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THE BITTER-SWEET DIFFERENCE BETWEEN RECOMBINANT AND ENDOGENOUS HCG – preliminary data –

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Summary – The gonadotropins luteinizing hormone (LH) and chorionic gonadotropin (hCG) stimulate gonadal steroid production, both in women and men. hCG is also used by some athletes to stimulate testosterone production and to normalize the testosterone/epitestosterone ratio during or after administration of testosterone and other anabolic steroids. The International Olympic Committee (IOC) imposed a ban on hCG in sports in 1987. Analytical procedures on this subject rely solely on immuno-affinity quantification (ELISA, etc.), and different assay methods for determining hCG in urine are poorly standardized for which results obtained may vary significantly. Furthermore, in 2001 recombinant hCG from CHO cells was introduced by Serono to complement the endogenous preparation from Organon (Pregnyl). As an important part of hCG is made up of carbohydrates (about 30%) and glycosylation is a post-translational process that is not directly genetically regulated, structural differences between both may be confined in this portion of the molecule and hence permit an unambiguous differentiation. In this study we have evaluated the structural properties of both endogenous and exogenous hCG at the peptide and glycosidic level. Following SDS – PAGE separation, bands corresponding to the α-subunit and β-subunit were digested with either trypsin or recombinant PNGase F. Peptides, analysed directly after digestion and purification using in-house made reversed phase tips revealed a possible difference in site-occupancy between endogenous and recombinant hCG at one of the asparagines of the β-subunit. N-glycans were purified and labelled with an aromatic functionality prior to mass spectrometric analysis. From these data it may be concluded that in both species bi-antennary glycans predominate and that the processing of the α-subunit N-

glycans is more efficient in CHO cells as no hybrid-type glycans were present in the recombinant hCG whereas these species were the most abundant in the endogenous molecules.

Introduction

Follicle stimulating hormone (follitropin, FSH), thyroid stimulating hormone (thyrotropin, TSH), and luteinising hormone (lutropin, LH) make up the pituitary glycoprotein hormone family. A fourth member consists of the placental hormone chorionic gonadotropin (choriogonadotropin, CG). All four are composed of a non-covalent heterodimeric complex: *i.e.* a α-subunit and a β-subunit (figure). As the amino acid sequence of the α-subunits are identical, the hormonal specificity is attributed to the β-subunit.

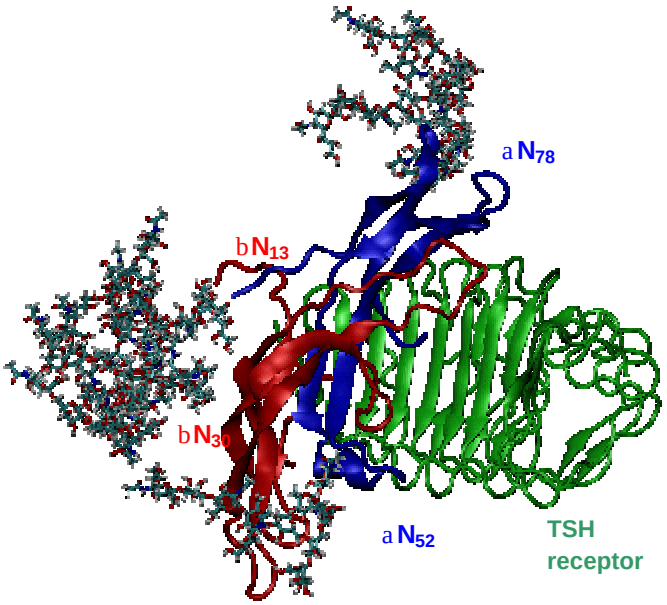

Figure 1. Model of hCG (α chain in blue and β chain in red. The four N-glycan chain are modelled-in with the GlyProt function (www.glycosciences.de) and indicated in the figure) interacting with the extracellular domain of the FSH receptor. The model was assembled with VMD software (v 1.8.3)[1].

