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Quality evaluation of highly purified human menopausal gonadotropin preparations by means of gel electrophoresis and mass spectrometry

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Human gonadotropins are glycoprotein hormones with a highly complex structure, which demands the application of sophisticated analytical methodologies to assess their quality. The principal objective of this study was a comparative evaluation of gel electrophoretic techniques and mass spectrometry-based methods for the quality study of the two urinary-derived, highly purified, human menopausal gonadotropin preparations, Menopur 75/75 I.U. and Meriofert 75 I.U. Molecular mass (*Mr*), isoelectric point (*pI*), and isoform pattern of studied compounds were estimated via SDS-PAGE and 2D gel electrophoresis, whereas matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used for the downstream characterization of peptides obtained after *in-gel* tryptic digestion of selected protein spots. Additionally, for the estimation of the glycosylation pattern of these biologics, the enzymatic release of oligosaccharides was performed, and the isoform pattern was studied. Gel electrophoresis showed a typical electrophoretic behaviour for protein biotherapeutics medicines consisting of extremely complex spot patterns migrating at different masses and *pIs*. MS analysis proved to be a powerful tool for the identification and detailed characterization of the gonadotropins and the relevant peptides were identified with high sequence coverages. The results of this study are not only useful for the quality assessment of this class of complex biopharmaceuticals but may also serve as a supporting platform for further development of biopharmaceuticals based on modulation of the glycosylation pattern to enhance efficacy or reduce side effects.

1. Introduction

Since the advent of recombinant insulin (Goeddel et al. 1979), the class of biopharmaceuticals has experienced a boom, by which nowadays recombinant biologicals and biosimilars complement products of natural origin. Recombinant protein expression technologies allow modulation of gene sequences as well as the expression of both non-glycosylated and glycosylated products. Efficacy and safety of biopharmaceuticals may depend on factors related to their carbohydrate moieties, such as non-glycosylation, hypo-glycosylation or glycosylation specific for the expression system. Further progress in this area aiming at improved properties will also depend on the knowledge of structural modifications of the carbohydrate moieties of biopharmaceuticals.

Many of the registered compounds of this class are a mixture of several active compounds, thus creating a challenge to maintain the required level of quality both concerning production and analysis. Along with a comprehensive analysis of recombinant erythropoietin (Schlags et al. 2002) it has been shown that 2D-gel-electrophoresis complemented by selective digestion is a suited method for quality assessment of recombinant proteins.

Gonadotropins, in particular FSH, LH and hCG, constitute a class of glycoproteins of very high pharmaceutical relevance. There is a considerable number of gonadotropin-based preparations on the market of either human menopausal gonadotropin (hMG)) of either urinary or recombinant origin. Their use is inevitable in women with hypogonadotropic hypogonadism (HH) as well as when other therapies have failed to achieve ovulation (Practice Committee of the American Society for Reproductive Medicine 2008). Assisted reproduction is of growing importance. It has remained a rather sophisticated approach with unfortunately still rather low and varying success rates. Among several other factors, the quality of the applied gonadotropins may be assumed to be a critical factor.

In this paper, a method will be presented along with the comparative analysis of two complex biomedicines of natural origin providing deep structural information. The protocol may help to understand even minor differences in drug properties.

Menopur 75/75 I.U. (Ferring Pharmaceuticals, Switzerland) and Meriofert 75 I.U. (IBSA Farmaceutici Italia Srl, Italy) are two clinically relevant biological formulations indicated for the treatment of infertility. Their active pharmaceutical ingredient (API) is menotrophin, a mixture of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in 1:1 ratio that is extracted from the urine of postmenopausal women. Besides FSH and LH, human chorionic gonadotropin (hCG) is added to both pharmaceutical formulations to contribute to the total LH activity (Ezcurra and Humaidan 2014).

Chemically, FSH, LH and hCG are glycoproteins, consisting of two noncovalently associated heterodimers, with a common alpha-subunit and distinct beta-subunit (Papkoff and Samy 1967; Pierce et al. 1971). The common alpha-subunit consists of 116 amino acids and at asparagine residues, Asn 76 and Asn 102 N-linked glycans are attached, whereas the beta-subunit is hormone-specific and determines gonadotropin's activity. The beta-subunit of FSH (hFSHβ) is composed of 129 amino acids and is glycosylated at positions Asn 25 and Asn 42. The beta-subunit of LH (LHβ) consists of 141 amino acids and has only one glycosylation site at

Asn 50. The beta subunit of hCGβ contains totally 165 amino acid residues and is the most glycosylated, because, despite N-linked glycans that are present in FSHβ and LHβ, it is also composed of four additional O-linked glycans at serines (Ser 141, Ser 147, Ser 152 and Ser 158) (Lapthorn et al. 1994) resulting in a longer half-life than LH (Nakav et al. 2005).

