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CHARACTERISATION AND PURIFICATION OF SOME GLYCOPROTEINS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Native and desialylated human chorionic gonadotrophin showed different elution profiles by size-exclusion high-performance liquid chromatography (HPLC) and the difference was more pronounced using a mobile phase of low ionic strength. Ion-pair reversed-phase HPLC separated components of human chorionic gonadotrophin with apparent molecular weights ranging from 14 000 to 58 000 daltons (using sodium dodecyl sulphate gel electrophoresis). An affinity support prepared by attaching serotonin to LiChrosorb Diol specifically bound components containing sialic acid and may be useful for purification of sialoglycoproteins such as the glycoprotein hormones, β-interferon and transferrin. Size-exclusion HPLC revealed that of two human luteinizing hormone preparations whose sialic acid content correlated with their published *in vivo* activities, that with higher *in vivo* activity contained a significant quantity of high-molecular-weight material (possibly aggregation) which was not evident in the sample of lower potency.

INTRODUCTION

Preparations of human glycoprotein hormones, chorionic gonadotrophin (hCG), luteinizing hormone (hLH) and follicle stimulating hormone (FSH), for use as standards or reagents, or as formulations for therapeutic use, are routinely measured by radioimmunoassay and bioassay (using both *in vivo* and *in vitro* systems). These preparations are heterogenous, in part because of variations in the sialic acid content of the components. Progressive removal of sialic acid residues from hCG reduces *in vivo* activity but not immunological[1] or *in vitro* activity[2]. Likewise, desialylation of hLH causes a reduction in the *in vivo* activity without lowering the immunological activity[3].

Electrofocusing of highly purified preparations of hCG has demonstrated at least eleven components, six of which were isolated. The p*I* values of these components correlated with both their sialic acid content and *in vivo* activity, whereas the *in vitro* biological and immunological activities were unchanged[4]. Progressive desi-

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alylation of hCG reduces the half life of the hormone in plasma, and it has been concluded that there is a consequential loss in the *in vivo* activity[1,5]. Comparisons between *in vivo* (seminal vesicle weight assay) and *in vitro* assays for hLH and hFSH have supported this deduction[6,7]. In addition, the presence of sialic acid in the β-interferons has been suggested as a contributory factor to the stability of this interferon whilst in the circulatory system of animals[8].

Rapid methods for examining heterogeneity and subunit content of small quantities of the glycoprotein hormones would be useful for their characterisation and may eventually provide alternatives to the current *in vivo* and *in vitro* assays. hCG has been separated previously by reversed-phase high-performance liquid chromatography (HPLC) into its respective subunits[9,10]; however, further separation of the heterogenous subunits was not attempted. Anionic oligosaccharides and glycopeptides have been fractionated on the basis of hexose and hexosamine content using HPLC[11] but this study was not extended to glycoproteins.

The present paper reports the characterisation and purification of some glycoproteins using HPLC procedures, employing different separation mechanisms, together with a sensitive assay to determine the sialic acid content.

EXPERIMENTAL

Materials

Ammonium acetate, urea, Tris and glycine (all of AnalaR quality), N,N'-methylenebisacrylamide (laboratory reagent) and acrylamide (Electron) were purchased from BDH, trifluoroacetic acid from Aldrich (Milwaukee, WI, U.S.A.), anhydrous triethylamine from Koch-Light and absolute alcohol (AR) from James Burrough.

Serotonin (5-hydroxytryptamine) hydrochloride, neuraminidase (Type X from *Clostridium perfringens*), human chorionic gonadotrophin (3000 IU mg^{-1}), luteinizing hormone (from equine pituitaries) and porcine follicle stimulating hormone were all obtained from Sigma (St. Louis, MO, U.S.A.). The following preparations: human chorionic gonadotrophin, human chorionic gonadotrophin alpha subunit and human chorionic gonadotrophin beta subunit (NIBSC reagents 75/533, 76/508 and 75/535 respectively) were kindly provided by Dr. P. L. Storring, β-interferon by Dr. A. Meager and human transferrin by Dr. E. Griffiths. Dac-cel hCG radioimmunoassay kits were purchased from Wellcome Reagents (Dartford, U.K.). A broad p*I* calibration kit (pH 3–10) was obtained from Pharmacia Fine Chemicals.

