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Journal of Chromatography A, 680 (1994) 217–224

JOURNAL OF CHROMATOGRAPHY A

Analysis of the microheterogeneity of the glycoprotein chorionic gonadotropin with high-performance capillary electrophoresis

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Abstract

Human chorionic gonadotropin (hCG) is a heteromeric glycoprotein hormone with a molecular mass of *ca.* 38 000. The carbohydrate side chains terminate with sialic acid and account for roughly 30% of the mass of the hormone. Glycoforms of hCG have been routinely identified with conventional methods of isoelectric focusing or chromatofocusing and exhibit varied bioactivity. In the present report, high-performance capillary electrophoresis (HPCE) was used to separate the glycoforms of hCG and its subunits. Optimal conditions for obtaining near-baseline resolution of the glycoforms were 25 m*M* borate, pH 8.8 containing 5 m*M* diaminopropane. The samples were separated in a 100 cm fused-silica capillary with an internal diameter of 50 μm at 25 kV and 28°C. In its native form, hCG migrated in less than 50 min as 8 distinct, highly resolved peaks. In the absence of diaminopropane, hCG migrated as a single, broad peak. When analyzed individually, the α subunit separated into four peaks and the β subunit resolved as seven peaks. The two subunits could also be separated when the heterodimer was incubated in 0.25% trifluoroacetic acid for 1 h prior to injection into the capillary. To illustrate the potential clinical application of this technique, four different sources of hCG were analyzed. The number of different isoforms was constant among the four samples; however, the relative concentration (amounts) of the isoforms varied. These results illustrate the potential utility of HPCE in the clinical diagnostic analysis of hCG microheterogeneity.

1. Introduction

Human chorionic gonadotropin (hCG) is a member of a family of glycoprotein hormones [1] which includes thyroid-stimulating hormone (TSH) and the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH). hCG is produced by the trophoblasts of the normal placenta and of choriocarcinoma tissue [2–4] and by non-trophoblastic neoplasia [5,6]. The hormone has an approximate molecular mass of 38 000 and consists of an α and β subunit that are non-covalently associated. Whereas both subunits have two N-glycosylated asparagines, the β-subunit contains an additional four O-glycosylated serine residues located within the C-terminal region. These carbohydrate modifications account for 30–35% of the mass of the hormone and result in several different glycoforms of the hormone [7].

The microheterogeneity of hCG and related glycoproteins has been examined by the methods of chromatofocusing and isoelectric focusing (IEF). The overall charge of hCG is acidic with a

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SSDI 0021-9673(94)00182-9

p*I* around 4 but it is comprised of 6 to 8 isoforms with p*I* values ranging between 3 and 6 [7]. The significance of the various isoforms is manifest in biological activity. More acidic variants of each of the hormones in this family tend to be more biologically active due to slower clearance from the circulation [7]. This relationship between the glycosylation pattern and the hormone's potency may be important in certain endocrine disorders and during the normal course of pregnancy. Studies of hCG from patients with trophoblastic disease have revealed that the glycosylation pattern is different from that of normal hCG [8–11]. These changes present an opportunity to develop diagnostic screening methods which discriminate choriocarcinoma from non-malignant trophoblastic diseases [12].

Although methods for analyzing heterogeneity of glycoproteins have provided valuable information, they are not without limitations. Both chromatofocusing and IEF are labor-intensive analyses and neither technique has provided quantitative or qualitative resolution of the different isoforms needed to obtain pure isoform preparations. In addition, with chromatofocusing a significant amount of the hormone remains on the column until elution with 1 *M* NaCl. The chemical nature of this highly charged material is not clear.

An alternative to the traditional methods of separation is the use of high-performance capillary electrophoresis (HPCE). This technique combines the advantages of simplicity, speed and reproducibility with high separation efficiency. Wide [13,14] used zone electrophoresis in agarose gel to analyze the heterogeneity of FSH, LH and TSH. Although the resolution was poor due to the limitations of the apparatus, Wide estimated that each of the hormones was composed of at least 20 different isoforms with minor differences in charge. The significance of this finding *versus* the number of isoforms identified with IEF is not known.

Recently, Landers *et al.* [15] reported a method for the efficient, high-resolution separation of the glycoprotein ovalbumin. In this method, the buffer additive diaminobutane was utilized to enhance separation that otherwise yielded a single broad peak. These studies present, for the first time, a facile method for the reproducible separation of hCG by HPCE and the improved baseline separation of its isoforms using a diaminoalkane as a buffer additive.