The purification process for human menopausal gonadotropins initially involved precipitation, ion exchange chromatography, and gel filtration techniques. These products contained both FSH and LH and exhibited a low degree of purity. To address this, immunoprecipitation techniques, particularly with monoclonal antibodies, were employed to remove LH from the formulation and reduce contaminants. This resulted in highly purified FSH formulations, which can be characterized using physicochemical techniques. (Jennings et al. 1996)

Currently, recombinant gonadotropins have overcome the disadvantages of urine-derived gonadotropins, including limited urine sources and batch-to-batch variability. (Lispi et al. 2006)

Due to their low purity initially, assessment of urine-derived products relied on biopotency testing in animal models and stability testing. However, for highly purified gonadotropins, physicochemical methods can also be employed for assessment. (Frydman et al. 2000; Lunenfeld et al. 2019)

Generally, the glycan structure is considered to be biantennary complex type, where on FSH and hCG they terminate in sialic acids, whereas on LH in sulphates (Baenziger and Green 1988). Therefore, LH is rapidly removed from circulation because it is readily recognized by a receptor in hepatic endothelial and Kupffer cells that binds glycans terminating in sulphates (Fiete et al. 1991). However, in regard to the biantennary glycan structure, Bousfield and Dias (2011) also reported both triantennary and tetraantennary glycans comprising the glycan structure as well as the fucose residues. Many studies have been conducted in terms of gonadotropins glycosylation pattern concerning their physiological functions. The presence of highly glycosylated hCG during pregnancy was reported (Cole and Khanlian 2007; Wide et al. 1994). On the other hand, alterations in the glycan structure have been reported to occur also during the menstrual cycle, with increasing age in female and men (Bousfield et al. 2014a).

Considering the physiological role of FSH which stimulates ovarian follicle formation, and since only the heterodimer is capable of triggering biological activity, which is determined by the β-subunit (Lapthorn et al. 1994), hFSHβ has a decisive role in female fertility (Kumar et al. 1997). There are four naturally occurring human pituitary hFSHβ glycosylation variants that have been reported. These glycoforms are named for their molecular mass in western blot analysis, where $hFSH\beta^{24}$ represents the fully glycosylated hFSHβ (at Asn 25 and Asn 42). Other two of four isoforms are known as hypo-glycosylated variants, which are a mixture of $hFSH^{18}$ and $hFSH^{21}$ representing the loss of glycosylation at Asn 25 and Asn 42, respectively ($hFSH^{21/18}$). The fourth glycoform is completely deglycosylated $hFSH^{15}$ (Davis et al. 2014). Concerning the bioactivity of these glycoforms, it has been revealed that hypo-glycosylated glycoforms ($hFSH^{21}$ and $hFSH^{18}$) are much more biologically active than fully-glycosylated $hFSH^{24}$ and their levels were found to be decreasing progressively with age (Bousfield et al. 2014a, b).

Technological advancements in the field of analytical instrumentation offer an in-depth understanding of many problems, especially regarding the quality of hMG-based preparations. Techniques like gel electrophoresis complemented with modern techniques such as mass spectrometry have shown to be suitable for the quality evaluation and structural characterization of the biopharmaceuticals (Nebija et al. 2011a; Stubiger et al. 2005; Nebija et al. 2014, 2011b). Consequently, this study was designed to assess the suitability of gel electrophoresis and MALDI-TOF MS analyses for the quality study of commercially available hMG preparations, in regard to identity, structural integrity, and glycosylation pattern.

2. Investigations and results

2.1. Protein concentration assay

Pierce 660 nm Protein Assay revealed a TPA of 169.5 µg/ml for Menopur 75/75 I.U. and 99.5 µg/ml for Meriofert 75 I.U. (R^2 = 0.9922). This difference in TPA may arise from the impurities or the change of biological activity of proteins depending on the production process. On the other hand, since both preparations are urinary-derived preparations, different glycosylation pattern can impart potential interference with the protein assays by whether under- or over-estimating the results (Fountoulakis et al. 1992).

