Clinical Chemistry 46:11
1796–1803 (2000)

Determination of the Glycoforms of Human Chorionic Gonadotropin β-Core Fragment by Matrix-assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

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Background: **Metabolism of human chorionic gonadotropin (hCG) in the serum and kidney yields the terminal urinary product hCG β-core fragment (hCGβcf), comprising two disulfide-linked peptides (β6-β40 and β55-β92) of which one (β6-β40) retains truncated N-linked sugars. Hyperglycosylated hCGβcf may indicate choriocarcinoma or Down syndrome, but the glycosylation profile of hCGβcf has not been thoroughly evaluated.**

Methods: **hCGβcf, purified from pregnancy urine, was reduced by "on-target" dithiothreitol (DTT) reduction and analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The mass ($[M+H]^+$) of the primary sequence of the glycosylated peptide β6-β40 was subtracted from the *m*/*z* values of the discrete peaks observed to give the masses of the carbohydrate moieties. Carbohydrate structure was predicted by sequentially subtracting the masses of the monosaccharide residues corresponding to N-linked carbohydrates of the hCG β-subunit reported in the literature.**

Results: **Mass spectra of hCGβcf revealed a broad triple peak at *m*/*z* 8700–11300. After reduction, the triple peak was replaced by a discrete set of peaks between *m*/*z* 4156 and 6354. A peak at *m*/*z* 4156.8 corresponded to the nonglycosylated peptide (β55-β92). The remaining nine peaks indicated that urinary hCGβcf comprises a set of glycoforms smaller and larger than the trimannosyl core.**

Conclusions: **hCGβcf comprises a wider set of glycoforms than reported previously. Peaks of highest mass indicate evidence of hyperglycosylated carbohydrate moieties. The data support previous reports that hCGβcf oligosaccharides lack sialic acid and galactose residues. No indication was found of a β6-β40 peptide that was entirely devoid of carbohydrate.**

The heterodimeric placental hormone human chorionic gonadotropin (hCG)[3] is a widely studied member of the glycoprotein hormone family that includes luteinizing hormone, thyroid-stimulating hormone, and follicle-stimulating hormone. Each consists of a common α subunit and a unique β subunit that confers specific hormonal activity. hCG sustains early pregnancy by binding luteinizing hormone receptors at the corpus luteum surface and stimulating progesterone secretion until the placenta is able to carry out this function itself. In addition to being universally used as an early marker of pregnancy, hCG is monitored in sports drug testing *(1)*. The hCG β-subunit (hCGβ) is also a marker of various epithelial and gestational trophoblast carcinomas *(2, 3)*, where it may exert independent stimulatory activity *(4, 5)*. The precise structure of hCG has been well characterized by HPLC-mass spectrometry *(6, 7)* and by crystallographic analysis *(8, 9)*. Peptide mass mapping by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), using trypsin for digestion, has also been found to be a suitable technique for the identification of hCG and its subunits *(10, 11)*.

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Received June 29, 2000; accepted August 25, 2000.

[3] Nonstandard abbreviations: hCG, human chorionic gonadotropin; hCGβ, hCG β-subunit; DTT, dithiothreitol; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; hCGβcf, hCG β-core fragment; SA, sinapinic acid; TFA, trifluoroacetic acid; dH_2O, distilled, deionized water; and GlcNAc, *N*-acetylglucosamine.

hCG, in common with other glycoprotein hormones, is characterized by sugar moieties on both subunits that determine receptor affinity and metabolic clearance. The currently accepted structural model of the N-linked carbohydrates found on hCGβ is of a complex biantennary moiety terminating in sialic acid based on a trimannosyl core *(12)*. Despite the model, wide carbohydrate heterogeneity has been found. Mono-, bi-, and triantennary N-linked sugars occur in hCGβ in both normal and abnormal pregnancies as well as in choriocarcinomas *(12–17)*. An increase in the triantennary form seems to be a feature indicative of an abnormal state and may be of diagnostic value. In normal pregnancies, Elliott et al. *(14)* found that the biantennary form accounted for 76.5% of the N-linked oligosaccharide content of urinary hCGβ, that 21.6% was triantennary, and that the remainder was monoantennary (Fig. 1). In gestational choriocarcinoma, by contrast, the triantennary content accounted for up to 63.6% of the total. A similar increase in hCG hyperglycosylation has been detected in Down syndrome by Cole et al. *(18)*. Indeed, an ELISA for hyperglycosylated hCG has been developed based on a monoclonal antibody raised to the hyperglycosylated variant *(19, 20)*. Prenatal diagnostic testing of maternal urine based on this ELISA is claimed to detect up to 97% of Down syndrome cases with a 5% false-positive rate when modeled with values of other markers *(21)*.

