CHROM. 16,781

BIOLOGIC ACTIVITY OF HUMAN CHORIONIC GONADOTROPIN FOLLOWING REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

JOHN W. WILKS* and SHARRON S. BUTLER
Fertility Research, The Upjohn Company Kalamazoo, MI 49001 (U.S.A.)
(Received March 29th, 1984)

SUMMARY

Human chorionic gonadotropin (hCG) was analyzed by reversed-phase high-performance liquid chromatography (HPLC) using mobile phases previously described in the literature, as well as newly developed solvent systems. Fractions of hCG collected following reversed-phase HPLC were bioassayed by activation of adenylate cyclase to determine their biologic potencies. hCG retained only 10–60% of its biologic activity following reversed-phase HPLC, depending on the chromatographic conditions employed. A portion of the reduced biologic activity was attributed to dissociation of the α- and β-subunits of hCG at the low pH of the mobile phases, since neutralization of the pH prior to lyophilization and bioassay increased the biologic potency of the chromatographed hormone. The remaining loss in biologic activity is presumably due to organic solvent denaturation.

INTRODUCTION

Human chorionic gonadotropin (hCG) is a hormone of placental origin comprised of two non-covalently associated dissimilar subunits, and containing 30% carbohydrate[1]. The α-subunit is identical for all the glycoprotein hormones (hCG, luteinizing hormone, follicle stimulating hormone and thyroid stimulating hormone). The β-subunit differs for each of these glycoprotein hormones, and confers the distinct biologic properties to each molecule. The complete amino acid sequences, and carbohydrate sequences of the N- and O-linked oligosaccharides, are known for the subunits of hCG[1]. A number of lengthy procedures for the purification of hCG have been described which employ affinity chromatography, ion-exchange chromatography and/or gel filtration[2–4]. More recently, two laboratories have described rapid procedures for preparing highly purified hCG and its subunits by reversed-phase high-performance liquid chromatography (HPLC)[5,6]. Neither laboratory addressed the issue of the effect of the reversed-phase HPLC procedure on the biologic activity of hCG. We studied a number of reversed-phase HPLC methods for the purification of hCG, and their influences on the biologic activity of the hormone. All the reversed-phase HPLC methods studied partially reduced the biologic activity of hCG.

EXPERIMENTAL

Materials

Highly purified hCG was obtained from Dr. Darrell N. Ward (Houston, TX, U.S.A.); the hCG had a biologic potency of 11,450 I.U./mg. HPLC grade solvents were obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). All other reagents were of analytical grade.

High-performance liquid chromatography

HPLC was performed on a Beckman liquid chromatograph equipped with Model 112 pumps, a Model 420 solvent programmer and a Model 165 detector (Berkeley, CA, U.S.A.). All experiments were performed using a solvent flow-rate of 1 ml/min, and one of the following columns: SynChropak RP-P (25 × 0.41 cm I.D., SynChrom, Linden, IN, U.S.A.), μBondapak C_{18} (30 × 0.2 cm I.D., Waters Assoc., Milford, MA, U.S.A.) and Ultrasphere Octyl (25 × 0.46 cm I.D., Beckman). All columns were operated with a guard column (4 × 0.41 cm I.D.) containing SynChropak RSC support (SynChrom). A variety of mobile phases and gradients were utilized; the details of these are given in the figure legends and Table I. In some experiments, a FLO-ONE radioactivity detector (Radiomatic Instruments & Chemical Co., Tampa, FL, U.S.A.) was used to detect ^{125}I-hCG. HPLC fractions were lyophilized prior to bioassay using a Savant Model SVC200H concentrator (Savant Instruments, Hicksville, NY, U.S.A.).

hCG bioassay

The biologic activity of HPLC fractions containing hCG was determined by their ability to activate adenylate cyclase in plasma membranes prepared from luteinized rat ovaries. Methods for the preparation of membranes and performance of the adenylate cyclase assays have been described[7,8]. The only modification to the published methods was the addition of 2.5 μM forskolin in 1.6% ethanol to the assay buffer for the adenylate cyclase assay. Forskolin increases the maximal response of the ovarian enzyme to hCG stimulation resulting in a steeper dose–response curve and increased assay precision. Standard hCG and HPLC fractions were assayed at 8–10 doses in each assay; each dose was tested in triplicate. The 3–6 doses falling on the linear portion of the dose–response curve were used in calculating potencies using standard bioassay statistics[9]. Since highly purified hCG was used throughout these studies, the standard for bioassay consisted of hormone which had not been subjected to HPLC. Potencies of HPLC fractions were expressed relative to the standard; a relative potency of 1.00 indicates a potency equal to the standard. Protein concentrations in the standard, and HPLC fractions, were determined by the binding of Coomassie Brilliant Blue G-250 to protein (Bio-Rad Protein Assay, Bio-Rad Labs., Richmond, CA, U.S.A.).