Chorionic gonadotropin has the role during early pregnancy to stimulate the *corpus luteum* to produce progesterone until the placenta itself acquires the ability to produce this pregnancy sustaining steroid[2]. Whereas FSH and TSH have their own receptor, both LH and CG interact with the same lutropin/choriogonadotropin receptor, a 7-transmembrane domain G-protein coupled molecule[3]. As such CG may stimulate the Leydig cells in men, giving rise to testosterone secretion. Hence, hCG was banned by the IOC in 1987 (males only), and recently (2005) the ban was extended to females by the WADA[4]. The pharmaceutical preparation of CG is available both as a purified product from human urine (Pregnyl, Organon) or as a recombinant molecule, expressed

in CHO cells (Ovitrelle, Serono). Both products have been attributed different properties related to the different degrees of purity and also due to structural differences. The working hypothesis is that these differences reside in the carbohydrate moiety and that unveiling these should provide unambiguous handles for discriminating between endogenous and exogenous CG.

Materials and Methods

Human recombinant chorionic gonadotropin (Ovitrelle) was obtained from Serono (Madrid, Spain). Human urinary chorionic gonadotropin (Pregnyl) was obtained from Dr. P. Erbel (Utrecht University, The Netherlands). Trypsin, 2-amino benzamide, 1,2-diamino-4,5-methylenedioxy-benzene, and urinary chorionic gonadotropin were purchased from Sigma. QMA quartz paper was from Whatman (Madrid, Spain). Graphitised carbon columns were from Alltech (Barcelona, Spain). Dihydroxy benzoic acid was from Bruker (Madrid, Spain). All other reagents were of the highest grade commercially available.

Sialic acid analysis – Sialic acids were released by hydrolysis in 3 M acetic acid, 3 h, 80 ºC and converted into fluorescent derivatives with 1,2-diamino-4,5-methylene-dioxy-benzene (DMB) as reported by Hara *et al.*[5]. Analysis of DMB labelled residues was performed by reversed phase HPLC on a Waters Nova-Pak column (3.9 x 150 mm) using acetonitrile-methanol-water (9:7:84) as isocratic eluent at a flow rate of 1ml/min. Fluorescence detection was carried out at an excitation λ=373 and an emission λ=448 nm[6].

SDS-PAGE – One dimensional SDS-polyacrylamide gel electrophoresis was performed according to the method described by Laemmli[7]. It was prepared using standard methods on the Bio-Rad Mini-Protean II system (Bio-Rad Laboratories, Hercules, CA, USA) (7 cm x 10 cm minigels). 15 % acrylamide gels of 0.75 mm thickness were used in all the experiments. Protein sample load was 1 µg in sample loading buffer [25 µl, 0.15 M Tris pH 6.8 (2.5 eq), glycerol 100% (1 eq), 3% SDS (2 eq), 2-mercapto-ethanol (0.5 eq), 0.3 % bromo phenol blue (0.2 eq), and water (0.4 eq)]. Gels were run at 100 V constant voltage in 25 mM Tris/ 190 mM glycine/ 0.1% SDS. After electrophoresis, the gel slab was fixed in 40% ethanol, 5% acetic acid, 50% water for 30 min. After rinsing, the gel was submerged in 0.25% silver nitrate solution and incubated for 20 min. After

incubation, the silver nitrate was discarded, and the gel slab was rinsed twice with water for 10 min and then developed in a solution of 12.5 g sodium carbonate, 0.1 ml formaldehyde 37% and 487 ml H_2O with vigorous shaking. After the desired intensity of staining was achieved, the development was terminated by discarding the reagent, followed by washing of the gel slab with a solution of 7.3 g EDTA·Na_2 in 500 ml H_2O. Silver-stained gels were stored in water at 4 ºC until analysed.

Enzymatic digestions – PNGase F digestions were performed both in gel and in solution. In general, glycoproteins were reduced with 50 µl of 10 mM dithiothreitol (DTT) in 50 mM phosphate buffer (pH 7.3) for 30 min at 56 ºC. Following DTT treatment, proteins were alkylated with 50 µl of 50 mM iodoacetamide (IAA) in 50 mM phosphate buffer (pH 7.3) for 30 min in the dark[8]. For solution-digestions, samples were centrifuged with 10 kDa filter 10 min at 6900 g, sonicated for 3 min and the retentate dissolved in 200 µl 50 mM sodium phosphate buffer (pH 7.3). In case of in-gel digestions solutions were simply aspirated, washes applied and the gel-pieces dehydrated prior to the enzymatic digestions. PNGase F (3 U) was added and the mixture incubated at 37 ºC for 16 h.