Chromatography

Apparatus. Model 110 A Altex pumps were used in conjunction with a Cecil 272 spectrophotometer at 225 nm and a Rheodyne 7125 loop injector for isocratic HPLC. The same equipment was used together with a solvent programmer and solvent mixer (both from Altex) for gradient systems.

Materials. A TSK G2000 SW size-exclusion column (300 mm × 7.5 mm I.D.) and Brownlee Lab Aquapore RP-300 (250 mm × 4.6 mm I.D.) were purchased from Toya Soda and Anachem (Luton, Bedfordshire, U.K.) respectively. Stainless steel columns (between 50 and 150 mm × 4.6 mm I.D.) were slurry packed at 300 bar with Hypersil ODS (Shandon Southern, Runcorn, U.K.) and serotonin derivatized LiChrosorb Diol (10 μm).

Size-exclusion HPLC. Other than when stated, the mobile phase was 0.1 *M* ammonium acetate and the flow-rate 0.5 ml min^{-1}.

Hypersil ODS. The solvent compositions were: A, 95% 0.04 *M* triethylamine adjusted to pH 8.9 with trifluoroacetic acid, 5% ethanol; B, 20% 8.8 m*M* trifluoroacetic acid, 80% ethanol. A linear gradient was run between 10 and 83% B over 20 min at a flow-rate of 1 ml min^{-1}.

Aquapore RP-300. The gradient conditions were the same as those used for Hypersil ODS but with a pH of 8.2 for A and a gradient time of 30 min.

Affinity HPLC. LiChrosorb Diol was derivatised using route C described by Borchert *et al.*[12] as follows: 1 g silica was mixed with 213 mg sodium periodate, suspended in 3.3 ml water and degassed thoroughly. The suspension was rotated gently for 3 h at room temperature, decanted into a number 4 sinter funnel and washed with approximately 200 ml water. The preparation was suspended in approximately 7 ml 0.1 *M* sodium bicarbonate pH 8.0 containing 25 mg serotonin and rotated for 24 h at room temperature. The suspension was adjusted to pH 10 with sodium carbonate, then 40 mg sodium borohydride in 1 ml water were added over a period of approximately 3 h with constant stirring. The suspension was decanted and washed successively with water, 1 *M* sodium dihydrogenphosphate, water and finally acetone. The derivatised silica was dried *in vacuo*.

The solvent composition of A was 90% 0.02 *M* triethylamine with the pH corrected to 8.9 using trifluoroacetic acid, 10% ethanol; B contained 50% 8.8 m*M* trifluoroacetic acid, 50% ethanol. A linear gradient was run between 10 and 45% B for 20 min at a flow-rate of 0.7 ml min^{-1}.

Sialic acid assay

This was carried out as described by Hess and Rolde[13]. A Perkin-Elmer fluorescent spectrophotometer MPF-4 was used for detection of fluorescent derivatives.

Radioimmunoassay

This was performed as described in the kit instructions. Counts were determined with a Beckman gamma counter.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

A Bio-Rad Protean double slab electrophoresis cell was used. The composition of the gels and conditions of electrophoresis were as described by Oxford *et al.*[14]. The gels were developed using a silver stain[15].

Isoelectric focusing

Polyacrylamide gels were run and stained as described in the instructions printed by LKB[21].

Enzymatic hydrolysis of sialic acid residues

A solution of neuraminidase in 0.1 *M* ammonium buffer pH 5 was added to the glycoprotein to give a final activity of 330 mU ml^{-1} and incubated at 37°C for 2 h. The enzyme was inactivated at 100°C for 30 min in a sealed tube as a control.

Dissociation of hCG

A 1-mg amount of glycoprotein was heated at 47°C for 1 h with 0.7 *M* urea, 1.4% trifluoroacetic acid in a total volume of 1.3 ml.

