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Immunoaffinity extraction and tandem mass spectrometric analysis of human chorionic gonadotropin in doping analysis

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Abstract

A confirmatory and quantitative HPLC–tandem mass spectrometry (MS–MS) method for human chorionic gonadotropin hormone (hCG) at concentrations as low as 5 IU/l following immunoaffinity extraction of the glycoprotein from urine was developed. The extraction method involved retention of urinary hCG in the immunoaffinity column via specific antigen–antibody interaction. A variety of eluents were then used to quantitatively elute hCG from the immunoaffinity column. Qualitative and quantitative analysis of hCG were undertaken using MS–MS by identifying the amino acid sequence of the marker peptide βT5 obtained from hCG by tryptic digestion and the peak areas of three product ions b_6^+, b_9^+ and y_{11}^+, respectively.

Keywords: Chorionic gonadotropin; Glycoproteins

1. Introduction

Human chorionic gonadotropin (hCG) is a glycoprotein hormone produced abundantly by the placenta during pregnancy. It is made up of two dissimilar subunits, alpha (α) and beta (β). Other hormones that belong to the same family with hCG are follicle stimulating hormone (FSH), lutropin (LH) and thyroid stimulating hormone (TSH) [1]. These hormones share similar α-subunits, but differ in the β-subunits. The variation in the β-subunits is especially significant for hCG which has an extension of a highly glycosylated region at its carboxyl terminal [2], and this has not only enabled the raising of antibodies specific for hCG [3] but has also provided advantages for its characterization from other glycoproteins via mass spectrometry (MS) [4,5].

The molecular masses of hCG, α-hCG and β-hCG are approximately 36 700, 14 500 and 22 200 [4], respectively. The carbohydrate is estimated to account for 30% of the total mass of the intact molecules; α-hCG contains two N-linked oligosaccharides, whereas the β-hCG contains two N-linked and four O-linked oligosaccharides [5,6].

hCG has been misused by male athletes to induce the secretion of testosterone from the testis. Its administration stimulates endogenous testosterone secretion without affecting the T/E (testosterone/

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epitestosterone) value, a criteria used for detecting exogenous testosterone administration [6]. Although the administration of hCG has been banned by the International Olympic Committee (IOC) in 1987, a definitive method has not been decided by the IOC for its detection [7]. Currently, immunoassay is the only recognized method for the detection of hCG in doping analysis. However, immunoassay does not provide evidential criteria because of its cross reactivity with other hCG-like materials and thus lack discriminatory power [8]. Alternative method such as tandem mass spectrometry (MS–MS) for analysis of performance-enhancing compounds was suggested [9].

For hCG doping control procedure to be accepted by the IOC where cut-off value was suggested to be at 10 IU/l [13], the analytical technique must be rapid, reliable, selective, specific and sensitive. Mass spectrometric detection requires sample clean-up especially for analytes in biological matrices such as urine or blood. Immunoaffinity clean-up procedure, which involves extraction of the analyte concerned by using a suitable solvent following the immobilization of selected antibodies onto a solid support, allows trace enrichment of hCG in a single step. This is crucial as complicated multi-step purification procedures might result in low overall yields of hCG. The confirmation of hCG was performed using MS–MS where the amino acid sequences of tryptic peptides of hCG were determined.

This study describes the high-performance liquid chromatography (HPLC)–MS–MS analysis of hCG peptides digested using trypsin enzyme, from which a peptide marker was selected. Immunoaffinity column procedure, employing polyclonal antibodies immobilized onto Sepharose for urinary hCG extraction and subsequently confirmatory and quantitative analysis by using MS–MS was developed. The use of peptide βT5 marker for the identification of hCG was also investigated.

2. Experimental

2.1. Chemicals

Human chorionic gonadotropin (lot CG-10), cyanogen bromide-activated sepharose 4B, guanidine hydrochloride, Tris base, sodium azide, ethanolamine, L-1-tosylamide-2-phenylethylchloromethyl ketone (TPCK) treated bovine pancreas trypsin and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, USA). Trifluroacetic acid (TFA), acetic acid and formic acid were obtained from R&M (Essex, UK) and Ajax (Sydney, Australia), respectively. Sodium hydroxide and citric acid were from J.T. Baker (Phillipsburg, NJ, USA) while HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). Dithiotreitol, iodoacetic acid, sodium dihydrogenphosphate (dihydrated) and magnesium chloride were obtained from Fluka (Buchs, Switzerland). Polyclonal antibodies against hCGβ raised in rabbits for the preparation of the immunoaffinity column were purchased from Sigma. An IMX hCG kit was purchased from Abbott Laboratory (Abbott Park, IL, USA). Ultra-pure water collected from an ELGA deioniser (Bucks., UK) at 18.2 MΩ was used throughout the experiment.