Trypsin digestions were performed in 0.1 M NH_4HCO_3 (pH 7.9) containing 20 ng of trypsin (ratio Enzyme:Substrate 1:50). Incubations were performed at 37 ºC for 16 h[9].

Glycan purification – Liberated oligosaccharides was separated from remaining detergent, protein and salts in a single step, on graphitised carbon columns[10]. Prior to use, the graphitised carbon black SPE columns (150 mg) were washed with 1.6 ml of 80% (v/v) acetonitrile in 0.1% (v/v) trifluoro acetic acid (TFA) followed by 1.6 ml of water. Next, aqueous sample solutions were applied to the columns and allowed to run into the absorbent. Salts were washed-off with 1.6 ml of water, while the glycans were eluted with 1.6 ml of 25% (v/v) acetonitrile in 0.05% (v/v) TFA. Finally column was washed with 1.6 ml of 80% (v/v) acetonitrile in 0.1% (v/v) TFA. Carbohydrate fractions containing acetonitrile were evaporated under a flow of N_2 for 1 h at 23 ºC, then they were frozen at –80 ºC and lyophilised. Prior to fluorescent labelling, lyophilised glycans were dried at 60 ºC in a vacuum oven for 30 min.

Fluorescence labelling of released glycans with 2-amino benzamide – Oligosaccharide samples were derivatised with 2-amino benzamide (2AB). Employing a fresly prepared solution of 0.35 M 2AB in 500 µl dimethylsulfoxide: acetic acid (70:30) containing 1 M $NaCNBH_3$. An aliquot 5-10 µl of this solution was added to each dried oligosaccharide sample and the mixture was incubated 4 x 30 min at 65 ºC with intermediate mixing[11]. In order to eliminate the excess 2AB, labelled samples were applied to Whatman QMA paper, allowed to dry and washed with 5 ml of acetonitrile. Carbohydrates were eluted with 1.8 ml of water. Purified oligosaccharides were frozen and lyophilised.

Mass spectrometric analysis – Samples (proteins, peptides or carbohydrates) were dissolved in water at varying concentrations. An aliquot was mixed with the corresponding matrix and <1 µl of this preparation applied to the polished stainless steel target and allowed to dry at room temperature. Sinapinic acid (10 mg/ml) matrix solution in CH_3CN:H_2O:TFA (50:50:0.1 v/v/v) was chosen for protein analysis. A solution of α-cyano-4-hydroxycinnamic acid 20 mg/ml in MeCN:H_2O:TFA (70:30:0.1 v/v/v) was used for peptide samples and DHB 10 mg/ml in MeCN:H_2O (50:50 v/v) for N-glycans. Experiments were carried out on a Voyager-DE™ STR Biospectrometry workstation (Applied Biosystems), equipped with a N_2 laser (337 nm). Typically, spectra were acquired in linear mode for negative polarity and in reflectron mode for positive polarity. The array detector was set to the high resolution position and mass scans were accumulated in the mass range (between 900-5000 Da depending on the sample). For data acquisition, the laser intensity was 2000-2500 V and the laser beam was moved manually over the sample in order to compensate for sample depletion under the laser beam. External calibration of the spectrometer was performed using a mixture of 2AB-labelled glucose oligomers in positive ion mode and 2AB-derivatised N-glycans from fetuin in negative mode. In the case of peptide spectra, calibration was carried out with Sequazyme™ Peptide Mass Standards Kit of the desired range (PerSeptive Biosystems). Recorded data were processed with Data Explorer™ Software (Applied Biosystems).

