

Reprint from

RECENT ADVANCES
IN DOPING ANALYSIS
(2)

M. Donike
H. Geyer
A. Gotzmann
U. Mareck-Engelke
(Editors)

Sport und Buch Strauß, Köln, 1995

C. Liu, L.D. Bowers:
Studies toward Confirmation of HCG using HPLC/MS
In: M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in doping
analysis (2). Sport und Buch Strauß, Köln, (1995) 235-242

Studies toward Confirmation of HCG using HPLC/MS

Sports Medicine Drug Identification Laboratory, Indiana University Medical Center,
Indianapolis, Indiana, United States

Introduction

There has been increasing interest in the development of methods which can definitively identify the presence of exogenous peptide hormones in urine. Recent advances in mass spectroscopy have made determination of peptides by mass spectroscopy a reality. Glycopeptides such as human chorionic gonadotrophin and erythropoietin present particular difficulties and opportunities for mass spectral analysis. Due to the presence of polysaccharide chains of varying lengths on these glycoproteins (microheterogeneity), a variety of species are present with different masses, all originating from the same primary sequence of amino acids. This presents a difficulty, since any particular fragment or intact glycopeptide can give rise to a number of mass signals, thus decreasing the observed signal for the compound. On the other hand, there may be particular glycoprotein components which are characteristic of pharmaceutical preparations of these glycoproteins, but which do not reflect a normal physiological distribution.[1, 2]

We report here on our efforts to characterize human chorionic gonadotrophin using electrospray (ESI) mass spectrometry following separation by HPLC. hCG is a glycoprotein consisting of two non-covalently linked subunits, α and β . The α subunit is shared with a number of other glycoproteins including TSH, LH and FSH. The β subunit, however, is unique to hCG. It contains 145 amino acids, of which the 24 residues at the C-terminal end, primarily differentiate hCG and LH. There are, however, several single amino acid substitutions in the LH sequence that should give rise to somewhat different fragments upon digestion with trypsin. The β subunit contains two N-linked carbohydrate sites and four O-linked carbohydrate sites (See Figure 1). It appears that the glycation of LH and hCG are also a means of differentiating between these two related glycoproteins. The β chain of hCG also has six internal disulfide bridges and the molecule is characterized by relatively tight folding.

Materials and Methods

A Sciex API III MS/MS equipped with an Ionspray interface was used throughout these experiments. The mass range of the quadrupole system was 10 - 2000 daltons. An HPLC system consisted of a Beckman Model 126 solvent delivery system, a Lee ViscoJet microliter mixing chamber, a Rheodyne 7185 microbore injection valve, and a Keystone 1x150 mm DeltaBond C-18 column. The entire effluent flow of 50 μ L/min was directed to the MS. Gradient elution was used throughout, with Solvent A consisting of 0.1% trifluoroacetic acid and Solvent B consisting of 0.1% trifluoroacetic acid in acetonitrile.

The hCG was obtained as the pharmaceutical preparation Pregnyl (Organon). The peptide was used without further purification or characterization. The enzymes trypsin, neuraminidase, glyconase, and glycosidase were obtained from Sigma and used without further purification.

Results and Discussion

A variety of breakdown products of hCG are excreted in the urine.[3] These compounds include intact hCG, free β chains, the β core fragment, the carboxy-terminal fragment and "knicked" hCG. There are reports in the literature that the knicked hCG derived from Pregnyl™ (Organon Company), contains non-physiologically knicked hCG.¹ The presence of these various peptides derived from hCG in the urine can give rise to some of the differences between immunoassay results for hCG. Although any of these compounds detected in urine could be an indication of exogenous hCG use in males, Wehmann and Nisula [4] found that after IV administration of purified hCG the predominant species in urine was the intact molecule. We, therefore, have initiated HPLC/MS studies to quantify and identify intact hCG in urine.

Our initial attempts to obtain a MS signal from the intact hCG molecule using the ESI were unsuccessful. We attributed this lack of success to an insufficient mass to charge ratio on the peptide since the Sciex API III has an m/z limit of 2400 Dalton. Similarly, signals from dissociated α and β subunits were not observable using this technique. We were able to obtain a signal from the α and β subunit after the subunits were denatured and reductively alkylated with iodoacetic acid or vinylpyridine. It was apparent from these studies, however, that the microheterogeneity of carbohydrate at the six potential

glycation sites gave rise to so many species that the detection limits for the molecule would be insufficient to obtain useful data from physiological fluids.

