile of the various peaks obtained when using sodium tetraborate buffer appears very symmetrically defined. However, when using a volatile buffer, such as ammonium bicarbonate, the resolving separation efficiency diminished significantly, making it difficult to identify the separated analytes. Furthermore, some urinary components separated by CE–MS produce a low UV signal

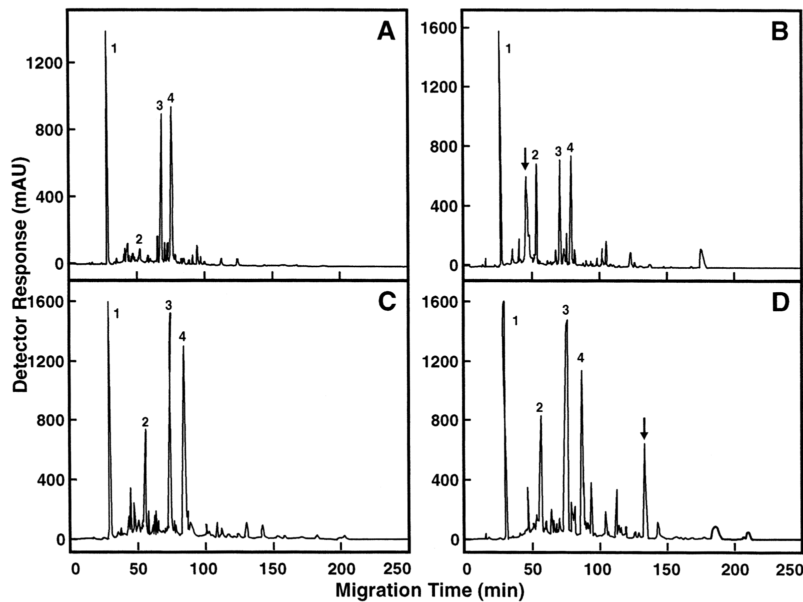

Fig. 8. Capillary electrophoresis profile of separated urine constituents. Electropherograms of 4 urine specimens monitored at 195 nm. Approximately 10 major constituents of urine specimens were detected by CE when monitored by UV. Four of these constituents were observed at large concentrations as determined by peak area. Capillary electrophoresis was performed utilizing 80 m*M* sodium tetraborate buffer, pH 9.1, containing 1% (v/v) acetonitrile. (**A**) Urine specimen obtained from a normal male subject. (**B**) Urine specimen obtained from a female subject. The arrow represents albumin. (**C**) Urine specimen obtained from a normal female subject. (**D**) Urine specimen obtained from a patient with benign prostatic hyperplasia. The arrow illustrates an unidentified urine constituent.

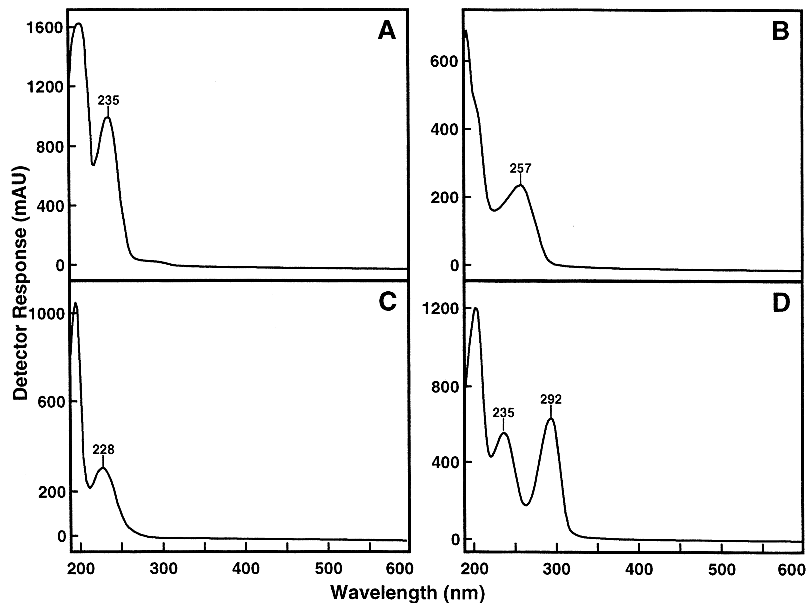

Fig. 9. UV spectrum characteristics of the 4 predominant peaks observed in Fig. 8. Each spectrum is unique (**A–C**), but the one corresponding to uric acid is unmistakable (**D**).