Similarly, carbohydrate heterogeneity remains in the terminal urinary degradation product of the β-subunit, the hCG β-core fragment (hCGβcf). This core fragment has a primary structure of 73 amino acids, consisting of two polypeptides: β6-β40 and β55-β92, joined by four cystine residues (Fig. 2). Only the β6-β40 chain is glycosylated, retaining the two complex N-glycans linked to asparagine residues 13 and 30. However, as a consequence of metabolism, these branched chain carbohydrates are truncated. Various studies of the carbohydrate structure of hCGβcf using a combination of immunoaffinity, gel-filtration, and ion-exchange chromatography, glycosidase digestion, hydrazinolysis, and periodate oxidation have been reported *(22–26)*. A population of shortened mono- and biantennary carbohydrate cores has been suggested, which may or may not be fucosylated *(22)*. There is disagreement over whether these moieties possess sialic acid or galactose residues, although there is some evidence that they do extend beyond the trimannosyl cores *(24)*. In an attempt to help resolve this disagreement, we investigated the structural variation in these oligosaccharide moieties, using an alternative approach that incorporates analysis by MALDI-TOF MS.

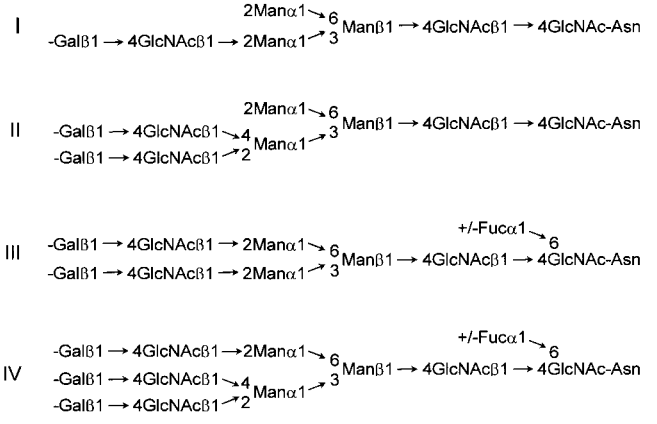


Fig. 1. Structures of the asialo asparagine-linked oligosaccharides of the hCGβ subunit from normal pregnancy, hydatidiform mole, choriocarcinoma (*I*, *III*, and *IV*, respectively), and gestational diabetes (*II*).
Adapted from Elliott et al. *(14)*.

MALDI-TOF MS is a powerful tool for determining the mass of macromolecules, as first reported by Karas and Hillenkamp *(27)*, yielding signals that correspond mainly to singly charged molecular ions. Chemical or enzymatic cleavage of posttranslationally modified proteins, followed by MALDI-TOF MS analysis, has been used for the purpose of structural characterization. Exoglycosidase digestion with MALDI-TOF MS analysis has been used to elucidate carbohydrate sequences and composition of glycoproteins *(28)*. Nonetheless, at first glance, this approach appears to be overcomplicated for investigating the glycoforms of hCGβcf, particularly because the branch chain sequence is unlikely to be affected by metabolism of the β-subunit, albeit that the chain is truncated. Indeed, exoglycosidase digestion could confound interpretation of results because absolute specificity for one substrate is rare and this may possibly lead to the presence of artifacts. However, direct analysis of hCGβcf by low-resolution MALDI-TOF MS is not sufficiently discriminating to identify individual glycoforms because of the lack of resolution between them. A simple but potentially useful approach could be to reduce the disulfide bonds, e.g., with DTT, to yield the two separate polypeptide chains before analysis. Separation of the β55-β92 chain, which is of no diagnostic value, from the β6-β40 chain is advantageous because the overall masses of the glycoproteins being analyzed are reduced, which is conducive to discriminating between them. The mass of each glycosylation variant on the β6-β40 chain can then be determined by calculating the difference in mass between each peak observed to the mass of its corresponding primary amino acid sequence, i.e., nonglycosylated β6-β40. The presence of residual triantennary sugars on hCGβcf has not been as well determined as for free hCGβ but should be characterized to determine any diagnostic potential. In this study, we used MALDI-TOF MS, for the first time, to directly observe and determine the number of glycoforms in a purified sample of pooled pregnancy hCGβcf.