2.2. Equipment

The following equipment was used: ion trap mass spectrometer, LCQ Classic, Finnigan MAT (San Jose, CA, USA); HPLC system, bin pump and degasser, Hewlett-Packard series 1100 (Palo Alto, CA, USA); incubation oven, UBS, Memmert (Schwabach, Germany); freeze dryer, CHRIST LDC-2, B. Braun (Osterode am Harz, Germany), syringe pump, A-9606, Harward Apparatus (Hallisston, Canada). Centrifuge, J6/M1, Beckman (Palo Alto, CA, USA).

2.3. Digestion of hCG using trypsin

A 100-μg amount of hCG was desalted using a protein concentrator column (C_{18}, 5 μm, 300 Å, 2 cm×4.6 mm I.D., Jones Chromatography). A 65-μl volume of denaturing buffer (6 *M* guanidine–HCl, 0.5 *M* Tris, 2 m*M* EDTA, pH 8.6) and 10 μl of 1 *M* dithiotreitol were added to the desalted hCG and incubated at 37 °C for 30 min. A 25-μl volume of 1 *M* iodoacetic acid in 1 *M* NaOH was then added and the mixture was further incubated for 30 min at room temperature. Excess reagents were removed from the protein sample using the protein concen-

trator column. The recovered hCG was then reconstituted in 50 μl of 50 m*M* NH_4HCO_3 solution and trypsin was added at a 50:1 protein-to-enzyme ratio, incubated at room temperature for 20 h followed by another addition of the same amount of trypsin. The mixture was further incubated for another 4 h at room temperature, making the final protein-to-enzyme ratio 25:1. The digested protein was lyophilized and stored at −20 °C before analysis.

2.4. HPLC separation

The tryptic peptides of hCG were reconstituted in 50 μl of deionized distilled water. A volume of 2 μl of the sample was injected into reversed-phase column (C_{18}, 300 Å, 5 μm, 250 mm×1 mm I.D., Vydac) and chromatographically separated using HPLC. The flow-rate was set at 1 ml/min and then split by a splitter to a 50 μl/min flow-rate. Mobile phase A was 0.05% TFA in deionized water and B was 0.05% TFA in acetonitrile. The gradient used was at 10–60% B for 60 min and held constant at 60% B for 5 min. The HPLC system was interfaced to an ion trap mass spectrometer.

2.5. Mass spectrometry

MS analysis was carried out using in the electrospray ionization (ESI) mode. The MS data were acquired at heating capillary temperature 200 °C, sheath gas flow-rate was 60 arb, spray voltage at 4 kV, tube lens offset was −60 V and the capillary voltage was at 38 V. The triple plays experimental method consisting of full scan MS, zoom scan and MS–MS scan was created for the analysis. Known molecular ions of hCG peptides were programmed to the parent ion list of data dependent scan (zoom scan and MS–MS scan). The parameters of the data dependent scan were default collision energy of 25, charge state of 2, minimum signal acquired was $1 \cdot 10^5$ counts, isolation width was 2 *m*/*z*. The masses of the tryptic peptides and product ions were calculated using PAWS program (version 8.5, freeware edition).

2.6. Preparation of immunoaffinity columns

The standard procedure provided by the manufacturer for coupling of antibody to cyanogen bromide-activated Sepharose 4B was followed without modification. About 2 ml of the antibody-coupled gel was packed in a polypropylene cartridge (230 mm×2.5 mm I.D., Bio-Rad) covered with PTFE fittings at both ends. The column was stored in 0.01 *M* phosphate-buffered saline (PBS) (7.5 m*M* Na_2HPO_4, 2.5 m*M* $NaH_2PO_4 \cdot 2H_2O$ and 0.2 *M* NaCl, pH 7.2) containing 0.1% BSA and 0.02% NaN_3.

2.7. Determination of column binding capacity

The columns were loaded with 3000 IU/l of hCG, incubated for 20 min and then washed with 0.1 *M* PBS (pH 7.2) containing 0.1% Tween 20. Washed fractions were collected and assayed for unbound hCG. The loading of the column was repeated until the column was unable to retain any more hCG. The difference in the amount of hCG loaded on column and the amount of unretained hCG gives an estimation of the column binding capacity.