Results and Discussion

The structural comparison of the different gonadotropins was carried out at three different levels: macromolecular characteristics, peptide mapping and glycosylation. Mass spectrometric analysis of the intact macromolecules revealed that both heterodimers have masses of ~ 35000 Da (figure 1), the recombinant material showing a slightly higher mass than the endogenous material. It appears evident from the mass spectra that the recombinant α-subunit is partially non-glycosylated. Two peak clusters (with microheterogeneity) are evident with a mass difference corresponding to a bi-antennary complex type structure. The low-intensity cluster represents ~30% of the total and corresponds to a single N-glycan containing α-subunit. The other cluster contains two N-glycans. The urinary species however, showed a single cluster with apparently two N-glycans.

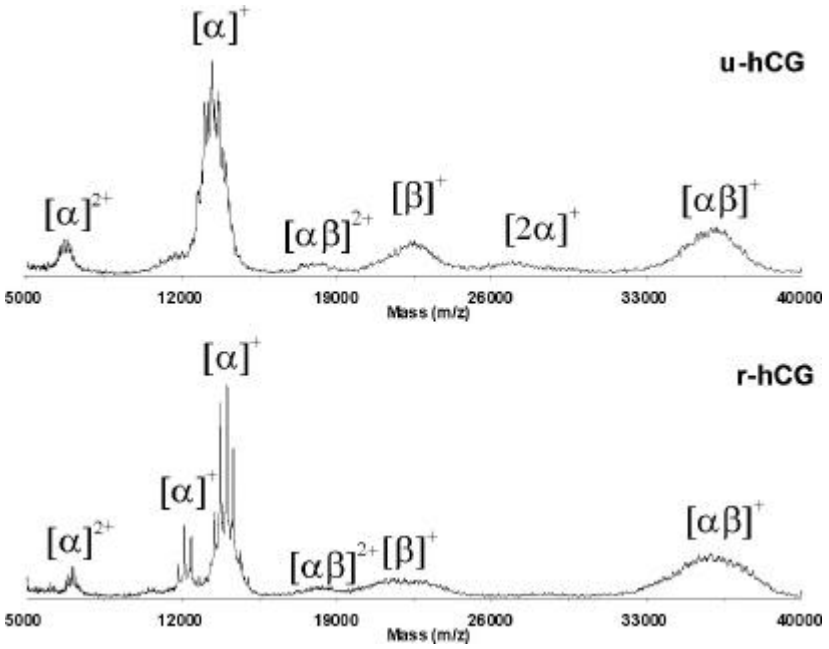

Figure 1. MALDI mass spectra of recombinant (lower panel) and endogenous (upper panel) human chorionic gonadotropin.

Peptide mass finger printing following tryptic digestions were performed on gel-separated sub-units and resulted in excellent sequence coverage for the β-subunits, with only the (glyco)peptides absent. Surprisingly, a partial glycosylation of the recombinant β-subunit is suggested from the observation non-glycosylated Asn30, reinforcing the hypothesis of potential differentiation through structural features. Poor sequence coverage was obtained for both α-subunits, albeit that also in this case proteolytic

peptides containing Asn52 and Asn78 were observed, reinforcing the observations with the intact molecules (Table I).

Table I. Peptide sequences of α- and β-hCG. U-hCG corresponds to the sample from Sigma; r-hCG corresponds to the sample from Serono; β-hCG corresponds to the sample from Organon. The peptide coverage for each sample is indicated in grey.

***a*-subunit**

u-hCG	**1**	APDVQDCPEC	TLQENPFFSQ	PGAPILQCMG	CCFSR AYPTP	LR SKKTMLVQ	**50**
r-hCG	**1**	APDVQDCPEC	TLQENPFFSQ	PGAPILQCMG	CCFSR AYPTP	LR SKKTMLVQ	**50**
β-hCG	**1**	APDVQDCPEC	TLQENPFFSQ	PGAPILQCMG	CCFSR AYPTP	LR SKKTMLVQ	**50**
u-hCG	**51**	K***N***VTSESTCC	VAKSYNRVTV	MGGFKVE***N***HT	ACHCSTCYYH	KS	**92**
r-hCG	**51**	K***N***VTSESTCC	VAKSYNRVTV	MGGFKVE***N***HT	ACHCSTCYYH	KS	**92**
β-hCG	**51**	K***N***VTSESTCC	VAKSYNRVTV	MGGFKVE***N***HT	ACHCSTCYYH	KS	**92**