Preparation of ^{125}I-hCG

Highly purified hCG was radiolabeled by a procedure developed in our laboratory. One milligram of 1,3,4,6-tetrachloro-3α-6α-diphenylglycouril (Iodo-gen, Pierce, Rockford, IL, U.S.A.) was dissolved in 25 ml of methylene chloride. A 50-μl volume (4.6 nmoles) of Iodo-gen was pipetted into the bottom of a 1-ml glass vial

and the solvent was evaporated under nitrogen. Subsequently 10 μl of 50 m*M* sodium phosphate buffer (pH 7.4) containing 30 μg of highly purified hCG were placed in the vial. One mCi of $Na^{125}I$ was added to the vial, and the reaction was allowed to proceed at room temperature for 10 min with gentle mixing on a laboratory rotator. The reaction was stopped by dilution in 0.75 ml of distilled water. Specific activity was determined by precipitating a small aliquot of the reaction mixture with trichloracetic acid to determine the percentage of counts which were protein bound. The remainder of the reaction mixture was desalted on a Bio-Gel P60 column (20 × 0.7 cm, Bio-Rad Labs.) using 0.1% bovine serum albumin in distilled water as the eluent. Those fractions containing ^{125}I-hCG were lyophilized and stored at −20°C. The mean specific activity of ^{125}I-hCG, prepared in this manner, was 24.4 ± 2.0 μCi/μg ($n=6$); the mean relative biologic potency of these radiolabeled hCG preparations in the adenylate cyclase assay was 1.08 (95% confidence limits = 1.01–1.16).

RESULTS

Our initial studies were conducted using the mobile phases and a gradient described by Putterman *et al.*[5], but substituting the SynChropak RP-P column for a Waters μBondapak C_{18} column. As shown in Fig. 1, highly purified hCG was resolved into multiple components with this procedure; more than a dozen peaks were visible. Bioassay of the material eluted from the column gave low biologic potencies for all the fractions (Fig. 1), except for those peaks pooled into fraction IV. Fraction IV gave a relative biologic potency of 0.92 (0.84–1.00, 95% confidence limits); however this fraction represented only 14% of the protein loaded onto the column. Use of the same mobile phases on the μBondapak C_{18} column resulted in a chromatographic profile nearly identical to that observed in Fig. 1. Changing mobile phase B to 5.7 m*M* trifluoroacetic acid (TFA) in methanol gave a similar chromatographic result (SynChropak RP-P column) and the biologic potencies for all fractions were low (pooled relative potency = 0.28).

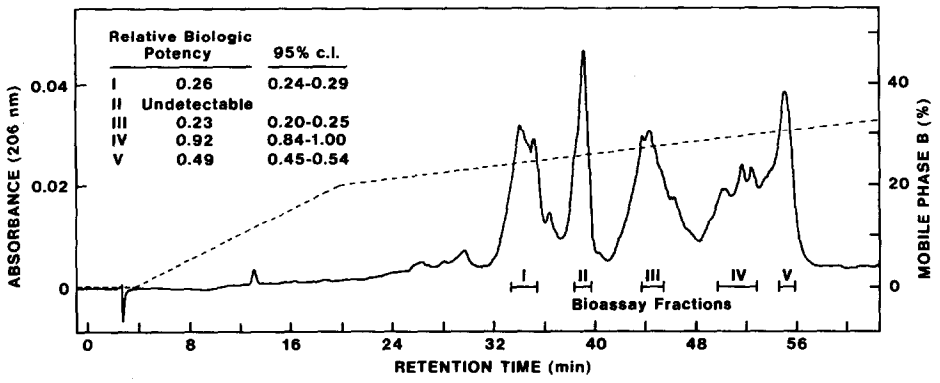

Fig. 1. Chromatography of hCG on SynChropak RP-P. Mobile phase A, 6.7 m*M* trifluoroacetic acid (TFA) in water; mobile phase B, 5.7 m*M* TFA in acetonitrile; the amount of sample loaded is 200 μg. The linear gradient for mobile phase B is indicated by the broken line. The biologic potencies of the fractions and their 95% confidence limits (95% c.l.) are indicated in the figure.