2. Materials and methods

2.1. Materials

Boric acid, sodium tetraborate and 1,3-diaminopropane (DAP) were obtained from Sigma (St. Louis, MO, USA). The neutral marker, dimethylformamide (DMF), was purchased from Aldrich (Milwaukee, WI, USA).

Four different preparations of hCG were used. The reference batch hCG CR127 was kindly supplied by the National Hormone and Pituitary Program (University of Maryland School of Medicine). Two separate batches of crude urinary hCG (Diosynth, Chicago, IL, USA) were purified using a previously published method [16]. This purification scheme results in the separation of nicked hCG and hCGβ fragment from intact hCG [17]. The resulting preparations were designated hCG 2292 and hCG 393. The fourth preparation of hCG, AK930, was purified from urine of a patient with metastatic choriocarcinoma using the method referenced above.

Pure subunits were obtained by treating hCG 2292 with 6 *M* guanidine hydrochloride for 18 h. The dissociated subunits were purified by reversed-phase HPLC on a Vydac C_8 column (Hesperia, CA, USA) in 0.1% aqueous trifluoroacetic acid (TFA) and a gradient of 5% to 48% acetonitrile in 0.1% aqueous TFA. Two major peaks were obtained, the first was the α subunit and the second was the β subunit (Fig. 1).

2.2. Capillary electrophoresis

All separations were performed on an Applied Biosystems Model 270A capillary electrophoresis system (Foster City, CA, USA) interfaced with a personal computer utilizing Beckman System Gold software (version 5.0; Beckman, Fullerton,

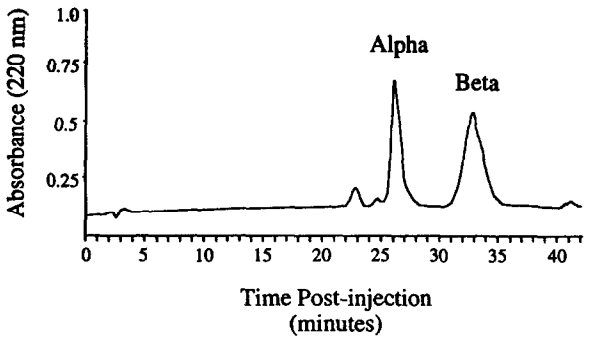

Fig. 1. Analysis and purification of hCG 2292 with reversed-phase HPLC following treatment with 6 *M* guanidine · HCl for 18 h. Conditions were 0.1% aqueous TFA with a gradient of 5 to 48% acetonitrile in 0.1% aqueous TFA.

CA, USA). Separations were performed with fused-silica capillaries which were 100 cm in length (78 cm to the detector) with an internal diameter of 50 μm. All electrophoretic separations were carried out at 25 kV constant voltage (inlet as the anode and outlet as the cathode) and the capillary temperature was maintained at 28°C. Detection was by absorbance at 200 nm.

Prior to each electrophoretic separation, the capillary was washed with 2 column volumes of 0.1 *M* NaOH followed by a 8-column-volume rinse with running buffer. The neutral marker DMF (1.5 m*M*) was then vacuum-injected for 1 s followed by the vacuum injection of each sample for 2 s. The estimated concentration of each hCG solution used for analysis was about 4 mg/ml.

In order to determine optimal conditions for the separation of hCG and its isoforms, several buffers of varying ionic strength and pH were analyzed. Six borate buffers at ionic strengths of 25 and 100 m*M* and pH values of 8.2, 8.8 and 9.2 were examined. In addition, 50 m*M* Tris–384 m*M* glycine, pH 8.3 and 20 m*M* ammonium formate, pH 8.8 were also tested. The buffer additive DAP was tested at various concentrations. All buffers were made with HPLC-grade water (EM Science, Gibbstown, NJ, USA) and filtered through a 0.2-μm filter (Gelman Science, Ann Arbor, MI, USA) before use.

2.3. Data analysis

In order to compare the distribution of the isoforms of the different preparations of hCG, the integration functions of System Gold were used to determine the area under the curve of the major peaks. Eight peaks were identified and the percent of the total area under the curve was calculated for each. Each of the four preparations of hCG was analyzed at least three times and the average percent of the total area for each peak was determined. Percent of total area for each peak were analyzed by ANOVA and the means were compared with the Scheffe *F*-test using the statistical program Statview (version 1.0.3).

