hCG β core fragment is a metabolite of hCG: evidence from infusion of recombinant hCG

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Abstract

The availability of recombinant human chorionic gonadotrophin (r-hCG) has allowed us to measure its metabolic and renal clearance rates and to study the origin of the β core fragment of hCG (hCG β cf). Serum and urine samples were collected from six subjects, after an intravenous injection of 2 mg (equivalent to 44 000 IU Urinary hCG) r-hCG, and assayed for hCG and the beta subunit (hCG β). Urine from four of the subjects was also subjected to gel chromatography and assayed for hCG β cf and hCG.

r-hCG, administered as an intravenous dose, was distributed, initially in a volume of 3.4 ± 0.7 l (mean $\pm$ s.d.) and then in 6.5 ± 1.15 l at steady-state. The disappearance of r-hCG from serum was bi-exponential, with an

initial half-life of 4.5 ± 0.7 h and a terminal half-life of 29.0 ± 4.6 h. The mean residence time was 28.6 ± 3.6 h and the total systemic clearance rate of r-hCG was 226 ± 18 ml/h. The renal clearance rate was 28.75 ± 6.2 ml/h (mean $\pm$ s.d.). hCG β cf was detected in all urine samples collected at 6 h intervals. Over the 138 h period of urine collection, 12.9% (range 10.1–17.3%) of r-hCG injected was recovered as the intact molecule and 1.7% (range 0.8–2.9%) was recovered as the hCG β cf, in 4 subjects. The molar ratio of hCG β cf to hCG in urine increased from $3.1 \pm 1.7\%$, on day 1, to $76 \pm 34.3\%$ (mean $\pm$ s.e.m.) on day 5, after r-hCG infusion, suggesting that hCG β cf is a metabolic product of the infused r-hCG.

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Introduction

Human chorionic gonadotrophin (hCG), produced by the syncytiotrophoblast cells of the placenta, consists of α and β subunits which are produced separately from distinct genes and subsequently combine to form the dimeric molecule. Throughout pregnancy the free α and free β subunits are secreted into the plasma in addition to the intact hCG. At present, it is generally considered that these subunits do not perform any significant biological activity.

Previous studies on the pharmacokinetics and metabolism of hCG have indicated a multi-exponential disappearance curve in serum (Midgely & Jaffe 1968, Yen *et al.* 1968, Rizkallah *et al.* 1969, Wehmann & Nisula 1981, Korhonen *et al.* 1997) and that only 17–28% of the serum hCG is excreted in the urine as the intact heterodimeric form (Wide *et al.* 1968, Wehmann & Nisula 1981). In addition to a wide range of heterogeneous forms of intact hCG, the urine of pregnant females contains free α and free β subunits, nicked hCG (missing linkages in the β 41–54 region) (Puisieux *et al.* 1990, Birken *et al.* 1991, Kardana *et al.* 1991) and C-terminal

forms, i.e. fragments of the β chain (Amr *et al.* 1983) and hCG β cf (Kato & Braunstein 1988, Birken *et al.* 1988).

The quantitatively major urinary product, the β core fragment of hCG (hCG β cf), has an apparent molecular weight of 12–14 kDa, with a protein core of 73 amino acids. Structurally, it consists of two polypeptide chains, the β amino acids 55–92 and β amino acids 6–40 covalently linked by disulphide bonds. However, it lacks the immunodeterminant of hCG β . hCG β cf has also been found in the urine of nonpregnant patients with trophoblastic disease or cancer, in the absence of detectable concentrations of plasma hCG (Kardana *et al.* 1988). Consequently hCG β cf has been used as a cancer marker, particularly for gynaecological tumours. The origins of hCG β cf are uncertain. It has been assumed that it originates from the renal metabolism of intact or free hCG β , as demonstrated in the rat (Lefort *et al.* 1986). However, there are reports of its secretion by placental tissue in culture (Cole & Birken 1988).

Previous studies have been complicated because pure hCG has not been available (all hCG was of urinary origin, where hCG β cf is a known contaminant). The recent

availability of recombinant hCG (r-hCG) has allowed us to investigate the production of hCG β cf from the metabolism of pure r-hCG. In addition, it has allowed us to study the pharmacokinetics of the recombinant hormone and compare the data with the results of previous studies that have used urinary-derived hCG.

