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Immunoaffinity trapping of urinary human chorionic gonadotropin and its high-performance liquid chromatographic–mass spectrometric confirmation

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Abstract

A method has been developed for confirmation of the glycopeptide human chorionic gonadotropin (hCG) in urine. A solid-phase immunoaffinity trapping technique utilizing a monoclonal antibody recognizing both intact hCG and free hCG β-subunits was developed for the extraction of hCG from urine. Recovery of hCG from a urine matrix was essentially quantitative. The hCG was quantitatively eluted with 6 *M* guanidine hydrochloride, reductively alkylated with vinylpyridine, and subjected to tryptic digestion. The tryptic digest was analyzed by HPLC–MS. Ions from three tryptic fragments were monitored with selected ion monitoring to provide specific detection of hCG. The signal observed for a concentration of 25 mIU/ml of hCG could be clearly distinguished from background with a signal-to-noise ratio of 12:1.

Keywords: Gonadotropin; Glycoproteins; Human chorionic gonadotropin; Chorionic gonadotropin

1. Introduction

Human chorionic gonadotropin (hCG) is a glycoprotein hormone normally produced by the trophoblatsic cells during the early stage of pregnancy [1]. Since hCG can stimulate general steroid production, its administration could enable athletes to avoid detection in drug testing for testosterone abuse while maintaining a high level of steroids [2,3]. The International Olympic Committee (IOC) has banned hCG administration for its potential ability to enhance performance. Although sensitive enzyme immunoassays (EIA) have been developed for hCG measurement, their admissibility in legal proceedings has been of concern due to their low discriminating power [2]. Validation of a urinary hCG method must include not only a consideration of potential matrix effects, but also potential cross-reactivity with other hormones, such as human luteinizing hormone (hLH), and their metabolic products.

We have reported on the HPLC–MS characterization of hCG [4] and peptides derived from hCG [5] to lay the foundation for development of a HPLC–MS confirmation method. Briefly, we were unable to detect intact hCG or its subunits with electrospray (ES) mass spectrometry due to the microheterogeneity of the oligosaccharides attached to the protein. Reductive alkylation with vinylpyridine and proteolytic digestion with trypsin provided peptide fragments that could be readily separated by reversed-phase HPLC and detected by MS. We were able to confirm the reported amino acid sequence and oligosaccharide substitution using this technique [4].

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Isolation and purification of hCG from urine have been established in various separation modes. The most frequently used schemes for isolation of large amounts of hCG and related materials involved either reversed-phase or bioaffinity solid-phase extraction followed by either gel permeation or reversed-phase chromatography [1]. Concanavalin A was linked to Sepharose for the purification of hCG and other glycopeptide hormones [6,7]. Birken et al. [8] reported the use of hydrophobic matrix for the fractionation of hCG and related materials from urine. These methods worked well for the preparation of a relatively large amount of hCG samples. When hCG concentration was very low, these methods would not provide sufficient recovery. For hCG concentrations as low as 10 mIU/ml (proposed IOC cut-off concentration: 10 mIU/ml or 45 fmol/ml), a more efficient trapping method must be used so that a high hCG recovery can be achieved.

Immunoaffinity chromatography is a separation technique in which the highly specific antibody–antigen interaction is incorporated into the separation process. Since the binding between the antibody and antigen is very specific and strong, the recovery in immunoaffinity chromatography, especially at trace concentrations, is much higher than any other separation methods [9,10]. Antibodies have been developed that are directed to specific epitopes on hCG [11]. The monoclonal antibody designated B108 is directed to an epitope found on intact hCG, hCG β-subunit, nicked β-subunit, β-core fragment and β-subunit carboxy-terminal fragment [12]. Although intact hCG was the primary urinary excretion product following administration of hCG [13], we selected an antibody with a broader specificity. In this paper, we report the development of an immunoaffinity trapping method to extract hCG and related materials from urine and the semi-quantitative detection of selected tryptic fragments of hCG β-subunit by HPLC–MS for the purpose of confirming its presence at trace concentrations.