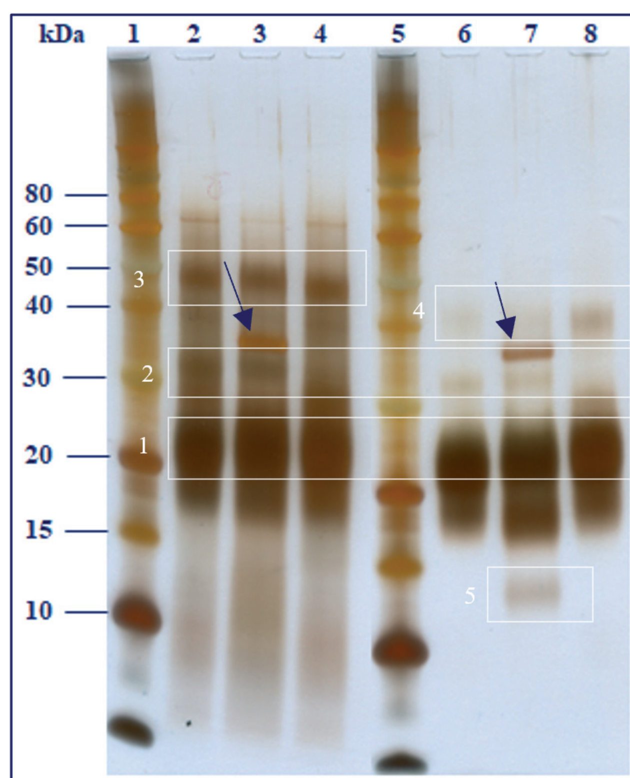

Fig. 1: *SDS-PAGE of Menopur 75/75I.U (TPA 847.5 ng) (B: P10570F) and Meriofert 75 I.U. (TPA 497.5 ng) (B: 170628).* Lane 1 and 5: Molecular weight marker, lane 2: Menopur untreated, lane 3: Menopur PNGase F treated, lane 4: Menopur Neuraminidase treated, lane 6: Meriofert untreated, lane 7: Meriofert PNGase F treated, lane 8: Meriofert Neuraminidase treated. Dark blue arrow: PNGase F.

2.2. SDS-PAGE

The main information obtained from SDS PAGE was regarding the characterization of heterogeneity and size of the proteins (Fig. 1). Migration pattern of both formulations showed higher sample complexity for Menopur than for Meriofert (lanes 2 and 6, respectively). Furthermore, the total protein amount in both samples was found to be different (169.5 µg/ml vs. 99.5 µg/ml for Menopur and Meriofert respectively). The higher amount of protein was loaded in lane 2 (Menopur) than in lane 6 (Meriofert). However, for both samples of pharmaceutical formulations, similar migration pattern could be noted with a more intensive band around 22 kDa, thus indicating for the most abundant proteins present in the sample. Also, a common band around 33 kDa has been observed. After *in-gel* digestion and MS analysis of respective bands, through MS and MS/MS data, it was confirmed that the bands around 22 kDa correspond to FSHβ, and alpha-subunit (Fig. 1, row 1), while the bands around 33 kDa represent hCGβ (Fig. 1, row 2). Such findings, based on SDS-PAGE and MS analysis were previously reported (Van De Weijer et al. 2003; Bassett et al. 2009). In addition, Giudice et al. (2001) and van de Weijer et al. (2003) reported that in SDS-PAGE, LHβ is not detectable in hMG preparations, because such preparations contain relatively small amount of LH, and the 1:1 relative *in-vivo* bioactivity ratio of FSH to LH

is due to hCG (10-fold higher) rather than LH. However, Bassett et al. (Bassett et al. 2009), using a monoclonal antibody to LH, confirmed that the bands around 22 kDa also correspond to LHβ. Moreover, our findings (based upon gel electrophoresis and MS analysis) support the presence of proteinaceous material (impurities) unrelated to gonadotropins (bands above 40 kDa), and also the existence of aggregated FSH or heterodimeric gonadotropins as was assumed (Fig. 1, row 3 and 4) (Bassett et al. 2009; Van De Weijer et al. 2003). Aforementioned factors including the protein aggregation could be the source of noted differences in the sample complexity and gel migration pattern of respective formulations.

In order to investigate the glycosylation pattern, Menopur and Meriofert were enzymatically deglycosylated and desialylated, using PNGase F and Neuraminidase respectively (Fig. 1, lanes 3, 4, 7 and 8, respectively). PNGase F treatment produced small differences in migration between untreated and treated samples. As seen in Fig. 1, in respective lanes 3 and 7, the most intensive bands around 22 kDa, (in both formulations after PNGase F treatment), the smears are larger, which can be indicative for the shift in mass. Moreover, in Meriofert (Fig. 1, lane 7) a new band appeared around 13 kDa, indicating possible glycan removal (Fig. 1, row 5). In the context of SDS-PAGE, desialylation mainly exerts its effects upon the electrophoretic migration rate. While the bands around 33 kDa (Fig. 1, row 2, lane 4 and 8), corresponding to hCGβ had a considerable decrease in their *Mr* (to about 29 kDa), other bands did not show any significant changes in their electrophoretic mobility. Other bands, around 22 kDa, for both medicines, appeared to have slower electrophoretic mobility after the Neuraminidase treatment, resulting from the removal of negatively charged sialic acids which were cleaved off by this enzyme.