TABLE I

RELATIVE BIOPOTENCIES OF hCG FOLLOWING REVERSED-PHASE HPLC

*Mobile phase**			*Gradient*	*Column*	*Relative biologic potency*	*95% Confidence limits*	*Number of experiments*
I	A =	water–ethylene glycol monomethyl ether–*n*-butanol–TFA** (87:10:3:0.05)	0–10% B in 1.5 min, 10–60% B in 21 min	SynChropak RP-P	0.12	0.10–0.14	1
	B =	ethanol–*n*-butanol–ethylene glycol monomethyl ether–TFA (70:20:10:0.012)					
II	A =	water–ethylene glycol dimethyl ether–*n*- butanol–TFA (87:10:3:0.1)	0–10% B in 1.5 min, 10–60% B in 21 min	Waters μBondapak C_{18}	0.64	0.57–0.72	3
	B =	ethanol–*n*-butanol–ethylene glycol dimethyl ether–TFA (70:20:10:0.025)		SynChropak RP-P	0.54	0.45–0.66	1
III	A =	water–ethylene glycol dimethyl ether–*n*-butanol–TFA (87:10:3:0.05)	0–10% B in 1.5 min, 10–60% B in 21 min	SynChropak RP-P	0.45	0.42–0.49	4
	B =	ethanol–*n*-butanol–ethylene glycol dimethyl ether–TFA (70:20:10:0.012)					
IV	A =	water–ethylene glycol dimethyl ether–*n*-butanol–TFA (87:10:3:0.025)	0–10% B in 1.5 min, 10–60% B in 21 min	SynChropak RP-P	0.20	0.18–0.23	4
	B =	ethanol–ethylene glycol dimethyl ether–*n*-butanol–TFA (70:20:10:0.006)					
V	A =	water–ethylene glycol dimethyl ether–TFA (93:7:0.012)	10–40% B in 10 min	SynChropak RP-P	0.18	0.11–0.27	1
	B =	1-propanol					
VI	A =	water–ethylene glycol dimethyl ether–TFA (93:7:0.012)	10–40% B in 10 min	SynChropak RP-P	0.23	0.17–0.31	2
	B =	2-propanol		Waters μBondapak C_{18}	0.33	0.26–0.42	2
VII	A =	water–ethylene glycol dimethyl ether–TFA (93:7:0.012)	15% B for 6 min, 15–25% B in 1 min, 25% B for 2 min, 25–15% B in 1 min	SynChropak RP-P	0.36	0.22–0.59	1
	B =	2-propanol					

* Mobile phases I–IV were patterned after Van der Zee and Welling[10].

** TFA = trifluoroacetic acid.

Moudgal and Li[9] reported that hCG chromatographed as a single peak using a C_8 column, 1 *M* pyridine–0.5 *M* acetic acid for mobile phase A, and 1-propanol for mobile phase B. Using this system to chromatograph ^{125}I-hCG, we observed many peaks. Changing mobile phase A to 0.5 *M* pyridine–0.5 *M* acetic acid resulted in a more satisfactory resolution of hCG with one major peak (Fig. 2). Bioassay of unlabeled hCG fractions, collected at the same retention time as ^{125}I-hCG, resulted in a mean relative biologic potency of 0.59 ($n=3$, 95% confidence limits = 0.49–0.71).

Subsequent experiments focused on finding mobile phases which would elute hCG off the reversed-phase columns as a single peak. Table I outlines the experimental conditions used, and representative chromatograms are shown in Fig. 3. Under the best conditions, hCG retained about 60% of its biologic activity with mobile phases patterned after those described by Van der Zee and Welling[10]. Substitution of ethylene glycol dimethyl ether for ethylene glycol monomethyl ether proved advantageous in terms of preserving biologic activity. Reduction of the TFA to one-fourth of that used by Van der Zee and Welling[10] was detrimental to the biologic activity of the hormone. Substitution of 1-propanol or 2-propanol for mobile phase B also resulted in reduced relative biologic potencies. There were no significant differences when results were compared between the SynChropak RP-P or the Waters μBondapak C_{18} column.