2. Experimental

2.1. Chemicals

Pregnyl, an hCG pharmaceutical preparation, was purchased from Organon (lot No. 0350193315, W. Orange, NJ, USA). Emphase AB1 affinity media and the bicinchonic acid (BCA) protein assay kit were purchased from Pierce (Rockford, IL, USA). The monoclonal antibody B108 (1 mg/ml) was purchased from Scantibodies Laboratory (Santee, CA, USA). Cyanogen bromide-activated Sepharose beads, dithiothreitol, vinylpyridine, sodium borate, sodium citrate and Tris buffer were obtained from Sigma (St. Louis, MO, USA). All reagents of were of analytical grade or better, and used as received. Solvents were all of HPLC Grade (Baxter) Water was purified through a Ultra-Pure water system from Millipore (Pleasanton, CA, USA).

Pregnyl ($10 \cdot 10^6$ mIU of hCG) was dissolved in 10 ml of 0.1 *M*, pH 7.5 phosphate containing 0.1 *M* NaCl (PBS). The Pregnyl solution was further diluted with 0.1% BSA in phosphate buffered saline (PBS) for the determination of the antibody binding constant and capacity. For the Scatchard analysis, nine standards between 10 and 1000 mIU/ml were added to 100 μl of gel. The unbound fraction was analyzed using the Abbott IMx EIA method. For recovery studies, the Pregnyl solution was diluted in a blank urine solution to obtain concentrations of 30 and 500 mIU/ml. The standards for the HPLC–MS assay were prepared by spiking Pregnyl into five fractions of 10 ml of normal male urine to achieve the following concentrations: 0 mIU/ml (blank), 10 mIU/ml, 50 mIU/ml, 100 mIU/ml and 500 mIU/ml. The hCG concentration in all of these solutions was established by IMx EIA.

[^{125}I]-hCG was purchased from DuPont NEN (Boston, MA, USA). The protein was purified prior to use by gradient reversed-phase HPLC using 50 mmol/l, pH 6.8 sodium phosphate buffer (A) and acetonitrile (B). The percentage of solvent A was linearly changed from 5% to 50% in 60 min. For radioisotope recovery studies, purified [^{125}I]hCG was added to the Pregnyl solution containing 30 and 500 mIU/ml. [^{125}I]hCG presented less than 10% of total hCG even at a concentration of 30 mIU/ml.

2.2. Instrumentation

Enzyme immunoassay (EIA) determinations of hCG were performed on the Abbott IMx system (Abbott Park, IL, USA). Radioisotope activity was counted on an Apex™ automatic gamma counter (MicroMedic Systems, Horsham, PA, USA). Solu-

tions were dried in a SpeedVac RT100 system (Savant, Farmington, CT, USA).

A PE-Sciex API-III^{Plus} quadrupole tandem mass spectrometer (Thornhill, Ontario, Canada) equipped with an articulated ion spray interface was used for the HPLC–MS analysis. The HPLC system consisted of a Beckman (Fullerton, CA, USA) Model 126 solvent delivery module and a Model 166 UV detector. The tryptic digest was injected through a Rheodyne (Cotati, CA, USA) Model 8125 injector into a 150×1 mm I.D. Vydac TP218 column (The Separations Group, Hisperia, CA, USA) and eluted with a linear gradient of 95:5 A–B to 50:50 A–B in 60 min at a flow-rate of 50 μl/min, where solvent A is 0.05% formic acid in water and solvent B is 0.05% formic acid in acetonitrile. A Valco (Houston, TX, USA) Tee was used post-column to split the effluent 1:10 with the majority of the effluent going through a length of 127 μm I.D. PEEK tubing to the UV absorbance detector and the minor portion flowing through 50 μm I.D. fused-silica tubing to the nebulizing needle of the ionspray interface of the mass spectrometer. Mass calibration was carried out with ammonium adduct ions of polypropylene glycol. The mass spectrometry was run in the selected ion monitoring (SIM) mode. The ions monitored were: m/z 1112.5, 1209.5 and 1258.5 from tryptic fragment T3 with oligosaccharide N4, N3, and N1 attached [4]; m/z 1042, 1229.5 and 1302.5 from tryptic fragment (T4+oligosaccharide N1)$^{4+}$, (T4+oligosaccharide N3)$^{3+}$ and (T4+oligosaccharide N1)$^{3+}$ [4]; and tryptic fragment T5^{3+} (m/z 659) and T5^{2+} (m/z 988) (see Fig. 1 and Fig. 2). The mass spectrometer settings were: dwell time, 100 ms; ionization voltage, 4000 V; orifice voltage: 75 V.