Materials and Methods

Protocol

Six healthy non-pregnant female volunteers aged 17–45 years, with normal menstrual cycles and no known liver or kidney disorders were recruited. Following informed consent, volunteers in their follicular phase received an intravenous bolus (2 mg; equivalent to approximately 44 000 IU urinary hCG) of r-hCG (Ovidrel; Serono Australia Pty. Ltd, Frenchs Forest, NSW 2086, Australia) via a saline drip over a 3 min period. The hormone was administered within 5 min from when the lyophilized material was reconstituted.

The first two subjects had blood samples collected from the opposite arm every 5 min for the first hour, every 10 min for the next 2 h, every 20 min for the next 6 h, at 2-hourly intervals for the next 24 h and at 6-hourly intervals for the next 72 h. Total urine excretion was collected at hourly intervals for the first 6 h, 2 hourly intervals for the next 26 h and at 12-hourly intervals up until 7 days after infusion. The other four subjects had blood samples collected every 5 min for the first 15 min, every 15 min for the next 45 min, every 30 min for the next 10 h, hourly for the next 3 h, followed by blood collection at 11, 15, 21, 29, 39, 51, 75, 87 and 105 h after infusion. Total urine samples were collected at 6 hourly intervals up to 7 days after infusion. All sera and urine samples were each aliquotted into four separate containers to avoid multiple freeze-thaw steps when each sample was assayed for hCG, hCG β or chromatographed and stored at -20°C .

The study was approved by the Human Ethics Committee at The Queen Elizabeth Hospital.

Gel filtration

r-hCG, hCG β (CR125; provided by the National Institutes of Health, National Institute of Child Health and Human Development, Bethesda, MD, USA) and purified hCG β cf (de Medeiros *et al.* 1992a) were initially chromatographed to establish their elution profile on a Superdex 75 column (1.6×60 cm; Amersham Pharmacia Biotech, Uppsala, Sweden) preequilibrated and run with 150 mM ammonium bicarbonate at a flow rate of 1 ml/min. One millilitre fractions were collected, desalted and assayed for the individual hormones. When r-hCG (167 μg in 1 ml 150 mM ammonium bicarbonate) was subjected to gel

filtration, fractions corresponding to the purified hCG β cf peak were pooled, desalted and assayed for hCG β cf. The pooled sample was then rechromatographed for confirmation of hCG β cf.

Twenty millilitres of each urine collection was concentrated 5 to 13 times using Macrosep centrifugal concentrators with a 3000 molecular weight cut-off membrane (Pall Filtron, Northborough, MA, USA). Concentration of the samples allowed the detection of the hormones in very dilute samples following gel filtration, as described above. The chromatography fractions were desalted by repeated lyophilisation and reconstitution with deionised water, and then were finally reconstituted in 50 mM phosphate buffer, pH 7.4, containing 0.5% bovine serum albumin and 0.05% sodium azide.

Immunoassays

hCG immunoreactivity in r-hCG, serum, urine and chromatography fractions was measured with the commercial Tandem-R hCG immunoradiometric assay (IRMA) (Hybritech Inc., San Diego, CA, USA) according to the manufacturer's protocol. The assay, which has a limit of detection of 1.5 mIU/ml and a working range of 5 to 400 mIU/ml, has been reported to detect both nicked hCG and non-nicked hCG (Cole 1997). hCG was assigned a molecular weight value of 38 kDa and a conversion factor of 1 IU = 1.2 pmoles was used to convert the results to molar equivalents.

