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Immunochromatographic analysis of bovine growth hormone releasing factor involving reversed-phase high-performance liquid chromatography–immunodetection

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Abstract

We have developed high-performance immunoaffinity chromatography (HPIAC) methods for the detection and quantitation of bovine growth hormone releasing factor (GHRF), which could also be applicable to its metabolites in biofluids. These approaches have involved a combination of IAC using immobilized antibody (Ab) to GHRF, together with reversed-phase high-performance liquid chromatography (RP-HPLC) separations of initially isolated and concentrated protein, followed by selective detection, involving on-line immunodetection (ID) schemes. ID methods involved HPIAC supports of the Ab, together with synthesized Ab-fluorescein isothiocyanate conjugates. We have demonstrated optimization methods for each step of the entire hyphenated technique (IAC–HPLC–ID), and then actually quantitated GHRF using this overall system. The minimum detectable concentration was about 1 ng/5 ml (200 ppt) with fluorescence detection (excitation wavelength, 490 nm; emission wavelength, 510–650 nm). We have also tested a single blind, spiked biological sample (bovine plasma), spiked with a known level of GHRF. Accuracy (7.4%) and precision (S.D.=±22%) were quite acceptable for a double immunoassay method.

Keywords: Immunodetection; Immunoaffinity columns; Detection, LC; Growth hormones; Antibodies; Antigens; Peptides

1. Introduction

Immunodetection (ID) is a novel, now-commercial technique that can be used, in part, to quantitate antibody–antigen (Ab–Ag) binding using conventional high-performance liquid chromatography (HPLC) or specialized equipment [1–7]. A commercially available instrument (Integral) can perform many aspects of immunoassays, on-line, as well as other multidimensional HPLC-based separations [7]. However, virtually any commercial HPLC instrumentation is also suitable for performing ID type assays. The ID technique can, as here, be based on the use of perfusion chromatography media [8,9], flow-through beads, which provide a large amount of accessible surface area, equivalent to thousands of interconnected microtiter wells [7,9–13]. Alternative supports exist for performing ID assays, such as silica gel, glass beads and polysaccharide materials.

There is a limited amount of literature describing the successful interfacing of gradient elution, reversed-phase (RP)-HPLC, with on-line, continuous ID detection for proteins from biofluids [14–17]. In this conceptual approach, species first separated by a

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variety of HPLC conditions, even with organic–aqueous eluting solvents, could be selectively trapped post-column on an ID support and then detected using various ID methods. Such a hyphenated technique, e.g., RP-HPLC–ID, would then provide the selectivity of the HPLC separation mode, together with enhanced, selective ID in various formats, using enzyme, fluorescence (FL) or chemiluminescence (CL) probes.

Growth hormone releasing factor (GHRF) is a peptide released from the hypothalamus that stimulates the chromopholac cells of the pituitary to secrete growth hormone (GH). The primary sequence of bovine GHRF consists of 44 amino acids. Both synthetic and recombinant varieties of GHRF for bovine growth hormone have been described and studied [18–27]. In this study, we have used a synthetic GHRF analog, obtained from The Pharmacia and Upjohn company, consisting of 30 amino acids (M_r=ca. 3500). Antibodies to this GHRF analog have been raised and provided by The Upjohn Company.

In this paper, we describe a new method for the eventual detection and quantitation of bovine GHRF (eventually its metabolites) in biofluids (bovine plasma). This has used a direct connection of an RP-HPLC column, for the eventual separation of GHRF analog and its metabolites, in series with an ID cartridge. We provide a practical approach for such an interfacing, first starting with typical RP-HPLC gradient elution conditions, for the pure protein (GHRF), but eventually for proteins from biofluids for in vivo metabolism and pharmacokinetic type studies. This RP-HPLC–ID approach was then used with an initial immunoaffinity (Ab), solid-phase extraction cartridge (IAC), in series with the RP-HPLC–ID system (IAC–RP-HPLC–ID=ICA), in order to capture, preconcentrate and clean-up GHRF from eventual biofluid samples.

Using this overall system, we have now quantitated ppt levels of GHRF standard. We have also tested a single-blind biological sample, spiked GHRF in bovine plasma, and obtained accuracy and precision that were quite acceptable. This has suggested that the final, optimized ICA approach should now be applicable for the semi-routine analysis of other biological samples.

2. Materials and methods

2.1. Chromatographic conditions

Optimization was performed on a Gilson Model 232 System (Gilson Medical Electronics, Middleton, WI, USA), which consisted of two Gilson Model 302 pumps, a Gilson Model 802B manometric module, a Gilson Model 811B dynamic mixer, a Gilson Model 115 variable wavelength UV detector and a Gilson Model 121 FL detector. In UV detection, the wavelength was set at 280 nm and a.u.f.s. was 0.1. Data was acquired on a Dell 386 computer (Dell Computer, Austin, TX, USA), using Gilson Model 715 HPLC controller, version 1.20, software. The HPLC injection valve was a Rheodyne Model 7125 (Rheodyne, Cotati, CA, USA). Three other solvent delivery pumps from Beckman (Beckman, Fullerton, CA, USA), Altex Scientific (Rainin Instruments, Woburn, MA, USA) and Eldex Laboratories (Menlo Park, CA) were interconnected at various points to the Gilson System, for performing RP-HPLC–ID (on/off) operations.

Two mobile phases were prepared for gradient elution RP-HPLC. The first consisted of 0.1% trifluoroacetic acid (TFA) (Aldrich/Sigma, Milwaukee, WI, USA) in water (EM Science, Gibbstown, NJ, USA) (A) and the second of 0.1% TFA in acetonitrile (ACN) (EM Science) (B). These solutions were filtered using a 0.45 μm Durapore membrane filter (Millipore, Bedford, MA, USA) prior to use and then degassed under vacuum. A Delta Pak C_4, 300×3.9 mm column (Millipore), and a Shodex RS Pak, 150×6 mm column (Millipore) were used for the RP-HPLC separations. The gradient elution conditions employed (time (min)/%B) were: 0–1/30–50%; 1–24/50%; 24–25/50–30%. The flow-rate was 0.25 ml/min and UV detection was at 280 or 494 nm. For the peptide mapping of digested GHRF, another gradient elution was employed, time (min)/%B: 0–100/0–37.5; 100–110/37.5–75.0; 110–115/75–0. Flow-rate was 1.0 ml/min and UV detection was at 214 nm.