2.3. Preparation of the immunoaffinity matrix

The antiserum containing 1 mg/ml B108 antibody was concentrated to 10 mg/ml using a CentriPrep3 membrane (Amicon, Beverly, MA, USA). A 1-ml sample of B108 concentrate was diluted in 10 ml of coupling buffer (0.1 *M*, pH 9.0 borate containing 0.6 *M* sodium citrate) and 500 mg AB1 beads was added. After vortex mixing for several seconds, B108 was coupled to AB1 beads using the procedures provided by the manufacturer [14]. The beads contained about 2.5 mg B108 protein/ml of gel.

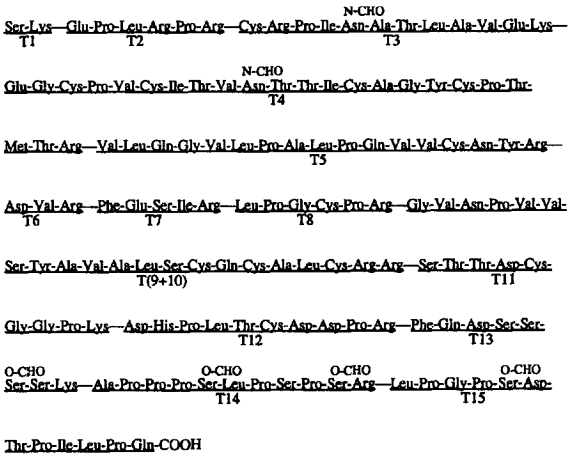

Fig. 1. Amino acid sequence of the β-subunit of hCG with standard tryptic fragments labeled with T#. CHO indicates the location of oligosaccharide attachment.

In one experiment, B108 was coupled to cyanogen bromide-activated Sepharose 4B beads to check the effects of support media on binding and elution efficiency. About 250 mg of Sepharose beads (a volume equivalent to the AB1 beads) was added to 12 ml of B108 coupling solution which was diluted from 1 ml of B108 concentrate in 11 ml of 0.2 *M*, pH 8.5 sodium bicarbonate containing 0.5 *M* NaCl. The coupling was performed according to the procedures provided by the manufacturer [15]. This

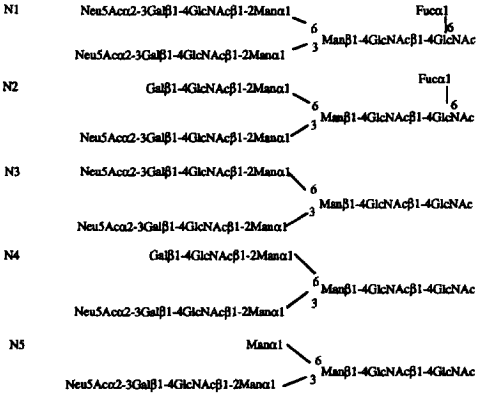

Fig. 2. Reported structures of the carbohydrates in T3 and T4, where Neu5Ac presents the acetylated neuraminic acid, Gal the galactose, GlcNAc the *N*-acetylgalactosamine, Man the mannose and Fuc the fucose.

again resulted in a matrix with approximately 2.5 mg protein/ml of gel.