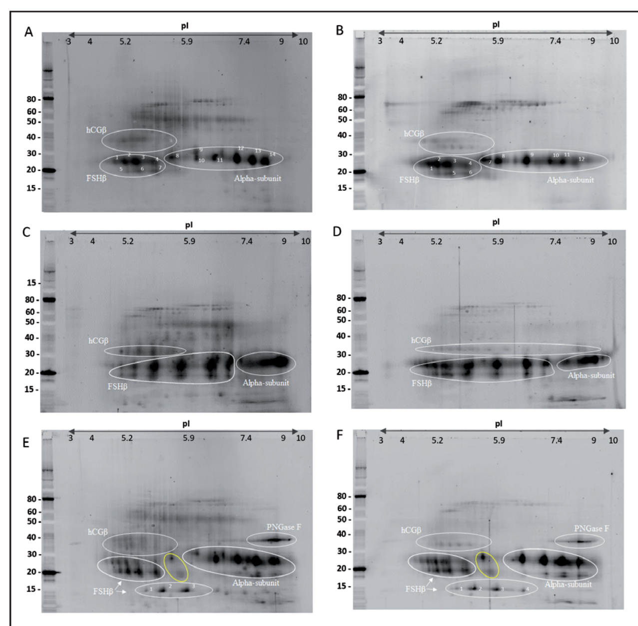

Fig. 2: 2-D gel electrophoresis of Menopur (TPA 2.5 µg) (B: P11340H) and Meriofert (TPA 2.5 µg) (B: 180610). **A.** Menopur untreated, **B.** Meriofert untreated; **C.** Menopur Neuraminidase treated, **D.** Meriofert Neuraminidase treated; **E**. Menopur PNGase F treated, **F.** Meriofert PNGase F treated. Spots circled in yellow, obtained mixed peaks corresponding to both alpha-subunit and to FSHβ

2.3. 2D gel electrophoresis

Regarding their complexity, biological products are expected to have a certain degree of heterogeneity and isoforms (Nebija et al. 2011a). Therefore, Menopur and Meriofert as two pharmaceutical formulations containing biological substances derived from the urine of postmenopausal women are expected to demonstrate a high extent of heterogeneity and isoforms. This was confirmed by our experiments of 2D gel electrophoresis of studied medicines (Fig. 2, A-F). In general, both formulations shared similar spot patterns. Experimental data revealed an extremely complex spot pattern at different *Mr* spanning over the whole *pI* range. The common alpha-subunit appeared around 24 kDa, between pI 5.9 and 9, while spots corresponding to FSHβ became visible between pI 5 – 5.5 and in two distinct mass ranges, around 24 kDa and 21 kDa. In the pI range between 4.5 – 5.5 and at *Mr* around 33 kDa, hCGβ was detected. These findings are in accordance with previously published data (Van De Weijer et al. 2003; Bassett et al. 2009).

Respective spots were excised, *in-gel* digested and resulting peptides were subjected to MALDI-TOF MS for identification purposes. As expected, untreated samples exhibited poorly resolved spot patterns with numerous spots of which 5 spots were more abundant (spots 2, 3, 12 – 14, Fig. 2A) for Menopur and up to 7 major isoforms (spots 1-3, 7-9, 11, Fig. 2B) for Meriofert. In case of the latter, 2-D electrophoresis demonstrated high complexity of spots, spread over a wide *pI* range but with no significant difference in *Mr*., which could be a result of the high degree of glycosylation. Desialylation demonstrated high impact on the pI of spots (Fig. 2, C and D). However, after deglycosylation using PNGase F, although generally spot pattern complexity was reduced, new spots emerged at lower *Mr* (spots 1-3 in the Fig. 2E and spots 1-4 in the Fig. 2F), which indicate the glycan removal. Furthermore, *pI* of spots was affected by deglycosylation and particularly desialylation. These documents the abundance of sialic acid residues. The removal of an N-linked glycan from the protein backbone converts the asparagine to aspartic acid (acidic amino acid). Given that gonadotropin glycans are rich in sialic acid residues (which imparts a negative charge to the molecule), the asparagine to aspartic acid conversion might compensate for the effects of the removed glycan in the *pI* value. Therefore, the shift in *pI* was not pronounced.

2.4. MALDI-TOF MS analysis

2.4.1. Identification of 1D gel bands.

Initial identification of protein components from 1D gels of pharmaceutical formulations, Menopur and Meriofert was achieved using MALDI-TOF MS analysis. The bands of interest were excised and subjected to *in-gel* tryptic digestion. Resulting peptides were extracted and subjected to MALDI-TOF MS analysis, which revealed that the bands around 22 kDa represent common alpha-subunit and FSHβ (Fig. 1, row 1).

Table 1: Identified peptides representing alpha-subunit in Menopur and Meriofert 1-D gel.

Start – End	Observed *m/z*	Calculated *m/z*	MC	Peptide
1 – 6	875.4993	875.4007	1	-.MDYYRK.Y
60 – 66	817.4713	817.4494	0	**R.AYPTPLR.S**
60 – 68	1032.5650	1032.5764	1	R. AYPTPLRS.K
76 – 87	1355.5824	1355.5857	0	K.NVTSESTCCVAK.S
88 – 99	1358.8387	1358.6813	1	K.SYNRVTVMGGFK.V

In bold – peptide identified by MS/MS fragmentation, MC – missed cleavages. 33 % sequence coverage, score 10 (threshold < 56).