2.8. Extraction procedure utilizing the immunoaffinity column

Approximately 11 ml of hCG-spiked urine was centrifuged at 1500 rpm for 5 min to remove particulate matter. An aliquot of 10 ml of the centrifuged urine was transferred to a clean polypropylene tube for hCG extraction. The immunoaffinity column was first flushed with 6 ml of distilled water to remove the storage buffer and then conditioned with 5 ml of 0.01 *M* PBS (pH 7.2). During this step, the column flow was adjusted to 7–9 drops/min. A 2-ml volume of the centrifuged urine was loaded onto the 2-ml immunoaffinity column. The sample was allowed to stand in the column for 20 min to allow antibody–antigen association to occur. The urine was then removed from the column by flushing with another 2 ml of 0.01 *M* PBS (pH 7.2). This process was repeated until all 10 ml urine had passed through the column. Finally, the column was washed with seven bed volumes of washing buffer (0.1% Tween 20 in 0.1 *M* PBS, pH 7.2) followed by 2 ml of elution buffer (1 *M* citric acid adjusted to pH 2.2 with 10 *M* NaOH). After the first 1 ml of the elution buffer had entered the gel, the collection of the eluate began. When all 2 ml of

the elution buffer had fully immersed in the gel, a 5-min equilibration time was allowed to dissociate the antibody–antigen complex completely. This was followed by 7 ml of elution buffer. A total of 9 ml eluate was collected. The pH of the eluate was then adjusted to 6.5–7.5 with 10 *M* NaOH. hCG recovery in the neutralized eluate was determined using the IMX hCG kit. The column was regenerated by first flushing with 0.1 *M* PBS (pH 7.2) until neutral followed by 0.01 *M* PBS (pH 7.2) before storing in 0.01 *M* PBS containing 0.1% BSA and 0.02% NaN_3.

When hCG was analyzed using MS, the neutralization step in the extraction procedure was omitted. However, the eluate from the immunoaffinity column was concentrated and desalted using the protein concentrator column; a syringe pump was used to pump the protein solution through the column at 1 ml/min, the column was then flushed with 10 ml distilled water and the protein was recovered with 0.1% formic acid in acetonitrile–water (70:30). The eluted protein was dried under a stream of nitrogen at 37 °C. The dried protein was digested with trypsin according to the method described above except a total of 1 μg of trypsin enzyme was added.

2.9. Quantitative analysis of hCG marker

The tryptic-digested hCG was first reconstituted in 25 μl of high-purity distilled water. An aliquot of 10 μl of the sample was then injected into a reversed-phase column (C_{18} 300 Å, 5 μm, 150 mm×1 mm I.D., Vydac) and separated chromatographically using HPLC. The flow-rate set at 1 ml/min was further split by a T-splitter to 20 μl/min through-column. Mobile phases A and B were 0.05% TFA in water and 0.05% TFA in acetonitrile, respectively. The gradient used was 5–95% B for 20 min and held at 95% B constant for 5 min. The HPLC system was interfaced to an ion trap mass spectrometer.

Data dependent experimental method consisting of full scan MS and MS–MS scan was created. Double charged molecular ion for βT5 peptide, $[964.3]^{2+}$ was programmed as the parent ion for data dependent MS–MS scan. The parameters set for data dependent MS–MS scan were collision energy of 25, charge state of 2, minimum signal acquired of $1{\cdot}10^4$ counts, and the isolation width of 2 *m*/*z*.

3. Results and discussion

3.1. Selection of hCG marker

Development of qualitative and quantitative analysis for hCG via mass spectrometry involved the selection of a hCG peptide marker formed from tryptic digestion of the glycoprotein. Although peptides derived from the β-subunit of hCG were investigated for their suitability as hCG marker, the selected marker of hCG must be distinctly different from the corresponding β-subunit of LH as the amino acid sequences of the β-subunits for LH and hCG are very similar.

The total ion chromatogram (TIC) of the hCG peptides analysis is shown in Fig. 1. The labeled peptides were identified and confirmed by zoom scan and MS–MS scan, the unlabeled peaks were resulted from autodigestion of trypsin. Amongst the peptides of β-subunit origin that were detected and were found not suitable to be used as marker were βT6, βT7 and βT8, which has the identical amino acid sequences as peptides derived from LH; βT1+2, which was formed via incomplete trypsin digestion on β-hCG; nicked hCG tryptic fragment, tryptic digestion of nicked β-hCG fragment where nicking occurs at position between 47 and 48 amino acid residue and is cleaved by enzyme human leukocyte elastase [11]; βT12, which has weak molecular ions intensities and βT9, which molecular ions may probably composed of metastable ions causing anomalous detection of the peptide by MS. Two glycopeptides (βT3 and βT4) derived from the β-subunit were also detected, however the MS–MS data obtained from these glycopeptides were unable to confirm their amino acid sequences, which makes the glycopeptides not a suitable marker for this study.