Two solutions were used for the purification and quantitation of Ab on an immobilized GHRF column. The loading buffer was 10 m*M* phosphate with 0.15 *M* NaCl, pH 7.2, and the desorption solution

contained 0.15 *M* NaCl with 0.5 ml HCl, pH 2.2. The RP-HPLC mobile phase was diluted after eluting from the RPLC column with a pH 10.7, 50 m*M* phosphate solution containing 0.15 *M* NaCl. These solutions were filtered using a 0.45 μm filter prior to use and stored at 2–8°C. The flow-rate of these solutions was 1.0 ml/min except when the Ab-fluorescein isothiocyanate (FITC) conjugate was injected onto the ID cartridge, when the flow-rate of the loading buffer was 0.2 ml/min.

2.2. Biotinylation of GHRF with N-hydroxysuccinimide (NHS)-LC-Biotin [28]

The following biotinylation steps were performed using the methods provided by Pierce (Rockford, IL, USA). About 1 mg of GHRF from Upjohn (The Pharmacia and Upjohn Company, Kalamazoo, MI, USA) was dissolved in 500 μl of 10 m*M*, pH 6.0, phosphate. NHS-LC-Biotin (1 mg) was dissolved in 1 ml of water and 37 μl of the solution was added to the tube containing the GHRF. The mixture was incubated for 30 min at room temperature. To remove unreacted biotin, the product was centrifuged at 2000 *g* for 15 min using Centricon-30 Microconcentrator from Amicon (Beverly, MA, USA). After centrifuging, the sample was diluted using 10 m*M* phosphate with 0.15 *M* NaCl, pH 7.2. To ensure complete removal of all excess biotin, the above process was repeated five more times. The binding affinity of the biotinylated GHRF was confirmed using ELISA methods, as below. Only active, biotinylated GHRF was used to prepare the affinity support for purification of its Abs.

2.3. Immobilization of GHRF to streptavidin column

Biotinylated GHRF was immobilized to a Poros (PerSeptive Biosystems, Framingham, MA, USA) streptavidin column using a recirculation device. Details of this device are available from PerSeptive [7,10,11]. The buffer was 10 m*M* phosphate with 0.15 *M* NaCl, pH 7.2, and the flow-rate was 1.0 ml/min for 40 min. The eluent was monitored at 280 nm.

2.4. Purification of Abs using immobilized GHRF column

Monoclonal Abs to the N-(NS-21) and C-terminal (CX-7) in ascites fluid, and polyclonal Ab in serum to GHRF from The Pharmacia and Upjohn Company were purified using an immobilized GHRF column. The GHRF column was equilibrated with the above described loading buffer and 100–300 μl of two-fold diluted ascites fluid in loading buffer was injected onto the GHRF column. The column was washed with loading buffer for 5 min, and then bound Ab was eluted by the above described HCl desorption solution. Eluted Ab was immediately neutralized with 1 *M* Na_2HPO_4, pH 8.7. The collected Ab solution was concentrated using an Ultrafree-MC Filter from Millipore [30 000 nominal molecular weight limit (NMWL)]. Concentrated Abs were stored at 2–8°C with 0.1% NaN_3 until used.

2.5. Biotinylation of Abs with NHS-LC-Biotin [28]

Purified Ab, ca. 1.0 mg, to GHRF, was dissolved in 0.5 ml of 50 m*M* sodium bicarbonate, pH 8.5. A 1 mg weight of NHS-LC-Biotin was dissolved in 1 ml of water and 37 μl of the solution was added to the tube containing the purified Ab. The mixture was incubated at room temperature for 30 min. To remove unreacted biotin, the product was centrifuged at 2000 *g* for 15 min using an Ultrafree-MC Filter from Millipore (30 000 NMWL). After centrifuging, the sample was diluted with 10 m*M*, pH 7.2, phosphate buffer with 0.15 *M* NaCl. To ensure complete removal of all excess biotin, the above process was repeated five more times.

2.6. Immobilization of antibodies to streptavidin column

Biotinylated Abs (50:50 (w/w) batches NS-21 and CX-7) were immobilized onto a Poros type streptavidin column using a recirculation device. The loading buffer was 10 m*M* phosphate with 0.15 *M* NaCl, pH 7.2, and flow-rate was 1.0 ml/min for 40 min. The eluent was monitored by UV at 280 nm.

2.7. Indirect antibody enzyme-linked immunosorbent assay (ELISA) [28]

ELISA Starter Kits and all chemicals used were obtained from Pierce and the following steps were performed according to the manufacturer's instructions. The coating buffer was prepared by dissolving 1 packet of BupH Carbonate to 500 ml with distilled water. The wash buffer was prepared by adding 2.5 ml of Surfact-Amps Tween 20 and 1 packet of BupH Dulbecco's phosphate-buffered saline (PBS) to 500 ml distilled water. The blocking buffer was prepared by bringing 10 ml of Blocker bovine serum albumin (BSA) in PBS to 100 ml of BupH Dulbecco's PBS. Ag solution containing GHRF was prepared at approximately 20 μg/ml in coating buffer. Primary Ab, monoclonal and polyclonal immunoglobulin (IgG) to GHRF, were prepared at approximately 20 μg/ml in the blocking buffer. Peroxidase labelled secondary Ab was diluted about 1600-fold in the blocking buffer. 2,2′-Azinobis(3-ethylbenzothiazoline)-6-sulfonic acid, diammonium salt (ABTS) reagent (Pierce) was used as a peroxidase substrate.

A 100 μl aliquot of the GHRF antigen solution was placed into each well of a microtiter plate. The plate was incubated for 1 h at room temperature. The plate was emptied and residual liquid was tapped on a paper towel. Each well was rinsed with 3×100 μl of wash buffer. The plate was emptied and residual liquid was again tapped on a paper towel. The plate was incubated for 1 h at room temperature with 100 μl of blocking buffer. The plate was again emptied and residual liquid was tapped on a paper towel. A 100 μl aliquot of primary Ab, IgG to GHRF, was added to each well. The plate was incubated for 1 h at room temperature. Each well was rinsed with 3×100 μl of wash buffer. The plate was emptied and residual liquid was tapped on a paper towel. A 100 μl aliquot of labelled secondary Ab was added to each well. The plate was incubated for 2 h at room temperature. Each well was rinsed with 3×100 μl of wash buffer. A 100 μl aliquot of wash buffer was added to each well and incubated for 5 min. The plate was emptied and residual liquid was tapped on a paper towel. A 100 μl aliquot of ABTS solution was added to each well. The plate was incubated at room temperature for 30 min. The reaction was stopped by adding 50 μl of 1% sodium dodecyl sulfate (SDS) solution.