2.4. Immunoaffinity trapping of hCG

About 100 μl of AB1-B108 beads (a total binding capacity of about 20 000 mIU of hCG) was added to each 10 ml urine or PBS-standard sample and the sample rocked for 2 h at room temperature. The beads were allowed to settle from the solution and the supernatants were carefully removed. To each tube containing the beads, 0.8 ml of reduction buffer (containing 6 *M* guanidine HCl, 0.5 *M* Tris and 2 m*M* EDTA) was added and the mixtures were again rocked at room temperature for 30 min. Alternately, the solution and beads after incubation were transferred to a 1-ml polypropylene syringe. In this case, the beads were washed with 1 ml of PBS and eluted with 0.4 ml of the reduction buffer. In some experiments, solutions and beads at each step were counted for radioactivity or assayed with EIA for hCG.

Reduction of the disulfide bonds was accomplished by adding dithiothreitol (5 mg) and incubating the solution for 3 h at room temperature. The resulting cysteine residues were alkylated with vinylpyridine (10 μl) for an additional 3 h. The sample was diluted to 5 ml with 50 m*M* ammonium bicarbonate, pH 8.0, and the reduction buffer salts were then removed by passing the mixture through a C_{18} cartridge which had been previously washed with 1 ml of methanol, 1 ml of H_2O, 1 ml of acetonitrile (0.1% TFA) and 1 ml of 50 m*M* ammonium bicarbonate, pH 8.0. The high concentration of unreacted vinylpyridine was washed out with a 10% methanol–ammonium bicarbonate solution. Elution of the hCG was carried out with 75% acetonitrile in 0.1% TFA. The solution was dried. In some experiments, the solution compositions were varied to determine the location of unrecovered hCG. Both the solutions and the beads were counted for radioactivity.

The final step was reconstitution of each dry sample in 40 μl of 50 m*M*, pH 8.0 ammonium bicarbonate and digestion with 2 μl of 1 mg/ml trypsin solution at room temperature for 3 h. The tryptic digests were then directly injected into the HPLC–MS system for identification and quantitation.

3. Results and discussion

Based on our previous work [4,5], we devised the scheme shown in Fig. 3 for the enrichment and detection of urinary hCG. Since reductive alkylation with vinylpyridine was a key step in preparing hCG for HPLC–MS detection, we designed the extraction scheme to have as many common reagents as possible to minimize transfer and dry down steps. Our selection of the monoclonal antibody (MoAb) B108 was based on its relatively high affinity and its ability to specifically bind intact hCG and the free β-subunit. The MoAb was coupled to the Emphase solid support using the azlactone function groups incorporated into the matrix. The binding of MoAb was essentially quantitative over the range of protein concentrations used. The desired loading of MoAb was determined by operational aspects of solid-phase manipulation rather than binding capacity of the beads. When 110 μg of B108 was coupled to 500 mg (4 ml) of Emphase AB1 beads, the binding capacity, as determined from a Scatchard plot, was 12 000 mIU/ml of beads. This was about 30% of the expected value, based on the amount of antibody used. The measured affinity constant was $1.32 \cdot 10^{10}$ M^{-1}, which is approximately what is observed in solution [12]. Although it has been suggested that the

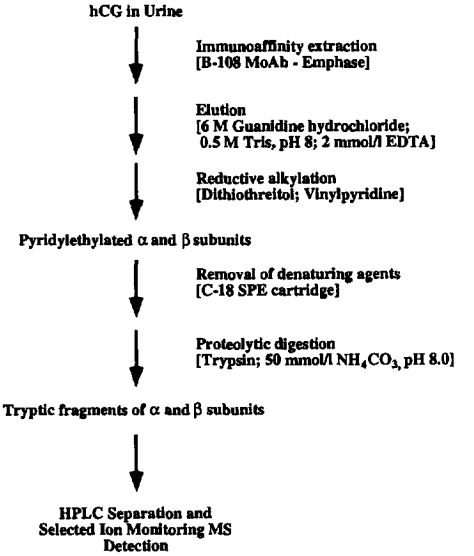

Fig. 3. Schematic diagram of the steps involved in the analysis of urinary hCG.

affinity constant for an antibody used for immunoaffinity chromatography should be about 10^{-8} M^{-1} [16], our interest was in batchwise concentration of trace amounts of protein which benefits from an increased affinity. Based on empirical urine-based hCG binding studies, the amount of MoAb bound to the support was adjusted to give a final capacity of 2×10^{5} mIU/ml of support.

