Research Article

Human chorionic gonadotrophin pharmaceutical formulations of urinary origin display high levels of contaminant proteins—A label-free quantitation proteomics study

To determine whether there is a measurable protein background in different formulations of urinary and recombinant human chorionic gonadotropin (hCG). Primary outcome measures: identification of contaminant proteins in urinary-derived formulations of hCG; secondary outcome measures: quantitative values of contaminant proteins in different batches of urinary-derived hCG formulations. It was found that urinary-derived batches have high presence of contaminant proteins beside the active substance. The relative amount of contaminant proteins and hCG differs strongly between different batches.

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Contamination / Human chorionic gonadotrophin / Pillar-arrayed / Proteomics / Urine

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1 Introduction

The human population is increasingly affected by infertility with estimated 1 in 6 of the total population [1]. Couples wishing children but unable to conceive on natural way require treatment with assisted reproductive techniques. The most commonly and widely used and conducted approach is the in-vitro fertilization (IVF). This method uses both exogenous follicle-stimulating hormone to induce follicular growth and human chorionic gonadotrophin (hCG) to induce final maturation of oocytes. The number of hCG injections depends on the individual health situation of the patient but several injections are necessarily.

Human chorionic gonadotrophin (hCG) is one of the most widely studied markers in embryonic development. It is used as an obstetric marker and it is often regarded as little more than a signal for maternal recognition of pregnancy. Human chorionic gonadotrophin is a member of the dimeric glycoprotein hormone family that also includes the follicle-stimulating hormone, luteinizing hormone, and thyroid-stimulating hormone. The members of this hormone family share a common subunit and have a unique subunit to each hormone. Additionally, each hormone shows a different level of glycosylation, which determines circulating half-life and receptor binding affinity [2].

The use of gonadotrophin derived from either animal or human tissues was not always without clinical danger (e.g., antibody formation from pregnant mare serum gonadotrophin; Creutzfeld–Jacob disease from human pituitary gonadotrophin). The hCG is extracted from urine (uhCG) of pregnant women for almost three decades and it is being used for induction of mid-cycle follicular maturation and ovulation in women undergoing an IVF treatment.

Due to their biological origin, uhCG products have shown large biological variability and significant batch-to-batch variability.
variation. Therefore, recombinant technology has been introduced for production of recombinant hCG (rhCG) with higher purity and higher batch-to-batch reproducibility, and the possibility to have a better control of the final amount in different doses. The possibility to precisely control the amount of active substance in recombinant products provides a good starting point to develop personalized therapy for patients, depending on their individual hormonal status.

Although a recombinant product is available on the market, urinary preparations are still manufactured and are widely used [3–5].

Analysis of commercially available, uhCG was already performed earlier [6] and discussions about the possible risks of infection transmission and development of Creutzfeld-Jacobs disease in treated patients were published [7–9].

Despite of the availability of the recombinant product, the use of urinary-derived products is widely spread due to lower costs and availability of these products, especially in countries with lower incomes but also within Europe and the USA. Furthermore, the demand for these products is rising due demographic facts such as aging population and rising age of women for child bearing. In addition to rising need for medical use, hCG is also misused for weight-loss treatment such as applying in “Cura Romana”. Therefore, a stringent analysis is needed to identify and classify possible contaminants that might not be removed from the raw material during the production process.

In this study, we have analyzed and compared different batches of both uhCG and rhCG formulations. Data are available via ProteomeXchange with identifier PXD010471.

2 Materials and methods
2.1 Source of analyzed material

Different batches of uhCG were purchased through the pharmacy of the General Hospital of Vienna and by direct purchase from pharmacies in Bosnia-Herzegovina and Serbia. Details on manufacturer and analyzed batches are shown in Supporting Information Table 1.

2.2 Sample preparation

Trypsin, for protein digestion, was purchased from Promega Inc. (Vienna, Austria). Methanol (MeOH), ACN, 2,2,2-trifluoroethanol, formic acid (FA), heptafluorobutyric acid (HFBA), iodoacetamide (IAA), triethylammonium bicarbonate (TEAB), and dithiothreitol were purchased from Sigma-Aldrich (Vienna, Austria). Digestion of hCG formulations was performed using the routine approach described in earlier publications [10].