RESULTS AND DISCUSSION

Assay of sialic acid

Since μg quantities only of glycoprotein hormones are present in reagents, standards and formulations, the commonly used assay for sialic acid of Warren[16] is not adequately sensitive. However, derivatization of sialic acid present in brain gangliosides with 3,5-diaminobenzoic acid to form a fluorescent product[13] is approximately 100-fold more sensitive as an assay procedure than the former method. In this laboratory, the fluorescent assay gave a linear response between 5 and 800 ng of free sialic acid. Spiked samples of hCG (5–400 ng sialic acid per μg hCG) also gave a linear response. Five replicates gave a coefficient of variation of 4.8% for sialic acid (25 ng each) and a coefficient of variation of 3.9% for hCG (20 μg each) indicating acceptable reproducibility. Sialic acid estimates for hCG recovered from a TSK G2000 SW size-exclusion column (injections of 25 μg each) gave a coefficient of variation of 10.9% for six injections and 5.8% for the five best samples.

Sets of sialic acid concentrations containing a constant amount of each component were assayed to detect possible interference by buffers and bulking agents. Each additive gave a linear response against an increased background, except for 20 m*M* sodium phosphate buffer pH 7.2 (containing 0.2 *M* sodium chloride) which gave

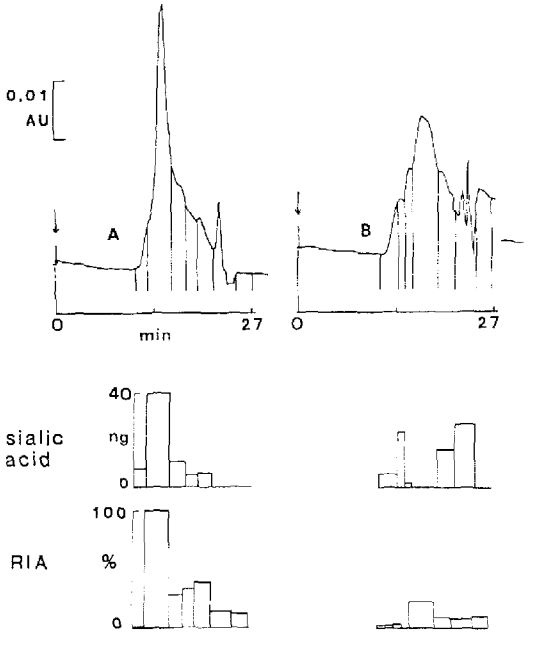


Fig. 1. Effect of desialylation of hCG on the size-exclusion elution profile and immunological activity. (A) hCG incubated with inactivated neuraminidase; (B) hCG incubated with active neuraminidase. For column, mobile phase and conditions, see text.

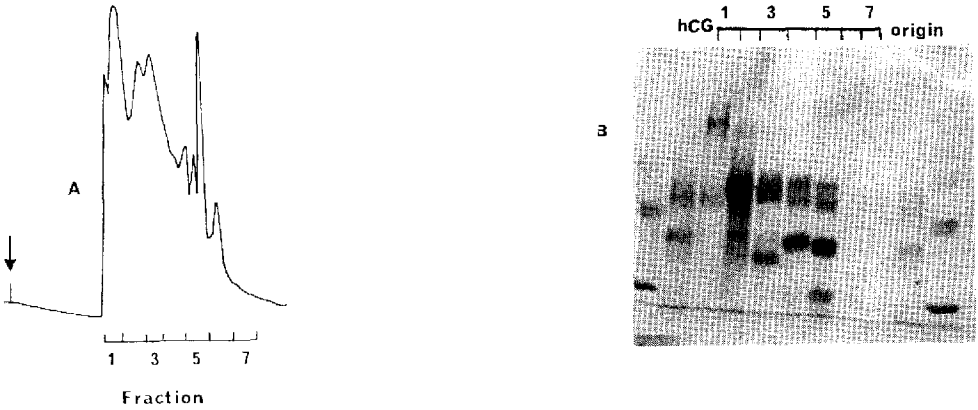


Fig. 2. Size-exclusion HPLC of dissociated hCG. (A) Size-exclusion elution profile; (B) SDS-PAGE of fractions. For column, mobile phase and flow-rate, see text.

a linear response with a reduced background fluorescence. Mannitol (500 μg per assay) showed minimal interference, whereas interference for albumin (714 μg per assay) was considerable, and lactose (1 mg per assay) charred.