3. Results and discussion

Addition of diaminopropane to the running buffer was necessary for the successful separation of hCG isoforms. When hCG was analyzed without DAP, a single peak with a broad tail and a migration time of under 20 min was observed (Fig. 2A). Adding DAP at various concentrations (Fig. 2B–E) resulted in the detection of multiple, well resolved peaks with a concomitant increase in the total migration time. Resolving power increased with additional DAP used; 1 m*M* DAP resulted in the appearance of multiple peaks with poor resolution, whereas the addition of 2.5 and 5.0 m*M* DAP led to the separation of eight clearly defined peaks in less than 50 min. Increasing the DAP concentration to 10 m*M* did not result in an increase in the number of peaks or improved resolution (data not shown).

Based on these results, all subsequent experiments were conducted with 5 m*M* DAP added to the running buffer. The improvement in resolution with 5 *versus* 2.5 m*M* DAP was sufficient to justify the additional migration time required for the analysis. The optimal conditions for separation were then determined and resulted in the choice of 25 m*M* borate buffer, pH 8.8 with 5 m*M* DAP as the running buffer. The current generated with this buffer in a 100 cm × 50 μm

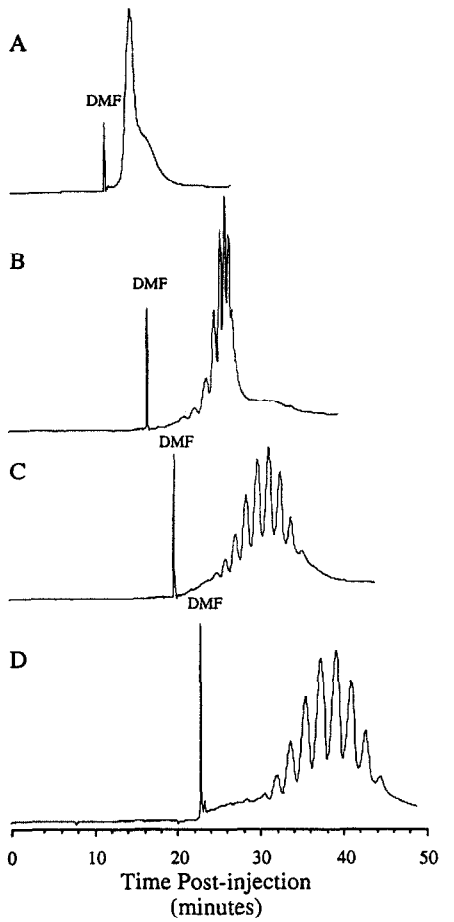

Fig. 2. Effect of various concentrations of diaminopropane on the electrophoretic migration and separation of hCG 2292 (4 mg/ml). Separations were carried out in a 100 cm × 50 μm fused-silica capillary with 25 m*M* borate, pH 8.8, 2 s injection of sample (4 mg/ml), 25 kV and 28°C. The DAP concentrations used were 0 (A), 1.0 (B), 2.5 (C) and 5.0 m*M* (D).

I.D. capillary at 28°C and 25 kV was 6 to 7 μA. Although borate was the buffer of choice, Tris-glycine and ammonium formate buffers containing 5 m*M* DAP yielded similar separations of hCG that had the same number of isoforms but without the enhanced baseline resolution obtained with borate buffer (data not shown).

It has been hypothesized that diaminoalkanes such as DAP enhance the separation of the glycoforms by decreasing the endoosmotic flow (EOF). This decrease in EOF results from the cationic amines interacting with the negatively charged capillary wall and thereby increasing the migration time of the sample. This increase in migration time allows for enhanced resolution of the different glycoforms. Other diaminoalkanes such as putrescine (1,4-diaminobutane) have been utilized as buffer additives for the analysis of microheterogeneity of glycoproteins, including tissue plasminogen activator (tPA) [18], ovalbumin and pepsin [15] and human recombinant erythropoietin [19]. Landers *et al.* [20] compared diaminoalkanes of different chain lengths (C_2–C_6) and demonstrated that diaminopropane and diaminobutane modified EOF and improved the resolution of ovalbumin to the same degree. In the present study, we also tested diaminobutane and diaminopentane and observed similar results (data not shown).