2.3 Chromatographic separation and detection

All separations were performed using the nanoRSLC Ultimate 3000 HPLC system coupled to the Q-Exacte Orbitrap mass spectrometer (ThermoScientific, Vienna, Austria). The chromatographic analysis was performed by sample loading on the C18 trap column (Acclaim PepMap C18, 300 μm id x 5 mm, ThermoScientific, Vienna, Austria) and the separation was performed using the Pillar-Arrayed-Column (μPAC) with 2 μm interpillar distance and 2 m separation path (PharmaFluidics, Gent, Belgium) in the column oven at 50°C. Separation method is described in the Supporting Information and the LC gradient can be seen in Table 1. The LC program is provided as Supporting Information. Peptide detection was performed using both UV at 214 nm and MS with positive electrospray ionization with a nano source and ionization needle of 20 and 10 μm tip ID. The 20 most intensive signals in each MS scan were selected for MS/MS (fragmentation) with HCD at NCE (normalized collision energy) set to 30. The decision for HCD was made due to the better performance of peptide fragmentation, as already described, among others, by Pejchinovskiy et al. and Jedrychowsky et al. [11, 12]. The Orbitrap family of mass spectrometers uses the HCD fragmentation approach and fragments ions in a collision cell rather than an ion trap. The benefit of this approach is that there is no low-mass cutoff and the ions are being detected at high resolution in the Orbitrap, which, finally, result in higher quality MS/MS spectra.

2.4 Data analysis

The database search was performed using Proteome Discoverer version 2.2 (ThermoFisher Scientific, Vienna, Austria). Search parameters can be viewed in the Supporting Information.

This study was performed within the Prot-HiSPRA, FP7 project and the decision of the Ethik-Kommission der Medizinischen Universität Wien (IRB of the Medical University of Vienna) EK Nr.: 1105/2010 covered all experiments performed.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE database.
Figure 1. A number of contaminant proteins originating from the starting product were identified in urinary samples, more than 200. A certain number of additional proteins were also identified in the recombinant sample.

partner repository with the dataset identifier PXD010471 https://doi.org/10.6019/PXD010471 [13–15].

3 Results and discussion

3.1 Identification of contaminant proteins in uhCG preparations

In addition to the main component, a number of other proteins were identified in all urine-derived samples, Fig. 1. An overview of the top five contaminant proteins identified in all urinary samples, and their relative quantities, is shown in Table 2. Table 3 shows the number of all identified proteins. Supporting Information Table 1 shows details on all analyzed formulations including initial concentration of total proteins. Supporting Information Table 3 shows the total number of identified proteins as exported from Scaffold.

Urine is relatively complex sample and the separation of tryptic peptides generated upon enzymatic digestion requires rather longer separation gradient as already shown earlier for samples of similar complexity [16–18]. The use of novel μPAC columns required adjustments for separation gradient, the column flow rate and the valve switching times (Supporting Information Table 2). Due to the relatively high void volume of the separation column of approximately 10 μL, a fast flow delivery at the gradients’ start of 800 nL/min was applied and 600 nL/min were used for the sample separation until the flashing phase. Currently possibilities of applying faster and shorter separation gradients using the shorter variant of the 50 cm μPAC, displaying significantly lower void volume, are being explored, Fig. 2.

In our laboratory, all samples are being separated using a nano LC system equipped with a trap and a separation column. TFA is used as an ion-pairing agent but other, perfluorinated or perchlorated, ions can provide significantly better ion-pairing [19–21]. In our case, we have identified the HFBA as the most useful organic acid for optimized trapping. The addition of HFBA causes strong increase of retention times [21, 22] for tryptic peptides and enables longer wash times for the trap column, and enables sample loading at higher temperatures.

3.2 Contaminant proteins in rhCG preparations versus urinary preparation

Analysis of recombinant hCG preparation showed that these contain less additional, contaminant, proteins in comparison to the urinary hCG preparations. In order to ensure that these proteins do not originate from previous injections of urinary formulations, blanks (buffer used for dissolving peptides) were analyzed before and after the injection of each sample and flushing procedures for injection path and the trap column were applied [10].

Although Thennati et al. have described the method for the quality control of the recombinant hCG through analytical steps using SDS–gel separation and MALDI-ToF analysis of the final product [23], the analysis of multiple batches purchased across country borders was not reported recently.

Current analysis showed that a large number of contaminant proteins, originating from the starting product, was identified in different batches of the final product, Table 3. A discussion on contaminant proteins in hCG formulations have already been published [23–26] and other publications addresses the possibility of the presence of harmful substances in commercial formulation [7,8,27–29] but no final decision was published, and the urinary-derived hCG formulations are still widely used although it has been shown that recombinant hCG can be used with the same success rate. Furthermore, a report on prion proteins, and possible danger of infection, that were detected in hCG formulations extracted from human urine were published by van Dorsselaer et al. [28].