Tryptic fragments of the β subunit of hCG have been relatively well characterized. Figure 2 shows the HPLC trace obtained from a tryptic digest (top). Assignment of the amino acid sequences to the various peaks is complicated by the fact that the presence of carbohydrate significantly increases the mass of the primary sequence. Using the method of Carr et al.[5], we were able to use the mass spectrometer to collisionally dissociate the molecule in the region of the orifice into the vacuum system. This collisional dissociation gives rise to characteristic carbohydrate fragments which can be used to differentiate glycosylated peptides from simple peptides. In the example shown in Figure 1 (bottom) the hexose, sialic acid, and asialated carbohydrate fragments are identified for a peptide sequence ⁹Cys through ²⁰Arg which has a carbohydrate group attached to ¹³Asn. Knowledge of the peptide sequence as well as the reported carbohydrate structure then allows assignment of the mass of the fragment. The triply charged ions labelled A1 through A4 are various polysaccharides attached to the amino acids and constitute an example of the previously mentioned microheterogeneity observed for glycosylated peptides. Using this technique, we have been able to clearly identify all of the tryptic digest fragments for amino acids 1—114 from the β subunit of hCG.

The response of the HPLC/MS system to the carboxy-terminal fragments (amino acids 115 to 145) has been problematic. There are four O-linked carbohydrate sites in this segment. The microheterogeneity observed for multiple carbohydrates on a single peptide fragment causes not only problems with the mass spectrometer but also with the broad HPLC peaks. In an attempt to improve the signal observed, we undertook two experiments to remove either the sialic acid from the terminus of the carbohydrate or complete removal of carbohydrate with the enzyme glyconase. Complete removal of the O-linked carbohydrate was not successful. Removal of the sialic acids with the enzyme neuraminidase greatly increased the signal for the expected tryptic fragments (¹²⁴Phe-¹³³Lys and ¹³⁴Ala-¹⁴⁵Arg). This step, however, adds complexity to the analytical scheme. Further studies will be required to definitively determine the importance of this analytical step.

In summary, we have developed a preliminary scheme (Figure 3) which could be used for the identification and quantification of hCG in urine samples. Although analytically feasible, the ultimate format of the technique will be dependent on the requirements for

identity and precision of quantitation required to satisfy both legal and scientific criteria. For example, is identification of two peaks from the tryptic digest sufficient to identify hCG or are multiple peak identifications required?

References

1. L. COLE: personal communication.
2. L. WIDE, C. BENGTTSSON, B. BERGLUND, B. EKBLUM: Detection of administered recombinant erythropoietin in human blood and urine samples. in *Blood Samples in Doping Control*, P. Hemmersbach and K.I. Birkeland, eds., On Demand Publishing, Oslo, 1994, pp 159-61.
3. L.A. COLE, A. KARDANA, P. ANDRADE-GORDON, et al.: The heterogeneity of hCG: III. The occurrence, biological, and immunological activities of nicked hCG. *Endocrinology* 1991; 129: 1559-67.
4. R.E. WEHMANN, B.C. NISULA: Metabolic and renal clearance rates of purified human chorionic gonadotropin. *J Clin Invest* 1981; 68: 184-94.
5. M.J. HUDDLESTON, M.F. BEAN AND S.A. CARR: Collisional fragmentation of glycopeptides by electrospray ionization LC/MS and LC/MS/MS: methods for selective detection of glycopeptides in protein digests. *Anal Chem.* 1993; 65: 877-84.