Peptide βT5, whose presence as the most abundant ion in the TIC (Fig. 1) appears to be the most reliable marker for hCG with regard to the uniqueness of its sequence. The chosen parent ion of peptide βT5 that exists at high ion intensities is also an advantage to the quantitative method as it reduces the detection limit of hCG.

Fig. 2 shows the spectrum of MS scan, Zoom scan and MS–MS scan for peptide βT5. The full MS scan data show that peptide βT5 was ionized mostly to

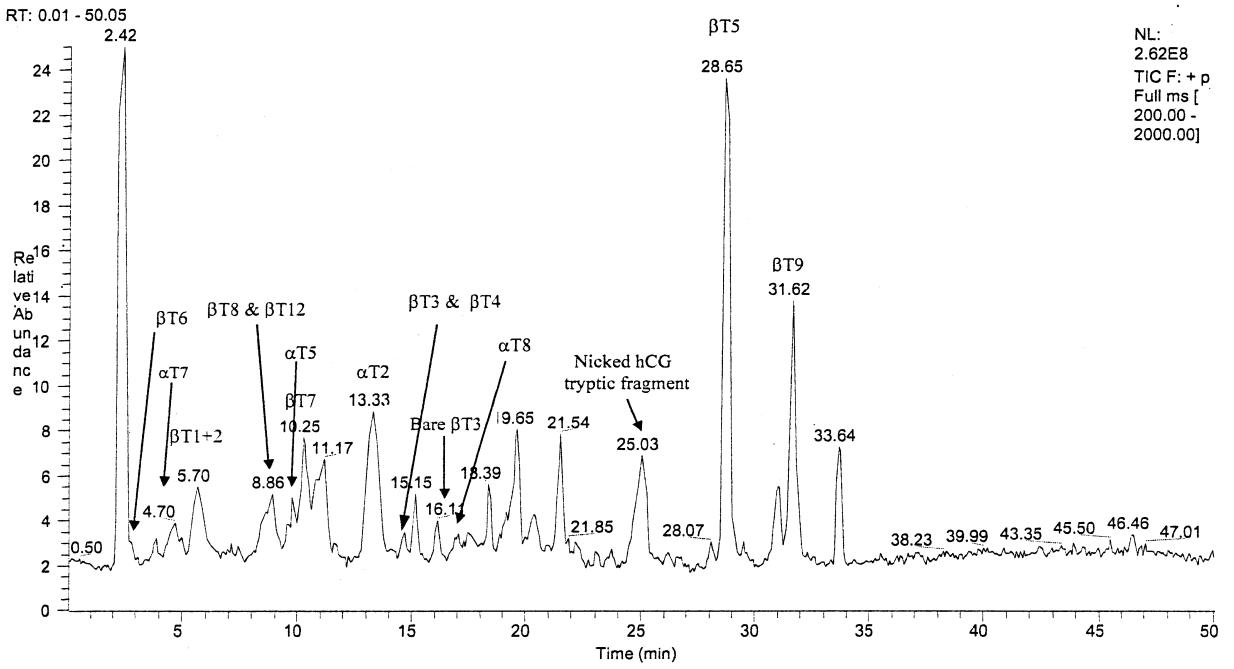

Fig. 1. Total ion chromatogram (TIC) for tryptic peptides of hCG.

double charged ion at m/z ratio 964.3 compared to single (1927.6) or triple charged (643.2) ions. Zoom scan spectrum confirmed the double charged state of the 964.3 ions where the separation of C^{12}/C^{13} isotopic ratio of the two adjacent peaks was 0.5 m/z. In addition, the amino acid sequence of βT5 was confirmed as VLQGVLPALPQVVCNYR by its product ions spectrum revealed in the MS–MS scan. The MS–MS scan of peptide βT5 produced comprehensive product ions spectrum that provides the information of its amino acid sequence.

As the amino acid sequence of the peptide, which is revealed in the product ion spectrum was used as the basis for the development of hCG confirmatory method, the long sequence (17 amino acid residues) of peptide βT5 enhances the specificity of the method and therefore eliminates the occurrence of false positives results. Besides, the m/z ratio of the parent ion of peptide βT5 at 964.3 reduces interferences from chemical noise, which occur at low m/z ratios.