The extraction efficiency of the immunoaffinity matrix was determined both by EIA of hCG in the eluate solutions and by [^{125}I]hCG radioisotope tracer studies. The [^{125}I]hCG as received from Dupont-NEN contained about 50% non-hCG radioactivity. The labelled hCG was purified by reversed-phase HPLC in a non-denaturing mobile phase prior to use. Both the EIA and the isotope tracer studies indicated that recovery of hCG from a urine matrix was 99.5(±0.5)% at 30 mIU/ml and 98.5(±1.0)% at 500 mIU/ml. The binding of hCG to immunoaffinity beads was performed in urine solution without any pH adjustment since the binding efficiency was about the same over the pH range 4–8.5 (data not shown). Initial experiments with unpurified [^{125}I]-hCG led us to test for support matrix effects, but no difference in recovery was found between Emphase AB-1 and Sepharose. The availability of excess binding capacity on the immunoaffinity support was confirmed by a study in which two samples of urine containing 5000 mIU hCG and radioisotopic tracer were added sequentially to the same beads. The beads were able to quantitatively remove all of the hCG from solution.

Guanidine-HCl (GuHCl) is a strong chaotropic reagent [17] and has been widely used for the cleaning of immunoaffinity beads. It was also used for denaturation of the hCG prior to alkylation [4]. We hypothesized that all the bound hCG could be eluted from the affinity matrix with this agent. In the batch mode, more than 90% recovery of [^{125}I]hCG was achieved in the reduction buffer. When the column elution mode was used, the recovery was increased to about 98–99%, probably due to volume retention in the beads in the batch mode. In addition to better recovery, the wash and elution procedure in column mode were much easier than those in batch elution. Increasing the elution volume from 400 μl to 1000 μl had no significant effect on recovery. Since a small elution volume was advantageous for the following reductive alkylation reaction and C_{18} desalting step, the elution volume used was 400 μl. It should be noted that 6 *M* GuHCl can decrease the radioisotope counting efficiency. This quenching effect was taken into account for all recovery studies.

Since the eluted hCG sample was to be digested by trypsin and analysed by HPLC–MS, it was necessary to remove the high concentration of denaturing salt after the reductive alkylation (see Fig. 3). After pyridylethylation, the hCG sample in reduction buffer was directly loaded onto a C_{18} BondElute cartridge. The high concentration salts were washed out with 50 m*M*, pH 8.0 ammonium bicarbonate. The reduced, alkylated hCG was then eluted with 75% acetonitrile in 0.1% TFA. The acidic buffer was chosen for elution after experiments in pH 8 ammonium bicarbonate gave less than 20% recovery, as measured by radioisotope tracer. The unrecovered protein was located on the stationary phase of the SPE cartridge. In the acidic buffer, 31% and 34% recovery were obtained at 30 mIU/ml and 500 mIU/ml concentrations respectively. In an attempt to understand the nature of the irreversible loss process, denatured hCG (not pyridylethylated) was passed through the SPE column and the recovery increased to 75%. We hypothesized that the protein loss was due to precipitation on the hydrophobic stationary phase, and that addition of pyridylethyl groups to the cysteine residues further decreased the solubility of the protein. If the GuHCl solution of reduced, pyridylethylated hCG was diluted with 50 m*M*, pH 8.0 ammonium bicarbonate to a final volume of 5 ml and run through the same SPE desalting procedure, the recovery was increased to 56% and 60% for 30 and 500 mIU/ml, respectively. Attempts to increase the recovery further were not successful. It is possible that at the low protein concentrations used, sample adsorption to the C_{18} beads limited the recovery of the sample. Bakerbond C_{18} SPE cartridges were evaluated to determine whether the loss was specific to the Analytichem SPE, but no improvement was observed. Another possibility under investigation is the use of restricted access extraction materials where the vinylpyridine and guanidine hydrochloride would be retained but the protein excluded from the hydrophobic matrix. We also considered the use of alternative reduction buffer systems. Since vinylpyridine is not soluble in

dilute buffers such as 50 m*M* ammonium bicarbonate, an alternate alkylation reagent might also need to be used. Because of the importance of vinylpyridine to detection of the tryptic fragments, we did not pursue this further.

