Article

Analytical identification of additional impurities in urinary-derived gonadotrophins

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Abstract

Advances in proteomic technology have enabled contaminant proteins to be identified from complex protein mixtures. The purity of two purified urinary gonadotrophin products, human menopausal gonadotrophin (u-HMG) and human FSH (u-hFSH), was compared with a preparation of recombinant human FSH (r-hFSH). After separation by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), western blot analysis showed that the recombinant preparation contained only FSH, whereas the urine-derived preparations exhibited several non-FSH or LH-related bands. These urinary components were further investigated by a proteomic approach using two-dimensional SDS–PAGE followed by mass spectrometric identification. The proteomic approach detected a total of 23 non-gonadotrophin-related proteins, at variable levels in different batches of the urine-derived preparations. Of these, 16 co-purified proteins have not been previously reported to be present in urine-derived gonadotrophins. These results indicate that the process used to purify urinary gonadotrophins may not remove all non-gonadotrophin proteins. By using a comprehensive proteomic approach, it has been shown that the recombinant FSH preparation has greater purity than either of the urine-derived preparations.

Keywords: *gonadotrophins, impurities, quality, r-hFSH, u-hFSH, u-HMG*

Introduction

Commercial gonadotrophins are used to stimulate the ovaries in ovulation induction cycles or during assisted reproduction. Since their introduction in the 1960s, there have been significant improvements in the quality and consistency of these therapeutic proteins. All of these improvements have relied on developments in available analytical techniques and manufacturing technology.

The first generation of gonadotrophins were crude extracts of pooled urine from post-menopausal and pregnant women donors, and had a gonadotrophin content of just 2–5% of the total product. The improvement in purity of FSH, either extracted from urine or produced by recombinant DNA technology, has been well documented. By appropriate application of advanced purification and production technology, it has become possible to supply FSH products with a purity of >95% (Giudice *et al.*, 2001; Bassett and Driebergen, 2005), or >99% in the case of recombinant human (r-h)FSH products, without detectable co-purified proteins (Lispi *et al.*, 2006; Herrler and Beneke, 2008).

Despite these advances, urinary products still contain non-gonadotrophin proteins and other urinary proteins, such as epidermal growth factor (EGF) and eosinophil-derived neurotoxin (EDN) (Kauffman *et al.*, 1999; Yarram *et al.*, 2004). The challenge for improving urinary products even further is to remove these proteins while maintaining the integrity of the FSH and LH molecules. To achieve this goal, suitable analytical tools that allow a physicochemical assessment of gonadotrophins in the presence of other proteins are essential. The availability and application of new high-resolution proteomic technologies now allows for improved assessment of the molecular integrity, purity and consistency of gonadotrophin proteins, with high sensitivity. Such information is necessary to stimulate further product improvements in the future.

This study used a comprehensive analytical approach to assess the purity and inter-batch quality of current formulations of commercially available purified urinary gonadotrophins and compare them with an r-hFSH preparation. This exploratory analysis includes samples from only a few batches, as a previous analysis has documented the lack of contaminants in r-hFSH and the presence and variability of contaminants in urinary human (u-h)FSH in a larger number of batches (Lispi *et al.*, 2006).

Materials and methods

Source of material

Between 19 and 30 vials from six commercial batches of urinary human menopausal gonadotrophin (u-HMG) 75 IU (Menopur; Ferring Pharmaceuticals, Denmark) and 14 and 73 vials from three batches of u-hFSH 75 IU (Bravelle; Ferring Pharmaceuticals, Denmark) were used. Both products are manufactured as a lyophilized powder in a single dose vial. Each vial is accompanied by a vial of sterile diluent containing 1 ml of 0.9% sodium chloride injection, USP. According to the supplier's product information, each vial of the u-hFSH product is overfilled to contain 82.5 IU to deliver the 75 IU dose; no overfill data are available for the u-HMG product.

Between 17 and 28 cartridges from one batch of solution-for-injection (GONAL-f; Merck Serono S.A. – Geneva, Switzerland, an affiliate of Merck KGaA, Darmstadt, Germany) were obtained directly from the manufacturing sites for each dose strength of r-hFSH (300 IU/0.5 ml, 450 IU/0.75 ml and 900 IU/1.5 ml; **Table 1**). In-house FSH and LH reference standards were used (Merck Serono S.A. – Geneva).

Analytical methods

Gel electrophoresis

Gel electrophoresis was performed using a pre-cast 4–12% bis–tris polyacrylamide gel (NuPAGE Novex; Invitrogen, Carlsbad, CA, USA), at 200 V for 35–40 min at room temperature.

Samples in reducing conditions were loaded in each lane at 6 IU as well as molecular weight markers (Mark 12 – Invitrogen Corporation, Carlsbad, CA, USA). Following electrophoresis, protein bands were visualized by silver staining (PlusOne; Amersham Biosciences, Uppsala, Sweden), according to the manufacturer's instructions.

Western blot analysis

After separation by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), proteins were transferred onto a nitrocellulose membrane by electric transfer. The membranes were incubated in blocking solution, and then incubated with a specific commercial antibody according to the test to be performed.

To detect the FSH-, LH- and human chorionic gonadotrophin (HCG)-related bands in u-HMG and r-hFSH, the following primary antibodies were used: a polyclonal antibody directed against FSH/LH (code AB928; Chemicon International, Temecula, CA, USA); a polyclonal antibody to β-HCG (code 3AR393; Scantibodies Laboratory, Santee, CA, USA); a monoclonal antibody to LH (clone 19/1; Merck Serono S.A. – Geneva); a monoclonal antibody to FSH (clone 9/14;

Table 1. Product details for all batches of gonadotrophins analysed in the study.

Product	*Batch*	*Expiry date*	*Country of origin*
u-HMG 75 IU	FHA0–15A	05/2007	USA
	FHA0–13A	05/2007	USA
	FHA0–15B	05/2007	USA
	51803C	04/2007	Austria
	53001D	06/2007	Hungary
	53101A	07/2007	Brazil
u-hFSH 75 IU	FMA013A	04/2004	USA
	FMA023B	04/2006	USA
	FMA025A-1	04/2006	USA
r-hFSH 300 IU	G1405	05/2007	Italy
r-hFSH 450 IU	G4406	05/2007	Italy
	G4407	05/2007	Italy
r-hFSH 900 IU	G9408	05/2007	Italy

r-hFSH, recombinant human FSH; u-hFSH, urinary human fFSH; u-HMG, urinary human menopausal gonadotrophin.

Merck Serono S.A. – Geneva). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) (code 170–6515; Bio-Rad, Hercules, CA, USA) was used as secondary antibody for detection of HCG-related bands, whilst HRP-conjugated goat anti-mouse IgG (code 170–6516; Bio-Rad) was used for LH- and FSH-related bands.

For all gonadotrophin preparations, immunoactivity detection was carried out using enhanced chemiluminescence (ECL; Amersham Biosciences, Little Chalfont, Buckinghamshire, UK), and Coomassie blue staining was used to detect the molecular weight markers.

Isoelectric focusing

This method was performed as described in a previous publication (Lispi *et al.*, 2006). Briefly, a commercial dried polyacrylamide gel was rehydrated in a solution buffered to create a pH gradient of 2.6–6.5. After transfer to a nitrocellulose membrane, immunoblotting and detection were performed to reveal proteins.