During the derivatization procedure, sugar residues from hCG may be released by hydrolysis and interfere with the assay. Each monosaccharide was derivatized and the following values obtained (mole for mole sialic acid): fucose, 0.9%; mannose, 2.3%; galactose, 0.5%; galactosamine, 0.7%; N-acetylgalactosamine, 2.7%; glucose, 1.7%; glucosamine, 0.2% and N-acetylglucosamine, 2.2%. Specific hydrolysis of hCG with neuraminidase and separation of free sialic acid by size exclusion chromatography (Fig. 1, see Size-exclusion HPLC) demonstrated that the principal fluorophore is formed from sialic acid.

Size-exclusion HPLC

hCG. Some glycoprotein hormones have been examined previously by size-exclusion HPLC[17,18]. Whilst using this separation to check for interference in the assay of sialic acid from sugar residues, two effects of desialylation of hCG became evident. First, enzymatic hydrolysis resulted in increased retention of some components in the glycoprotein preparation (Fig. 1) (the amount of enzyme used for the hydrolysis was too low for detection by this procedure). This increase in retention was more pronounced using a mobile phase at half the ionic strength where the main peak had split into two peaks. Secondly, the immunological activity of desialylated hCG fractions was reduced considerably. Two control samples, one of which was incubated without enzyme and the other incubated with heat inactivated neuraminidase, showed no change in UV absorbance, immunoactivity or sialic acid profiles, thus demonstrating that these two effects were not artifacts produced by the conditions of incubation. The possibility that these two effects were a result of contaminating protease activity was also discounted since separate digests of albumin with neuraminidase and trypsin and subsequent examination by size-exclusion HPLC and SDS-PAGE showed no degradation of the albumin with neuramidase. Incubation of synthetic chromogenic substrates, designed for arginine-specific peptidases, with neuraminidase[19] revealed negligible levels of other enzymes. It was therefore concluded

that the increase in retention and reduction in immunological acitivity of the hCG fractions were a result of desialylation.

Since components of desialylated hCG eluted in the position expected for the subunits, the elution profile of denatured hCG was examined by SDS-PAGE (Fig. 2) revealing the expected order with decreasing molecular weight: fraction 1 contained high-molecular-weight material (possibly aggregates) and the β-subunit, fraction 2 contained β-subunit and fraction 5 α-subunit. In addition, the α-subunit fractions contained small proportions of the β-subunit which may have been carry-over from the earlier peaks or β-subunits containing fewer sialic acid residues. The increased retention of desialylated hCG has been demonstrated previously where it was suggested that the carbohydrate content of the β-subunit was a significant determinant in the expanded molecular size of hCG[17].

hLH. Two hLH preparations were examined, one of which contained approximately two thirds more sialic acid than the sample of lower *in vivo* activity (5740 and 4190 IU mg $^{-1}$ protein respectively, using the seminal vesicle weight gain assay[6]). Size-exclusion HPLC demonstrated that the more active sample contained totally excluded material which may have been aggregates (Fig. 3) whereas the less active sample contained little such material.

Reversed phase

Elution of hCG from a reversed-phase packing (Hypersil ODS) using an ammonium acetate–ethanol gradient gave some separation. However, gel electrophoresis (SDS-PAGE) demonstrated that material higher in molecular weight than expected for hCG but present in the unfractionated glycoprotein, bled from the column. Recovery measured immunologically was low.

