Clinica Chimica Acta 413 (2012) 232–236

Contents lists available at SciVerse ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Loss of human chorionic gonadotropin in urine during storage at −20 °C

Anna Lempiäinen [a,b,*], Kristina Hotakainen [a,b], Henrik Alfthan [a], Ulf-Håkan Stenman [a,b]

[a] Department of Clinical Chemistry Helsinki University Central Hospital Laboratory Division (HUSLAB), Haartmaninkatu 2, FIN-00290, Helsinki, Finland
[b] Helsinki University, Haartmaninkatu 2, FIN-00290, Helsinki, Finland

ARTICLE INFO

Article history:
Received 6 May 2011
Received in revised form 3 August 2011
Accepted 24 September 2011
Available online 4 October 2011

Keywords:
hCG
Storage
Stability
Doping
Glycerol
Urine

ABSTRACT

Background: Quantitative determination of human chorionic gonadotropin (hCG) in urine is used in population studies of pregnancy disorders and in doping control. For these purposes samples are often stored at −20 °C, which in our experience may cause loss of hCG.
Methods: We redetermined hCG in 17 pregnancy urine samples stored at +4, −20 and −80 °C for 3–10 months and in 74 cancer urine samples stored at −20 °C for 5–14 years. We further studied the effect of added urea, pH and four preservatives on hCG immunoreactivity during storage at +4, −20 and −80 °C.
Results: At −20 °C, twenty to 100% of hCG immunoreactivity was lost in 15 of 17 pregnancy urine samples and in 43 of 74 cancer urine samples. At −20 °C, added urea (0.2–1.0 mol/L) caused a rapid decrease in hCG immunoreactivity, which glycerol (5–10%) prevented.
Conclusion: hCG immunoreactivity is lost in many urine samples during storage at −20 °C. Urea most likely plays a role in this process, but other mechanisms contribute to the loss. Urine should not be stored at −20 °C before analysis of hCG.

1. Introduction

Human chorionic gonadotropin (hCG) is a glycoprotein hormone composed of an α-(hCGα) and a β-subunit (hCGβ). The α-subunit is common to the other glycoprotein hormones, e.g. luteinizing hormone (LH), follicle-stimulating hormone (FSH) and thyroid-stimulating hormone (TSH), while the β-subunit is hormone specific and determines the biological function of each glycoprotein hormone. hCG is expressed by placental trophoblasts during pregnancy and stimulates steroid hormone production in the corpus luteum. Based on its ability to stimulate gonadal function, hCG is used in assisted reproductive treatment as a replacement for LH. hCG is also used in doping to restore suppressed gonadal function after use of anabolic steroids [1].

Pregnyl® is a hCG preparation that is widely used in assisted reproduction and it has also been used for doping. After injection of 10 000 IU/L of Pregnyl® to women, urine hCG concentration peaks on the first day, declines steadily with a mean half-life of 1.5 days and remains above the upper reference limit (15 pmol/L or 5 IU/L) for 7–11 days. The concentrations of the β-core fragment of hCG (hCGβcf) in urine peak after 1–2 days and after that the decline rate is similar to that of hCG. The concentrations of hCGβcf decrease below the upper reference limit (<7 pmol/L) 2–3 days earlier than those of hCG [2].

Clinically, analysis of hCG in urine is a convenient, noninvasive method for diagnosing and monitoring of pregnancy and of hCG-producing tumors [3]. For these purposes, long-term storage is seldom needed, but urine samples used for doping control are often stored at −20 °C before assay. The B sample, which is used to confirm a positive result, is stored at this temperature [4]. Storage at −20 °C has also been used for population studies on pregnancy disorders [5–8].

In some earlier studies, hCG has been found to be stable during storage at −20 °C [8,9], but in our experience this causes a variable loss of hCG in some samples. This may be accompanied by a simultaneous increase in hCGβ concentration, but in some samples the concentrations of all hCG forms decrease. Storage at −20 °C has also been shown to cause loss of urinary LH and FSH [10–12], which are similar to hCG in structure. Inconsistent recovery of urinary hCG during storage at −20 °C was recently reported, but the frequency or magnitude of the loss was not described [13].