Determination of total protein content of drug preparations

The total protein content of u-HMG and r-hFSH was determined by Bradford assay using the Coomassie Plus Protein Assay Kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions for the Micro Microplate Protocol, with a working range of 1–25 μg. Bovine serum albumin was used as standard.

Immunoassays

Methods for quantification of gonadotrophins, EGF and EDN were all performed as described previously (Lispi *et al.*, 2006), using commercial kits (a diagnostic enzyme-linked fluoroimmunometric assay, an EGF DEG00-Quantikine immunoassay and an EDN enzyme-linked immunosorbent kit respectively).

FSH in-vivo bioassay

This method was described in detail in a previous publication (Lispi *et al.*, 2006). Each sample was tested independently in triplicate, and the activity of FSH *in vivo* was assessed by the rat ovarian weight gain method of Steelman and Pohley (1953).

During the test, samples were handled as follows. Each vial of u-HMG and u-hFSH was reconstituted with 1 ml of diluent contained in the package (NaCl 0.9%) using a sterile syringe. The reconstituted sample was withdrawn from the original vial within 60 s of reconstitution, using the same syringe used in the reconstitution phase. The reconstituted solution was injected directly into the tube containing phosphate buffer solution and 0.1% bovine serum albumin. The resulting solution was diluted to obtain three doses of FSH (0.33, 0.66 and 1.33 IU/ml) corresponding to the final cumulative doses of FSH of 1, 2 and 4 IU per rat; the volume of solution injected was 3 ml for each dose. Each dose sample was assayed in triplicate and compared with the FSH in-house reference standard, to provide an estimated ratio of bioactivity for each preparation. For measurement of u-hFSH in-vivo bioactivity, a urinary-derived FSH standard was used and for measurement of r-hFSH in-vivo bioactivity, a recombinant-derived FSH standard was used; both were calibrated against international standards (Lispi *et al.*, 2006).

Size exclusion–high performance liquid chromatography

Chromatography was performed using a size exclusion–high performance liquid chromatography (SE–HPLC) column (Biosep S2000; Tosoh Bioscience GmbH, Stuttgart, Germany) at room temperature, using a sodium phosphate/sodium sulphate buffer at pH 6.7, at a flow rate of 1 ml/min for FSH content and 0.7 ml/min for FSH purity. Detection was carried out at 214 nm. Protein content and purity were determined by calibration against an in-house reference standard, quantified by amino acid analysis.

Reverse phase–high performance liquid chromatography

The reverse phase–high performance liquid chromatography (RP–HPLC) method has been described in a previous publication (Bassett and Driebergen, 2005).

The two subunits of r-hFSH were separated, and the peaks relative to the oxidized alpha subunit were quantified as a proportion of the intact alpha subunit. To assess FSH and LH content, the beta subunits of FSH and LH were separated, and the peaks relative to each subunit were quantified by reference to FSH and LH in-house reference standards.

Two-dimensional SDS–PAGE and mass spectrometry

Two batches of both u-HMG and u-hFSH were analysed. A sample (of approximately 1050–1875 IU) of each urinary product was pretreated by ultracentrifugation to remove non-protein product excipients. Approximately 100–400 μg of protein were loaded onto a two-dimensional (2D) SDS–PAGE gel. Proteins were separated on a first dimension according to their isoelectric point (pI) at 86,000 HV, and then on a second dimension according to their molecular weight. Following 2D separation, the gels were stained by silver or Coomassie blue staining. Detected proteins were removed from the gel using a gel cutter (PROTEINEERsp; Bruker Daltonik GmbH, Bremen, Germany), washed, reduced, alkylated and dehydrated automatically with a MassPrep robot (Micromass; Manchester, UK). The gel pieces were digested with porcine trypsin (Promega; Madison, WI, USA). The resulting tryptic peptides were extracted with 60% acetonitrile in 5% formic acid and analysed by mass spectrometry (MS), either with a matrix-assisted laser desorption ionization (MALDI) or electrospray (ES) ionization source.

Mass spectrometry

MALDI time-of-flight (TOF) MS measurements were acquired using the Ultraflex TOF/TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). The samples were prepared by standard dried droplet preparation on stainless steel

MALDI targets using alpha-cyano-4-hydroxycinnamic acid as matrix (Luche *et al.*, 2004). External calibration of MALDI mass spectra was carried out using singly charged monoisotopic peaks of a mixture of bradykinin 1–7 (m/z = 757.400), human angiotensin II (m/z = 1046.542), human angiotensin I (m/z = 1296.685), substance P (m/z = 1347.735), bombesin (m/z = 1619.822), renin (m/z = 1758.933), adrenocorticotrophic hormone (ACTH) 1–17 (m/z = 2093.087) and ACTH 18–39 (m/z = 2465.199). To achieve mass accuracy, internal calibration was performed with tryptic peptides obtained from autolysis of trypsin, with monoisotopic masses at m/z = 842.510, m/z = 1045.564 and m/z = 2211.105.

Nanoflow liquid chromatography and tandem MS (NanoLC–MS/MS) analysis with ES ionization was performed using a CapLC (Waters, Manchester, UK) coupled to a hybrid quadruple orthogonal acceleration TOF tandem mass spectrometer, Q-TOF 2 (Micromass). An external calibration was performed using a solution of 0.01% phosphoric acid in 50% acetonitrile.

Database searches to identify proteins

Monoisotopic peptide masses were automatically annotated using Flexanalysis 2.0 software (Bruker Daltonics, USA) and used for database searches.

Proteins were identified by peptide mass fingerprinting using the MASCOT program (Matrix Science, London, UK) matching against peptides from the SwissProt and TrEMBL databases for all species [see http://www.ebi.ac.uk/uniprot/ (accessed 6 July 2009)]. One missed proteolytic cleavage per peptide was allowed and variable modifications, such as carbamidomethylation for cysteine and oxidation for methionine, were taken into account. For MALDI–TOF, a mass tolerance of 50 ppm was used, and for NanoLC–MS/MS, a fragment tolerance of 0.2 Da was used.

Results

SDS–PAGE to assess purity level of HMG, urinary FSH and recombinant FSH

The u-HMG, u-hFSH and r-hFSH preparations were analysed by 4–12% bis–tris gel electrophoresis and silver staining to visualize the protein profile (**Figures 1** and **2**). All preparations showed the presence of a protein band at a molecular weight of approximately 22 kDa, corresponding to the FSH, LH and HCG reduced alpha and FSH beta subunits. The band present in u-HMG batches at 33 kDa corresponded to the HCG beta subunit. All batches of urinary products (u-HMG and u-hFSH) presented additional multiple protein bands at molecular weights greater than 36 kDa (**Figures 1** and **2**). Apart from the major FSH band, no other bands were detected in the recombinant preparations.

Western blot to compare protein profiles of HMG and recombinant FSH

The u-HMG and r-hFSH preparations were also analysed by western blot using both polyclonal and monoclonal antibodies to visualize the FSH, LH and HCG protein profiles. Western blot analysis was not performed on u-hFSH as immunoassay results showed that LH and HCG were not present in sufficient quantities.