In Table 1, identified peptides of the common alpha-subunit in Menopur and Meriofert are presented. The peaks related to the common alpha-subunit-related obtained from MS measurements of 1D gels were not prominent, which could suggest a prevalence or might indicate a dominance of FSHβ peptides. Additionally, due to the limited number of arginine (R) and lysine (K) residues larger peptides are produced after tryptic digestion. However, it is evident that the bands around 22 kDa correspond to common alpha-subunit (Fig. 1, row 1). In contrast to common alpha-subunit, seven peptides corresponding to FSHβ were identified in both formulations, thus covering 80 % of the protein sequence (Table 2) (Fig. 3). The smears around 22 kDa demonstrate the presence of the most abundant natural glycoforms of FSHβ. In addition to that, the identification of peptide FCISINTTWCAGYCYTRDLVYK, which can potentially carry the glycosylation site at Asn 42 supports the presence of hFSH[21].

Table 2: Identified peptides representing FSHβ in Menopur and Meriofert 1-D gel.

Start – End	Observed *m/z*	Calculated *m/z*	MC	Peptide
1 – 14	1971.9554	1971.9246	1	-MKTLQFFFLFCCWK.A + Oxidation (M)
37 – 58	2791.2773	2791.2604	1	R.FCISINTTWCAGYCYTRDLVYK.D
54 – 64	1301.7595	1301.7139	1	R.DLVYKDPARPK.I
68 – 80	1645.8764	1645.8181	1	**K.TCTFKELVYETVR.V**
73 – 80	1008.5634	1008.5287	0	K.ELVYETVR.V
81 – 104	2729.2479	2729.1945	0	**R.VPGCAHHADSLYTYPVATQCHC-GK.C**
105 – 115	1315.5346	1315.4817	0	**K.CDSDSTDCTVR.G**
116 – 129	1561.7098	1561.6589	1	**R.GLGPSYCSFGEMKE.**
116 – 129	1577.7017	1577.6538	1	**R.GLGPSYCSFGEMKE.- + Oxidation (M)**

In bold – peptide identified by MS/MS fragmentation, MC – missed cleavages. 80 % sequence coverage, score 179 (threshold < 56).

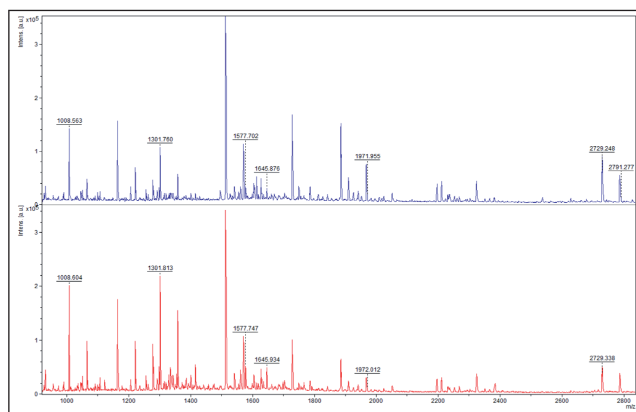

Fig. 3: Peptide mass fingerprint spectra of FSHβ in Menopur (top) and in Meriofert (bottom) from 1-D gel in Fig. 1.

Moreover, the identity of the bands around 33 kDa in both formulations was revealed after MS measurements and turned out to be hCGβ (Fig. 1, row 2). As presented in Table 3, totally 8 peptides were identified from the protein sequence of hCGβ, with a high score and high sequence coverage (around 66 %) (Fig.4 and Table 3).

Table 3: Identified peptides representing hCGβ in Menopur and Meriofert 1-D gel.

Start – End	Observed *m/z*	Calculated *m/z*	MC	Peptide
2 – 22	2324.2034	2324.2320	0	M.EMFQGLLLLLLLSMGGTWASK.E + Oxidation (M)
2 – 22	2382.2110	2382.2375	0	M.EMFQGLLLLLLLSMGGTWASK.E + Acetyl (N-term); 2 Oxidation (M)
23 – 28	1926.1929	1925.0557	0	K.EPLRPR.C
64 – 80	1926.1057	1926.0557	0	**R.VLQGVLPALPQVVCNYR.D**
64 – 80	1967.9331	1968.0663	0	R.VLQGVLPALPQVVCNYR.D + Acetyl (N-term)
81 – 88	1021.5634	1021.5352	1	R.DVRFESIR.L
81 – 94	1701.9056	1701.8781	2	R.DVRFESIRLPGCPR.G
84 – 94	1331.7237	1331.6816	1	**R.FESIRLPGCPR.G**
95 – 114	2224.1097	2224.0599	0	**R.GVNPVVSYAVALSCQCALCR.R**
95 – 115	2380.1907	2380.1610	1	R.GVNPVVSYAVALSCQCALCRR.S
95 – 115	2381.1907	2381.1450	1	R.GVNPVVSYAVALSCQCALCRR.S + Deamidated (NQ)
116 – 134	2128.9291	2128.8950	1	R.STTDCGGPKDHPLTCDDPR.F
125 – 134	1225.5643	1225.5193	0	K.DHPLTCDDPR.F
154 – 165	1235.5731	1235.6445	0	R.LPGPSDTPILPQ.- + Deamidated (NQ)

In bold – peptides identified by MS/MS fragmentation, MC – missed cleavages. 66 % sequence coverage, Score 147 (threshold < 56).