The choice of buffer may be critical for optimizing resolution of the different glycoforms. In their analysis of ovalbumin, Landers *et al.* [15] found that borate was superior to phosphate at putrescine concentrations of 1 m*M*. However, separation of the isoforms was possible in phosphate with a higher concentration of putrescine (5 m*M*). In contrast, Taverna *et al.* [18] found that putrescine was not efficient with borate but was with tricine for the analysis of tPA glycopeptides. In the present study, 25 m*M* borate, pH 8.8 containing 5 m*M* DAP provided the best results in terms of the resolution of hCG glycoforms. Tris-glycine and ammonium formate, on the other hand, yielded separation of the isoforms, albeit with resolution that was inferior to that seen with borate. Since we did not attempt to optimize the conditions with Tris-glycine and ammonium formate buffers it is not known if under different conditions these buffers would perform similar to borate. The borate ion may be inherently better for analyzing glycoproteins [15] because of its potential to form a borate–sugar diol complex as proposed by Novotny and co-workers [21,22]. The carbohydrates on hCG are of the high-mannose variety [23–26] which would allow for this type of complexation. Use of borate *versus* other buffers for the analysis of glycoproteins warrants further study.

The pattern of isoform separation is interest-

ing and merits discussion. Because HPCE separates molecules based on their charge-to-mass ratio, unlike IEF which separates proteins based on isoelectric point, the identity of the isoforms that correspond to each peak is not inherently obvious. Since the isoforms separated into peaks with a normal distribution, it suggests that the predominant forms are those with an "average" charge-to-mass ratio. The minor forms thus deviate from this ratio in both directions. Unfortunately, it is not possible to label each peak with a corresponding p*I*; this will require additional studies using fraction collection to obtain the individual forms and then determining the p*I* with IEF.

In addition to the high-resolution separation of the native heterodimer, the purified subunits were also analyzed for heterogeneity (Fig. 3). The α subunit is 92 residues in length and contains two sites for N-linked glycosylation whereas the β subunit is 145 residues long and has four sites for O-linked glycosylation in addition to two sites for N-linked glycosylation. Upon electrophoresis, the HPLC-purified α subunit resolved into four discernable peaks with migration times between 25 and 32 min (Fig. 3A). The first peak was detected in less than 3 min after the neutral marker DMF and appeared to be heterogeneous. Altogether, four major species of the α subunit were separated.

The separation pattern for the β subunit of hCG had a similar profile to the one for the intact dimer with fewer peaks (Fig. 3B *vs.* 3C). There were five major and two minor peaks (a total of seven) arranged in the same Gaussian or normal distribution as the native heterodimer; however, the peaks for the β subunit migrated faster than those for the intact dimer.

Since the subunits of hCG are not covalently linked, it was possible to dissociate them in acid and show that the separation profiles from the subunits analyzed individually were similar to the profile of the hormone following dissociation. Fig. 4 illustrates the effect of 0.25% TFA on the disassociation of the hCG subunits. The dissociation profiles represent 0, 60 min and 5 h after the addition of 0.25% TFA. As the amount of time increased following the addition of TFA, the

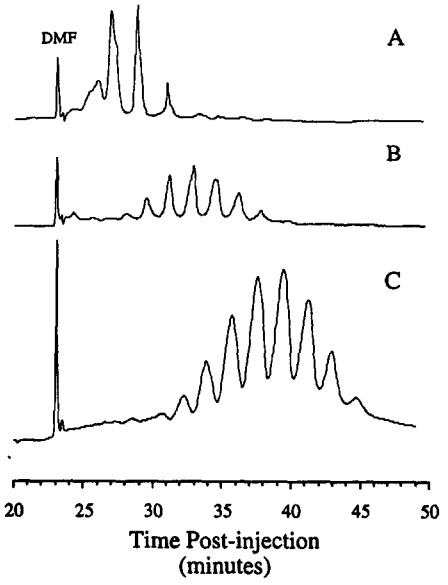

Fig. 3. Electropherograms of the individual α (A) and β (B) subunits of hCG 2292 (C). Separations were carried out in a 100 cm × 50 μm fused-silica capillary, with 25 m*M* borate plus 5 m*M* DAP, pH 8.8, 2 s injection of sample (4 mg/ml), 25 kV and 28°C.