Western blot with polyclonal antibody reactive to the whole FSH molecule, and, according to the manufacturer, cross-reactive with LH, gave three main bands in the u-HMG samples and only one in the r-hFSH samples (**Figure 3A**). The band at a molecular weight of approximately 22 kDa corresponds to the reduced alpha and beta subunits of FSH. The two additional bands identified in the u-HMG samples at approximately 20 and 33 kDa correspond to LH and HCG respectively. Additional bands, visible at approximately 50 kDa and higher molecular weights, may be assignable to aggregated FSH, which has recently been reported to be present in pituitary-derived FSH (Bousfield *et al.*, 2008).

Using a monoclonal antibody specific for the FSH beta subunit (**Figure 3B**), it was confirmed that only the 22 kDa bands were assignable to FSH. Some non-specific signals were present at higher molecular weights and can be assigned to aggregated FSH.

Using a polyclonal antibody to HCG (**Figure 3C**) and a monoclonal antibody to LH (**Figure 3D**), it was confirmed that the bands at 33 kDa and 20 kDa in the u-HMG samples were the HCG and LH beta subunits respectively.

Isoform profiles of HMG, urinary FSH and recombinant FSH

Isoform profiles for the u-HMG, u-hFSH and r-hFSH preparations were determined (**Figures 4** and **5**). The urinary preparations showed an isoform range of pI 3.0–5.2. The recombinant preparation showed an isoform range of pI 3.5–6.0. The difference in isoform profile between urinary and recombinant FSH products was also clearly seen.

Total protein content of HMG and recombinant FSH

The total protein content was determined by Bradford assay (**Table 2**). The mean protein content and between-batch variability [expressed as coefficient of variation (CV)] were 11.85 μg and 24.6% ($n = 6$) respectively for u-HMG 75 IU, and 76.7 μg and 2.9% ($n = 3$) respectively for r-hFSH.

The r-hFSH batches used in the present study were analysed previously (Lispi *et al.*, 2006). Between-batch variability in protein content (expressed as CV) was 1.9% ($n = 8$) for the r-hFSH solution for injection.

FSH content of HMG, urinary FSH and recombinant FSH

The amount of FSH protein in u-HMG, u-hFSH and r-hFSH was assessed using SE–HPLC and RP–HPLC (**Table 3**). For u-HMG, the RP–HPLC method was used (as this separates FSH from LH and HCG), and the mean FSH protein content per container and between-batch variability (expressed as CV)

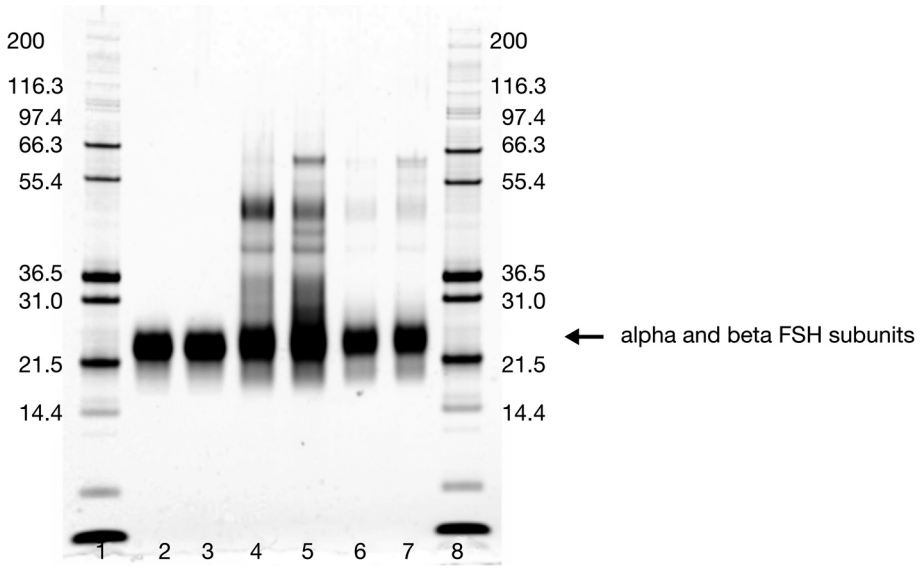

Figure 1. Protein profiling of urinary human FSH (u-hFSH), urinary human menopausal gonadotrophin (u-HMG) and recombinant human FSH (r-hFSH). Samples (6 IU) were loaded on a 4–12% bis–tris gel in reducing conditions and silver stained. Lane 1: molecular weight markers (Mark 12); lane 2: r-hFSH 300 IU, batch G1405; lane 3: r-hFSH 900 IU, batch G9408; lane 4: u-HMG 75 IU, batch FHA0–13A; lane 5: u-HMG 75 IU, batch 53001D; lane 6: u-hFSH 75 IU, batch FMA023B; lane 7: u-hFSH 75 IU, batch FMA025A-1; lane 8: molecular weight markers (Mark 12).

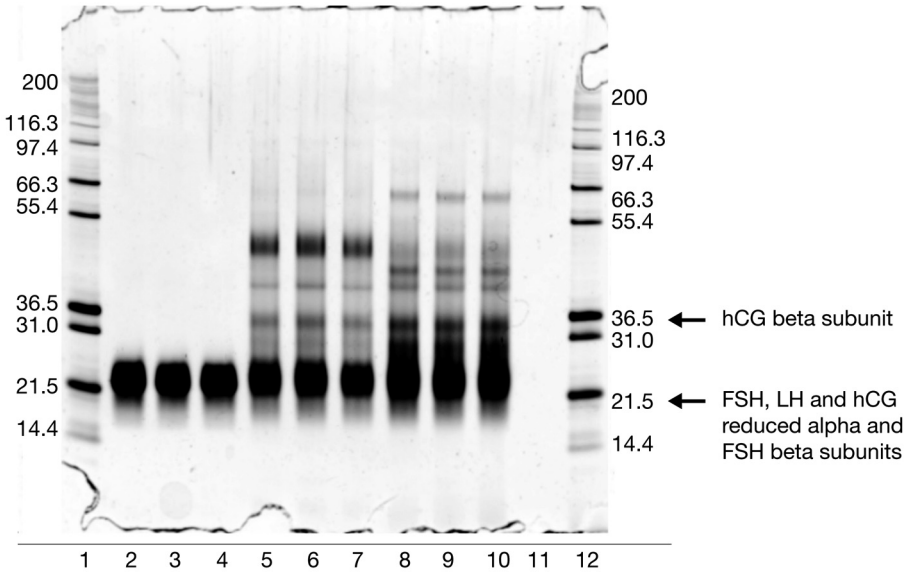

Figure 2. Protein profiling of urinary human menopausal gonadotrophin (u-HMG) and recombinant human FSH (r-hFSH). Samples (6 IU) were loaded on a 4–12% bis–tris gel in reducing conditions and silver stained. Lane 1: molecular weight markers (Mark 12); lane 2: r-hFSH 300 IU, batch G1405; lane 3: r-hFSH 450 IU, batch G4407; lane 4: r-hFSH 900 IU, batch G9408; lane 5: u-HMG 75 IU, batch FHA0–15A; lane 6: u-HMG 75 IU, batch FHA0–13A; lane 7: u-HMG 75 IU, batch FHA0–15B; lane 8: u-HMG 75 IU, batch 51803C; lane 9: u-HMG 75 IU, batch 53001D; lane 10: u-HMG 75 IU, batch 53101A; lane 11: sample buffer; lane 12: molecular weight markers (Mark 12). HCG = human chorionic gonadotrophin.