Our aim was to evaluate the stability of hCG, hCGβ, hCGα and hCGβcf in urine during storage at various temperatures. Because urine contains fairly high concentrations of urea (0.2–0.8 mol/L) that has been suggested to cause degradation of LH at −20 °C [11], we studied the effect of added urea on hCG loss. We also evaluated the protective effect of different additives; glycerol, which has been shown to prevent the degradation of other gonadotropins [10–12], ethylene diamine (EDA), which protects proteins against carbamylation caused by urea-derived cyanate, EDTA, and bovine serum albumin (BSA).

* Corresponding author at: Biomedicum Helsinki, Room A418a, Haartmaninkatu 8, P. O. B. 700, FIN-00029. Tel.: +358 50 5701369; fax: +358 9 4717 1731.
E-mail address: anna.lempiainen@helsinki.fi (A. Lempiäinen).

0009-8981/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.cca.2011.09.038

2. Material and methods

2.1. Reagents

Reagents were purchased as follows: partially purified urinary hCG (Pregnyl®) from N. V. Organon (Oss, the Netherlands), urea, BSA and EDA from Sigma-Aldrich (St. Louis, Missouri), glycerol from Merck AG (Darmstadt, Germany), protein buffer (DELFIA® Multibuffer) from PerkinElmer Wallac (Turku, Finland), 15-ml polypropylene tubes (Falcon®) from Becton-Dickinson (Franklin Lakes, New Jersey) and 2-ml polypropylene tubes (Safe-Seal micro tube®) from Sarstedt (Nümbrecht, Germany). WHO International Reference Reagent (IRR), IRR 99/650 for hCGβ, IRR99/720 for hCGα and IRR99/708 for hCGβcf, were provided by the National Institute of Biological Standards and Control (NIBSC, South Mimms, UK) [14,15].

2.2. Samples

Urine samples from nonpregnant and pregnant subjects were donated by apparently healthy women, who gave verbal informed consent. Urine from patients with hCG-producing tumors was leftover clinical samples stripped of personal identifiers. Exemption for obtaining informed consent from cancer patients was provided by the National Supervisory Authority for Welfare and Health. The sampling of biologic material was carried out according to the Helsinki declaration.

2.3. Assay methods

All samples were run in duplicate and each set of samples was analyzed in a single run. hCG in urine was determined by a time-resolved immunofluorometric assay (IFMA) (AutoDELFIA®, Perkin-Elmer Wallac) with a monoclonal antibody (MAb) against hCGβ for capture and a MAb against hCGα for detection [16]. This assay is specific for intact hCG and does not recognize hCGβ. To facilitate comparison of the concentrations of various hCG forms, the concentrations of hCG expressed in IU/L were converted to pmol/L by multiplying by 2.93 [17]. The detection limit of the hCG assay was 1.5 pmol/L (0.5 IU/L) and the intra-assay coefficient of variation (CV) 4–8% at concentrations between 2.1 and 15 000 pmol/L in serum samples [18]. Mean CV of duplicate samples in this study was 3% (range 0–16%). Glycerol (added up to 50% of the sample volume) did not affect results (data not shown). The AutoDelfia method, has not been validated for quantitative determination of hCG in urine. However, the AutoDelfia assay and the in-house assays of hCGβ and hCGβcf have been thoroughly characterized for analysis of both urine and serum [18].

hCGβ, hCGα and hCGβcf in urine were determined by in-house IFMAs using MAbs 9C11, 2G11 and 3C11 as capture antibodies and MAbs 1B2, 7E10 and 1B2 as detection antibodies, respectively [18]. We have characterized the epitopes of these antibodies by comparing with reference antibodies for which the epitopes have been characterized before [19]. 9C11 detects a β-chain epitope hidden in intact hCG, 7E10 and 2G11 an α-chain epitope and 1B2 a β-chain epitope exposed both on hCG, hCGβ and hCGβcf and 3C11 an epitope present only on hCGβcf. In the assay, 25 μL of samples or calibrators and 200 μL assay buffer are incubated in the antibody-coated wells and after 1 h the wells are washed three times and 200 μL of labeled antibody (50 ng) added. After further incubation for 30 min, the unbound antibody is removed by washing, enhancement solution is added and after 5 min fluorescence measured in a Wallac Victor (PerkinElmer Wallac) time-resolved fluorometer. The lower limit of detection was 0.3 pmol/L for hCGβ [18], 2.8 pmol/L for hCGα [H. Alfthan, unpublished results] and 0.4 pmol/L for hCGβcf [18]. The intra-assay CVs were 2–10% at concentrations above 3 pmol/L for hCGβ and hCGβcf [18]. In this study mean CV of duplicate samples was 5% for hCGβ (range 0–30%), 4% for hCGα (range 0–23%) and 4% for hCGβcf (range 0–33%). The hCG assay was calibrated against the 4th international standard (IS) (75/589), the hCGβ assay against WHO IRR 99/650, hCGα against IRR 99/720 and hCGβcf assay against IRR 99/708 [14,15]. The hCG assay has previously been calibrated against the 3rd IS, which is virtually identical with the 4th IS [15].