2.8. Streptavidin coated ELISA plates [28]

The stability of GHRF and Ab in organic solvents was tested using ELISA methods, in order to determine the conditions for connecting the RP-HPLC column and ID cartridge. Tris-buffered saline (TBS) was prepared with 25 m*M* Tris and 150 m*M* NaCl, pH 7.6. Wash buffer was prepared with TBS, 0.1% BSA and 0.05% Tween 20. Biotinylated GHRF was incubated in 50% ACN aqueous solution with 0.1% TFA for 1 day. The primary Ab, biotinylated monoclonal (m)-Ab to GHRF, was incubated in 200 μl of prepared solutions, the ratios of 50% ACN with 0.1% TFA and loading buffer was 1:0, 1:1, 1:2, 1:3, 1:4 and 1:5 diluted, respectively. Enzyme-labelled secondary Ab (Ab-En) was 1000-fold diluted with wash buffer. A 100 μl volume of biotinylated GHRF was added to each well and incubated for 2 h at room temperature. Each well was rinsed with 4×200 μl of wash buffer. A 100 μl aliquot of the primary Ab was added to each well of the microtiter plate. The microtiter plate was incubated for 30 min. Each well was rinsed with 4×200 μl of wash buffer. A 100 μl volume of the enzyme-labelled secondary Ab was added to each well of the microtiter plate. The microtiter plate was incubated for 30 min. Each well was rinsed with 4×200 μl of wash buffer. A 100 μl aliquot of ABTS solution was added to each well. The plate was incubated for 30 min at room temperature. The reaction was stopped by adding 50 μl of 1% SDS solution.

2.9. Tryptic digest of GHRF for peptide mapping

Approximately 1 mg of GHRF was dissolved in 0.88 ml of 125 m*M* Tris–HCl with 1.25 m*M* $CaCl_2$, pH 8.5. To the GHRF solution, 0.02 ml of trypsin (0.5 mg/ml) in 1 m*M* HCl was added. The mixture was incubated for 24 h at 37°C. The reaction was stopped by adding 0.1 ml of 10% (v/v) TFA. For a control reaction, the same reaction mixture in the absence of GHRF was prepared using the same procedure.

2.10. Synthesis and purification of m-Ab-FITC conjugates [28–31]

To Ab (500 μg/50 μl) solution, 50 μl of 0.5 *M* carbonate buffer, pH 9.5, was added. A 8.4 μl volume of 1 mg/100 μl of freshly dissolved FITC in 0.5 *M* carbonate buffer, pH 9.5, was added to the Ab solution. The mixture was incubated for 30 min in the dark at room temperature with stirring. FITC labelled Ab was purified using Centricon-30 from Amicon (30 000 NMWL).

2.11. Interfacing of IAC and RP-HPLC (Fig. 1)

The schematic diagram for interfacing of IAC and RP-HPLC is shown in Fig. 1. Solution A was loading buffer, 10 m*M* phosphate with 0.15 *M* NaCl, pH 7.2, and solution B was desorption solution, 0.15 *M* NaCl, pH 2.2, adjusted with HCl. Solutions E and F were 0.1% TFA in water and 0.1% TFA in ACN, respectively. Parts C, D, G, H and I, J, and K represent injector, IAC cartridge, mixer for mixing of E and F solutions, switching valves, RP-HPLC column and UV detector, respectively. Using this system with tryptic digested GHRF, the recognition properties of mixed monoclonal and polyclonal Ab IAC cartridges to GHRF were compared.

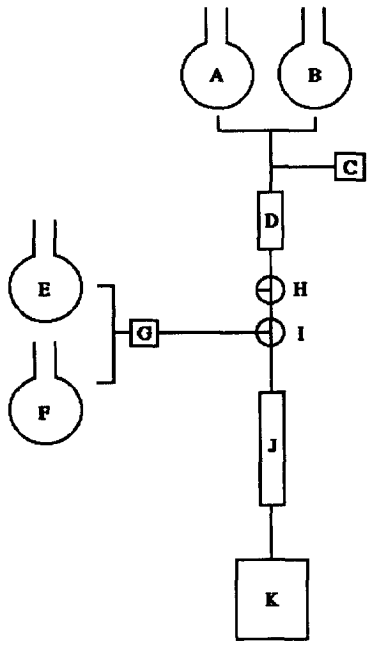

Fig. 1. Schematic diagram of the IAC–RP-HPLC–UV instrumental arrangement for initial analysis of GHRF using different IAC immobilized Ab supports: loading buffer, 10 m*M* phosphate with 0.15 *M* NaCl, pH 7.2 (A); desorption solution, 0.15 *M* NaCl, pH 2.2 (B); injector (C); IAC column (D); 0.1% TFA in H_2O (E); 0.1% TFA in ACN (F); mixer (G); switching valves (H) and (I); RP-HPLC column (J) and UV detector (K).

2.12. Evaluation and development of elution conditions for interfacing of RP-HPLC and ID (Fig. 2)

The specific buffers used in Fig. 2 to optimize RP-HPLC elution conditions and make them compatible with ID capture and Ab–Ag complex formation

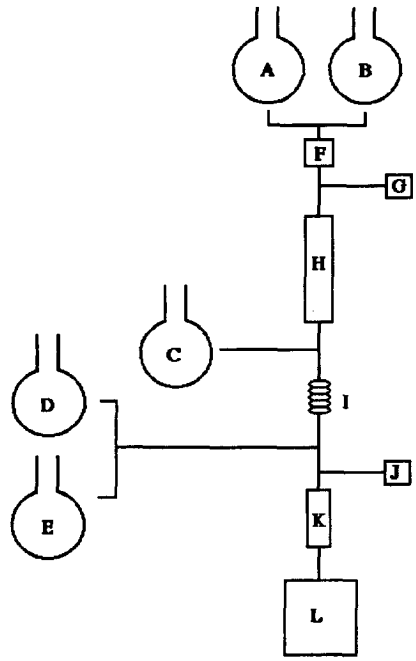

Fig. 2. Schematic diagram of RP-HPLC–ID instrumentation used to evaluate and improve the recognition ability of the ID column for GHRF. The formation and stability of the Ab–Ag complex was investigated against changes of %ACN and pH: 0.1% TFA in H_2O (A); 0.1% TFA in ACN (B); diluent solution, 50 m*M* phosphate with 0.15 *M* NaCl, pH 10.7 (C); loading buffer, 10 m*M* phosphate with 0.15 *M* NaCl, pH 7.2 (D); desorption solution, 0.15 *M* NaCl, pH 2.2 (E); mixer (F); injectors (G) and (J); RP-HPLC column (H); reactor coil (I); ID column (K) and UV detector (L).