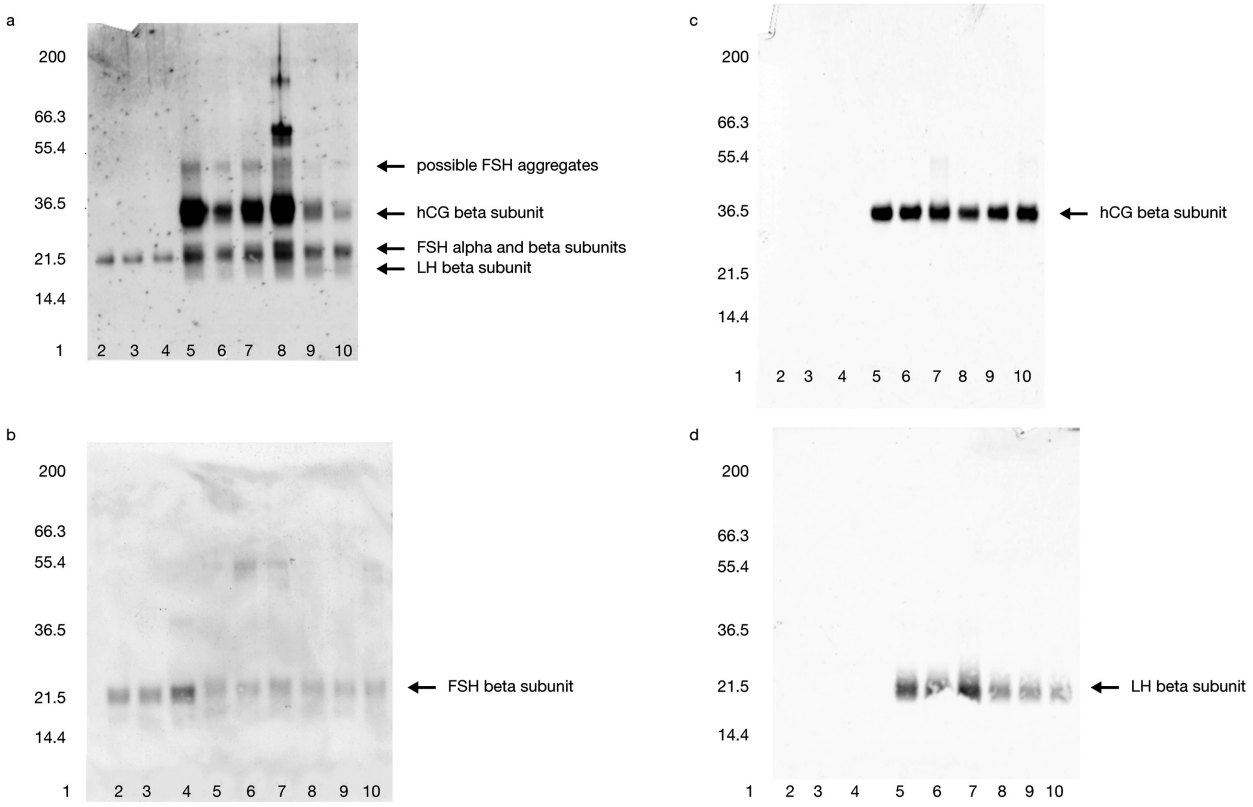

Figure 3. Immunochemical analysis of specific proteins in urinary human menopausal gonadotrophin (u-HMG) and recombinant human FSH (r-hFSH). (**a**) Antibody to the whole FSH molecule. (**b**) FSH beta subunit. Samples (6 IU loading) were separated by SDS–PAGE (4–12% NuPAGE® gel) and detected by western blot analysis using monoclonal antibody to the FSH beta subunit. (**c**) Human chorionic gonadotrophin (HCG). Samples (6 IU loading) were separated by SDS–PAGE and detected by western blot analysis using polyclonal antibody to rabbit beta HCG. (**d**) LH. Samples (6 IU loading) were separated by SDS–PAGE and detected by western blot using monoclonal antibody to LH. Lane 1: molecular weight markers (Mark 12); lane 2: r-hFSH 300 IU, batch G1405; lane 3: r-hFSH 450 IU, batch G4407; lane 4: r-hFSH 900 IU, batch G9408; lane 5: u-HMG 75 IU, batch FHA0–15A; lane 6: u-HMG 75 IU, batch FHA0–13A; lane 7: u-HMG 75 IU, batch FHA0–15B; lane 8: u-HMG 75 IU, batch 51803C; lane 9: u-HMG 75 IU, batch 53001D; lane 10: u-HMG 75 IU, batch 53101A.

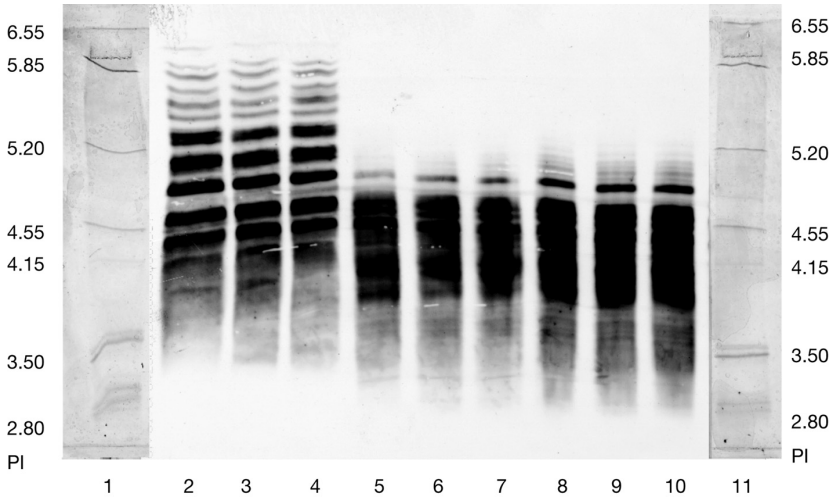

Figure 4. Isoelectric focusing separation (pH range 2.5–6.5) of recombinant human FSH (r-hFSH) and urinary human menopausal gonadotrophin (u-HMG) batches (20 IU sample loading) detected on western blot analysis by monoclonal antibody to FSH. Lane 1: isoelectric point markers (2.5–6.5); lane 2: r-hFSH 300 IU, batch G1405; lane 3: r-hFSH 450 IU, batch G4407; lane 4: r-hFSH 900 IU, batch G9408; lane 5: u-HMG 75 IU, batch FHA0–15A; lane 6: u-HMG 75 IU, batch FHA0–13A; lane 7: u-HMG 75 IU, batch FHA0–15B; lane 8: u-HMG 75 IU, batch 51803C; lane 9: u-HMG 75 IU, batch 53001D; lane 10: u-HMG 75 IU, batch 53101A; lane 11: isoelectric point markers (2.5–6.5).