Urine density was measured with a refractometer (UG-1, Atago CO Ltd, Bellevue, WA, US) and pH with the PHM92 (Radiometer Analytical, Lyon, France). Urinary tract infection was ruled out with a urinary dip stick test (Multistix® 8 SG, Siemens AG, Münich, Germany).

2.4. Stability of hCG in urine after up to 14 years of storage

We re-determined hCG in 74 hCG-containing urine samples from cancer patients after storage at −20 °C for 5 to 14 years. The initial hCG concentration, to which the results were compared, had been determined before storage with the same assay. We analyzed the effect of urine density (n = 28, determined before storage) and urinary creatinine concentrations (n = 30, retrieved from the patient charts) on hCG recovery. We re-determined urinary density in twelve samples.

2.5. Stability of hCG in urine during 1 week–10 months of storage

We studied hCG stability in fourteen consecutive urine samples (median density 1.017, range 1.012–1.029) from a single pregnancy obtained at pregnancy weeks 5–36. Aliquots were stored at +4 and −20 °C, respectively. Quantitation of hCG, hCGβ and hCGβcf was performed simultaneously after 3–10 months of storage. Recovery of hCG was calculated by comparing concentrations in samples stored at −20 °C to those stored at +4 °C. hCG has previously been shown to stable at +4 °C for at least 3 months [13].

The stability of urine hCG at different storage temperatures (+4, −20 and −80 °C) and its variation between individuals were studied using three urine samples obtained from different pregnant women containing 180, 22 500 and 28 000 pmol/L of hCG (densities 1.024, 1.015 and 1.013), respectively. Four aliquots were stored at +4, −20 and −80 °C each. The concentrations of hCG, hCGβ, hCGα and hCGβcf were determined before storage, after 1 week, 1 month, 3 months and 6 months of storage.

2.6. Effect of urea, pH, and preservatives on hCG immunoreactivity

We studied the effect of urinary pH, urea concentration and preservatives on hCG immunoreactivity by diluting pregnancy urine (hCG 7300 pmol/L, pH 7.7, density 1.009) 25-fold into nonpregnancy-urine (density 1.005) in order to achieve hCG concentration around 300 pmol/L. Aliquots were adjusted to pH 6 and pH 7 with 2 mol/L acetic acid and to pH 9 with 1.3 mol/L $NaHCO_3$,. Each pH-adjusted pool was further aliquoted and urea (5 mol/L) was added to concentrations of 0, 0.2 mol/L and 1 mol/L. Urine pools adjusted to pH 7 were further aliquoted and glycerol (5% and 10%), EDA (10 and 100 mmol/L), EDTA (5 and 10 mmol/L) or BSA (0.05, 0.1, 0.5, 1 and 5 g/L) was added. Two aliquots were stored in 2-ml polypropylene tubes at +4 °C, −20 °C and −80 °C. The concentrations of hCG, hCGβ, hCGα and hCGβcf were determined before storage, after 1 week and 1 month of storage.

We also studied whether the loss of immunoreactivity was dependent on hCG concentration, and whether the dissociated subunits were able to reassociate into intact hCG after reduction of urea concentration. We serially diluted partially purified hCG (Pregnyl®) into nonpregnancy urine in order to achieve hCG concentrations in the range 80–20 000 000 pmol/L (n = 7), and added urea to all samples to a concentration of 1 mol/L. hCG was determined after 1 week of storage at +4 °C and −20 °C. In samples with initial hCG concentrations of 3 500–20 000 000 pmol/L, hCG was redetermined after dilution of the aliquots with assay buffer (1:100–1:10 000, added urea <0.01 mol/L) and storage at +4 °C for 1 week.