A gradient of both triethylamine–ethanol and pH using the same packing separated a number of components from the hCG preparation without any bleed evident (checked by SDS-PAGE). Both the sialic acid content and immunological activity were predominantly associated with the main peak (results not presented). The same gradient was run on a 300-Å packing (Aquapore RP-300) and a similar separation

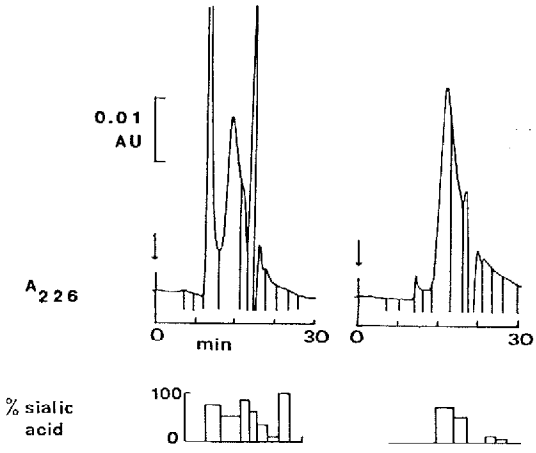


Fig. 3. Size-exclusion HPLC of hLH. For column, mobile phase and flow-rate, see text.

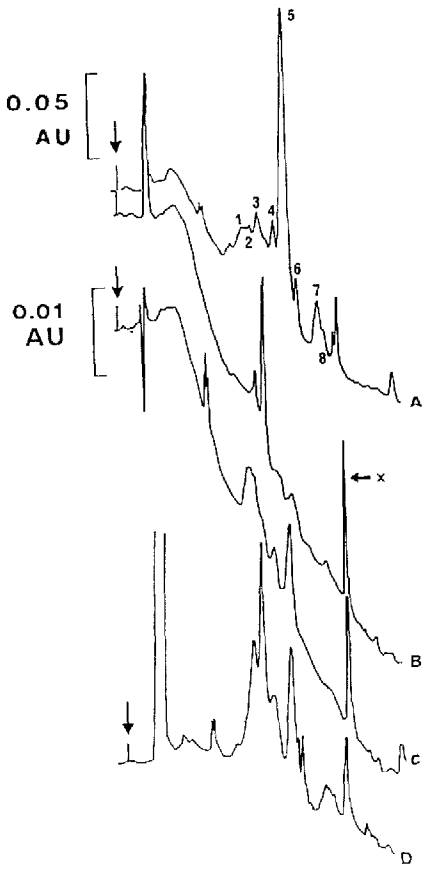


Fig. 4. Reversed-phase chromatograms of dissociated hCG. (A) hCG; (B) α-hCG preparation; (C) β-hCG preparation; (D) dissociated hCG. X = Artifact present in blank. For presumptive identification of peaks 1–5, see relevant text. Column: Aquapore RP-300. For mobile phase and flow-rate, see text.

was obtained with improved resolution and again the immunological activity was principally associated with the main peak. Since glycoprotein hormones are susceptible to dissociation into the respective subunits, the main hCG peak was reinjected into the same system and also examined by size-exclusion HPLC. Evidence of dissociation was minimal in the former and absent in the latter system.

Dissociation of an hCG formulation (using trifluoroacetic acid) resulted in increased peak height of some components and reduced height of the main peak (Fig. 4). The continued presence of the main peak in the dissociated preparation may have resulted from incomplete dissociation, reassociation on the column, co-elution with one of the subunits or an impurity. From the retention times of the subunit preparations (prepared by size-exclusion fractionation of dissociated hCG) the peaks were presumptively identified as the following: 2 and 3, α-subunits (both co-eluted with two peaks from the α-subunit NIBSC reagent), 1, 2 and possibly 5, β-subunits (the two shoulders at position 4 and peak 5 eluted in the same position as for the three peaks of the NIBSC β-subunit reagent). The identity of the β-subunits was supported

by the radioimmunological profile, the reaction of which is targeted against this component. The remaining peaks of the dissociated preparation may be subunits or impurity. The reversed-phase fractions were examined by isoelectric focusing. Although the glycoprotein gave poor staining, the following results were obtained: the solvent front, p*I* 7.2; peak 5, p*I* 6.3, 5.4 and 4.9; peak 6, p*I* 5.4, 5.0, and 4.9; peaks 7 and 8 combined, p*I* 4.6 and 3.8. Thus the separation was in part dependent on the