requirements are outlined below. Solution A was 0.1% TFA in water. Solution B was 0.1% TFA in ACN. Solution C was composed of 50 m*M* phosphate containing 0.15 *M* NaCl, pH 10.7. Solution D was a loading buffer, 10 m*M* phosphate with 0.15 *M* NaCl, pH 7.2, for washing the ID cartridge and reducing the background absorptivity after eluting various mixtures of A and B. Solution E was a desorption solution, 0.15 *M* NaCl, pH 2.2 adjusted with HCl, for eluting the bound GHRF from the ID cartridge. Parts F, G and J, H, I, K and L represent mixers for mixing of A and B solutions, injectors, RP-HPLC column, 1 ml reactor coil (Rainin), ID cartridge and UV detector, respectively.

2.13. Interfacing of IAC–RP-HPLC–ID (Fig. 3)

The schematic diagram of the IAC–RP-HPLC–ID instrumental arrangement for the final analysis of GHRF and eventually its metabolites from biofluids is shown in Fig. 3. Solutions A and B were 0.1% TFA in water and 0.1% TFA in ACN. Solution C was the diluent for diluting the gradient solutions, A and B, and was composed of 50 m*M* phosphate containing 0.15 *M* NaCl, pH 10.7. Solution D was the loading buffer, 10 m*M* phosphate with 0.15 *M* NaCl, pH 7.2, and solution E was the desorption solution, 0.15 *M* NaCl, pH 2.2 adjusted with HCl. Parts F, G, J and N, K, L, O, and P represent mixer, IAC cartridge, injectors, RP-HPLC column, reactor coil, ID cartridge, and UV or FL detector, respectively. Parts H, I, and M represent switching valves.

2.14. Verification of IAC–RP-HPLC–ID system (Fig. 3) using a single blind test

The accuracy and precision of the overall system was demonstrated using a single blind, spiked sample of bovine plasma (instrumental arrangement as in Fig. 3). Before quantitation of the single blind sample, a calibration plot was prepared for GHRF in the range of 10 to 20 ng/5 ml. This is the expected (relevant) concentration range for GHRF following its administration. The equation of this (external standard) calibration plot was $y=27168+3326x$, with a correlation coefficient (r) of 1.000. GHRF was then spiked at a level unknown to the analyst, within the range of 40 to 80 ng in 20 ml of bovine plasma

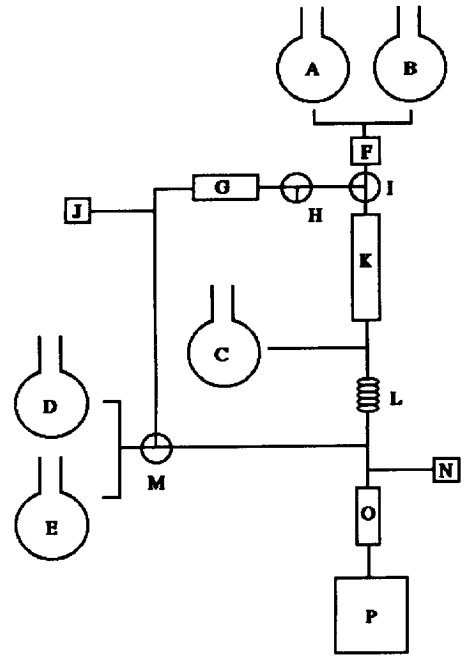

Fig. 3. Schematic diagram of the IAC–RP-HPLC–ID instrumental arrangement for final analysis of GHRF and its metabolites from biofluids using mixed m-Ab IAC supports: 0.1% TFA in H_2O (A); 0.1% TFA in ACN (B); diluent solution, 50 m*M* phosphate with 0.15 *M* NaCl, pH 10.7 (C); loading buffer, 10 m*M* phosphate with 0.15 *M* NaCl, pH 7.2 (D); desorption solution, 0.15 *M* NaCl, pH 2.2 (E); mixer (F); injectors (J) and (N); switching valves (H), (I) and (M); IAC column (G); RP-HPLC column (K); reactor coil (L); ID column (O) and UV or FL detector (P).

solution. A 5 ml volume of the sample was injected three times.

3. Results and discussion

3.1. Interfacing of immunoaffinity chromatography (IAC) with RP-HPLC (Fig. 1)

The ability to interface an IAC cartridge prior to HPLC, Fig. 1, has already been described [14–16,32]. In this arrangement, GHRF was first introduced into the IAC cartridge, isolated and preconcentrated, and then eluted with a suitable buffer (Section 2) onto the RP-HPLC column, where it was again peak compressed before the gradient elution run began. This arrangement, Fig. 1, thus permitted the direct interfacing of an IAC preconcentration

column with conventional RP-HPLC separation conditions.

Mixed m-Ab (ca. 50:50, w/w) and polyclonal (p)-Ab columns were compared with regard to their recognition ability for GHRF, using this system. The calibration plots of the mixed m- and p-Ab column for GHRF in the range of 1 μg to 100 μg showed the recognition properties of these columns (Fig. 4). The mixed m-Ab column had a wide linear range, 1 to 50 μg, and the detection limit was about 1 μg with UV at 280 nm. On the other hand, the p-Ab column showed an S-shape calibration plot (Fig. 4B) and the column did not recognize GHRF well in the lower concentration range, below 10 μg.