The HPLC–MS analysis was based on our previous experience on the characterization of hCG by HPLC–MS [4]. The tryptic fragments T3, T4 and T5 were selected for the identification and quantitation of hCG in urine (Fig. 4). Since Wehman et al. [13] have shown that the major excretion product of injected hCG is the intact form, the methodology for detecting athletic urinary hCG should be able to differentiate intact hCG from any other potential interfering species such as hLH, physiological excreted intact hCG, free hCG β-subunit, nicked hCG β-subunit and β-core fragment. In order to demonstrate that hLH does not interfere in this assay, the β-subunit of hLH was assayed using the HPLC–MS method. The substitution of a tryptophan for arginine at position 8 in the peptide chain results in a 17 amino acid fragment (β3–20) rather than T3 (β9–20) from hCG. Thus T3 would be missing from the chromatographic trace of hLH (Fig. 5). Amino acid substitutions in the β44–60 fragment (T5 in hCG) result in mass shift of 30 daltons for hLH, and thus this fragment from hLH would not be detected either.

Other potentially interfering species are physiologically excreted: intact hCG, free hCG β-subunit, nicked β-subunit and β-core fragments. In healthy individuals, the level of intact hCG and free β-

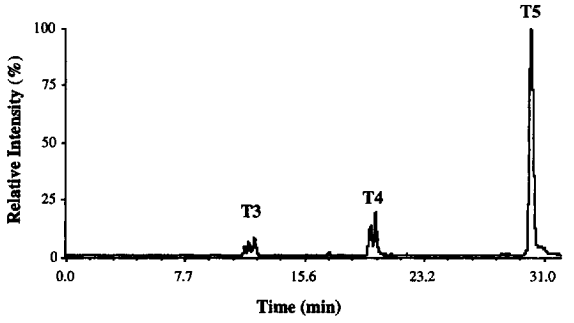

Fig. 4. HPLC–MS chromatogram of selected ion monitoring of T3, T4 and T5 from the tryptic digest of standard hCG β-subunit. The multiplicity of peaks observed for T3 and T4 is the result of the resolution of some of the different oligosaccharides attached to the T3 or T4 peptide backbone.

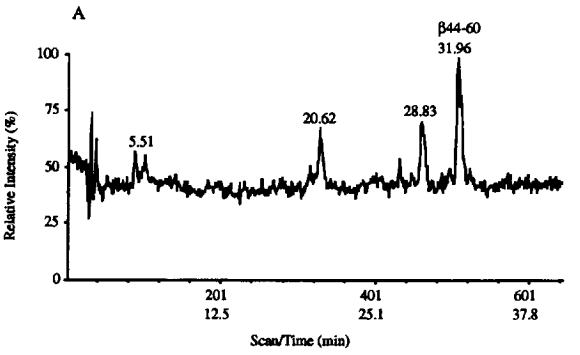

Fig. 5. HPLC–MS chromatogram of the tryptic digest of pyridylethylated hLH. The location of the T3, T4, and T5 peaks monitored for hCG is marked and is notable for the absence of any peak from hLH.

subunit is normally very low (<1 mIU/ml) and will not present a problem in hCG detection. The concentration of nicked β-subunit and β-core fragment, however, could be elevated at abnormal physiological conditions. Since T3, T4 and T5 will be used as the identification criteria, nicking at any other position other than β43–44 can be differentiated by HPLC–MS (see Fig. 1 for amino acid sequence references). For example, when a nicking occurs at β44–45, two fragments, T4 and β45–60 will be produced after trypsin digestion. T5 will not be observed. The relative intensity of T5 to T4 or T3 could also be used to differentiate Pregnyl from other nicked β-subunits. Similarly, only β21–40 and β55–60 will be produced when β-core fragment is digested with trypsin. Therefore, HPLC–MS analysis based on these three tryptic fragments will provide a specific method for the determination of hCG administration.