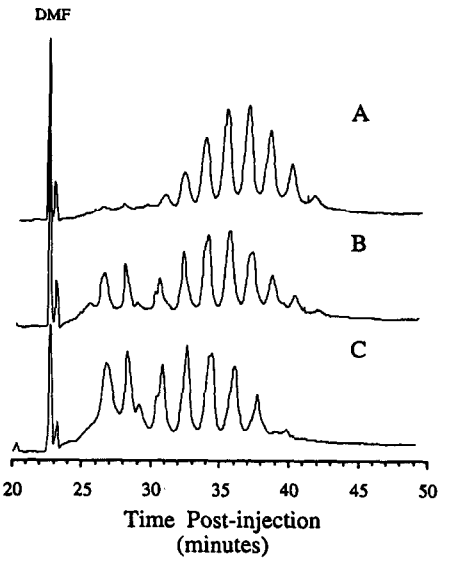

Fig. 4. Separation and analysis of microheterogeneity of the α and β subunits of hCG 2292 following addition of 0.25% TFA to the intact dimer. Figures represent 0 (A), 60 min (B) and 5 h (C) after the addition of TFA. Conditions as in Fig. 3.

magnitude of the peaks in Fig. 4C clearly decreased whereas peaks in positions similar to those for α and β appeared and increased in magnitude. The dissociation of hCG was examined after 24 h and did not exhibit any significant changes from the profile at 5 h.

The behavior and number of isoforms of the α subunit were expected based on its known glycosylation pattern. An important distinction to make, however, is that the results obtained here are for α subunit isolated from the dimer and are not the free subunit isolated from urine. This is a significant point because free subunit produced during pregnancy is more acidic than dimer α, may have a sialylated, serine-linked oligosaccharide, and consequently cannot form a heterodimer with the β subunit [27].

The oligosaccharides of the α subunit are characterized by a lack of fucosylation and exist predominantly in the monosialylated (50%) and asialylated (30%) forms [23]. The relative low amount of sialylation indicates that amount of acidity due to the oligosaccharides is minimal. In addition, based on the limited number of different possibilities that exist for oligosaccharides on the α subunit, Grotjan and Cole [7] estimated that two to three major isoforms of α exist. Results of the present study confirm these observations and predictions. The fact that the α subunit isoforms migrated more quickly than the β subunit and the intact dimer is probably due to the fact that it is smaller and less negatively charged than the other species. In addition, the number of peaks agrees nicely with Grotjan and Cole's estimate. In contrast, studies of the microheterogeneity of the α subunit using IEF have reported the presence of seven to eight peaks [27–29]. The reason for the differences between these reports and the present study is not known.

The similarity between the separation pattern for the β subunit and the one for the intact dimer is intriguing although not surprising. In general, the number of glycoforms should be influenced by the number of sialic acids present on the oligosaccharides. Since the β subunit contains six glycosylation sites and most of these contain oligosaccharides with one or two sialic acids, most of the heterogeneity of the dimer should be the result of the β subunit. Indeed, Cole [25] proposed this very idea based on studies of the O-glycosylation of the β subunit. The number of major peaks is in agreement with earlier IEF studies [30–32] and estimates by Grotjan and Cole [7]. Since the shape of the electropherogram is similar to that of the dimer, the contribution of the α subunit to dimer heterogeneity appears to be small, possibly increasing the total number of dimer isoforms by one or two.

In order to further investigate the nature of the separations, four unique preparations of hCG were analyzed and the results compared. Fig. 5 depicts the profiles of the four different