The used of the peptide βT5 as a marker in hCG doping analysis has an added advantage as the surface loop of peptide βT5 in hCG molecule interacts with the hCG receptor in the testis to stimulate steroidogenesis [4,11,12]. Thus, any change in the amino acid sequence within this region may reduce or obliterate steroidogenic activity, which may not bring about the desired doping effects. The used of peptide βT5 as hCG calibrant and marker were also being highlighted in the studies conducted by Laidler et al. [4] and Liu and Bowers [5], respectively.

On the other hand, peptide βT5 of LH (VLQAVLPPLPQVVCTYR), which differs from peptide βT5 of hCG by three amino acids (underlined) presents a completely different molecular ion mass and product ions spectrum. Thus, any contamination from LH in hCG samples will be easily determined. Given the reasons above, peptide βT5 was found to be the most suitable qualitative as well as quantitative marker for hCG.

3.2. Immunoaffinity extraction method

The concentration of human urinary hCG a day after administration of 5000 IU hCG may rise to 200 IU/l and drop thereafter to an undetectable con-

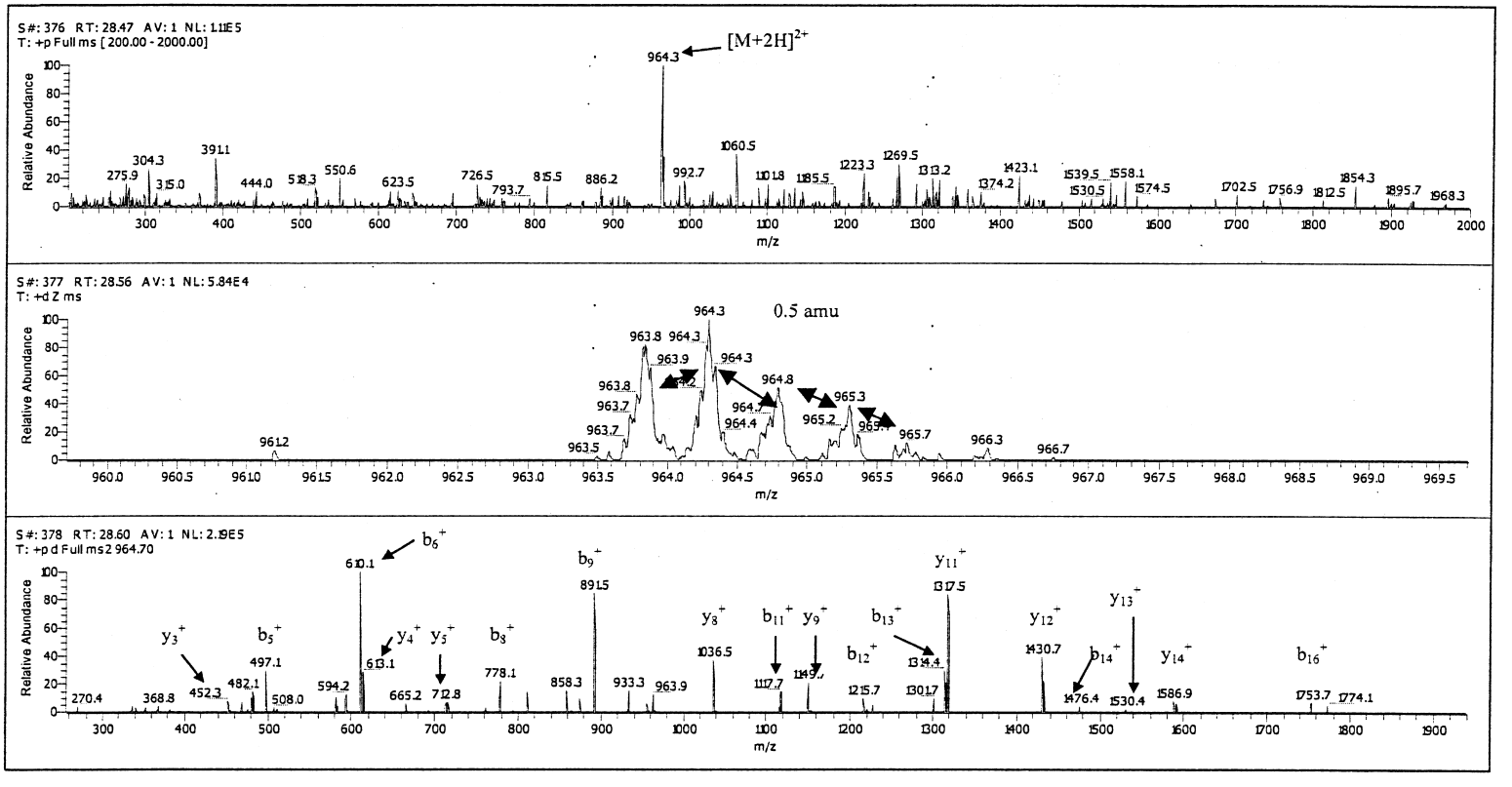

Fig. 2. Mass spectrum of the double charged ions of peptide βT5; upper panel: full scan MS, middle panel: zoom scan MS, lower panel: full scan MS–MS.