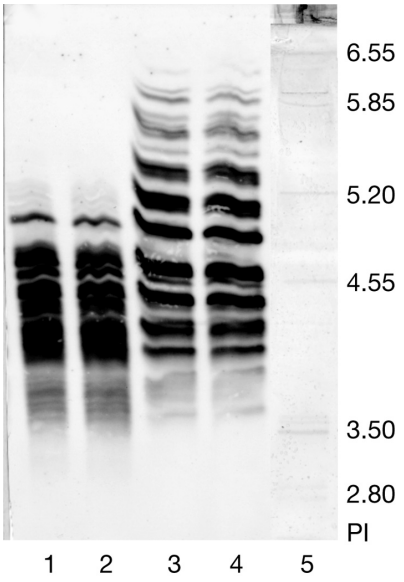

Figure 5. Isoelectric focusing separation (pH range 2.5–6.5) of recombinant human FSH (r-hFSH) and urinary human FSH (u-hFSH) batches (20 IU sample loading) detected by western blot analysis using monoclonal antibody to FSH. Lane 1: u-hFSH 75 IU, batch FMA023B; lane 2: u-hFSH 75 IU, batch FMA025A-1; lane 3: r-hFSH 450 IU, batch G4406; lane 4: r-hFSH 900 IU, batch G9408; lane 5: isoelectric point markers (2.5–6.5).

Table 2. Total protein content of urinary human menopausal gonadotrophin (u-HMG) and recombinant human FSH (r-hFSH) preparations assayed using the Bradford method.

Gonadotrophin product	*Batch*	*Total protein content, µg/ml*	*Mean (CV) total protein content µg/ml (%)*	*Normalized total protein content µg/75 IU*
u-HMG 75 IU	FHA0–15A	9.09	11.85 (24.6)	11.85
	FHA0–13A	10.30		
	FHA0–15B	8.51		
	51803C	14.73		
	53001D	15.11		
	53101A	13.37		
r-hFSH 300 IU	G1405	79.02	76.7 (2.9)	9.59
r-hFSH 450 IU	G4407	74.55		
r-hFSH 900 IU	G9408	76.72		

CV, coefficient of variation.

Table 3. FSH content of urinary human menopausal gonadotrophin (u-HMG), urinary human FSH (u-hFSH) and recombinant human FSH (r-hFSH) preparations, determined using size exclusion–high performance liquid chromatography (SE–HPLC) or reverse phase (RP)–HPLC.

Product[a]	*Batch*	*FSH content (CV) µg/vial (%)*	*Mean (CV) FSH content µg/vial (%)*	*Normalized FSH content µg/75 IU*
RP–HPLC				
u-HMG 75 IU	FHAO–15A	7.23 (0.97)	7.49 (3.35)	7.49
	FHAO–15B	7.28 (0.18)		
	51803C	7.47 (0.17)		
	53001D	7.74 (1.28)		
	53101A	7.77 (0.01)		
SE–HPLC				
u-hFSH 75 IU	FMA023B	6.35 (1.89)	6.56 (NA)	6.56
	FMA025A-1	6.77 (0.03)		
r-hFSH 900 IU	G9408	49.39[b] (0.41)	48.90[b] (NA)	6.11
r-hFSH 450 IU	G4406	48.50[b] (NA)		

CV, coefficient of variation; NA, not applicable for $n = 2$
[a]Neither u-HMG batch FHAO–13A nor r-hFSH 300 IU was tested.
[b]Units are µg/ml.

were 7.49 μg and 3.35% (n = 5) respectively. The mean FSH protein content was 6.56 μg per container for u-hFSH (n = 2), and 48.90 μg/ml (normalized value = 6.11 μg/75 IU) for r-hFSH (n = 2).

FSH immunoactivity of HMG, urinary FSH and recombinant FSH

The FSH immunoactivity of the three gonadotrophins was assessed using a commercially available FSH immunoassay (**Table 4**). The between-batch variability (expressed as CV) for FSH immunoactivity was 6.3% for u-HMG (n = 6) and 3.3% for r-hFSH (n = 4). Values for mean FSH immunoactivity were 36.9 IU/ml for u-HMG, 39.1 IU/ml for u-hFSH, and 398.5 IU/ml (normalized value = 49.8 IU/75 IU biopotency) for r-hFSH.

The r-hFSH batches used in the present study were analysed previously (Lispi *et al.*, 2006). Between-batch variability (expressed as CV) in immunoactivity was 2.5% (n = 8) for r-hFSH solution for injection.

Evaluation of delivered dose of HMG and urinary FSH

The FSH bioactivity for urine-derived HMG and FSH was assessed by an in-vivo bioassay. The delivered dose of FSH, estimated by preparing each sample according to the product leaflet, and the percentage recovery of FSH potency, evaluated by comparing the delivered dose to the claimed potency, are shown in **Table 5**. The total content of the vial was also assessed and compared with the claimed potency.

The mean delivered FSH bioactivity for u-HMG 75 IU was 64 IU, and for u-hFSH 75 IU was 66 IU. The between-batch variability (expressed as CV) for delivered FSH bioactivity in u-HMG 75 IU was 3.3% (n = 5). The mean percentage recovery of delivered FSH for u-HMG was 85%, and for u-hFSH was 88%.

The between-batch variability (expressed as CV) for total FSH bioactivity in batches of u-HMG 75 IU was 3.7% (n = 6). The mean percentage recovery per vial of FSH bioactivity was 89% for u-HMG and 92% for u-hFSH (**Table 6**).

This in-vivo bioassay was not performed for r-hFSH, as it had been carried out previously and is reported in Lispi *et al.* (2006).

LH and HCG content of HMG and urinary FSH

Commercially available immunoassays were used to quantify additional gonadotrophins (LH or HCG) in each urinary preparation (**Table 7**). For u-HMG 75 IU, the mean LH content and between-batch variability were 0.47 IU and 24.8% (n = 6) respectively and the mean HCG content and between-batch variability were 11.3 IU and 13.9% (n = 6) respectively. For u-hFSH, the mean LH and HCG content were 0.0089 IU/vial and 0.035 IU/vial respectively. No traces of other gonadotrophins were present in the recombinant preparations analysed.

Protein impurity content of HMG and urinary FSH

Commercially available immunoassays were used to determine the potential presence of the protein contaminants, EGF and EDN. EGF was not detected in the u-hFSH or u-HMG tested, whereas EDN was present in all urinary batches tested (**Table 8**).

SE–HPLC to assess the purity of u-hFSH and r-hFSH samples

The presence of high molecular weight (HMW) proteins was assessed in u-hFSH and r-hFSH batches using an SE–HPLC method to assess the purity. HMW proteins were present in the two u-hFSH batches at 1.31 and 5.26%, but were absent from r-hFSH. These HMW proteins are probably related to the impurities observed in u-hFSH (**Figure 1**).

Evaluation of oxidation level of HMG, urinary FSH and recombinant FSH

An RP–HPLC method was used to evaluate the oxidation level of u-HMG, u-hFSH and r-hFSH, and mean levels of oxidized gonadotrophin were 49.0, 51.0 and 1.6% respectively (**Table 9**).