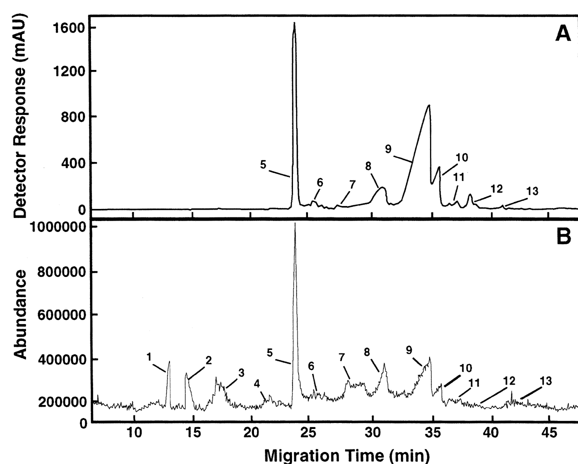

Fig. 10. Capillary electrophoresis mass spectrometry profile of the constituents obtained from a urine sample. (**A**) Electropherogram of the urine specimen used to generate data depicted in Fig. 8C, but the separation conditions were different. Capillary electrophoresis was performed utilizing 60 m*M* ammonium bicarbonate buffer, pH 8.0, containing 1% (v/v) acetonitrile. (**B**) Total ion–current electropherogram of the separated urinary components.

response, but a much higher ionization response (Fig. 10B, peaks 1–4). On the contrary, some separated urinary components had a high UV absorbance but poor ionization response in the positive mode (Fig. 10B, peak 9).

Since the migration velocity of the analytes separated by CE is in the nl/min scale, there was a delay of at least 10–15 min in obtaining mass signal after the analytes passed by the UV detector. This delay is influenced by various factors, including the dimensions of the capillary (I.D. and length) and the nature of the chemical substance. In order to match the profile of the UV detector response (Fig. 10A) with that of the mass spectrometer response (Fig. 10B), a software program was used that made it possible to align the UV response with the MS response. Furthermore, to corroborate the appropriate alignment response, it was necessary to use well-characterized reference standards (of known structure, UV spectrum and mass), as spiked markers, to compare to those suspected as urine constituents separated by CE–MS.

As illustrated in Table 3, the molecular mass (average of three consecutive determinations) of all analytes represented by the 4 major peaks is reported. Their masses are shown as MH^+ values. So far, creatinine (molecular mass, 113.0 Da) (Fig. 11A), urea (molecular mass, 60.0 Da) (Fig. 11A) and uric acid (molecular mass, 167.9 Da) (Fig. 11D) are confirmed urinary constituents. Fig. 11C shows an MH^+ value of 103.9, corresponding closely to the masses of malonic acid (molecular mass, 104.06 Da), hydroxyisobutyric acid (molecular mass, 104.10 Da) and hydroxybutyric acid (molecular mass, 104.11 Da). Nevertheless, other substances are not discarded since biological factors and/or conditions need to be studied to determine the final molecular composition of the analyte under study. Further investigation is currently in progress in our laboratory to identify urinary constituents not yet characterized.

Table 3
Major urinary constituents observed by capillary electrophoresis–mass spectromerty

Peak number	Analyte	Normalized migration time[a] (min)	Molecular mass (Da)
1	Urea	23.8	60.0
	Creatinine		113.0
2	Unknown	31.4	295.0
3	Unknown	34.8	103.9
4	Uric acid	36.1	167.9

[a] Normalized migration time is referred to as the migration time of the peaks of the mass spectrometer response after they have been aligned with the peaks of the UV detector response, using the appropriate software package.

A similar profile of resolution of the major peaks was obtained when using short capillaries (33 cm in total length and an I.D. of 50 μm). As shown in Fig. 12, an electropherogram of the constituents of a urine specimen, derived from a second patient suffering of benign prostatic hyperplasia, was obtained in less than 12 min. Peak area corresponding to peak 3 was elevated when compared with the same peak derived from control urine specimens analyzed with the same capillary (data not shown). Profiling of various urine samples from several diseased subjects is currently under investigation in our laboratory.