centration by the 13th day [10]. The cut-off value for hCG in doping analysis has been set at 25 IU/l, however Laidler et al. [13] suggested that this value should be reduced to 10 IU/l to eliminate false negative results. At this concentration range, an extraction method that combines both sample clean-up as well as trace enrichment of hCG from urine is highly desirable especially when the mode of detection is MS. Immunoaffinity chromatography which retains compounds selectively via specific antibody–antigen interactions will be hence the method of choice for sample preparation. This highly specific and selective method which does not involve numerous steps is relatively simple to perform.

An immunoaffinity column with a 2-ml volume capacity was packed in our laboratory, the antibody-coupling efficiency and the specific antibody-binding capacity for the column were found to be 2.93 mg Ab/2 ml gel and 1785.5 mIU hCG/mg antibody, respectively whilst the hCG-binding capacity for the column was 5231.6 mIU hCG/column. The column binding capacity would allows quantitative extraction of 10 ml (0.01 l) urine at the hCG concentration of 200 IU/l, which is also the highest concentration of hCG that may be found in urine after the administration of hCG. For the method developed here, 10 ml urine/sample was subjected to hCG doping analysis.

The optimum incubation period for antibody–antigen association was approximately 20 min. A decrease in the incubation period resulted in incomplete column retention of the hCG loaded within the column capacity. The optimum equilibration period for dissociation of antibody–antigen was 5 min. Continuous elution without equilibration required 17 ml of eluent to completely elute hCG from the immunoaffinity column compared to 9 ml of eluent used with equilibration.

The elution profile of hCG from the immunoaffinity column shows that out of the total 9 ml eluate collected, the third ml of eluate was found to contain the highest percentage (~40%) of the recovered hCG with the percentage of hCG decreasing gradually in the subsequent fractions to the 10th ml of eluate, which was undetectable.

A study conducted to measure column life span showed that a properly maintained column could be used for at least 55 extraction cycles before showing signs of reduced binding activity. However, for the first four extractions of hCG from urine using new immunoaffinity columns, the recoveries were lower as compared to the subsequent extractions. This could possibly be due to irreversible binding of hCG to immunoaffinity columns.

Ten different eluents comprising chaotropic salts, organic chaotropes and eluents of extreme pH were tested for eluting hCG from the immunoaffnity column. The choice of an eluent in immunoaffinity extraction was based on three criteria namely: (i) maximum column reusability, (ii) recovery of hCG in the smallest volume possible and (iii) rapid elution process. Table 1 shows that 1 *M* citric acid (pH 1.3), 1.5 *M* citric–NaOH (pH 2.2) and 1 *M* citric–NaOH (pH 2.2) were the eluents of choice as they fulfilled the second stated criteria. However, 1 *M* citric–NaOH (pH 2.2), which is the mildest eluent with minimal detrimental effects on the column, was selected. The recovery of hCG using 6 *M* guanidine–HCl was not determined due to the loss of hCG activity upon treatment with the denaturant. Besides, 6 *M* guanidine–HCl is highly viscous causing extremely slow flow-rate, thus prolonging the extraction process. The acidic eluent used may cause the dissociation of the hCG into its subunits, thus reducing the activity of hCG. To restore its activity, the pH of the eluate was adjusted to neutral before hCG was assay using IMX and fluorescence detection. The quenching of hCG activity by the

Table 1
Eluents that were used to elute hCG from the immunoaffinity column

Eluent	pH	hCG recovery in 9 ml (%)
1 *M* Citric acid	1.3	>99
1.5 *M* Citric–NaOH	2.2	>99
1 *M* Citric–NaOH	2.2	>99
0.5 *M* Citric–NaOH	2.2	48
2 *M* Acetic acid	2.2	60
0.1 *M* TFA	1.7	70
2 *M* Glycine–HCl	2.2	10
4 *M* $MgCl_2$	6.5	85
2.5 *M* NaI	7.5	6
3 *M* NH_4SCN	7.5	10
6 *M* Guanidine–HCl		Not detectable

1 IU of hCG was loaded onto the immunoaffinity column, different eluents were used to quantitatively elute hCG from the column. Incubation time was 20 min and equilibration time was 5 min, the smallest volume of eluent needed for complete elution of hCG from the column is 9 ml.

eluent was taken into consideration when determining hCG recovery.