The major active component, hCG was identified in all analyzed samples and, in addition to it, human serum albumin (HSA), lutropin subunit beta (LSHB), and glycoprotein hormones alpha chain (CGA), which is common to all gonadotropins, were also identified, Fig. 3. The presence of HSA in the final product, raises concerns in terms of product safety since HSA transports a number of other proteins or protein segments, which can cause adverse reactions and be potentially harmful [30–32].
3.3 Contaminant proteins in urinary derived samples

As for the contaminants in urinary-derived products, uromodulin, which is also the major urinary protein, was a major protein identified following hCG. Although this is a logical identification since it is the most abundant protein in human urine, its presence in an injectable product could be a matter of concern. Additionally, other proteins, such as alpha-1-microglobulin or apolipoprotein D, and prostaglandin were also identified with high confidence and with high relative abundances. The contaminants identified in urinary-derived hCG formulations resemble urinary proteins identified in urine-only samples, which were described in a recently published study on stress-induced urinary incontinence [33] and are already known to be involved in important biological functions and pathways. They act as growth factors, proteolytic enzymes, binding proteins and are members of the cytokine family, which have been reported to influence, both directly and indirectly, inflammation and immune response. Especially, accumulation effects of these impurities in patients after multiple treatments, usually practiced during IVF-procedure, might be considered as potential source responsible for development of adverse reactions. In addition to common adverse effects, also declared in the product safety sheets by the manufacturer, only women undergoing IVF treatment after administration of urinary-derived products have reported serious anaphylactic reaction. Koh et al. [34] reported a case of IgE-mediated hypersensitivity reaction in patient observed shortly after application of urinary-derived products. Patient described appearance of severe symptoms like shortness of breath, wheezing, flushed skin, generalized weakness, and vertigo, which required emergency life-treatment and had consequently immediate interruption of the IVF treatment. Skin test performed weeks after occurring treatment and had consequently immediate interruption of weakness, and vertigo, which required emergency life-like shortness of breath, wheezing, flushed skin, generalized products. Patient described appearance of severe symptoms patient observed shortly after application of urinary-derived preparations in comparison to negative ELISA results for high-purified gonadotropin products. Phipps et al. [35] reported about the same type of allergic reaction whereas Redfearn et al. [36] mentioned delayed type of hypersensitivity following treatment with urinary-derived products. All reports agreed on, and strongly suggested, that nongonadotropin proteins in urinary-derived products are the direct cause of described IgE-mediated hypersensitivity reaction.

The presence of nongonadotropin proteins in urinary-derived preparations mentioned in previous paragraph might be also an explanation for variable treatment efficiency observed for urinary and recombinant formulations [37]. It also could be an acceptable explanation for potential different immunological properties of these products regarding the influence on maturation of dendritic cell, production of pro-inflammatory and anti-inflammatory cytokines and regulation of T-cell response. The most studies researching immune modulatory effects of urinary-derived or recombinant hCG products are contradictory and show a lack of information relating to the source of the product used. Poloski et al. [38] and Dauven et al. [39] found that both formulations have the same ability to increase the number of human T-regulatory cells regardless of the concentration used. However, stronger suppression effect on the maturation of murine dendritic cells was observed when higher concentration of recombinant hCG was used. Dauven et al. [39] reported no changes in secretion and production of any pro-inflammatory or anti-inflammatory cytokines, especially the IL-10, independent of the recombinant hCG concentration. In comparison to this finding, Wan et al. [40] reported increased levels of IL-6, IL-10, and IL-12 after treatment of activated bone marrow dendritic cells with urinary-derived hCG product. Additionally, authors also described reduced potential of dendritic cells to induce T-cell
proliferation in presence of uhCG through effects on down-regulation of MCH class II expression and elevated IL-10 and IDO expression suggesting these effects as a key factor of fetal tolerance. Phimister et al. [41] described potential immunosuppressive and fetus protective role of uromodulin in amniotic fluid against T-cell mediated allogeneic rejection.

As already mentioned, uromodulin is the major contaminant protein observed in all urinary formulations, it is produced and secreted in the kidney and it is the most abundant protein in normal urine [42]. It was reported that urinary levels of uromodulin can be used as predictive biomarker for preeclampsia at the twenty-eighth gestational week [43].

Uromodulin can cause skin irritation (ear swelling response) if injected subcutaneous due to the binding of a ninemer peptide F991 that binds to the Ig light chain and, therefore, prevents the binding of the Ig light chains to their putative receptors.

Recent publication [44] highlighted the ability of urinary uromodulin to serve as an allergen epitope for the activation of allergy-associated T-cells in mouse allergy, and acting through the binding to the EGF-like receptor on the surface of polymononuclear cells, activate different signalling pathways and causing cytoskeletal rearrangement and phosphorylation of nuclear factor-kB that forward induce phagocytosis of polymononuclear cells [45].