Materials and Methods

MATERIALS

The hCGβcf used in this study was isolated from crude urinary hCG (Sigma) and characterized previously *(29)*. DTT, for the reduction of disulfide bonds, and NH_4HCO_3

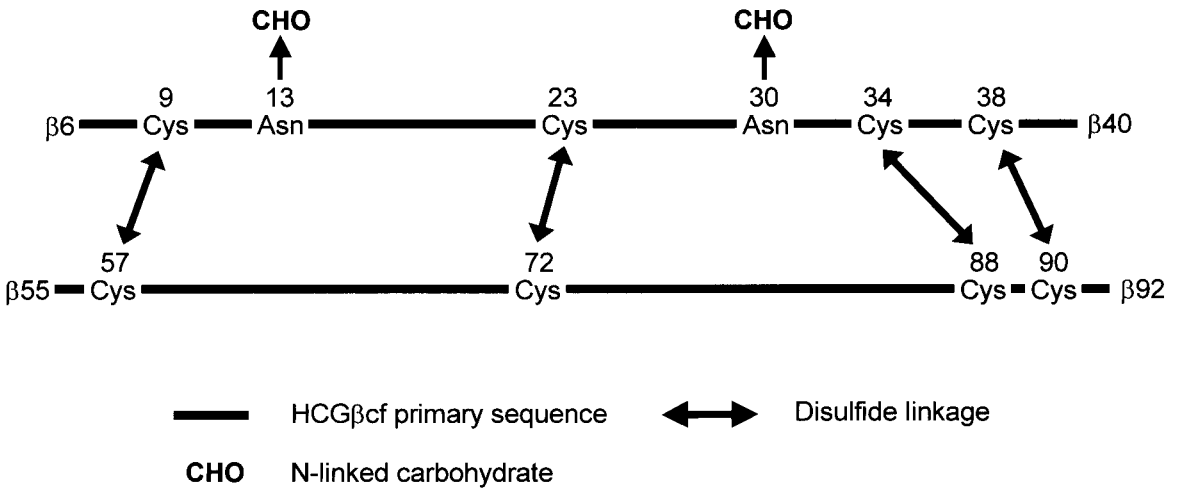


Fig. 2. Schematic diagram of the primary amino acid sequence of hCGβcf, showing positions of disulfide bonds and N-linked carbohydrates.

were obtained from Sigma-Aldrich. The MALDI-TOF MS matrix 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid; SA) was also purchased from Sigma-Aldrich and used without further purification. Acetonitrile and trifluoroacetic acid (TFA) were of analytical grade and obtained from Merck.

MALDI-TOF MS

To acquire spectra of the nonreduced molecule, we applied 0.5 μL of hCGβcf [20 μmol/L in distilled, deionized H_2O (dH_2O)] to a stainless steel MALDI-TOF MS target and mixed it with 1.0 μL of SA [20 g/L in 70:30 (by volume) acetonitrile-1 mL/L TFA in dH_2O] and air dried. The instrument was calibrated externally using horse heart cytochrome C (M_r 12 360.1; Sigma-Aldrich). Spectra of reduced glycoforms were obtained by first mixing, on a MALDI-TOF MS target, 0.5 μL of hCGβcf (20 μmol/L in dH_2O) with 0.5 μL of DTT (100 mmol/L in 100 mmol/L NH_4HCO_3, pH 8.8) *(30)*. When this mixture was virtually dry, 0.5 μL of 1 mL/L TFA in dH_2O was added to the remaining droplet. After the mixture was again allowed to partially dry, 1.0 μL of SA [20 g/L in a 70:30 mixture (by volume) of acetonitrile-1 mL/L TFA in dH_2O] was added, and the sample was air-dried as before. Mass spectrometric analysis was carried out on VG TofSpec E (Micromass) and LaserMAT 2000 (Finnigan MAT) instruments, with 1.5 m and 0.5 m flight tubes, respectively, and both operating at an accelerating voltage of 20 kV. A pulsed nitrogen laser (λ_{max} = 337 nm) was used to desorb ions from the samples, which were detected by a microchannel plate detector at a sampling rate of 500 MHz. Spectra were generated by summing 30–40 laser shots. Data were acquired from both instruments operating in the positive linear mode.