The free β -subunit of hCG (hCG β) in the same samples was determined by the Amerlex-M free hCG β IRMA kit (Biclon Australia Pty. Ltd, Marrickville, NSW, Australia), which has a sensitivity greater than 0.35 mIU/ml, a working range of 5 to 150 mIU/ml and no cross-reaction with hCG up to 50 000 mIU/ml. The molecular weight of hCG β was assumed to be 22 kDa and a conversion factor 1 IU = 45.45 pmoles was used to convert results to molar equivalents.

hCG β cf was estimated by an IRMA which has been previously described (de Medeiros *et al.* 1992a). A specific polyclonal antibody (DeM3) was used as the capture antibody and iodinated monoclonal antibody 32H2, which is directed against epitopes of both hCG β and hCG β cf, was used for detection. This assay has a sensitivity of 1.5 pmol/l, a working range of 5 to 1500 pmol/l and cross-reaction, on a molar basis, with hCG, hCG β , hCG α and hLH β of 2.1, 5.3, 0.6 and 0.018% respectively.

Creatinine was determined in urine using the Beckman creatinine reagent kit with the Beckman Creatinine Analyser 2 (Beckman Instruments Inc., Fullerton, CA, USA).

Pharmacokinetics

The serum hCG concentrations were initially plotted on semilogarithmic graph paper and pronounced distribution

kinetics were noted. The decline in the serum hCG concentrations (C) could be described mathematically by either a bi- (1) or a tri-exponential equation (2),

$$C = Ae^{-\alpha t} + Be^{-\beta t} \quad (1)$$

$$C = Ae^{-\alpha t} + Be^{-\beta t} + Ce^{-\gamma t} \quad (2)$$

where A, B and C are the coefficients and α , β and γ are the exponents of the equations. These equations were fitted to the serum hCG concentration–time data by non-linear least squares regression (Prism v.2, GraphPad Software Inc., San Diego, CA, USA) using both unweighted and weighted $1/C^2$ procedures. The bi-exponential equation with weighting $1/C^2$ was chosen as the most appropriate model based on the analysis of the relative residuals, minimisation of the sums of squares deviation and standard errors of the estimates of the coefficients and exponents. The model independent pharmacokinetic parameters of total systemic clearance, volume of distribution at steady-state and terminal half-life ($t_{1/2\beta}$) were derived from calculation of the area under the serum hCG concentration–time curve (AUC) by the linear trapezoidal method and the terminal slope. The distribution phase half-life was calculated as $\ln 2/\alpha$ and the initial volume of distribution (V_C) as dose/A+B. The fraction of the r-hCG dose excreted unchanged was the amount excreted in urine to 138 h divided by the dose administered and the renal clearance was the product of the fraction excreted unchanged and the total systemic clearance. Mean residence time (MRT) was calculated as AUMC/AUC where AUMC is the area under the product of serum hCG concentration $\times$ time and time curve.

Results

Serum and urinary hCG and hCGβ

Serum collected prior to infusion of recombinant hCG had no detectable hCG or hCGβ. Human chorionic gonadotropin was detected in the first sample collected, 5 min after infusion, rising to a mean peak level after 10 min and then declining over a period of 105 h (Fig. 1a). The maximum concentration of hCG excreted in the urine (754 ± 177 pmoles/mmol creatinine; mean $\pm$ s.e.m.) was found to occur in the first of the 6-hourly urine samples from 4 subjects (Fig. 1b). Although hCGβ immunoreactivity was detected in both the serum and urine samples (results not shown), the levels were below the r-hCG crossreaction in the assay, which we found to be 1.96%, and therefore irrelevant.

Gel filtration

Gel filtration of the r-hCG on Superdex 75 resulted in one major hCG immunoreactive peak in fractions 51–67 and

Figure 1 hCG concentration (mean $\pm$ s.e.m.), measured by immunoradiometric assay, in (a) the serum of six healthy female subjects and (b) the urine of four of the subjects, after an intravenous injection of r-hCG (2 mg).

relatively minor hCGβcf immunoreactivity (0.006% of hCG activity, calculated on a molar basis) in fractions 79–90, which corresponded to the elution fractions of purified hCGβcf. Rechromatography of the pooled fractions 79–90 resulted in 70% recovery of the hCGβcf immunoreactivity. The true nature of the low molecular weight peak has not been characterized. Its presence has not been reported by the manufacturer and we are unsure of its origin. However, in comparison to the levels measured in the urine of the subjects, the estimated 3 pmoles of hCGβcf immunoreactivity present in the 2 mg r-hCG injected, was insignificant.