2.4.2. Mass spectrometric analysis of 2D gels

The study of spot patterns provided deeper insight regarding macro- and microheterogeneity of the hormones present in the sample. After the enzymatic treatment, differences could be distinguished.

The identification of glycopeptides through the database is challenging, because of the complex glycosylation (Hu et al. 2016). hCGβ is the most glycosylated hormone among gonadotropins (composed of N- and O-linked sugars) (Fig.4). On the 2D gels of untreated Menopur and Meriofert, it was not possible to identify any peptide that carries N-linked glycans, suggesting the intact structural integrity and no macroheterogeneity.

In regard to the common alpha-subunit, a peptide that carries the glycosylation site at Asn 76 (*KTMLVQKNVTSESTCCVAK*, 2 missed cleavages) was identified, which suggest glycosylation heterogeneity. Moreover, it has been reported that glycosylation

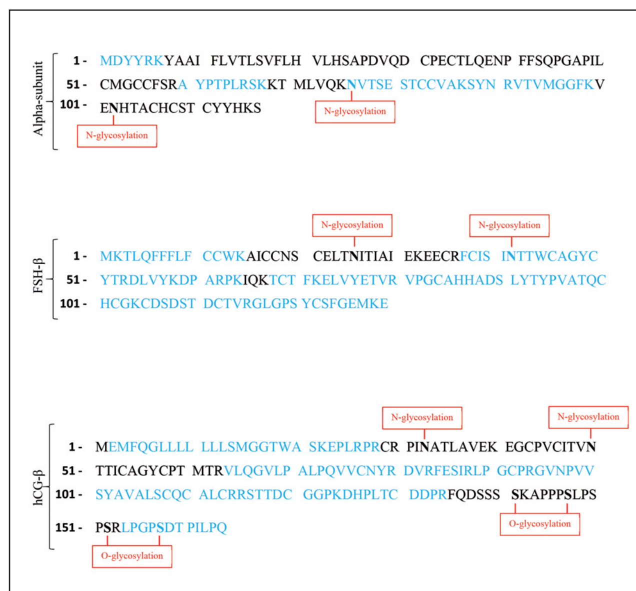

Fig. 4: Alpha-subunit, FSH-β and hCG-β protein sequence. Blue letters – identified peptides from SDS-PAGE of Menopur 75/75 I.U. and Meriofert 75 I.U. via MS and MS/MS analysis.

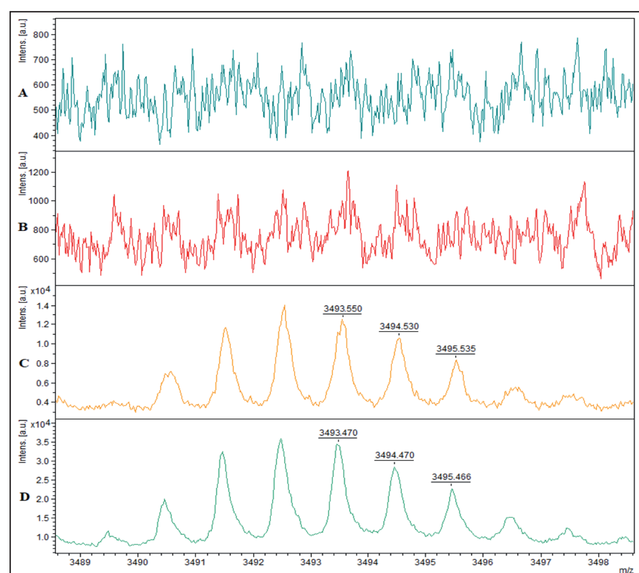

Fig. 5: Alpha-subunit peptide mass fingerprint of 2-D spots indicating the presence of new peaks after the PNGase F treatment. A. Meriofert untreated, B. Menopur untreated, C. Menopur PNGase F treated, D. Meriofert PNGase F treated.