TREATMENT OF RESULTS

Initially, the peak relating to the nonglycosylated hCGβcf peptide (β55-β92) was internally calibrated to the average molecular mass of its given primary sequence, $[M+H]^+$ 4156.8 (calculated using the Protein Abacus computer software; Finnigan MAT). This facilitated the automatic correction of the mass values of the remaining peaks to their correct values.

To determine the inferred mass values of the hCGβcf carbohydrate moieties, we subtracted the average molecular mass ($[M+H]^+$ 3752.4) of the primary sequence of the glycosylated hCGβcf polypeptide (β6-β40) from the mass ($[M+H]^+$) values of the acquired peaks (Table 1). The carbohydrate content of these peaks was then deduced by the sequential subtraction of the masses of the sugar residues present in hCG β-subunit carbohydrates (Table 2). Carbohydrate structures (Table 3) could then be predicted from the deduced monosaccharide content, taking into account the very small difference between the calculated and the observed masses (≤0.15%). Different combinations of carbohydrate structures are shown where the mass of a peak indicated several monosaccharides from which several permutations could be construed. Although this study did not yield any conformational information, the anomeric glycosidic linkages within the carbohydrate structures in Table 3 have been described previously *(14)* and were used in our analysis.

Results

SA was selected as the MALDI-TOF MS matrix of choice for this investigation because other standard matrices used in protein analysis, α-cyano-4-hydoxycinnamic acid and sDHB [a 9:1 (by weight) mixture of 2,5-dihydroxybenzoic acid and 5-methoxysalicylic acid], did not yield intense spectra for hCGβcf (data not shown). A typical spectrum of hCGβcf obtained by MALDI-TOF MS using SA is shown in Fig. 3. The spectrum is characterized by a broad triple peak at *m/z* 8700–11300 with discernible peaks at *m/z* 9110, 9930, and 10770. After reduction with DTT (Fig. 4), the triple peak disappeared and was replaced by a set of resolved peaks between *m/z* 4156 and

Table 1. Peptides of hCGβcf released after DTT reduction, detected by MALDI-TOF MS.

Peak	Observed mass, $M+H^+$	Mass of inferred primary sequence, $M+H^{+a}$	Mass − primary sequence	Predicted mass of residual CHO	Δ[b]	Δ, %	% by mass[c]
1	4630.2	3752.4	877.8	876.8	1.0	0.02	8.1
2	4819.6	3752.4	1067.2	1064.0	3.2	0.07	9.2
3	5000.8	3752.4	1248.4	1242.2	4.5	0.13	3.7
4	5358.2	3752.4	1605.8	1607.5	−1.7	−0.03	17.9
5	5517.6	3752.4	1765.2	1769.6	−4.42	−0.08	20.5
6	5677.9	3752.4	1925.5	1931.8	−6.3	−0.11	17.3
7	5821.1	3752.4	2068.1	2077.9	−9.18	−0.15	3.7
8	6179.0	3752.4	2426.6	2420.3	6.3	0.10	15.6
9	6353.8	3752.4	2601.4	2598.4	3.0	0.05	4.0

[a] Mass of the primary sequence of the glycosylated hCGβcf peptide, β6-β40.

[b] Difference between the calculated mass of the expected total carbohydrate and the inferred mass after subtraction of the mass of the primary sequence from the peak mass.

[c] Where the total area under peaks 1–9 in Fig. 2 equals 100%.

6354. The initial peak (Fig. 4), at *m/z* 4164.2, was presumed to arise from the nonglycosylated hCGβcf peptide chain (β55-β92) because the *m/z* value is within the 0.5% error allowed for the linear mode of the MALDI instrument. To obtain corrected values for the remaining peaks, this peak was internally calibrated to the average molecular mass of the given primary sequence, $[M+H]^+$ 4156.8 (calculated using the Protein Abacus computer software). Despite the fact that hCGβcf asparagine-linked carbohydrate moieties were not observed directly, the low percentage errors between the observed and the expected mass values of the peaks acquired show that it is likely that real glycoforms were detected (Table 1).