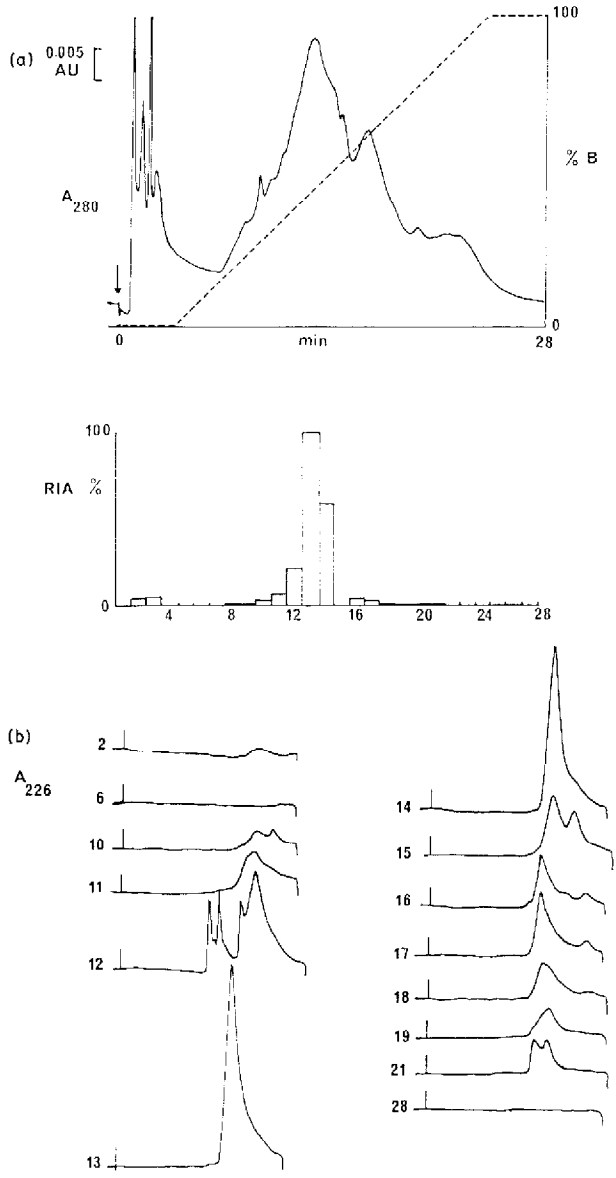


Fig. 5. Anion-exchange chromatography of hCG. (a) UV and radioimmunoassay elution profiles. Fraction 15 not measured by RIA. (b) Size-exclusion HPLC of anion-exchange fractions. For column, mobile phase and flow-rate, see text.

p*I* value, and so on the ionic character of the components, which may explain the apparent presence of more than one of each subunit. SDS-PAGE of the reversed-phase fractions confirmed that the peaks contain species of more than one apparent molecular weight, ranging from 14000 to 58000 daltons (measured using protein markers only). Hence the peaks of this separation are heterogenous with respect to both size and charge and the latter may be due to variation in sialic acid content.

When measured over a period of 3 days, the coefficient of variation for four injections of hCG was 3.3% and for five or more injections was 7.3%. Injections of varying amounts of the formulation (125–1000 IU, labelled claim) gave corresponding variations in peak height which yielded a plot (not shown) that was both linear and passed through the origin.

Two batches of an hCG formulation used therapeutically gave elution profiles that were not identical in this HPLC system. The components of a urinary preparation of human FSH and LH (1st International Standard) were resolved as were samples of equine LH and porcine FSH.

Anion exchange

Elution of hCG from an anion-exchange packing (Mono Q) gave a broad main peak containing several shoulders (Fig. 5a). Fractions containing appreciable UV absorbance were assayed for immunological activity. The activity predominated in the main peak. Examination of the fractions by size-exclusion HPLC indicated that the front and leading shoulder contain high-molecular-weight material and both subunits, the main Mono Q peak native hCG and the tail shoulder a range of components (Fig. 5b). The reappearance of material with molecular weights similar to the subunits and native hCG implies separation by charge and may reflect differences in sialic acid content.