Several attempts were made to renature hCG fractions following reversed-

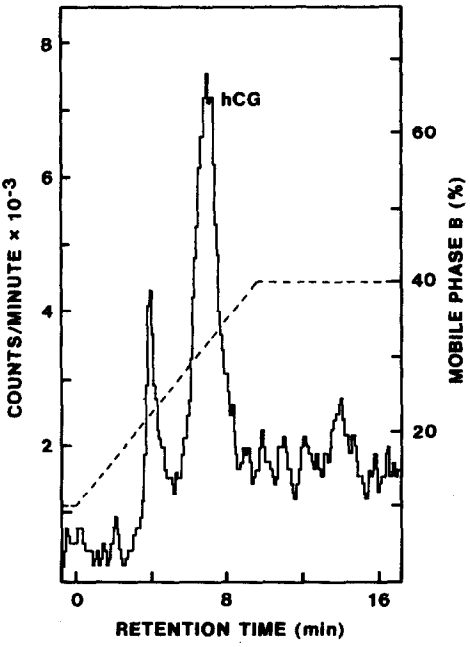

Fig. 2. Chromatography of ^{125}I-hCG on Ultrasphere Octyl. Mobile phase A, 0.5 *M* pyridine–0.5 *M* acetic acid; mobile phase B, 1-propanol; the amount of sample loaded is 50,000 cpm ^{125}I-hCG in 25 μg unlabeled hCG. The broken line indicates the linear gradient of 10–40% 1-propanol. Bioassay of unlabeled hCG fractions collected with the same retention time as ^{125}I-hCG yielded a relative biologic potency of 0.59.

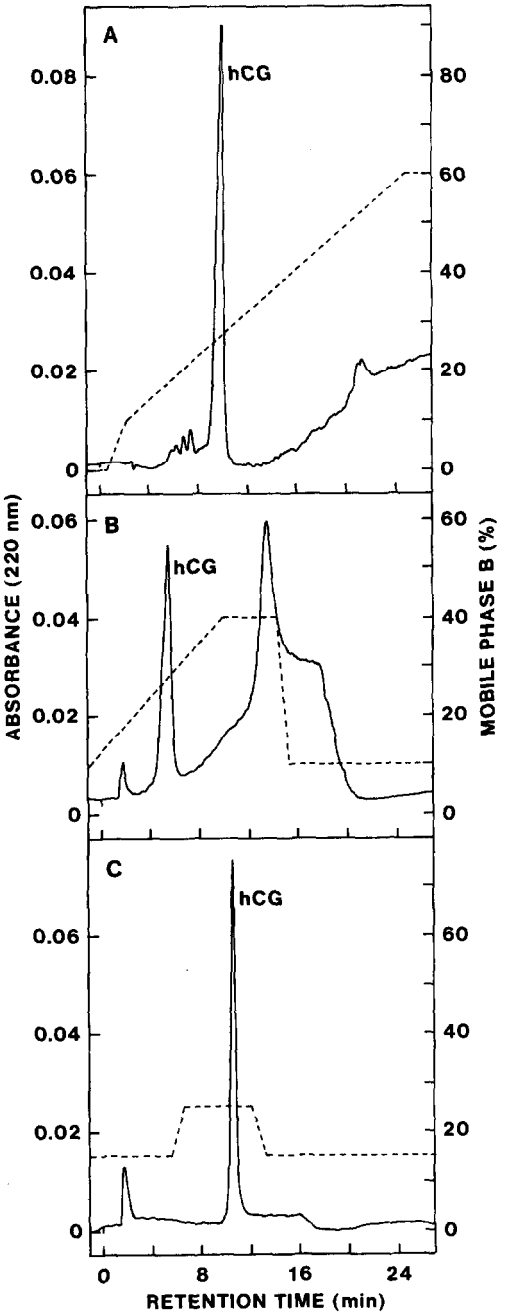

Fig. 3. Chromatography of hCG of SynChropak RP-P; sample loaded in each case is 50 μg. (a) Mobile phase A, water–ethylene glycol dimethyl ether–*n*-butanol–TFA (87:10:3:0.05); mobile phase B, ethanol–*n*-butanol–ethylene glycol dimethyl ether–TFA (70:20:10:0.012). (b) Mobile phase A, water–ethylene glycol dimethyl ether–TFA (93:7:0.012); mobile phase B, 1-propanol. (c) Mobile phase A, water–ethylene glycol dimethyl ether–TFA (93:7:0.012); mobile phase B, 2-propanol. The linear gradients for mobile phases B are indicated by the broken lines.

TABLE II

EFFECT OF pH ADJUSTMENT AND INCUBATION ON THE RELATIVE BIOLOGIC POTENCIES OF hCG FOLLOWING REVERSED-PHASE HPLC

c.l. = confidence limit.