Carbohydrate moieties, shown in Table 3, were determined as described above. Peak 1 (*m/z* 4630.2) arises from a polypeptide possessing a single truncated and fucosylated monoantennary oligosaccharide ($Man_2GlcNAc_2Fuc$) with a predicted mass of 876.8, whereas the polypeptide giving rise to peak 2 (*m/z* 4819.6) has two shortened fucosylated monoantennary sugars ($ManGlcNAc_2Fuc$ and $GlcNAc_2Fuc$) with a predicted mass of 1064.0. Peaks 6 (*m/z* 5677.9) and 7 (*m/z* 5821.1) apparently arise from hCGβcf glycoforms containing two N-linked trimannosyl core carbohydrates ($Man_3GlcNAc_2$). In peak 6, a single trimannosyl core possesses fucose (with a predicted mass of 1931.8), whereas both are fucosylated in peak 7 (with a predicted mass of 2077.9). The combination of monosaccharide residues (with a predicted mass of 2420.3) that make up the glycoform observed at peak 8 (*m/z* 6179.0) can be configured to produce an identical pair of sugar structures that are shortened versions ($GlcNAc_2Man_2GlcNAc_2$) of carbohydrate structures II or IV found by Elliott et al. *(14)*. The number of monosaccharide residues calculated for the polypeptides giving mass spectral peaks 3–5 and peak 9 precludes the determination of a single pair of glycoforms, and for these alternative structures have been suggested. The predicted mass of 1242.2 for the carbohydrate moiety of the glycoform at peak 3 (*m/z* 5000.8) indicates either a single fucosylated trimannosyl core attached to a monoantennary *N*-acetylglucosamine ($Man_3GlcNAc_3Fuc$) or a trimannosyl core ($Man_3GlcNAc_2$) at one glycosylation site and a single GlcNAc at the other, one of which is fucosylated. Peak 4 (*m/z* 5358.2) indicates a glycoform with a predicted carbohydrate mass of 1607.5, which can translate to two shortened core sugars ($Man_2GlcNAc_2$) differing by a fucose residue. An alternative combination includes a trimannosyl core and a shortened core ($ManGlcNAc_2$), one of which is fucosylated. Similarly, the predicted carbohydrate mass (1769.6) for the glycoform seen at peak 5 (*m/z* 5517.6) indicates a monosaccharide content from which may be constructed a trimannosyl core ($Man_3GlcNAc_2$) and a truncated core oligosaccharide ($Man_2GlcNAc_2$), one of which is connected to a fucose residue. Finally, two extended trimannosyl cores ($Man_3GlcNAc_4$) can be inferred from a predicted carbohydrate mass of 2598.4 of the glycopeptide at peak 9 (*m/z* 6353.8). Structures construed from this mass may be pairs or a combination of truncated oligosaccharides II, III, or IV in Fig. 1. There is an additional peak in the mass spectra of DTT-reduced hCGβcf between peaks 3 and 4 that we assume to be an additional glycoform but for which the data analysis software was unable to acquire a value.