For a 2-ml capacity immunoaffinity column, five incubation cycles were necessary to extract hCG from 10 ml of urine. An experiment was performed to extract 10 ml of 370, 150 and 100 IU/l hCG using five incubation cycles at 2 ml urine/cycle. For all hCG concentrations tested, no hCG was detected in the column wash indicating complete retention of hCG by the column. The volume of eluent used to recover hCG from the immunoaffinity column is proportional to the amount of the hCG retained by the column. At an eluent volume of 9 ml, the recoveries achieved in three experiments were approximately 95, 99 and 99% for 370, 150 and 100 IU/l hCG concentrations, respectively.

The whole immunoaffinity extraction process developed can efficiently extract hCG from 10 ml urine within 3.5 h. An increase in the column gel volume in order to reduce extraction time resulted in an undesirable increase in the elution volumes.

As the mode of immunoaffinity column extraction is through the specific antibody–antigen interactions, using of controls besides hCG was not possible in this study. However, the problem can be overcome by the use of fully calibrated hCG as internal standards in between extractions of hCG samples.

3.3. Qualitative and quantitative analysis of hCG using product ions marker

The method developed here provides both qualitative and quantitative information in a unique product ions spectrum for any marker. The qualitative data of hCG were revealed in the product ions spectrum produced by MS–MS of marker peptide βT5. The product ions spectrum provides information on the peptide's amino acid sequence to avoid the possibility of producing false positive results, which is more likely to occur in the selected ion monitoring (SIM) data acquisition method as it depends solely on the acquisition of the molecular ion masses and their retention times that may result in the loss of qualitative information of the compounds.

The quantitative data of hCG were obtained from the extracted product ions chromatograms of peptide βT5 which quantify the intensity of the selected product ions of the peptide. The quantitative method using product ions spectrum allows relatively low quantitation limit compared to the SIM method as the MS–MS scan eliminates all the chemical noises. Three of the most abundant product ions of peptide βT5, namely b_6^+, b_9^+ and y_{11}^+ with the m/z ratios of $[610.3]^+$, $[891.5]^+$ and $[1317.8]^+$, respectively, were chosen as the quantitative markers. These ions can be evaluated by their individual peak areas (Fig. 3). The peak ratios by area for b_9^+/b_6^+ and y_{11}^+/b_6^+ were 0.58 ± 0.06 and 0.98 ± 0.02, respectively, in the range of 5–30 IU/l hCG concentration ($n = 18$). An advantage of monitoring three independent quantitative data points obtained from a sample is that it provides more proves for positive doping case, which gives greater protection to the athlete for false positives due to interferences. Quantitation limit can be also reduced by using the summation of three product ions intensity as the signal-to-noise ratio is increased 2–3-fold. Table 2 shows the quantitative data used for constructing a standard curve based on b_6^+ product ions, the intercept was $-457\,221$, x variable was 1 628 623 and r^2 value was 0.999647. Nevertheless, the negative systematical error displays in curve may need further technical improvement.

The method described here enabled trace enrichment and mass spectrometric analysis at 5 IU/l urinary hCG concentration with minimal if any, background interferences that display in the product ion chromatogram. Furthermore, the qualitative and quantitative analysis of hCG was performed simultaneously in single analysis. Further improvement of the method may result in a quantitative method that satisfies the IOC requirement for the detection of misuse of hCG by athletes in sports.

4. Conclusion

The immunoaffinity extraction procedure developed enables quantitative, rapid and selective isolation of hCG from crude urine. The high reusability of the immunoaffinity column has made it a relatively economical extraction method. The specificity of data dependent mass spectrometry method allows the confirmation of hCG to be performed with confidence. The product ions spectrum of peptide