4. Conclusion and future trends

The monitoring of peptide concentrations during both disease episodes and therapy has proven dif-

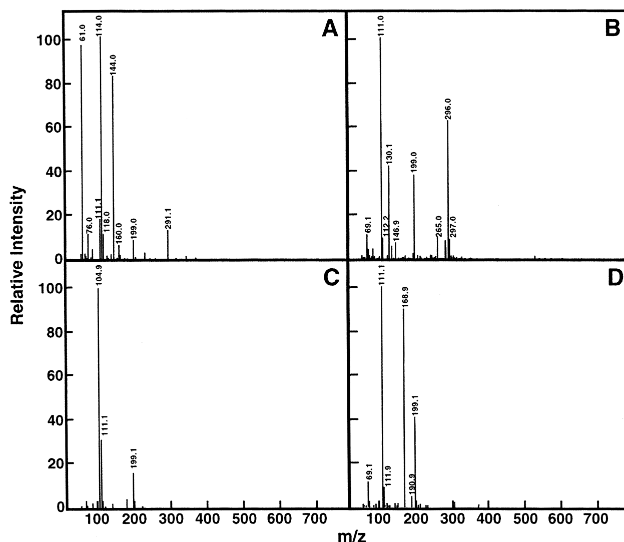

Fig. 11. Mass spectra data (shown as MH^+) corresponding to the four predominant peaks identified in Fig. 8. (**A**) Profile of two components identified as urea and creatinine (molecular mass, 60.0 Da and 113.0 Da respectively). (**B**) Unknown. (**C**) Unknown. (**D**) Profile of a component identified as uric acid (molecular mass, 167.9 Da).

ficult due to a lack of suitable procedures available for correlating laboratory evaluations with disease activity. A significant number of peptides are sensitive molecules that may not be able to resist the variety of stresses to which they are subjected. Such is the case of a labile peptide that is present in a biological fluid or the homogenate of a disrupted cell or tissue. The peptide may be exposed to denaturation, inactivation of a catalytic site, proteolysis, or other modifications of the molecule. In plasma, a significant number of peptides have a very short half-life. Therefore, time can be a critical factor for the quantification of peptides in complex matrices.

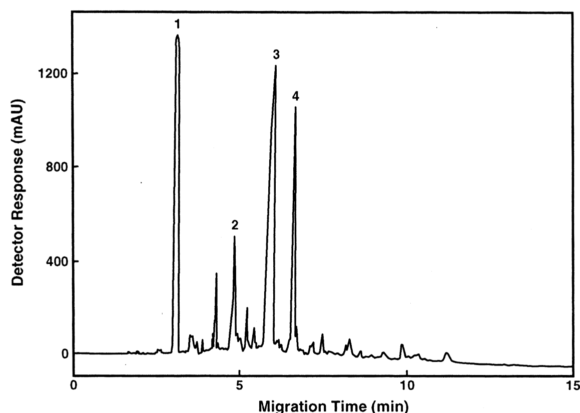

Fig. 12. Electropherogram of urine specimen derived from a second patient with benign prostatic hyperplasia. Capillary electrophoresis was performed utilizing 80 m*M* sodium tetraborate buffer, pH 9.1, containing 1% acetonitrile. The capillary was 50 μm I.D.×33 cm in total length.

In an attempt to overcome this problem, a technique based on the fast isolation of a peptide from freshly collected complex matrix was developed. This method of enrichment (and possible steric protection of the peptide) by affinity may be quite useful for the determination of intact peptide concentrations in hostile environments such as biological fluids, cells, or tissue specimens, particularly, if the peptide is found at low concentrations in the matrix and it is not detected by other CE detection methods. GnRH is readily accessible to protease activity since it is not bound to circulating proteins. Degradation of GnRH appears to be mediated by a combination of Tyr^5-Gly^6 endopeptidase activity as well as by enzymes that hydrolyze the $pyroGlu^1$ or cleave the carboxyl side of Pro^9 [66]. Further investigaton is currently in progress in our laboratory to decipher the degradation process of GnRH in physiological and pathophysiological conditions. The use of various types of antibodies and of a large number of

urine specimens obtained from several patients may be helpful in identifying the presence of a more representative marker(s).

In summary, immunoaffinity methods have been widely used in a variety of applications, mainly due to the specificity of the antigen-antibody reaction. The coupling of highly specific immunoaffinity methods to capillary electrophoresis and microchip technology and further interfacing to mass spectrometry, is becoming the method of choice for many clinical and forensic applications. High sensitivity, selectivity, reproducibility, speed, convenience and cost effectiveness are among the attractive features of this hybrid technology.

Another important factor, when compared with other immunoaffinity procedures (e.g., radioimmunoassay, ELISA, etc.), is the lack of false–positive or false–negative results. The superior selectivity of the immunoaffinity method, combined with the high-resolution power of CE and the accurate mass determination of the peptide of interest by MS, provide an additional dimension of this hybrid technology for the reliable characterization of analytes in diagnostic analysis. Immunoaffinity capillary electrophoresis coupled to mass spectrometry is a hybrid technology that has the potential to revolutionize chemical and biological analysis, impacting the Human Proteomic Project, forensic science, environmental science, as well as other important industries, including pharmaceutical, biotechnology and food.

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