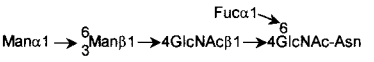
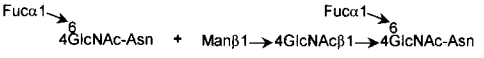
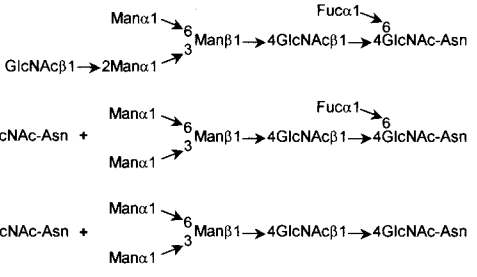
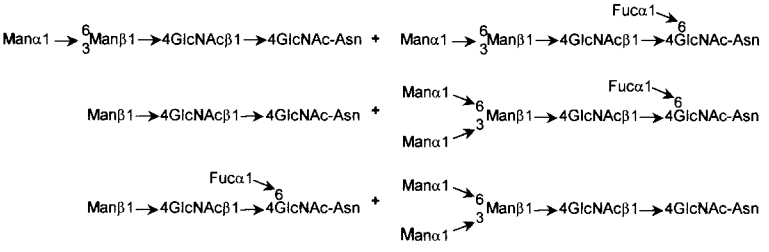
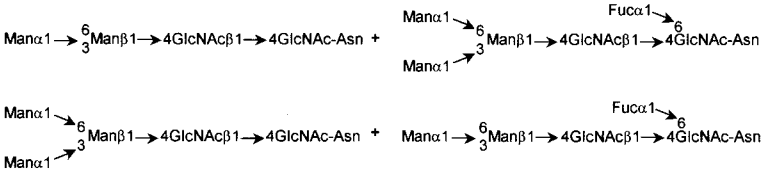
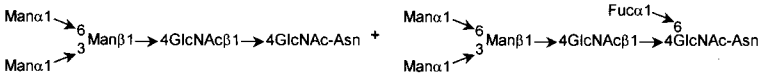
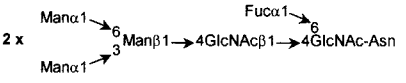
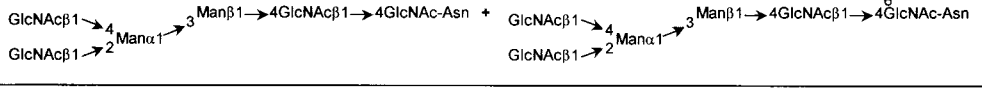
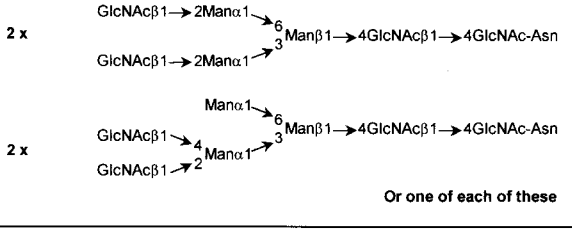
Table 2. Relative molecular mass (RMM) of the monosaccharides expected to be present in hCGβ subunit N-linked carbohydrates.

Monosaccharide	RMM	RMM − H_2O
Sialic acid	309.27	291.25
Galactose	180.16	162.14
GlcNAc	221.21	203.19
Mannose	180.16	162.14
Fucose	164.16	146.14

Discussion

Direct carbohydrate analyses of hCGβcf in previous studies have generally suggested that the metabolite retains shortened hCGβ asparagine-linked oligosaccharides that

Table 3. Proposed structures of the oligosaccharides in the glycoforms contributing to the microheterogeneity of hCGβcf.[a]

Peak	Proposed structure	Inferred sugar mass, *m/z*
1		876.8
2		1064.0
3		1242.2
4		1607.5
5		1769.6
6		1931.8
7		2077.9
8		2420.3
9		2598.4

[a] Where the masses found give rise to a variety of carbohydrate combinations on any one peptide, these are shown.