r-hCG and hCGβ immunoreactive peaks could not be resolved by chromatography on the Superdex 75 column (Fig. 2a). Therefore, when urine samples were chromatographed, fractions containing both these forms (51–70) were pooled prior to hCG and hCGβ assay. However, hCGβcf immunoreactive fractions (79–90) were well resolved from r-hCG and hCGβ as shown in both the elution profiles of chromatographed standards (Fig. 2a) and

Figure 2 Elution profiles of (a) r-hCG, hCG β and hCG β cf standards and (b) a urine sample collected after injection of r-hCG, when subjected to gel chromatography on a Superdex 75 (16/60) column, equilibrated and run with 150 mM ammonium bicarbonate.

a urine sample collected from a subject after injection of r-hCG (Fig. 2b). Therefore, hCG β cf assay of these fractions, after pooling, avoided any crossreaction with urinary hCG. The values were corrected for recovery by multiplying the total hCG β cf detected in the chromatographed urine by the total hCG in the equivalent volume of urine, prior to concentration and chromatography, and dividing by the hCG detected after chromatography. The hCG β cf excretion profiles for the four subjects showed considerable intersubject variability (Fig. 3a–d). The mean amount of hCG β cf excreted in the urine of 4 subjects over the 138 h period after r-hCG infusion was 912 pmoles, ranging from 423 to 1503 pmoles (Fig. 3e). That is, the quantity of hCG β cf produced is only 1.7% (range 0.8–2.9%) of the r-hCG injected and 12.2% (range 8.5–21.7%)

of the hCG dimer excreted in the urine. The ratio of hCG β cf to the intact hCG excretion increased from $3.1 \pm 1.7\%$ (mean $\pm$ S.E.M.) in urine collected on day 1 to $76.0 \pm 34.3\%$ (mean $\pm$ S.E.M.) on day 5, after r-hCG infusion (Fig. 3f).

Pharmacokinetics

The bi-exponential equation with weighting of the reciprocal of the square of the concentration provided the best and simplest mathematical description of the decline in serum hCG concentrations, following the intravenous dose. The total systemic clearance (Cl) was 226 ± 18 ml/h (mean $\pm$ S.D.), the initial volume of distribution (V_c) was 3.4 ± 0.7 litres, the volume of distribution at steady state

Figure 3 (a, b, c, d) The cumulative sum of hCG β cf detected in six-hourly urine samples, collected from four healthy female subjects, after an intravenous injection of 2 mg r-hCG. The urine was subjected to gel chromatography prior to being assayed. The value of hCG β cf was corrected for recovery as described in Results. (e) The cumulative sum of the total hCG β cf excreted in six-hourly urine samples of the four subjects (means $\pm$ S.E.M.). (f) hCG β cf excreted daily in urine samples collected from the same four subjects and expressed as a percentage (mean $\pm$ S.E.M.) of hCG detected in the same sample.

(V_{SS}) was 6.5 ± 1.15 litres, the initial half-life ($t_{1/2\alpha}$) was 4.5 ± 0.7 h, the terminal half-life ($t_{1/2\beta}$) was 29.0 ± 4.6 h and the mean residence time (MRT) was 28.6 ± 3.6 h (Table 1). The fraction of dose excreted unchanged (f_e) and the renal clearance rate (Clr) in the 4 subjects were calculated to be $12.9 \pm 3.4\%$ and 28.75 ± 6.2 ml/h respectively (Table 1).

Discussion

In this study, we have injected a recombinant preparation of hCG into healthy, non-pregnant female volunteers to demonstrate that the urinary hCG β cf is produced by the metabolism of the intact r-hCG molecule. Our observation of the appearance of hCG β cf in the urine of four female subjects, after receiving a dose of r-hCG, supports

the view that it is derived from the metabolic processing of hCG. Previous studies of pregnant subjects have shown that hCG β cf concentrations are 2–10 times that of the hCG dimer, on a molar basis, in pregnancy urine (Birken *et al.* 1988, Blithe *et al.* 1988, Krichevsky *et al.* 1988, Wehmann *et al.* 1989, 1990, de Medeiros *et al.* 1992b). However, in this study, the hCG β cf measured was only 12.2% of the amount of hCG excreted in urine, suggesting that, in pregnancy, there may be more than one pathway responsible for the production of hCG β cf. Trophoblastic tissue has been associated with the production of hCG β cf (Udagawa *et al.* 1998) as well as degradation of hCG (by nicking) to its subunits (Cole *et al.* 1993), which could account for the higher output of hCG β cf in pregnancy. The overall low excretion of hCG β cf in urine may reflect the fact that it is only an intermediate metabolite, which is metabolised further to fragments that are undetectable