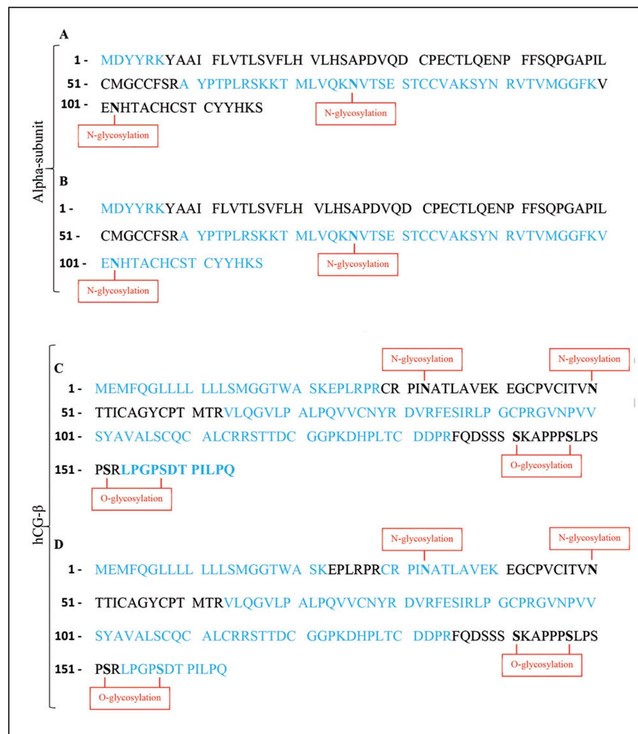

Fig. 6: Alpha-subunit and hCG-β protein sequence. Blue letters – identified peptides from 2D gel electrophoresis of Menopur 75/75 I.U. and Meriofert 75 I.U. via MS and MS/MS analysis. A. Identified peptides of alpha-subunit in untreated Menopur and Meriofert; B. Identified peptides of alpha-subunit after PNGase F treatment of Menopur and Meriofert; C. Identified peptides of hCG-β in untreated Menopur and Meriofert; D. Identified peptides of hCG-β after PNGase F treatment of Menopur and Meriofert.

has protective effects on the proteolytic degradation, which means that the glycan provides a steric hindrance and consequently prevents the enzyme (trypsin/lys C) to have access on the protein cleavage site (Sola and Griebenow 2009; Wang et al. 1996).

After the treatment with PNGase F, there were no significant changes in the obtained spectra for Meriofert and Menopur. In both formulations, because of the glycan removal, a peptide containing the glycosylation site at Asn 102 (*SYNRVTVMGGFKVE**N**HTACHCSTCYYHK)* was identified (Figs. 5 and 6).

As mentioned above in relation to the naturally occurring hFSHβ glycoforms, hFSHβ^{24}, hFSHβ^{21}, hFSHβ^{18}, hFSHβ^{15}, in figure 2 (A-F), the most abundant glycoforms (hFSHβ^{24}, hFSHβ^{21}) could be noticed and this is the first evidence about the presence of glycosylation macroheterogeneity. The second evidence was obtained after mass spectrometry-based analysis of the two regions of

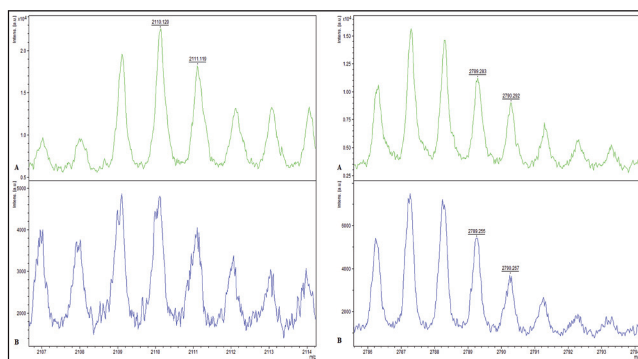

Fig. 7: Peptide mass fingerprint spectra of FSHβ showing the peaks corresponding to Asn 25 (left) and Asn 42 (right) N-linked glycan carrier peptide. Left. Up: the m/z peak from the untreated Menopur spot pattern around 21 kDa; Down: the same peak missing in the spot pattern around 24 kDa. Right. Up: The m/z peak from the untreated Menopur spot pattern around 21 kDa. Down. the m/z peak from the untreated Menopur spot pattern around 24 kDa.

spot patterns (around 24 kDa and around 21 kDa) corresponding to hFSHβ. In Menopur, in the mass region around 21 kDa, a peptide that carries N-linked glycans at Asn 25 (AICC**N**SCELTNITIAIEK) was identified, which was not identified in the spot pattern around 24 kDa (observed *m/z* 2110.1205 and 2111.1187) (Fig. 7). Such peptide was not identified in Meriofert. Moreover, in both mass ranges, the peptide containing the glycosylation site at Asn 42 (observed *m/z* 2789.2, 2790.2) was identified (Fig. 7). The macro- and microheterogeneity of FSHβ has been well-reported (Bousfield et al. 2014b), therefore it is expected to have a large number of such isoforms, which were evaluated after the enzymatic deglycosylation and desialylation. PNGase F demonstrated the presence of glycosylation macroheterogeneity (Fig. 8), whereas desialylation (Neuraminidase digestion) documented the considerable microheterogeneity (Fig. 2, C and D).

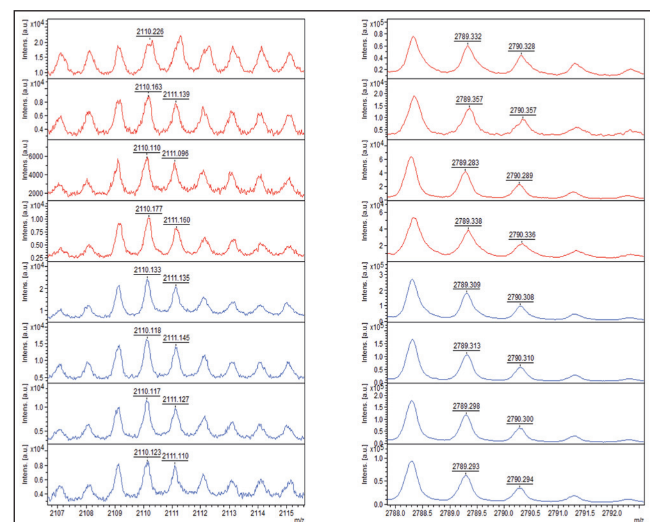

Fig. 8: Peptide mass fingerprint spectra of FSHβ showing the peaks corresponding to Asn 25 (left) and Asn 42 (right) N-linked glycan carrier peptide among all spots after PNGase F treatment. Red spectra – Menopur, blue spectra – Meriofert.