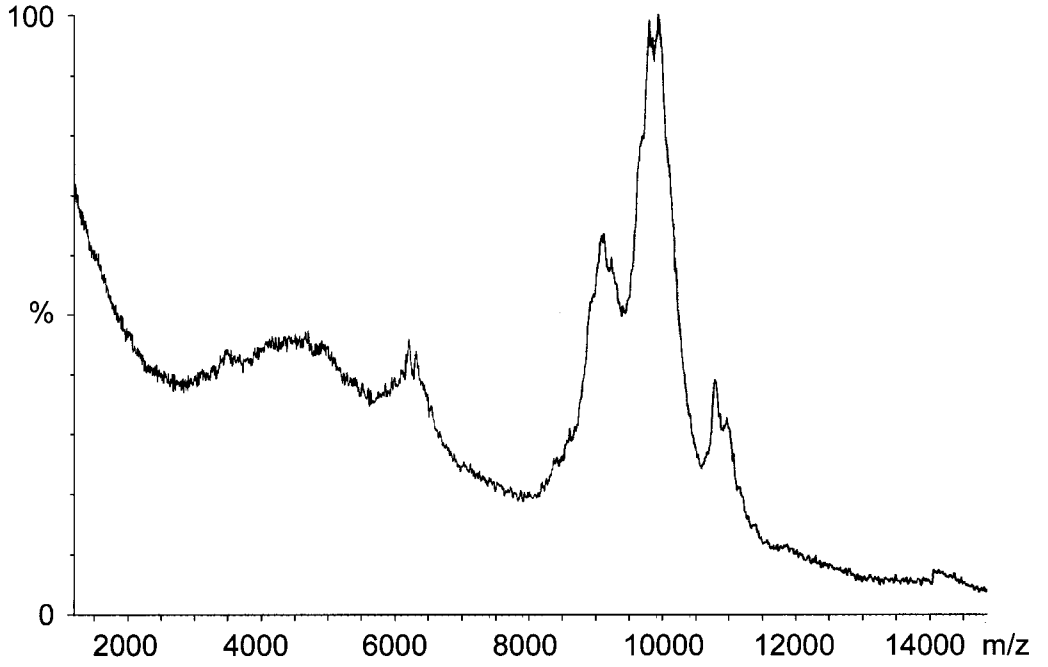


Fig. 3. MALDI-TOF mass spectrum of purified hCGβcf in SA matrix generated by summation of the signals from 40 laser shots and calibrated externally using horse heart cytochrome C (M_r 12 360.1).

have been trimmed back to their trimannosyl cores and smaller sugars *(22–26)*. In addition, de Medeiros et al. *(24)* found that 22–44% of hCGβcf molecules were not bound by the lectin concanavalin A, and of those 88% were completely deprived of any carbohydrate content. After DTT reduction, our MALDI-TOF MS data indicated the presence of only one nonglycosylated peptide at *m/z* 4156.8 within the dipeptide molecule. If the usually glycosylated peptide (β6-β40) had been completely lacking in carbohydrate, then a peak at *m/z* 3752.4 should have been apparent in the mass spectrum of reduced hCGβcf. This peak was not observed at all in any of the spectra

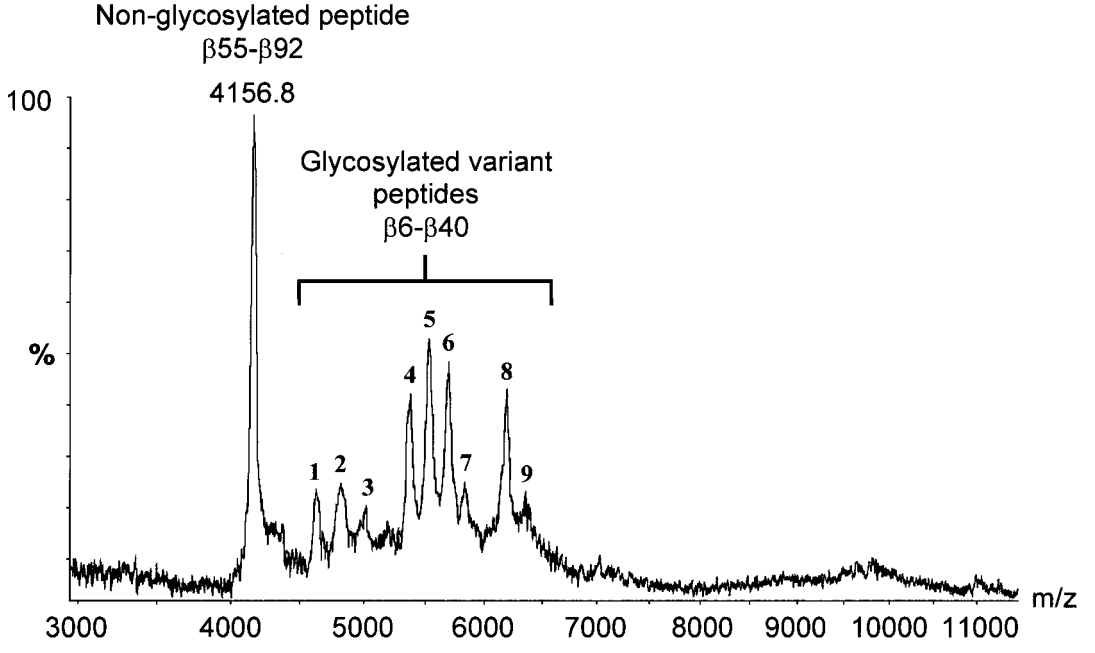


Fig. 4. MALDI-TOF mass spectrum of hCGβcf after "on-target" DTT reduction and using SA matrix.

The spectrum was generated by summation of the signals from 30 laser shots and calibrated internally using fragment mass $[M+H]^+$ 4156.8. *Peaks 1–9* are described in the text.

acquired from reduced hCGβcf. Additionally, our MALDI analysis of hCGβcf confirms the lack of sialic acid as expected but also the lack of any galactose in this preparation.