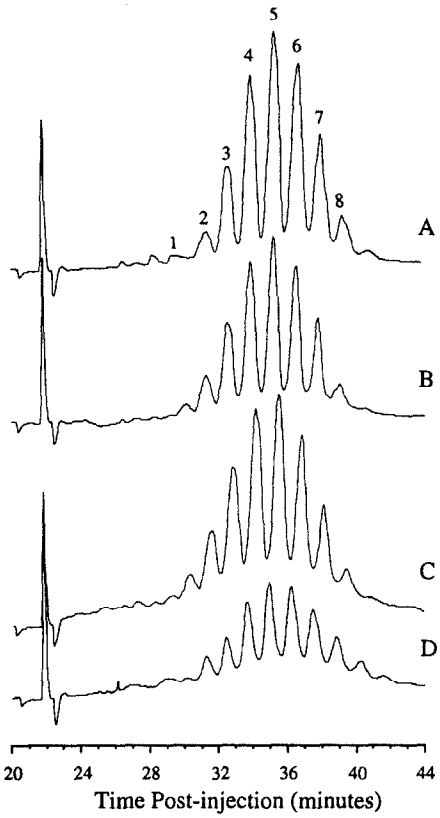

Fig. 5. Comparison of microheterogeneity among four separate preparations of hCG. (A) hCG CR127, (B) hCG 393, (C) hCG 2292 and (D) hCG AK930. Conditions as in Fig. 3. Peak numbers are referred to in Table 1.

preparations and illustrates an overall similar pattern of separation. Thus it appears that each of the four samples consists of roughly the same eight major isoforms. However, the relative amounts of each of the different isoforms, as manifested in the size of the peaks, was different among the different preparations. In order to compare the relative proportions of each of the peaks, it was necessary to quantitate them. The data in Table 1 shows the average peak area for each of the eight isoforms isolated from four different hCG samples. The data shown are the mean of three separate analyses for each preparation and are expressed as a percentage of the total area for all eight peaks. For the sake of comparison, Table 1 also depicts the mean values for the first four and last four peaks. Overall, the data show that there is a remarkable difference in the composition of the different hCG preparations. Nearly two thirds (64.9) of the isoforms of CR127 migrate as peaks 5–8, whereas peaks 5–8 represent 57.2% of hCG 393 and 51.7% of hCG 2292. The peaks for the choriocarcinoma preparation, AK930, migrated in a manner that did not allow an accurate comparison to the peak isoforms of the other hCG samples. If the data are calculated for AK930 in two ways so that the peaks align with either the previous or subsequent peak, then the relative amount of AK930 that is represented by peaks 5–8 is either 48.2% or 65.5%. Future studies using fraction collection and determination of the p*I* values of the different isoforms will provide a more complete picture of the characteristics of these different preparations.

In summary, seven to eight isoforms of hCG were separated with baseline resolution but only in the presence of the EOF modifier, DAP. Isoforms of the individual subunits were also separated with this procedure. Although we have not determined the p*I* of the different forms, variances in the different isoforms among differ-

Table 1
Comparison of the relative amounts of the glycoforms identified for each of four different preparations of hCG (%)

Sample[a]	Peak[b]							
	1	2	3	4	5	6	7	8
CR127	0.7 ± 0.1[c]	2.6 ± 0.1	10.5 ± 0.1	21.2 ± 0.2	26.6 ± 0.1	22.5 ± 0.1	12.0 ± 0.3	3.8 ± 0.1
		35.1 ± 0.5[d]				64.9 ± 0.6[e]		
393	1.4 ± 0.1	5.6 ± 0.1	13.4 ± 0.3	22.4 ± 0.1	25.1 ± 0.1	19.5 ± 0.1	10.1 ± 0.1	2.6 ± 0.2
		42.8 ± 0.6[d]				57.2 ± 0.5[e]		
2292	2.3 ± 0.1	7.4 ± 0.1	15.4 ± 0.1	23.2 ± 0.1	24.2 ± 0.1	17.5 ± 0.1	8.0 ± 0.1	1.9 ± 0.1
		48.3 ± 0.4[d]				51.7 ± 0.4[e]		
AK930	5.5 ± 0.3	8.7 ± 0.5	16.9 ± 0.5	20.7 ± 0.4	19.9 ± 0.3	15.0 ± 0.6	9.1 ± 0.3	4.2 ± 0.7
		51.8 ± 1.7[d]				48.2 ± 1.9[e]		

[a] Source of hCG (see Materials and methods section).
[b] Peak number in Fig. 5.
[c] Area under the curve for peak 1 divided by the total area under the curve for the entire electropherogram. Expressed as a mean % ± standard error.
[d] Sum of peaks 1–4.
[e] Sum of peaks 5–8.

ent preparations of hCG were clearly present and suggest that HPCE may be of great utility for the comparison of hCG microheterogeneity in normal tissues and neoplasms.

4. Acknowledgement

The authors wish to thank M. Cristine Charlesworth for the purification of the different hCG preparations.

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