The purpose of this study was to quantitate GHRF and (eventually) its metabolites, so it was necessary to determine how many different sites on GHRF might be recognized by these different Ab columns. It was not possible, in advance, to obtain the GHRF metabolites, since these have yet to be determined. As an approximation of these possible metabolites, we generated the trypsin digested products of GHRF. The mixture of peptide fragments of GHRF was injected onto the mixed m- or p-Ab column, in-

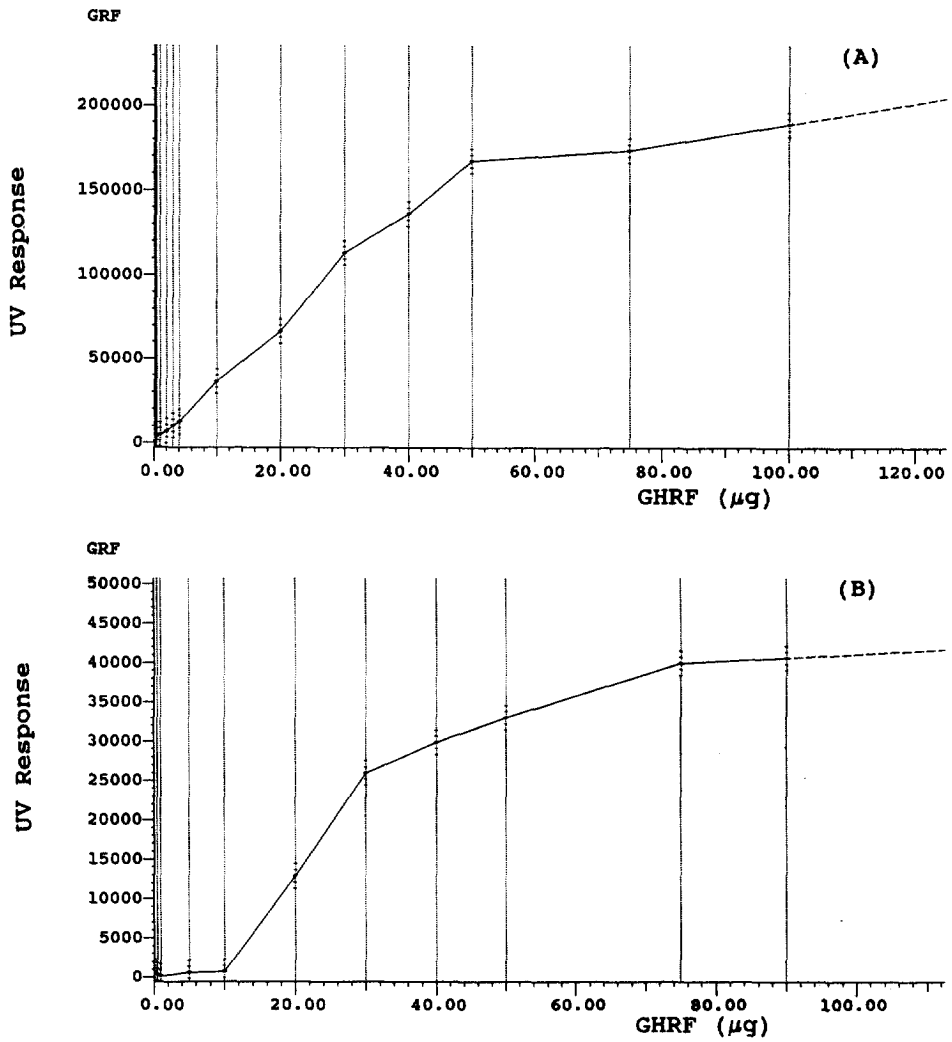

Fig. 4. Calibration plot for GHRF obtained using mixed m-Ab column (A) and p-Ab column (B). Mobile phase: 0.01 *M* phosphate with 0.15 *M* NaCl, pH 7.2, for loading; 0.15 *M* NaCl, pH 2.2, for desorption. Detection: UV at 280 nm. Flow-rate: 1.0 ml/min.

dividually, and some peptide fragments were captured on each column. Some of these peptide species were not captured and immediately eluted from the IAC cartridge. After washing the Ab column with loading buffer, captured peptide fragments were eluted into the RP-HPLC column, with the usual desorption buffer. The peptide fragments were separated via the RP-HPLC column, Fig. 5. The mixed m-Ab column appeared to recognize and capture several peptide fragments from digested GHRF, Fig. 5(C). On the other hand, the p-Ab column appeared to recognize only one fragment, Fig. 5(D), perhaps because of low affinity and low titer of the p-Ab. Thus, for the quantitation of biofluid levels of GHRF, we chose the mixed m-Ab column for both IAC and ID applications.

3.2. Interfacing of RP-HPLC With on-line ID, (Fig. 2)

It was anticipated that the RP-HPLC conditions might not be compatible with Ab–Ag binding in the ID, flow-through format. Before connecting the ID cartridge to the RP-HPLC column, the stabilities and recognition of Ag and Ab were tested using an RP-HPLC solution which contained ACN (50%) and 0.1% TFA. This involved off-line, ELISA type assays. According to such test results, the Ab–Ag complex was stable in a 15% ACN solution for several days at room temperature. GHRF was soluble and stable (bioactive) in its native state in the above acidic, aqueous ACN solution for several weeks. GHRF in a 50% ACN solution containing 0.1% TFA was injected onto the ID cartridge with or without 1 ml of a reactor coil in front. As shown in Fig. 6, the area of GHRF injected through a knitted open tubular (KOT), Teflon reactor coil and onto the ID column, after elution was larger than that injected without a reactor coil in-place. This suggested that under direct RP-HPLC conditions, without sufficient adjustment and equilibration of the now-eluted GHRF, the Ab on the ID column was not able to recognize and capture all of the injected GHRF. It was also possible that the Ag and/or Ab were not bioactive under these mobile phase (RP-HPLC) conditions.

In order to evaluate and improve the recognition ability of the ID column for GHRF, the Ab–Ag complex was investigated against changes of %ACN and pH using the apparatus in Fig. 2. When the ratio of the mixture of solutions (A+B) and C was 1:4, the area of the bound GHRF was more than 80%, as compared to a GHRF area obtained from the best loading conditions, using only an ideal loading buffer. The pH of the mixture was about 8.5–9.0, higher than normal, for direct ID loading conditions [7,10,11,13]. According to these results, slightly basic conditions were favorable to Ab–Ag binding in the RP-HPLC, ACN containing solutions. This suggested that the structure of GHRF may have changed slightly in the less polar, ACN solution. Importantly, these results showed that neither GHRF nor the immobilized Ab were denatured under the final RP-HPLC elution conditions, but that these needed to be modified to permit recognition and capture on the ID support. In all likelihood, the RP elution conditions were unsuitable for recognition and binding of Ab–Ag.