***b*-subunit**

u-hCG	**1**	SKEPLRPR CR	PI***N***ATLAVEK	EGCPVCITV***N***	TTICAGYCPT	MTR VLQGVLP	**50**
r-hCG	**1**	SKEPLRPR CR	PI***N***ATLAVEK	EGCPVCITV***N***	TTICAGYCPT	MTR VLQGVLP	**50**
β-hCG	**1**	SKEPLRPR CR	PI***N***ATLAVEK	EGCPVCITV***N***	TTICAGYCPT	MTR VLQGVLP	**50**
u-hCG	**51**	ALPQVVCNYR	DVR FESIRLP	GCPR GVNPVV	SYAVALSCQC	ALCR RSTTDC	**100**
r-hCG	**51**	ALPQVVCNYR	DVR FESIRLP	GCPR GVNPVV	SYAVALSCQC	ALCR RSTTDC	**100**
β-hCG	**51**	ALPQVVCNYR	DVR FESIRLP	GCPR GVNPVV	SYAVALSCQC	ALCR RSTTDC	**100**
u-hCG	**101**	GGPKDHPLTC	DDPRFQDSSS	SKAPPPSLPS	PSRLPGPSDT	PILPQ	**145**
r-hCG	**101**	GGPKDHPLTC	DDPRFQDSSS	SKAPPPSLPS	PSRLPGPSDT	PILPQ	**145**
β-hCG	**101**	GGPKDHPLTC	DDPRFQDSSS	SKAPPPSLPS	PSRLPGPSDT	PILPQ	**145**

Sub-units of the individual samples were separated by SDS-PAGE and enzymatically de-N-glycosylated in-gel. Released glycans were purified, labelled and analysed by mass spectrometry revealing different glycosylation patterns as a function of the origin of the chorionic gonadotropin (see figures 2-3). All β-subunits show predominantly bi-antennary complex-type N-glycans with 1 or 2 sialic acid residues in equal amounts. Identical profiles, albeit at ~ 50% of the intensity, were observed for the same structures devoid of the core-fucose. In case of the endogenous hCG also small amounts of mono-antennary complex type N-glycans were visualised. These were absent in the recombinant species.