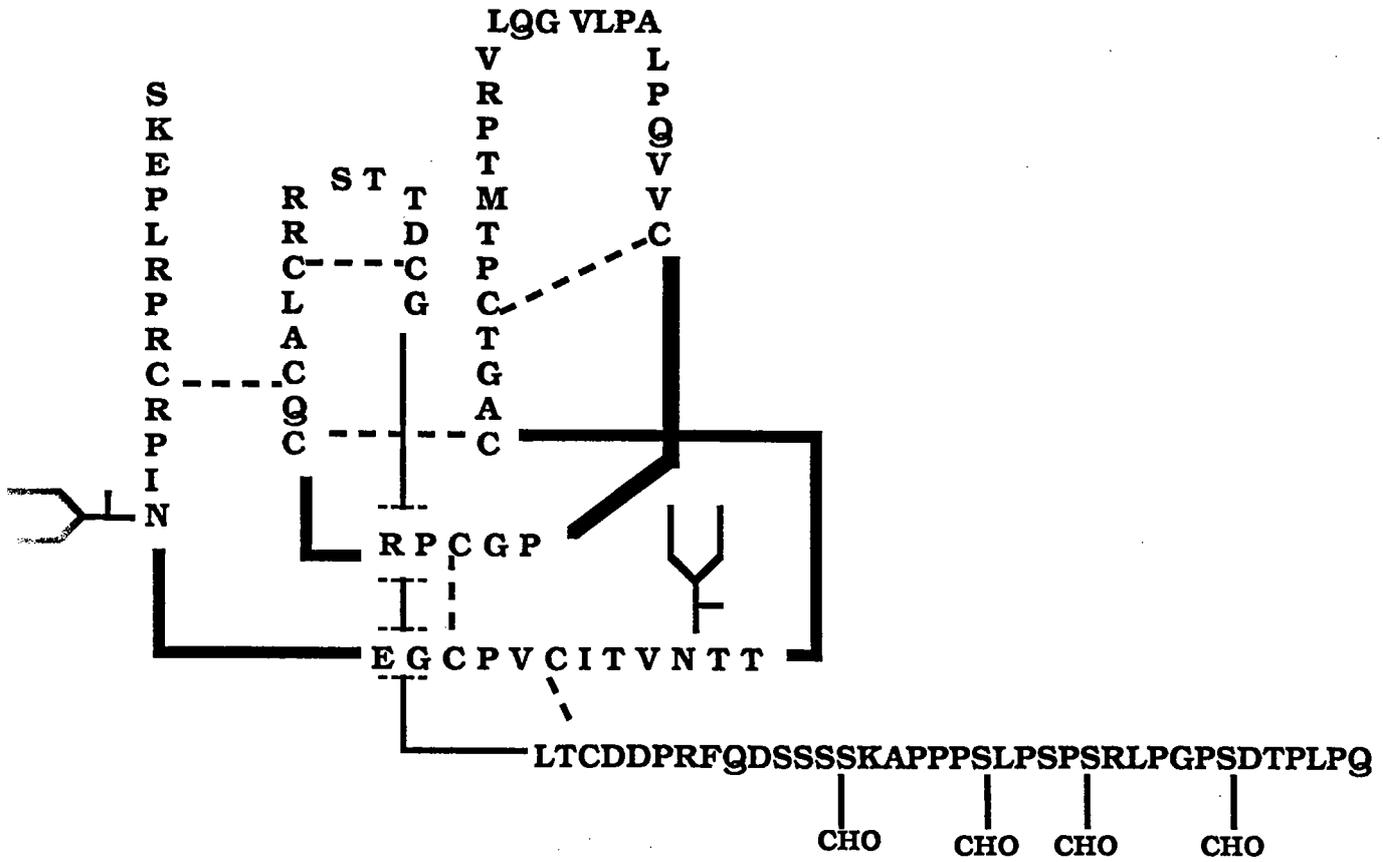
Figure Captions

Figure 1. Schematic representation of the β -subunit of hCG. The locations of "nicking" and of many of the tryptic cleavage sites are shown. Intra-molecular disulfide bonds are shown as dotted lines. The line structures at ¹³Asn and ³⁰Asn and at ¹²¹Ser, ¹²⁷Ser, ¹³²Ser, and ¹³⁸Ser represent the two types of glycosylation structures and their locations.

Figure 2. HPLC/MS trace (top) of the tryptic digest of the β -subunit of hCG. The mass spectrum of the peak at 20.1 minutes, corresponding to the sequence ⁹Cys—²⁰Lys, is shown below. The fragments below 450 m/z indicate the presence of glycosylation sites on the peptide.[5] The microheterogeneity of this sequence of hCG is illustrated by the four signals arising from triply-charged (A1-A4) observed at higher mass. A1 refers to a specific carbohydrate sequence attached to ¹³Asn on hCG.

Figure 3. Scheme for HPLC/MS confirmation of hCG in urine. The arrows indicate analytical sequences under consideration.

β - hCG Structure



HPLC/MS Analysis of a Tryptic Digest of β -chain of hCG