Fig. 7 is a RP-HPLC–ID–UV chromatogram, with specific conditions indicated, using the instrumentation described in Fig. 2. The above interfaced approach utilized direct GHRF injections onto the RP-HPLC column, and at fairly high concentration levels, as indicated (>10 μg per injection). Using a direct on/off ID approach, detection limits were perhaps the highest possible. Improved analyte detectability can result from several formats in ID [7,10–13,17,28,33].

3.3. Interfacing of IAC with RP-HPLC and on-line ID, (Fig. 3)

The above results demonstrated the basic ability to successfully interface gradient elution, RP-HPLC conditions with on-line ID capture for specific protein species, followed by a wide variety of ID schemes (on/off, sandwich, double sandwich, etc.). Fig. 8 shows a chromatogram (actual spiked, bovine plasma sample, see below) obtained using a simple on-line, sandwich IAC–RP-HPLC–ID format, wherein a m-Ab-FITC conjugate was first prepared (Section 2) having improved UV/FL detection over the m-Ab or GHRF alone. This ID method involved capturing the GHRF from the RP-HPLC column, then adding an excess of the Ab-FITC to the ID column, Fig. 3 (through N), washing excess Ab-

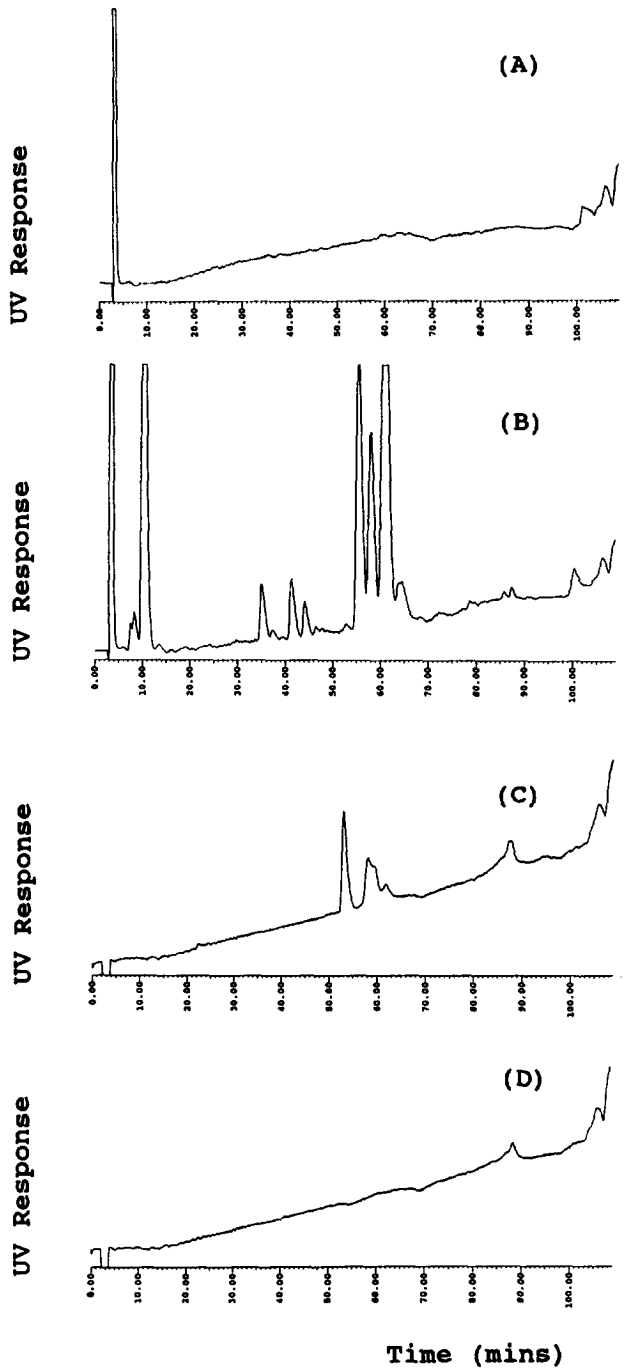

Fig. 5. Peptide maps of tryptic digested GHRF obtained using IAC–RP-HPLC system described in Fig. 1. Blank (A), entire peptide map (B), peptide map of mixed m-Ab column bound fraction (C), peptide map of p-Ab column bound fraction (D). Column: Delta Pak, 15 μm, C_4, 300 Å, 3.9×300 mm. Mobile phases: 0.1% TFA in H_2O (a) and 0.1% TFA in ACN (b). Gradient elution time (min)/%b: 0–100/0–37.5; 100–110/37.5–75.0; 110–115/75.0–0. Detection: UV at 214 nm.