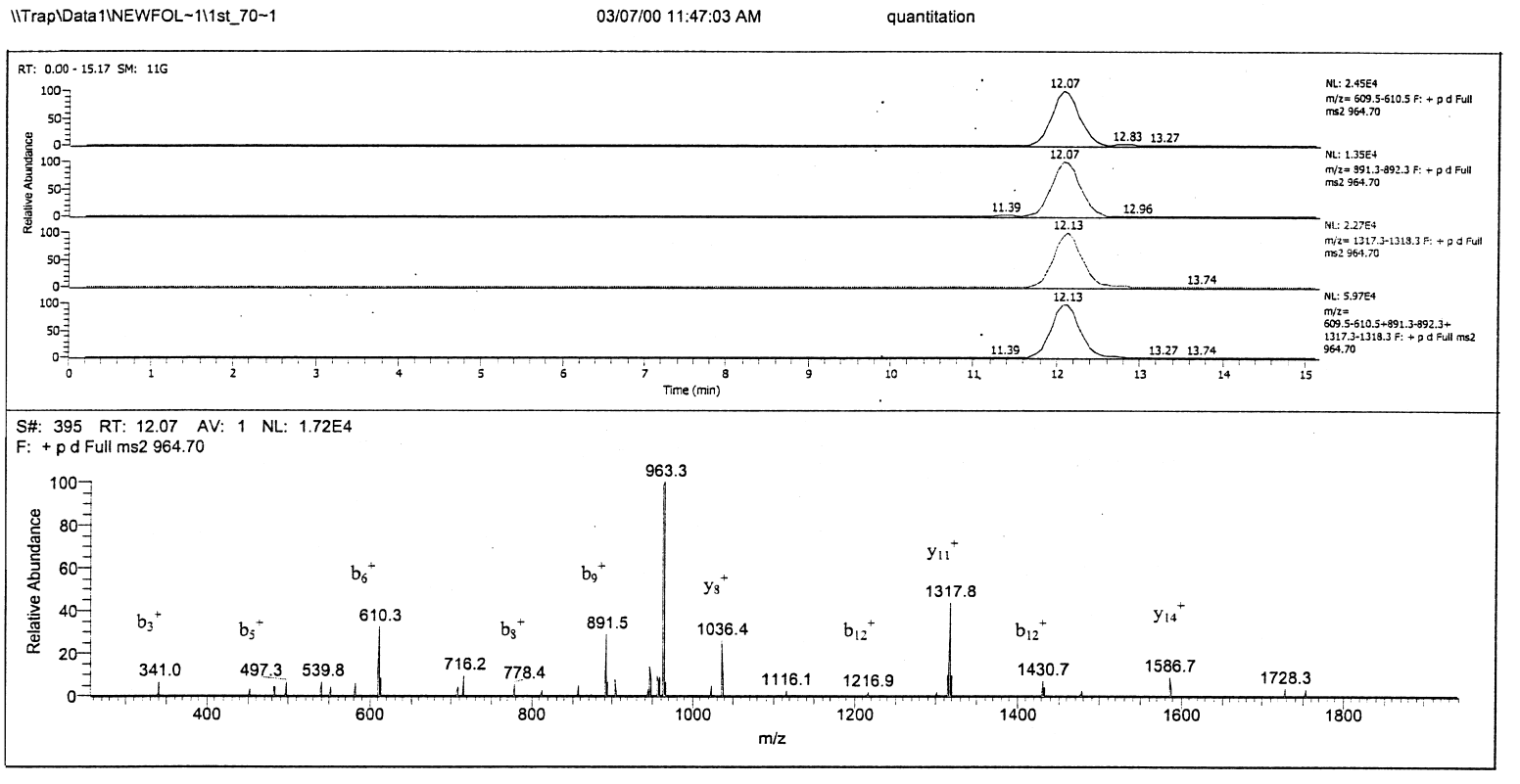

Fig. 3. Data of quantitative and qualitative analysis of hCG at 5 mIU/ml. Upper panel: extracted product ions chromatogram of βT5; lower panel: qualitative data confirms the amino acid sequence of peptide βT5, the selected marker for hCG.

Table 2
Standard curve data quantify using the b_6^+, $[610.0]^+$ product ion

hCG (mIU/ml)	Peak area			Mean	SD	RSD (%)
	(i)	(ii)	(iii)			
5	364 576	395 481	367 517	375 858	17 057.52	5
8	856 239	894 572	826 756	859 189	34 004.11	4
10	1 173 659	1 127 543	1 055 078	1 118 760	59 776.41	5
15	1 995 623	1 973 176	2 060 943	2 009 914	45 595.35	2
20	2 748 931	2 865 973	2 762 191	2 792 365	64 090.25	2
30	4 468 624	4 487 696	4 341 096	4 432 472	79 706.43	2

Different concentrations of hCG were spiked in control urines, extracted using the immunoaffinity column and digested with trypsin. The hCG peptides were then chromatographically separated and analyzed using LC–MS–MS. Product ions spectra were obtained and the intensities of the b_6^+ product ions were calculated from their product ions chromatograms.

βT5, a specific fingerprint for hCG, is also very useful in identifying and confirming the presence of hCG in urine. This specificity is particularly useful for developing rapid quantitative assays for hCG. Hence, the current method enables quantification and confirmation of urinary hCG at as low as 5 IU/l.

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