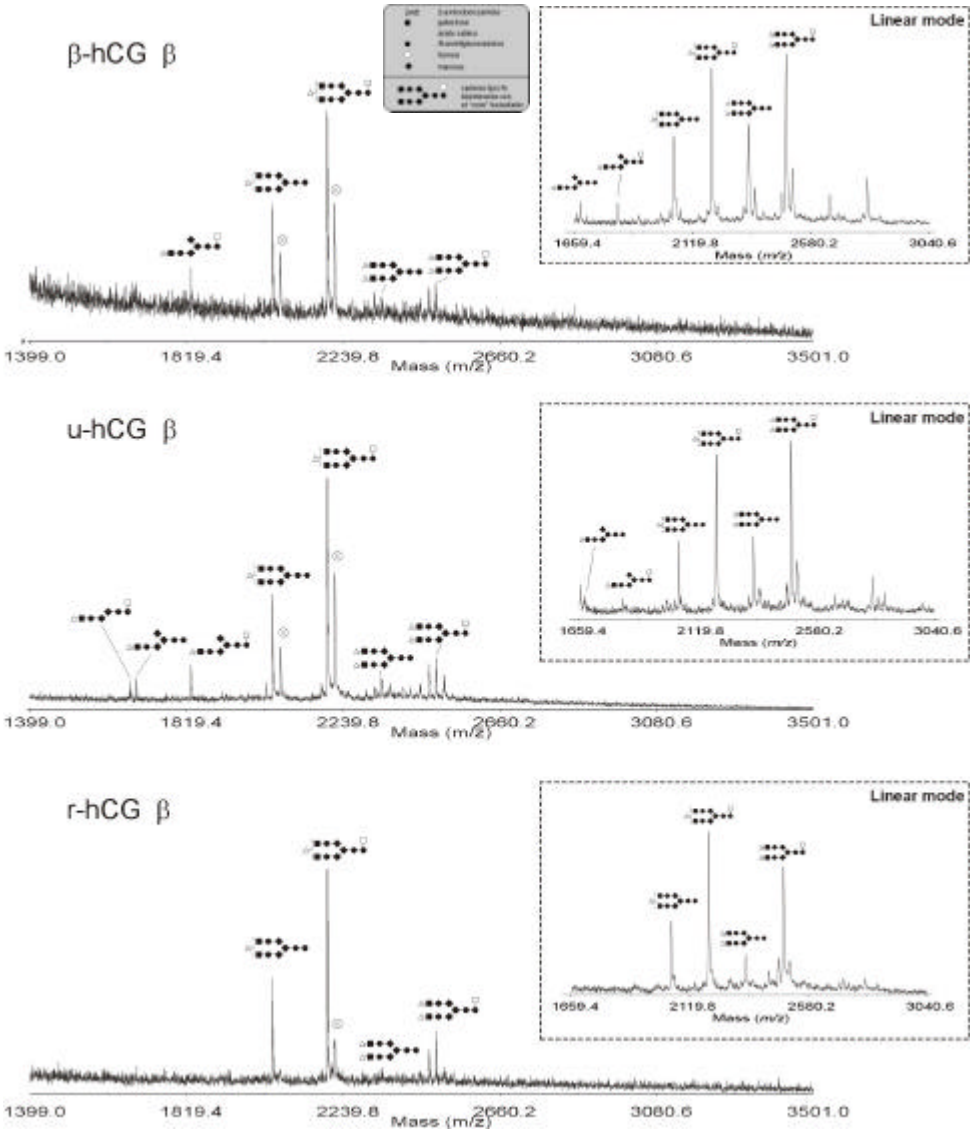

Figure 2. Negative ion-mode MALDI-TOF mass spectra of the N-glycans from the β-subunits of u-hCGs (upper panel), u-hCG (central panel) and r-hCG (lower panel). Mass spectra were recorded in reflector mode and inserts correpond to linear mode. The structures depicted in the spectra were derived from the mass values in combination with literature date. Peaks marked with a ⊗ originate from metastable fragmentation.

The differences in glycosylation were more pronounced in the α-subunits. Whereas the endogenous species displayed both hybrid type and complex type, mono- and bi-antennary N-glycans, all devoid of the core-fucose, the recombinant molecules contained only complex type N-glycans. Predominantly bi-antennary structures were observed but also small amounts of tri-antennary glycans could be seen.