Identification of non-gonadotrophin proteins in u-HMG and u-hFSH using electrophoresis and MS

After 2D gel electrophoresis (**Figure 6**), all proteins found to be present in the molecular weight range of approximately 25–100 kDa in u-HMG and u-hFSH were analysed by MS (ES–MS–MS and MALDI–MS), and identified from internationally available databases (**Table 10**).

In both batches of u-HMG the presence of the HCG beta subunit was confirmed. Non-gonadotrophin protein impurities were identified in both batches of both urinary products (**Table 10**). The most abundant proteins found in both batches of u-HMG were plasma serine protease inhibitor (protein C inhibitor), insulin-like growth factor binding protein 7 (IGFBP7), alpha-2-antiplasmin and apolipoprotein D. Plasma serine protease inhibitor (protein C inhibitor) and alpha-2-antiplasmin were also found in both batches of u-hFSH.

Discussion

Using a combination of highly sensitive analytical techniques, it is now possible to characterize fully the quality and precise composition of commercially available gonadotrophins. This study provides a comprehensive analytical comparison of two urinary gonadotrophin products and compares the profiles with that of a recombinant gonadotrophin product. After separation by SDS–PAGE, western blot analysis showed that the recombinant preparation contained only FSH, whereas the urine-derived preparations exhibited several non-FSH or LH-related bands.

Table 4. FSH immunoactivity of urinary human menopausal gonadotrophin (u-HMG), urinary human (u-hFSH) and recombinant human FSH (r-hFSH) preparations.

Product	*Batch*	*FSH content IU/ml*	*Mean (CV) FSH content IU/ml (%)*
u-HMG 75 IU	FHA0–15A	35.4	36.9 (6.3)
	FHA0–13A	37.0	
	FHA0–15B	35.2	
	51803C	40.8	
	53001D	38.3	
	53101A	34.7	
u-hFSH 75 IU	FMA025A-1	38.7	39.1 (NA)
	FMA023B	39.4	
r-hFSH 300 IU	G1405	414	398.5 (3.3)
r-hFSH 450 IU	G4406	404	
r-hFSH 450 IU	G4407	385	
r-hFSH 900 IU	G9408	391	

CV, coefficient of variation; NA, not applicable for $n = 2$.

Table 5. Delivered doses for urinary human menopausal gonadotrophin (u-HMG) and urinary human FSH (u-hFSH) preparations, determined using an in-vivo bioassay.

Product	*Batch*	*Labelled activity IU/vial*	*Labelled delivered activity IU/injection*	*Actual delivered activity IU/injection (95% confidence interval)*	*Mean delivered bioactivity IU (% recovery)*
u-HMG 75 IU	FHA0–15A	75	75	66 (63–69)	64 (85)
	FHA0–13A			63 (59–68)	
	FHA0–15B			66 (63–69)	
	51803C			ND	
	53001D			61 (57–66)	
	53101A			64 (60–69)	
u-hFSH 75 IU	FMA023B	82.5	75	67 (60–74)	66 (88)
	FMA025A-1			65 (60–71)	

ND, not determined.
The between-batch variability (expressed as coefficient of variation) for delivered FSH bioactivity in u-HMG 75 IU was 3.3% ($n = 5$).

Table 6. Bioactivity of urinary human menopausal gonadotrophin (u-HMG) and urinary human FSH (u-hFSH) preparations, determined using an in-vivo bioassay and a urinary-derived FSH standard.

Product	*Batch*	*Labelled activity IU/vial*	*Evaluated bioactivity IU/vial (95% confidence interval)*	*Mean bioactivity per vial IU (% recovery)*
u-HMG 75 IU	FHA0–15A	75	70 (67–73)	66.7 (89)
	FHA0–13A		71 (67–74)	
	FHA0–15B		68 (65–72)	
	51803C		66 (61–71)	
	53001D		62 (58–67)	
	53101A		63 (59–67)	
u-hFSH 75 IU	FMA023B	82.5	77 (70–84)	76 (92)
	FMA025A-1		75 (69–80)	

The between-batch variability (expressed as coefficient of variation) for total FSH bioactivity in batches of u-HMG 75 IU was 3.7% ($n = 6$).

Table 7. Human chorionic gonadotrophin (HCG) and LH (hLH) content of urinary human menopausal gonadotrophin (u-HMG), urinary human FSH (u-hFSH) and recombinant human FSH (r-hFSH) preparations, determined by immunoassay.

Product	*Batch*	*HCG content IU/vial*	*Mean (CV) HCG content IU/vial (%)*	*hLH content IU/vial*	*Mean (CV) hLH content IU/vial (%)*
u-HMG 75 IU	FHA0–15A	10.00	11.3 (13.9)	0.35	0.47 (24.8)
	FHA0–13A	9.49		0.41	
	FHA0–15B	10.31		0.35	
	51803C	12.42		0.51	
	53001D	13.16		0.59	
	53101A	12.71		0.61	
u-hFSH 75 IU	FMA025A-1	0.033	0.035 (NA)	0.0086	0.0089 (NA)
	FMA023B	0.037		0.0091	
r-hFSH 300 IU	G1405	ND	–	ND	–
r-hFSH 450 IU	G4407	ND		ND	
r-hFSH 900 IU	G9408	ND		ND	

CV, coefficient of variation; ND, not detectable; NA, not applicable for $n = 2$.

Table 8. Epidermal growth factor (EGF) and eosinophil-derived neurotoxin (EDN) content of urinary human menopausal gonadotrophin (u-HMG), urinary human FSH (u-hFSH) and recombinant human FSH (r-hFSH) preparations, determined by immunoassay.

Product[a]	*Batch*	*EGF content pg/vial*	*EDN content ng/vial*	*Mean (CV) EDN content ng/vial (%)*
u-HMG 75 IU	FHA0–13A	ND	1.09	1.59 (51.3)
	FHA0–15B	ND	0.70	
	53001D	ND	2.33	
	53101A	ND	2.22	
u-hFSH 75 IU	FMA025A-1	ND	0.78	0.74 (NA)
	FMA023B	ND	0.70	
r-hFSH 450 IU	G4406	ND	ND	–
r-hFSH 900 IU	G9408	ND	ND	

CV, coefficient of variation; ND, not detectable; NA, not applicable for $n = 2$.
[a]u-HMG 75 IU batches FHA0–15A and 51803C were unavailable for testing.

Table 9. Proportion of oxidized forms of gonadotrophins present in urinary human menopausal gonadotrophin (u-HMG), urinary human FSH (u-hFSH) and recombinant human FSH (r-hFSH) samples.

Product[a]	*Batch*	*Oxidized forms, %*	*Mean % oxidized forms*
u-HMG 75 IU	FHA0–15A	44.97	49.0
	FHA0–13A	54.58	
	FHA0–15B	48.30	
	51803C	48.96	
	53001D	48.83	
	53101A	48.17	
u-hFSH 75 IU	FMA023B	47.9	51.0
	FMA025A-1	54.1	
r-hFSH 450 IU	G4406	1.56	1.6
r-hFSH 900 IU	G9408	1.56	

[a]Sample r-hFSH 300 IU was not tested.