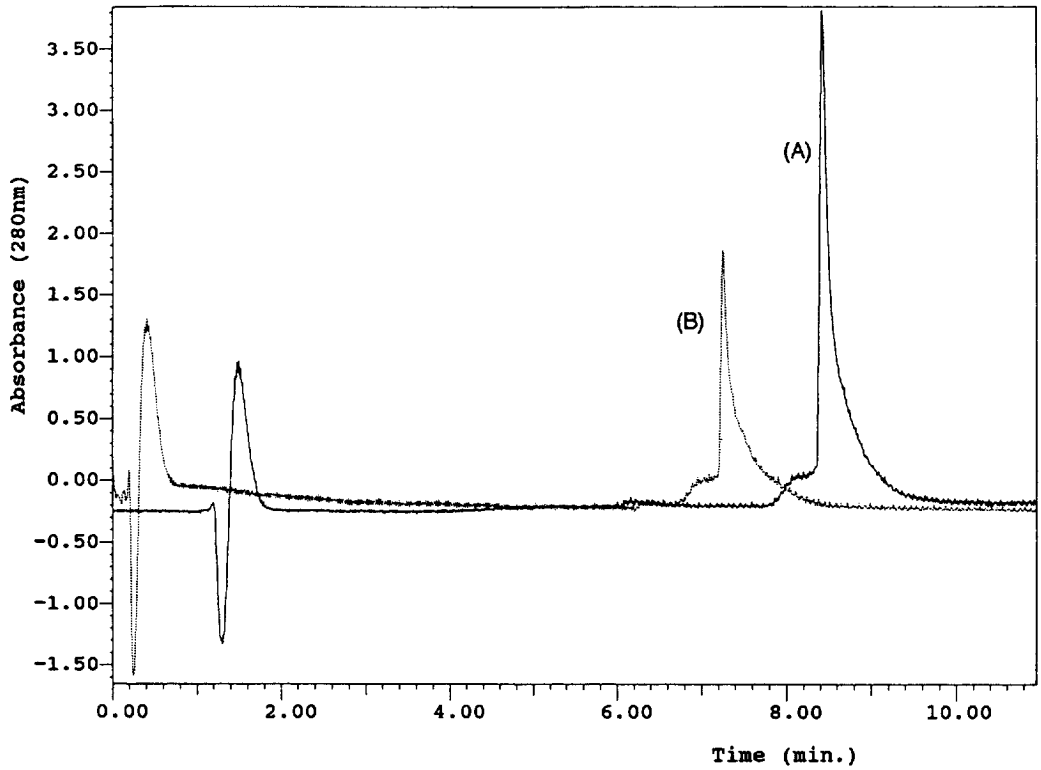

Fig. 6. Comparison of peak areas for the same mass of GHRF in 50% ACN–H_2O with 0 1% TFA injected through (A) and without (B) a knitted open tubular, Teflon reactor coil before the ID column. Mobile phase: 0.01 *M* phosphate with 0.15 *M* NaCl, pH 7.2, for loading; 0.15 *M* NaCl, pH 2.2, for desorption. Detection: UV at 280 nm. Flow-rate: 1.0 ml/min.

FITC from this column with loading buffer (D), and then eluting the Ag–Ab-FITC complex with desorption solution (E) through the FL detector (excitation wavelength; 490 nm, emission wavelength; 510–650 nm). Minimum detectable concentration with FL detection was ca. 1 ng/5 ml (200 ppt) injected onto the IAC–RP-HPLC–ID instrumental arrangement. The equation for the (external standard) calibration plot was $y=5323+135x$, with a correlation coefficient (r) of 0.98 within the range of 1 ng/5 ml to 50 ng/5 ml.

There was a substantial amount of background (nonspecific adsorption caused) response in the immunoassay. Such background responses (blanks) have been described and discussed elsewhere, with possible solutions suggested [7,10,11,13]. This also restricts the detection limit, quantitation limit, and overall linear range of a calibration plot. We (and others) have spent a great deal of time and effort in order to correct or overcome this nonspecific adsorption using various surfactants, salts, BSA, nonfat dry milk, urea and so on. We also used Fab fragments instead of whole Abs to make FITC conjugates. In the current work, such materials and approaches had almost **no** effect on reducing the background absorbance. The best way for reducing non-specific adsorption was to reduce the adsorption sites; in other words, to reduce the column volume. Initially, we used a Peek column (3 cm×2.5 mm I.D.) from PerSeptive Biosystems, but that column had too high background adsorption to be useful in quantitating biofluid levels. Thus, we changed to a Microbore Guard Column (2 cm×1 mm I.D.) from UpChurch Scientific (Oak Harbor, WA, USA) for the ID cartridge. The signal/noise ratio was greatly increased, 1.9 (Peek column) to 21.6 (Microbore guard column) for 5 μg of GHRF in the small volume column format. We acknowledge the invalu-

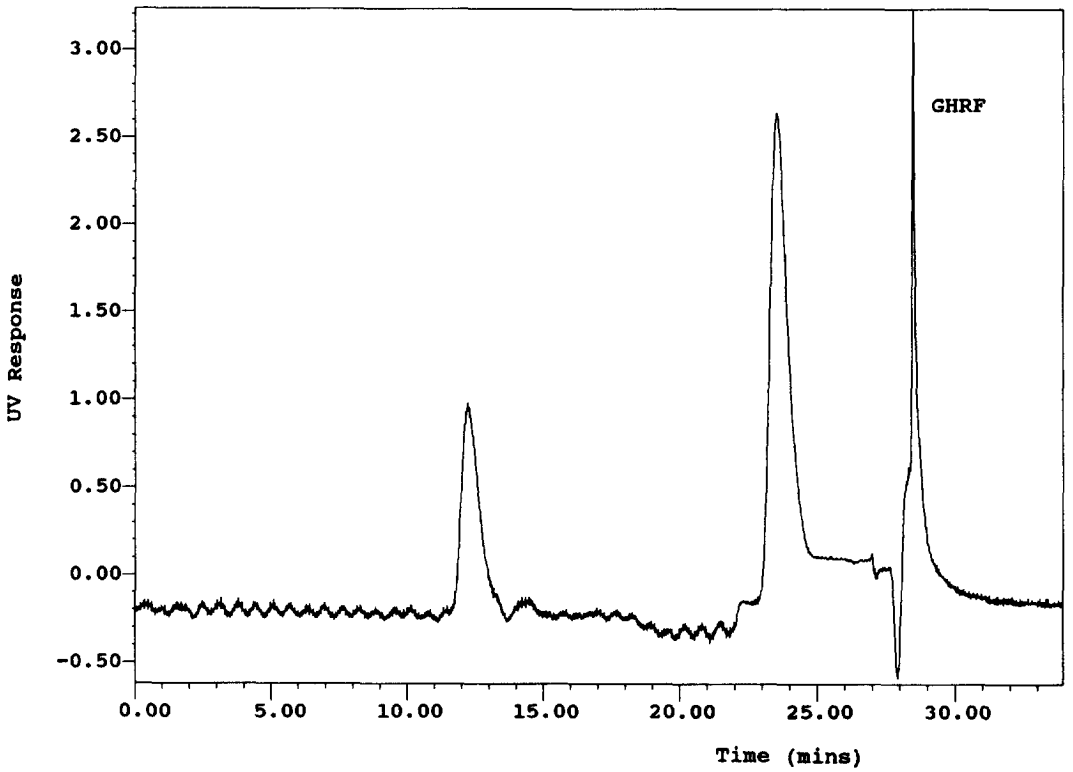

Fig. 7. An RP-HPLC–ID–UV chromatogram using the instrumentation described in Fig. 2. Standard GHRF injected onto the RP-HPLC column, separated, captured, eluted and detected. RP-HPLC–ID–UV conditions as described in Section 2.

able assistance and collaboration of Dr. Marty Vanderlaan of PerSeptive Biosystems in providing us with suggestions on how to lower nonspecific adsorption and background absorbance in ID formats.