Experimental conditions	*Number of experiments*	*Treated samples*	*Control samples*
Adjust pH to 7.5	3	0.54 (95% c.l. = 0.48–0.60)	0.38 (95% c.l. = 0.34–0.42)
Incubate at 37°C for 16 h at pH 7	2	0.12 (95% c.l. = 0.11–0.14)	0.13 (95% c.l. = 0.12–0.15)

phase chromatography; the relative biologic potencies of hCG following these procedures are shown in Table II. Since the mobile phases described in Table I had pH values of 2–3, the pH of fractions containing hCG was adjusted to 7.5 prior to lyophilization of the protein. Adjustment of the pH improved the relative biologic activity of the hormone. Recombination of the dissociated subunits of hCG is commonly achieved by incubation at 37°C for 16 h in a 10 m*M* sodium phosphate buffer at pH 7[2]. We performed incubations for 16 h following lyophilization of hCG fractions, but this procedure did not improve the relative biologic potency.

DISCUSSION

Denaturation and precipitation of proteins following reversed-phase HPLC is common[11]. It is therefore not surprising that our results should show a loss of biologic activity for hCG following reversed-phase HPLC. Our result is consonant with the observations of Bristow *et al.*[12] showing that thyroid-stimulating hormone retained only 58% of its biologic activity following reversed-phase chromatography. Moudgal and Li[6] used HPLC to purify the β-subunit of hCG. Although these authors did not consider the effect of the chromatographic procedure on the biologic activity of the protein, the figures in their paper show that the β-subunit of hCG obtained by HPLC possessed about 10% of the biologic activity present in the unchromatographed β-subunit[6].

It is well known that conditions of low pH can dissociate the subunits of hCG[2], and indeed Putterman *et al.*[5] observed dissociation of the intact hCG molecule under the conditions employed for reversed-phase HPLC purification of the glycoprotein hormone. The reduced biologic activity of hCG exposed to conditions of low pH can be fully restored following neutralization of the pH to 7[13]. Likewise, we were able to partially restore the biologic activity of hCG by pH adjustment following reversed-phase HPLC. The fact that biological activity could not be fully restored suggests that denaturation of a more detrimental type also occurred, perhaps as a result of the molecular forces required to elute the protein off the columns, or exposure of hCG to relatively high concentrations of organic solvent.

Van der Zee and Welling[10] recently described a mobile phase for the reversed-

phase HPLC of larger peptides and proteins which they reasoned would be less likely to precipitate proteins than other commonly used organic solvents. We found mobile phases patterned after their suggestion to give hCG fractions with the greatest retention of biologic activity (relative biologic potency of approximately 0.60). The pyridine–acetate system derived from the mobile phase used by Moudgal and Li[6] gave hCG fractions with equally high biologic potencies. All of these methods should prove useful for the purification of hCG by reversed-phase HPLC, however the protein purified by these procedures will have limited utility for biological investigations.

REFERENCES

1 J. G. Pierce and T. F. Parsons, *Ann. Rev. Biochem.*, 50 (1981) 465.
2 R. E. Canfield and F. J. Morgan,, in S. A. Berson and R. S. Yalow (Editors), *Methods in Investigative and Diagnostic Endocrinology*, Elsevier, New York, 1973, Ch. 1, p. 727.
3 S. Birken and R. E. Canfield, in K. W. McKerns (Editor), *Structure and Function of the Gonadotropins*, Plenum Press, New York, 1978, Ch. 3, p. 47.
4 P. Manjunath and M. R. Sairam, *J. Biol. Chem.*, 257 (1982) 7109.
5 G. J. Putterman, M. B. Spear, K. S. Meade-Cobun, M. Widra and C. V. Hixson, *J. Liq. Chromatogr.*, 5 (1982) 715.
6 N. R. Moudgal and C. H. Li, *Proc. Nat. Acad. Sci. U.S.*, 79 (1982) 2500.
7 L. Birnbaumer, P.-C. Yang, M. Hunzicker-Dunn, J. Bockaert and J. M. Duran, *Endocrinology*, 99 (1976) 163.
8 Y. Salomon, C. Londos and M. Rodbell, *Anal. Biochem.*, 58 (1974) 541.
9 D. J. Finney, *Statistical Method in Biological Assay*, Hafner Pub. Co., New York, 2nd ed., 1964, p. 302.
10 R. van der Zee and G. W. Welling, *J. Chromatogr.*, 244 (1982) 134.
11 M. T. W. Hearn, *Adv. Chromatogr.*, 20 (1982) 1.
12 A. F. Bristow, C. Wilson and N. Sutcliffe, *J. Chromatogr.*, 270 (1983) 285.
13 E. J. Umberger and G. H. Cass, *Science*, 129 (1959) 1738.