Apolipoprotein D (APOD) was identified in all urinary-derived samples that were analyzed. This 29-kDa glycoprotein is a prominent member of the lipocalin family and it is present in almost all tissues. This protein is secreted, and has a prominent role, in transport of small hydrophobic ligands, e.g., cholesterol, progesterone, pregnenolone, bilirubin, and arachidonic acid. It was reported that APOD can be found as predominantly associated with high-density lipoproteins in plasma [46–48].

Recent publications have highlighted a role for APOD in aging processes and the stress resistance suggesting that APOD actually plays a protective role in cellular and oxidative stress and it can also have been shown that this protein can mediate nerve repair [49]. There are also some reports that APOD can be used as a marker for breast cancer and bladder cancer but there is no report on possible effects of APOD if injected as in case of subcutaneous injection of hCG formulations [50].

In all analyzed samples, APOD was identified as highly expressed protein, which relative abundance varied between different product batches.

Alpha–1–microglobulin (AMBp) is a serum protein that belongs to the group of protease inhibitors. It was identified in urine of patients with kidney pathologies and its structure and physical properties have been well investigated. However, its function is still not really properly understood.
Some authors suggest that AMBP has an important part in regulating the immune system but is also being expressed during embryogenesis [50]. In cases of tubular dysfunction, in the early stages of diabetic nephropathy and in the late stages of chronic kidney disease [51], its excretion is increased. Its presence in hCG formulations in large amounts might also be explained by recent findings that have discussed an involvement of AMBP in the remodeling process of the uterine spiral arteries. These findings propose an association between elevated maternal plasma levels, which also explains high urine levels, of AMBP with the forthcoming development of preeclampsia. There is no information about the source of the raw material used for hCG isolation, but it can be speculated that some of the donors might have been in the preeclamptic state at the time of urine donation. Since increased level of AMBP can be observed long before the manifestation of clinical disease, AMBP is being applied as clinical biomarker [52].

Another protein identified under the top ten major contaminants of uhCG is the insulin-like growth factor (IGF)-binding protein-7 (IGFBP7). This secreted protein has primarily been located in follicular fluid. It has been described to be involved in cellular proliferation, adhesion, and angiogenesis, and it may suppress estrogen production by granulosa cells. Tamura at al. [53] investigated the effect of IGFBP7 on steroidogenesis in equine granulosa cells. It was shown that the culturing of GCs with exogenous supplemented IGFBP7 has led to the downregulation of Cyp19a1 mRNA expression, which inhibited estrogen production of GCs. Furthermore, IGFBP7 has been discussed as a possible candidate for cell cycle regulator of GC proliferation in rat ovarian follicles [54]. Furthermore, IGFBP7 has been recently proposed, by Wu et al. as a biomarker for the prediction and diagnosis of recurrent spontaneous abortion [55].

Ribonuclease pancreatic-RNASE1 and the non-secretory ribonuclease -RNASE2, which is also named eosinophil-derived neurotoxin, were identified in urinary-derived samples. Human RNases are secreted, small, catalytically active enzymes able to degrade RNA. One of the most important functions of the RNase1 regarding female reproduction seems to be the possible protection of the fetus through antiviral activity of this enzyme against human immunodeficiency virus HIV-1 [56, 57]. The same antiviral activity effect in the host cell defense against HIV-1 has been observed for the RNase1 isolated from the commercially available hCG formulation purified from the urine of pregnant women as well as recombinant RNase1. It has been also suggested that the RNase1 participate as a very potent activator of inflammation and immune response due to its ability to influence maturation and activation of dendritic cells to produce different cytokines like IL6, IL12 and TNFα [58].

3.4 Contaminant proteins in recombinant samples

Products originating from the production process employing recombinant method have significantly less contaminant proteins of human origin. In order to insure that these contaminations are not the carry-over from previous injections the separation system was flushed using the previously published method [10] and blank runs were analyzed. Although hCG was identified as the major component, contaminants such as keratin and human serum albumin (HSA) were also present in recombinant products. Upon manual inspection
of data for recombinant products, it was possible to exclude a significant number of putatively contaminant proteins identified in recombinant samples and identify them as a minor carry-over from urinary samples despite vigorous wash procedures.

4 Concluding remarks

Formulations of hCG, of both urinary and recombinant origin, contain contaminant proteins that originate from either starting material or has been introduced during the manufacturing process. The contaminant proteins were not present in blank samples that were treated in the same way as the hCG formulations.

Recombinant product showed traces of proteins other than hCG, which were not listed in the product’s leaflet. No proteins were identified in blank samples (sample’s buffer), which were analyzed as controls.

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The authors have declared no conflict of interest.

Both authors contributed to the experimental design. T.P.J. prepared all samples and performed data analysis. G.M. performed LC-MS analysis and data analysis.

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