Table 1 Pharmacokinetic parameters of r-hCG, following intravenous dosing in 6 healthy subjects

Subject	Cl (ml/h)	V _c (L)	V _{ss} (L)	t _{1/2α} (h)	t _{1/2} (h)	MRT (h)	fe (% dose)	Cl _r (ml/h)
1	214	2.76	6.07	4.2	33.6	28.4	17.3	37
2	216	2.76	4.83	4.8	25.1	22.7	10.1	22
3	214	3.45	5.75	5.7	23.9	26.9	13.7	29
4	260	4.6	7.82	3.9	26.3	29.9	10.5	27
5	225	3.22	6.90	4.0	30.0	30.4	*	*
6	228	3.68	7.59	4.5	34.9	33.2	*	*
Mean	226	3.4	6.50	4.5	29.0	28.6	12.9	28.75
S.D.	18	0.69	1.15	0.7	4.6	3.6	3.4	6.2
% CV	8	20	18	16	16	13	26	22

Cl, total systemic clearance; V_c, initial volume of distribution; V_{ss}, volume of distribution at steady state; t_{1/2α}, initial half life; t_{1/2}, terminal half life; MRT, mean residence time; fe, fraction of dose excreted unchanged in urine and Cl_r, renal clearance.
*Urine volume not available.

by the hCGβcf immunoassay, as suggested by Wehmann *et al.* (1989), when they recovered only 8% of injected hCGβcf in the urine of eight subjects.

r-hCG, injected i.v., exhibited a multi-exponential serum disappearance curve, as reported in previous studies for the disappearance of endogenous hCG, post-partum (Midgely & Jaffe 1968, Yen *et al.* 1968, Korhonen *et al.* 1997) and urinary hCG (Rizkallah *et al.* 1969, Wehmann & Nisula 1981). The studies, where urinary hCG was injected i.v., reported biphasic disappearance curves, with a fast component, t_{1/2} 5–6 h, and a slow component, t_{1/2} 24–32 h. Similarly, the studies of postpartum hCG reported a fast component of 3.6–11 h and a slow component of 18–37 h. Our data using r-hCG are similar to these values and suggest that there is no difference in the pharmacokinetics of r-hCG compared with hCG derived from endogenous material. In addition, Korhonen *et al.* (1997) reported a third component, with t_{1/2} = 53 h.

The percentage of injected hCG excreted in the urine, as the dimer, has been reported to vary from 17% to 28% (Wide *et al.* 1968, Wehmann & Nisula 1981). We also found a variable fraction of r-hCG excreted (10.1–17.3%), with a lower mean percentage. This may reflect a variation in the glycosylation of the recombinant protein, compared with the urine-derived hCG used in the previous studies. Alternatively, the difference may be the result of the immunoassays used in the studies, and their ability to detect the various isoforms of hCG.

The renal clearance rate determined by Wide *et al.* (1968) was 0.77 ml/min in the first 6 h and 0.64 ml/min in the 7–24 h period, when an immunoassay was used, and 0.39 ml/min and 0.34 ml/min, for the same respective periods, when calculated from bioassay results. Wehmann & Nisula (1981), using highly purified urinary hCG, reported the renal clearance rate to be 0.7 ml/min. The lower renal clearance rate determined in our study (0.48 ml/min) may reflect either the difference in the abundance of various isoforms, or that the urinary

preparations of hCG, used in the other studies, may contain higher amounts of nicked hCG, which has a shorter half-life.

In conclusion, we have demonstrated for the first time, that the pharmacokinetic parameters of r-hCG are similar to those of urinary hCG. In addition we report that hCGβcf is a metabolite of r-hCG.

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