Affinity HPLC

Sepharose bound serotonin has been prepared previously and the specificity of this ligand for sialic acid-containing components demonstrated[20]. A serotonin-

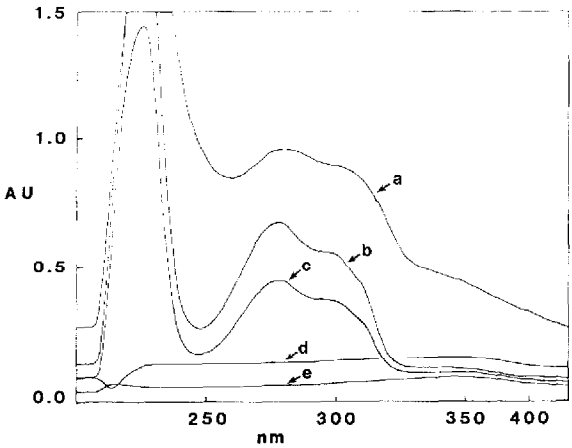
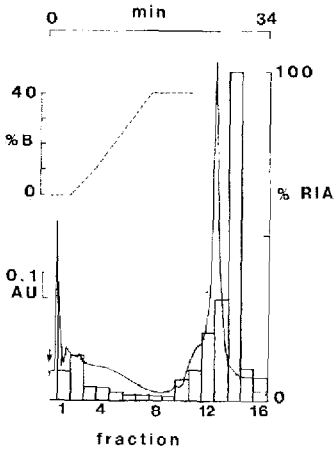


Fig. 6. Spectrum of LiChrosorb bound serotonin. Samples in a saturated solution of sucrose: (a) LiChrosorb bound serotonin; (b) LiChrosorb and serotonin; (c) serotonin; (d) LiChrosorb; (e) blank.



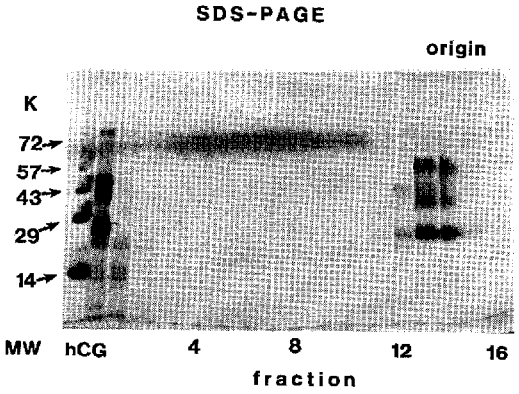


Fig. 7. Elution profile of hCG on serotonin-derivatized LiChrosorb. For column (100 mm × 4.6 mm), mobile phase and flow-rate, see text.

Fig. 8. SDS-PAGE of the hCG fractions eluted from serotonin-derivatized LiChrosorb. For conditions, see text.

LiChrosorb Diol support was prepared, the successful derivatization of which was demonstrated by the absorption spectrum (Fig. 6). The serotonin content of the derivatized silica was approximately 0.6 μmol m^2 as determined from the absorption profile.

Elution of an hCG preparation from the derivatized LiChrosorb showed most of the material emerging as one sharp peak, which was not retained on silica that had been prepared using the same procedure as the derivatized silica but without addition of serotonin. Specificity of the serotonin derivatized silica for sialic acid-containing components was demonstrated in the following way. Elution of specifically hydrolysed (using neuraminidase) hCG from the packing gave a peak that was reduced in area by 65% compared with that for a control sample of hCG that had been treated with heat-inactivated neuraminidase. The residual peak from the specifically hydrolysed hCG may have been due to the presence of sialic acid linkages that are resistant to cleavage by this enzyme. The hCG eluting with the single peak was immunologically active (Fig. 7). Further fractionation of the material within the peak by SDS-PAGE and size-exclusion chromatography demonstrated that the hCG components had been further purified (Figs. 8 and 9).

Specific retention and further purification of β-(fibroblast) interferon was obtained using the serotonin-derivatized silica (Fig. 10). The first of the two major peaks contained antiviral activity characteristic of β-interferon but with a lower titre than that of the starting material, which may have been due to inactivation during chromatography or incomplete elution. The second peak showed a very low proportion of antiviral activity.