Finally, we tested the accuracy and precision of the system using a single blind, spiked sample in a biological fluid, viz., bovine plasma, Fig. 8 (instrumental arrangement as in Fig. 3). The same sample was injected three times, and the average value obtained was 58±12.8 ng/20 ml [2.9±0.64 ppb (standard deviation, S.D.)]. The concentration of the single blind, spiked sample was actually 54 ng/20 ml (2.7 ppb). Thus, there was excellent agreement (accuracy) between found and spiked levels in this bovine plasma sample, with acceptable precision of the measurements (S.D.) for an immunoassay based approach. In general, the prior literature using ICA and on-line ID approaches together with HPLC for quantitation have reported precisions in the same range as here.

4. Conclusions

In the above approaches, it has been possible to separate at least one protein species under typical RP-HPLC conditions, and postcolumn adjust the final elution solvents and pH so that these became compatible with on-line ID capture and simple on/off, direct UV or alternative ID assays. Labeling of GHRF with Ab-FL labels can substantially lower detection limits using this on/off type assay in RP-HPLC–ID. Direct labeling of GHRF with FL labels, not reported here, is another approach to lower detection limits in the ID formats.

Ideally one would wish to employ an on-line, (Ab–En) enzyme enhanced immunoassay, postcolumn in HPLC, such as an ELISA method. To the best of our knowledge, this approach has not yet been described in the open literature, though it has been suggested as a possible format in certain technical literature [7,10,11,13]. We have now dem-

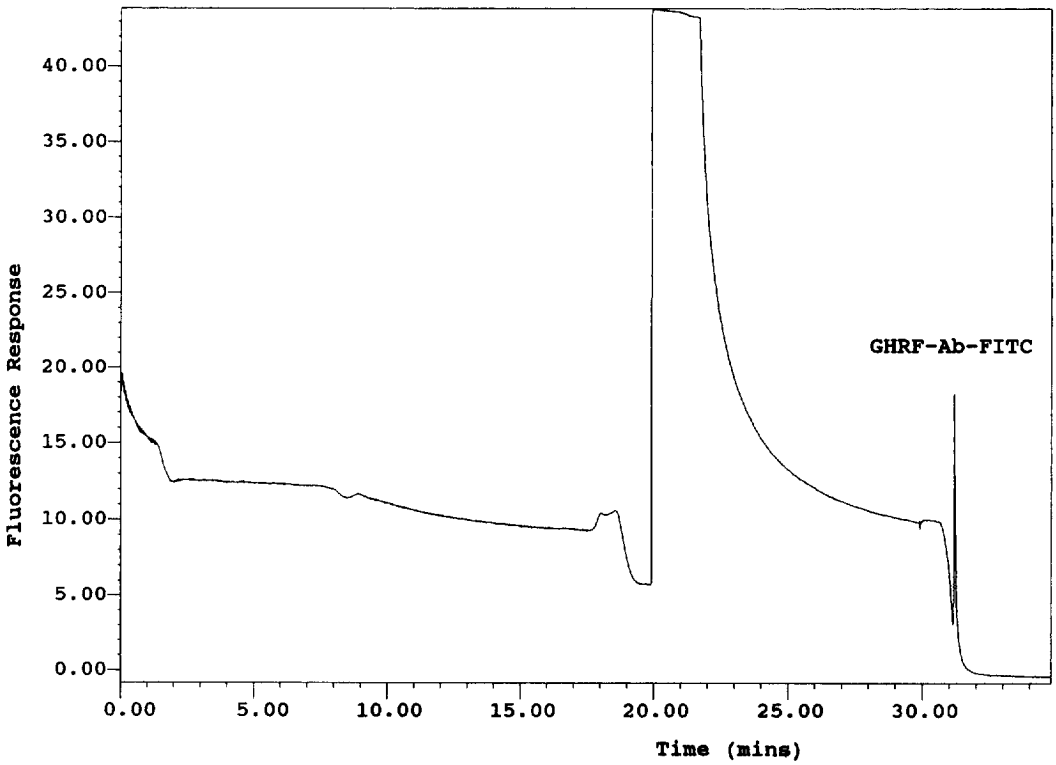

Fig. 8. IAC–RP-HPLC–ID GHRF chromatogram of a spiked, bovine plasma sample, with a simple on-line, sandwich ID format, using m-Ab-FITC conjugates with FL detection (instrumental arrangement as in Fig. 3). IAC–RP-HPLC–ID–FL conditions as described in Section 2.

onstrated an optimization method for each step of the entire connection and have actually quantitated GHRF in a biofluid using this final, hyphenated system. The ICA optimization approach described here should be helpful in its application to other biological/biomedical analytes in biofluids.

5. Glossary

Ab	= antibody
Abs	= antibodies
Ag	= antigen
Ab–Ag	= antibody–antigen complex
ABTS	= 2,2′-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid, diammonium salt
ACN	= acetonitrile
BSA	= bovine serum albumin
CL	= chemiluminescence detection
En	= enzyme
FITC	= fluorescein isothiocyanate
FL	= fluorescence detection
GHRF	= growth hormone releasing factor (bovine)
HPLC	= high-performance liquid chromatography
HPIAC	= high-performance immunoaffinity chromatography
IAC	= immunoaffinity cartridge
ICA	= immunochromatographic analysis (IAC–HPLC/RP-HPLC–ID)
ID	= immunodetection
IgG	= immunoglobulin
KOT	= knitted open tubular, Teflon reactor coil
LC	= long chain
m-Ab	= monoclonal Ab
M_r	= molecular mass
NaCl	= sodium chloride

NHS = N-hydroxysuccinimide
NMWL = nominal molecular weight limit
ppb = parts-per-billion
ppm = parts-per-million
ppt = parts-per-trillion
p-Ab = polyclonal antibody
PBS = phosphate buffered saline
RP-HPLC = reversed-phase liquid chromatography
S.D. = standard deviation (precision)
SDS = sodium dodecyl sulfate
TBS = tris-buffered saline
Tris = tris(hydroxymethyl)aminomethane
TFA = trifluoroacetic acid
UV = ultraviolet detection

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