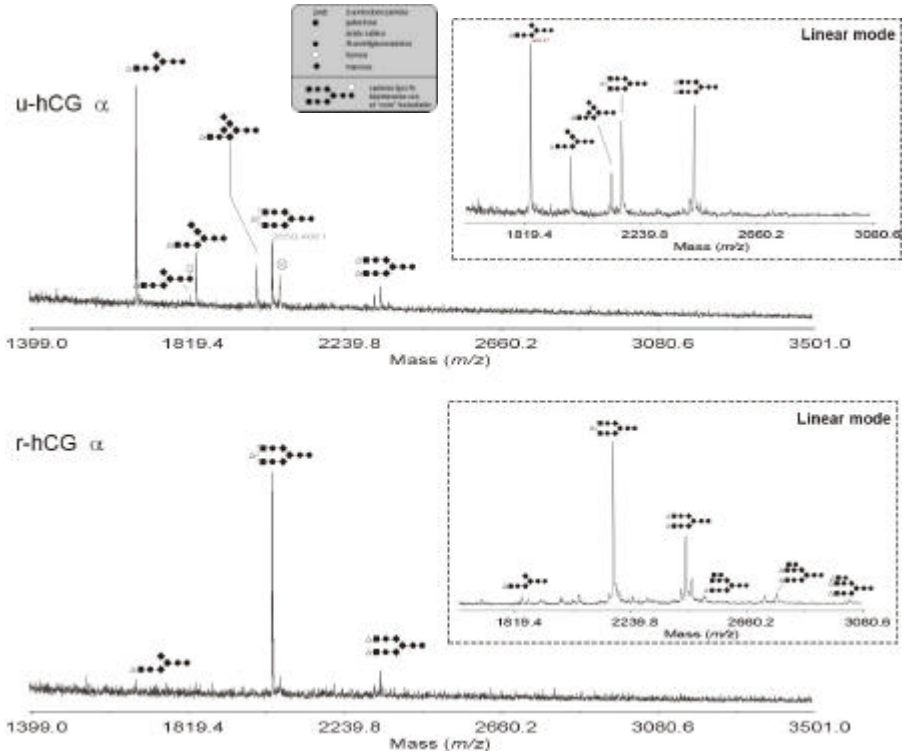

Figure 3. Negative ion-mode MALDI-TOF mass spectra of the N-glycans from the α-subunits of u-hCGs (upper panel), u-hCG (central panel) and r-hCG (lower panel). Mass spectra were recorded in reflector mode and inserts correpond to linear mode. The structures depicted in the spectra were derived from the mass values in combination with literature date. Peaks marked with a ⊗ originate from metastable fragmentation.

Finally, the monosaccharide composition of the distinct preparations was examined and in particular the sialic acid constituents. Following release, DMB-labelling and HPLC analysis, the chromatograms (figure 4) displayed in the case of urinary derived CG a single sialic acid species (Neu5Ac). In addition to the peaks derived from the reagent used. In the case of the recombinant molecule the presence of a second sialic acid was observed. As anticipated, the retention time of this residue corresponded to Neu5Gc and this species accounted for ~ 1.5% of the total.

Conclusions

Our studies on the structural characteristics of human chorionic gonadotropin derived from both human urine or from recombinant expression in Chinese hamster ovary cells show that clear structural differences exist between both species and that they could be employed to discriminate. At the peptide level only differences in site occupancy in the α-subunit glycosylation is observed, i.e. ~ 30% of the recombinant α-subunit is not glycosylated.

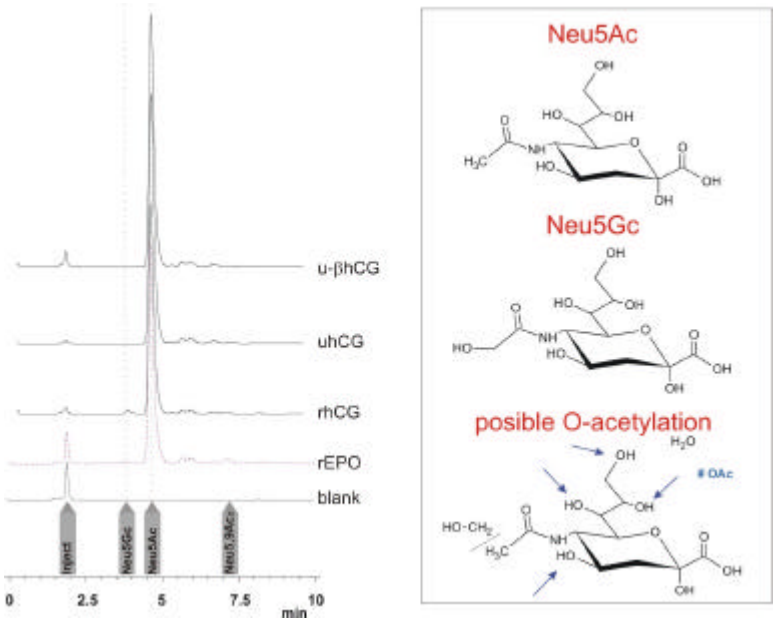

Figure 4. Panel on the left: from top to bottom: urinary derived hCG from Organon, urinary derived hCG from Sigma, recombinant hCG from Serono, recombinant erythropoietin (European Pharmacopoeia), Blank simple. The retention times of the distinct sialic acid residues are indicated. Right panel: structure of the most frequently occurring sialic acid residues.

At the glycosidic level differences in glycan maturation are seen. Again, the most prominent differences reside in the α-subunit and comprise the presence of hybrid type N-glycans only in the endogenous molecule and the presence of higher-antennary complex-type N-glycans only for the recombinant species. Finally, recombinant hCG derived from CHO cells, as does rEPO from CHO cells, contains ca. 1.5% of Neu5Gc. This residue is a true marker of the exogenous origin of hCG as the human system is genetically unable to produce this compound.

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