The galactose content of hCGβcf has been reported differently. Direct carbohydrate analyses after acid hydrolysis *(24)* or conversion of sugars to glycamines *(23)* has detected small amounts of galactose. Conversely, indirect analysis has found hCGβcf N-linked sugars lacking in the monosaccharide *(22, 25)*. The observation that hCGβcf is not bound by galactose-specific *Ricinus communis* agglutinin-agarose affinity matrix *(23, 25)* has been attributed to the inability of the matrix to bind one galactose residue per N-linked sugar, whereas contaminating glycoprotein is thought to account for hCGβcf galactose detected directly *(22)*. This direct MALDI-TOF MS analysis indicated that no galactose was present in the N-linked sugars of the sample we investigated. It is uncertain where the degradation of hCG carbohydrates occurs. In their study of hCGβ separated from intact urinary hCG, Liu and Bowers *(7)* detected a range of both sialated and asialo mono-, bi-, and triantennary N-linked oligosaccharides. It would therefore seem likely that the majority of sialic acid and galactose residues are lost as hCG is processed to hCGβcf after renal reabsorption. Whether or not this is the case, our results indicate that hCGβcf possesses neither sialic acid nor galactose residues. In fact, the glycoforms that appear to be present in hCGβcf range from a single GlcNAc residue to more complex structures extending beyond the trimannosyl core. The largest carbohydrate structures inferred by our results, peaks 8 and 9 in Fig. 4, may arise from degraded forms of the sugars II and IV in Fig. 1, determined by Elliott et al. *(14)* in hCGβ.

Absolute quantification of the relative amounts of each N-linked carbohydrate moiety was not possible using this method. Semiquantification of relative amounts of glycoform pairs was possible by determining the areas under the peaks of the reduced peptides. Unfortunately, many peaks could have arisen from multiple combinations of sugars. However, the monosaccharides determined from peak 8 (see Table 3) give rise to a combination of only two carbohydrates moieties that may arise from shortened triantennary oligosaccharides. This peak comprised 15.6% of the carbohydrate content of this sample, which is in agreement with the triantennary content of normal pregnancy hCG β-subunits as determined by Elliott et al. *(14)*. Because the crude preparation from which this hCGβcf was prepared may have contained urinary hCGβ from not necessarily normal pregnancies, it is impossible to say whether the larger sugar moieties we observed originated from either the triantennary carbohydrate (IV in Fig. 1) seen in normal or aberrant pregnancies or the biantennary sugar (II in Fig. 1) observed almost exclusively in gestational diabetes *(14)*.

In conclusion, the percentage abundance of other glycoforms detected by MALDI-TOF MS indicates that it is likely that the microheterogeneity of the hCGβ N-linked carbohydrates found by Elliott et al. *(14)* is conserved in hCG β-core fragments from all normal and aberrant pregnancies. Although not precisely defining hCGβcf glycosylation, the MALDI-TOF mass spectrum of DTT-reduced hCGβcf produces what appears to be a distinctive "fingerprint". For example, peaks of higher mass attributable to the increased triantennary sugar content of hCGβcf would be expected to increase in area in the spectra of hCG β-core fragments from individuals with abnormal or Down syndrome pregnancies and choriocarcinoma. Until now, definitive testing for hyperglycosylation in hCG in patient urine has required multiple precipitation and chromatographic steps *(31)* followed by thiol reduction, glycan release, and HPLC *(14)*. This is an approach that may be considered too time-consuming for routine clinical biochemistry. Although an extract of hCGβcf was analyzed in our current investigation, MALDI-TOF MS analysis of unextracted urine samples from pregnant women shows a broad peak corresponding to protonated hCGβcf, indicating that its concentration in pregnancy urine is sufficiently high for detection purposes. Further study will help to elucidate whether the reductive technique described here can be applied to pregnancy or choriocarcinoma urine, possibly after desalting and concentration by centrifugal ultrafiltration *(10)*. The potential clinical utility for distinguishing between normal pregnancy and Down syndrome pregnancy by this approach needs to be demonstrated and is currently under investigation.

This ongoing project is funded by the Joint Research Board of St. Bartholomew's Hospital, London.

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