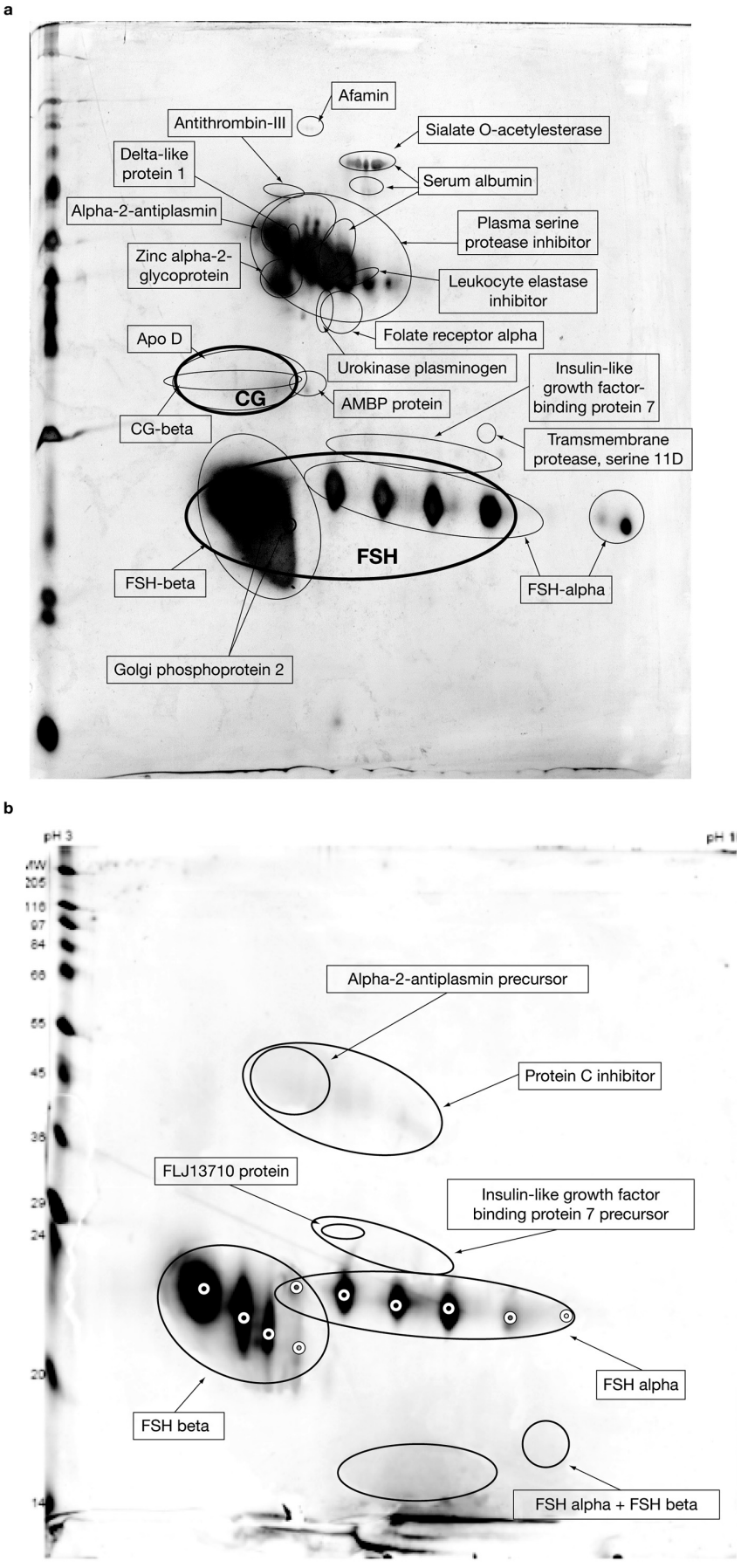

Figure 6. Identification of non-gonadotrophin proteins in urinary human FSH (u-hFSH) and urinary human menopausal gonadotrophin (u-HMG) preparations. (**a**) 2D gel of u-HMG: 1125 IU of batch 51803C. (**b**) 2D gel of u-hFSH: 1875 IU of batch FMA023B. AMBP = alpha-1-microglobulin/bikunin; Apo D = apolipoprotein D.

Table 10. Non-gonadotrophin proteins identified per batch of urinary human menopausal gonadotrophin (u-HMG) and urinary human FSH (u-hFSH) using electrophoresis and mass spectrometry. For u-HMG, the major co-purified proteins are distinguished.

Impurity	*u-HMG*		*u-hFSH*	
	51803C	*FHA015A*	*FMA013A*	*FMA023B*
Plasma serine protease inhibitor (protein C inhibitor)[a,b]	++	++	+	+
Leukocyte elastase inhibitor[a,b]	++	+	+	
Insulin-like growth factor binding protein 7[b,c]	++	++		+
Alpha-2-antiplasmin[b,c]	++	++	+	+
Urokinase plasminogen activator surface receptor	++			
Human serum albumin[b,c]	++	+*		
Zinc-alpha2-glycoprotein[a,b]	++	+		
Apolipoprotein D (apo D)	++	++		
Folate receptor alpha	++			
Sialate *O*-acetylesterase	++			
Delta-like protein 1	+			
Fibulin-1	+			
Kininogen-1	+			
Afamin	+	+		
Alpha-1-microglobulin/bikunin	+			
Antithrombin-III	+			
Alpha-1-antitrypsin	+			
Golgi phosphoprotein 2	+	+		
Transmembrane protease, serine 2	+	+		
Reversion-induced cysteine-rich protein with Kazal motifs	+			
Calmodulin-like protein 5	+			
Clusterin	+			
Hypothetical protein FLJ13710[b,c]				+

+ protein is present; ++ protein is present at high abundance (for u-HMG only); *N-terminal fragment.
[a]Proteins previously identified as contaminants of u-HMG (van de Weijer *et al*., 2003).
[b]Proteins previously listed as contaminants (Herrler and Beneke, 2008).
[c]Protein previously identified as a contaminant of u-hFSH (Lispi *et al*., 2006).

It has been shown that recombinant FSH has high product purity with no other detectable proteins, together with a uniform isoform profile and low oxidation level (1.6%). This confirms previous findings showing that r-hFSH preparations consistently deliver the expected dose from batch to batch, free of undetectable proteins (Bassett and Driebergen, 2005; Lispi *et al*., 2006).

The results show that, like many urine-derived gonadotrophins (Giudice *et al*., 1994, 2001; van de Weijer *et al*., 2003; Bassett *et al*., 2005), u-HMG contains additional protein contaminants which vary in both identity and quantity between batches. Non-gonadotrophin proteins were also found in u-hFSH. Using HPLC, specific immunoassays, 2D-gel electrophoresis and ES-MS, 24 urine-derived protein contaminants were identified in u-HMG and u-hFSH, including 16 contaminants not previously described. Previously published details of the purification process have suggested that similar co-purified proteins should be found in each of these two products as they share similar extraction and purification processes (Reichl *et al*., 2002; Wolfenson Band *et al*., 2006). However, the MS results indicate that the contaminants present in u-HMG and u-hFSH are quite different. Although both products contain protein C inhibitor, leukocyte elastase inhibitor, alpha-2-antiplasmin and IGFBP7, u-HMG also contains human serum albumin together with many other proteins that are not present in u-hFSH (**Table 10**). Conversely, the hypothetical protein FLJ13710 is only present in u-hFSH.