Sialic acid-containing transferrin eluted from the serotonin-derivatized silica towards the end of the gradient cycle as a broad peak, possibly due to the presence of more than one component. The specificity of the material within the peak for binding iron was demonstrated.

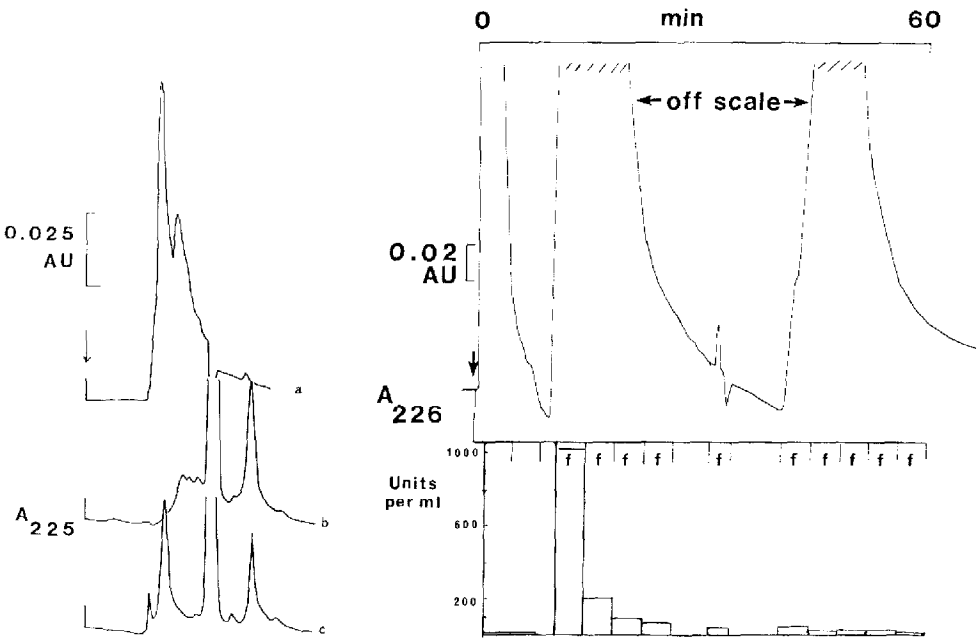


Fig. 9. Size-exclusion HPLC of hCG eluted from serotonin-derivatized LiChrosorb. (a) Unfractionated hCG; (b) solvent front; (c) peak from serotonin-derivatized LiChrosorb. For column, mobile phase and flow-rate, see text.

Fig. 10. Elution profile of β-interferon on serotonin-derivatized LiChrosorb. f = Fraction. For column (100 × 4.6 mm), mobile phase and flow-rate, see text. Units per μl = interferon activity.

CONCLUSIONS

The differences found between the total sialic acid content of the two hLH preparations studied support the suggestion that discrepancies between *in vivo* (using the seminal vesicle weight gain assay) and *in vitro* estimates of potency are due to differences in the sialic acid content[6]. The ratio of *in vivo* to *in vitro* potency estimates for the high and low potency preparations were 0.64 and 0.32 respectively[6]. Therefore the assay of sialic acid in conjunction with the *in vitro* assay may provide a more accurate assessment of biological activity than the *in vitro* system alone and may offer an alternative to the *in vivo* bioassay.

The elution profile of hCG separated on a size-exclusion support using a mobile phase of low ionic strength should indicate the proportion of desialylated glycoprotein present. This approach might be of use in the quality control of preparations for therapeutic use. Further characterisation of the constituents of such preparations is possible by judicious application of other modes of HPLC (reversed-phase, ion-exchange) and combination of these separations with radioimmunoassay (RIA).

Affinity HPLC of sialoglycoproteins using the newly developed serotonin-LiChrosorb Diol support, allows specific separation of sialylated hCG and possibly other sialic acid-containing hormones and consequently of material of high *in vivo* activity. Such separations should be useful for purification and have application in assay of glycoproteins.

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