In order to increase the detection sensitivity, the mass spectrometry was performed in selected ion monitoring mode. The monitoring of smaller number of ions will allow a longer data acquisition time for each ion, therefore a higher signal-to-noise ratio should be achieved. All three fragments were monitored simultaneously, requiring a total of ten ions to be monitored. The dwell times were limited to 50 ms/ion in order to achieve reasonable sampling across the chromatographic peaks. The use of time-programmed SIM to extend the dwell time for each ion, which was not compatible with our computer

hardware, should allow improvement of the signal-to-noise ratio.

Before running the hCG urine extraction sample, a standard hCG β-subunit tryptic digest was injected and analyzed by multiple ion monitoring so that the retention times for these three fragments could be obtained. Three peaks were observed at about 12 min, 20 min and 30 min which corresponded to T3, T4 and T5 respectively (Fig. 4). A formic acid-containing mobile phase was used because it increased the MS sensitivity by four-fold compared to the corresponding TFA-containing mobile phase [4]. Note also that the various oligosaccharide chains (microheterogeneity) at T3 and T4 were separated using this formic acid-containing mobile phase system. This could further help the confirmation of hCG based on the relative ratio of the oligosaccharide chains. When a blank sample with no Pregnyl spiked was treated through the whole process, no signal was found at 12 min and 20 min. This indicated that no interference was observed for the detection of T3 and T4 from the urine background. However, an intense peak from the urine matrix was observed at 30 min which interfered with the detection of T5. If this signal came from an impurity in the mobile phase, its accumulation during the separation process could give a false signal which affect the quantitation of hCG in urine. Extensive washing of the column with a high percentage of methanol did not completely remove this peak. When hCG concentration was greater than 50 mIU/ml, the ion intensity showed a sharp increase. When a urine sample spiked with Pregnyl was analyzed, two major ions, m/z 1209.5 and 1258.5, were observed for T3, corresponding to T3 attached with N1 and N3 respectively (see Fig. 2 for *N*-linked carbohydrate structures). No significant signal was observed for fragment T3 attached to N4. For T4, only peptide fragment T4 attached with N1 (m/z 1302.5) was present in high abundance.

Due to the decreased response from glycopeptides, a non-glycosylated peptide should be most useful for quantitation. Since the detection of T5 was interfered with by the background signal, it may be possible that, by adding a low concentration of volatile buffer such as ammonium acetate to the mobile phase, the retention time of T5 will shift to a position where no interference ion from the background is observed. Therefore, the background interference might be eliminated. Another approach is to monitor another tryptic fragment which is unique to hCG, such as T(9+10). If there is no background interference to this fragment, it could be used for quantitation. At present, no quantitative evaluation of hCG was attempted. Spiking 25 mIU/ml of hCG into a blank urine resulted in a signal that could be clearly distinguished from background with a signal-to-noise ratio of 12:1.

In summary, our results show that peptide hormones such as hCG can be detected and confirmed in urine using electrospray HPLC–MS. Matrix-assisted laser desorption mass spectrometry has also been used to characterize the tryptic fragments of hCG [18]. By judicious selection of the pyridylethylated tryptic peptides monitored, interference from other peptide hormones such as hLH can be avoided due to differences in mass and tryptic fragment length. At present, detection of 25 mIU/ml of hCG can be achieved using 10 ml of urine. We anticipate that improvements in desalting methods to improve the recovery of hCG through the entire cleanup procedure and in the selected ion data acquisition should also improve the limit of detection for hCG to the 10 mIU/ml limit suggested by Laidler et al. [19].

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