Previous studies have indicated the presence of as yet uncharacterized non-gonadotrophin proteins in urinary gonadotrophins (van de Weijer *et al*., 2003). The current study enabled identification of some of these co-purified proteins. IGFBP7 was highly abundant in both batches of u-HMG tested, and was identified in one batch of u-hFSH. IGFBP7 is secreted into the follicular fluid and may inhibit granulosa cell differentiation by suppressing oestrogen production (Tamura *et al*., 2007). Alpha-2-antiplasmin was detected in both batches of u-hFSH and u-HMG in this study. Alpha-2-antiplasmin inactivates plasmin, which is involved in early follicular responses to ovulatory stimuli (Tsafriri *et al*., 1989).

In addition to reporting the identification of several co-purified proteins in the urinary gonadotrophin preparations that were not identified previously, the study confirmed the presence of those previously found in u-HMG, such as leukocyte elastase inhibitor, plasma serine protease (protein C) inhibitor and zinc-alpha2-glycoprotein (van de Weijer *et al*., 2003). With the u-HMG preparation reported to comprise no more than 70% gonadotrophin protein (van de Weijer *et al*., 2003), it is estimated that most of the remaining 30% non-gonadotrophin proteins distributed in the molecular weight range 25–100 kDa

have been identified (**Figures 1** and **2**). The biological relevance of many of these protein impurities remains unknown, although studies in mice have demonstrated that gonadotrophins derived from urine may have negative effects on gene expression during the peri-implantation period (Sibug *et al.*, 2005, 2007). In addition, a serine protease inhibitor has been shown to inhibit sperm penetration of hamster oocytes (Moore *et al.*, 1993) and an elastase inhibitor complex was associated with a poorer blastocyst development rate and a higher number of arrested embryos (Zorn *et al.*, 2004). Furthermore, it has been shown that urinary and recombinant gonadotrophins induce differing patterns of cumulus cell protein expression in patients undergoing IVF cycles (Hamamah *et al.*, 2006).

The glycoprotein EDN is a common contaminant of commercial urinary gonadotrophin preparations (Kauffman *et al.*, 1999). Indeed, concentrations of 96 ± 60 ng/vial have been reported in preparations of u-HMG (Menogon; Ferring) (Kauffman *et al.*, 1999). Although concentrations were lower than those present in Menogon, EDN was detected in both u-hFSH and u-HMG in this study (0.70–2.33 ng/vial). High concentrations of hyperglycosylated EDN have been reported to be present in the urine of post-menopausal women with ovarian cancer, but this was not specifically investigated in this study (Ye *et al.*, 2006). EDN has also been shown to have a putative inhibitory effect on mouse oocyte maturation (Sakakibara *et al.*, 1991, 1992).

EGF was not detected in either u-hFSH or u-HMG. This is believed to be because the purification process is able to remove EGF but not EDN from urinary gonadotrophin preparations.

The compositional analysis confirmed previous findings that FSH and HCG are the major gonadotrophins in u-HMG (van de Weijer *et al.*, 2003), and demonstrated that u-hFSH contained only trace amounts of both LH and HCG.

Both u-HMG and u-hFSH contained 49–51% of oxidized protein. Interestingly, this is greater than the 30–40% of oxidized protein previously reported in urinary gonadotrophins, and not surprisingly considerably higher than that reported for recombinant FSH in this study (<2%). The oxidized gonadotrophin content presumably reflects the oxidative stress of the rigorous extraction and purification processes to which all urine-derived products are subjected. However, the presence of non-gonadotrophin proteins indicates that thorough processing is unable to eliminate all unwanted proteins from urinary preparations. Moreover, the high levels of oxidation observed in u-HMG and u-hFSH suggest that associated non-gonadotrophin proteins may also be oxidized. As oxidation alters the immunogenicity of therapeutic proteins (Hermeling *et al.*, 2004) this could, theoretically, lead to undesired immunogenicity of non-gonadotrophin proteins in urinary preparations.

It was shown that the isoform profile of urinary gonadotrophin preparations was more acidic (pI 3.0–5.2), than that of the recombinant product (pI 3.5–6.0), which is consistent with many previous findings. More acidic isoforms are released during the early follicular phase of the menstrual cycle while more basic isoforms, with high in-vivo activity and shorter half-life, are released in the mid-cycle phase (Wide and Bakos, 1993; Flack *et al.*, 1994; Anobile *et al.*, 1998). Post-menopausal women have more acidic FSH glycoforms than women at any stage of the menstrual cycle (Anobile *et al.*, 1998). Only 17% of glycoforms following the menopause have a pI >4.3, compared with approximately 50% at mid-cycle, and 36, 37 and 29% at early-to-mid-follicular, late follicular and luteal phases respectively (Anobile *et al.*, 1998).

In this study, two techniques were used to assess protein content: the Bradford assay and HPLC-based methods. The Bradford method is generally more suitable for unknown proteins and complex mixtures such as urine-derived gonadotrophin preparations. Although this method is not generally used to measure the protein content in purified preparations of urinary FSH or r-hFSH, which are more suited to HPLC methods, it does allow an assessment of total protein content variability between batches of product. The discrepancy between protein content estimates derived from the Bradford and the HPLC methods are primarily due to differing analytical methodology. Total protein content estimates (as determined by the Bradford assay) for the six batches of u-HMG 75 IU varied by 24.6%. This variability may have been due to the between-batch variability of the gonadotrophin protein and co-purified proteins present, although a larger number of batches would need to be tested to confirm this.

Urine-derived gonadotrophin preparations contain both intact FSH dimers and inactive FSH subunits. Therefore, analytical methods may not fully capture the level of biological activity provided by the FSH molecules present in the final product. For any pharmaceutical gonadotrophin preparation, the Steelman Pohley in-vivo bioassay is the standard method for determining the FSH content based on the activity of the molecule. Using this bioassay, the delivered biological dose of FSH obtained from u-HMG batches demonstrated low variability (3%), but with only 85% of the 75 IU labelled potency. Similarly, the delivered FSH dose for u-hFSH also showed only 88% recovery of the labelled potency of 75 IU.

The major barrier to improving the quality of urine-derived gonadotrophins is at the level of control of the raw material, pooled menopausal urine. This is extremely challenging, as it is dependent on individual contributors. Furthermore, any variation in the purification process, however slight, will inevitably produce batches of urinary gonadotrophins that vary in the concentration and nature of each contaminant. This combination of variability from both source and during processing may lead to the between-batch variability of impurity composition reported, and which was particularly evident for u-HMG.

In addition to revealing the identity of all contaminant proteins present in a complex mixture, current technologies allow the accurate quantification of contaminants in each individual batch of purified urinary gonadotrophins. Larger studies assessing a greater number of batches of gonadotrophin preparations and utilizing similar protein quantification methodology will reveal the true extent of any compositional variability of urinary-derived gonadotrophin products.

In conclusion, recent developments in laboratory analytical techniques can enable a more detailed assessment of gonadotrophin preparations used for multi-follicular stimulation than was previously possible. This study showed that both u-HMG and u-hFSH are highly oxidized, and contain variable

and inconsistent concentrations of urine-derived co-purified proteins. In contrast, the recombinant production process results in a pure FSH preparation with no urine-derived proteins, and with a low oxidation level.

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