Gonadotropins, in general, are rich in sialic acid residues especially FSHβ. On the mass spectra, in both formulations, *m/z* signals starting from the spots on the acidic side to those headed towards the basic side started being more intensive, indicating the shift due to the removal of sialic acids. Also, as close as the FSHβ spots approach to the common alpha-subunit, signals corresponding to the latter started to appear (Fig. 2, C and D).

As a summary, orthogonal approaches which are capable to analyse products of high structural complexity such are biopharmaceuticals were employed as analytical methodologies for the comparative analysis of Menopur and Meriofert, two highly complex biologicals of human origin. Due to their inherent complexity, a single analytical method would not be sufficient for the assessment of their quality, purity and structural integrity. Migration behaviour of their components was studied using 1D and 2D gel electrophoresis. Products under study demonstrated high structural complexity, typical for biological medicines, which after protein backbone synthesis in organism undergo glycosylation process that imparts their additional complexity. After their separation in 2D GE, proteins were tryptically *in-gel* digested and resulted peptides were subjected to MALDI-TOF MS, where the identification of the main active components FSH, hCG and common alpha-subunit was achieved, with a high sequence coverage. Not surprisingly significant differences in the results of the product could be shown, which might deserve specific deepened studies. The study results demonstrate that 2D GE/MALDI TOF MS analysis is a suitable method for the rapid identity confirmation of biological medicines. In our opinion, apart from its use for in-process control purposes, speed, simplicity, and specificity of this method also providing insight on the carbohydrate moieties, will allow its application as a tool to improve properties of biomedicines, both related to drug efficacy and safety.

3. Experimental

Menopur® 75/75 I.U. (Menotrophin), (Ch.-B.: P10570F; P11340H), sterile freeze-dried powder and solvent for solution for injection and Meriofert® 75 I.U. (Menotrophin), (Ch.-B.: 170628; 180610), sterile freeze-dried powder and solvent for solution for injection were purchased at a pharmacy shop in Vienna (Austria) and stored in dark under 23.3°C. The analyzed batches of pharmaceutical formulations were manufactured during the years 2018-2019. All chemicals were of analytical grade.

3.1. *Protein concentration assay*

Pierce 660 nm Protein Assay Reagent kit (Thermo Scientific, USA) based on the formation of dye-metal complex to protein in acidic conditions was used to assess the protein content of the Menopur and Meriofert vials, prior reconstituted in 50 µL water (18.2 MΩcm at 25 °C, provided in-house by Simplicity water purification system, Merck-Millipore, USA) according to (Antharavally et al. 2009).

3.2. *One dimensional gel electrophoresis*

To perform SDS-PAGE analysis, dried sample aliquots containing 847.5 ng (Menopur) and 497.5 ng (Meriofert) of total protein amount (TPA) were reconstituted in water and each sample was further mixed in a 1:4 ratio with 4x LDS (lithium dodecyl sulphate) sample buffer and 10% DTT (dithiothreitol). The mixtures were heated (95 °C, 5 min) and cooled down to room temperature. Electrophoresis was performed on NuPAGE® 10% Bis-Tris Mini Gel, 1.0 mm x 12 wells using MES buffer as running buffer (both from Invitrogen), at constant voltage (200V) in an XCell SureLock Mini-Cell (Life Technology). The gel was silver stained according to (Shevchenko et al. 1996).

3.3. *Isoelectric focusing*

Prior to isoelectric focusing (IEF), the samples were reduced and alkylated. A sample aliquot, containing 2.5 µg of TPA was diluted to 50 µl with water. Urea (final concentration 1M) and tris(2-carboxyethyl) phosphine (TCEP, final concentration 10 mM) (Sigma-Aldrich, USA) were added. The mixture was vortexed and spun down, followed by incubation at 37 °C, for 45 min on a thermomixer (600 rpm). Afterwards, iodoacetamide (IAA, final concentration 30 mM) (Sigma-Aldrich, USA) was added and again the mixture was incubated for 10 min at RT, in dark. After 10 min, DL-dithiothreitol (DTT, final concentration 20 mM) (Sigma-Aldrich, USA) was added and the mixture, once again, was incubated for 10 min at RT. Subsequently, the sample was dried in a vacuum centrifuge (Univapo).
The IEF was carried out in SERVA IPG BlueStrip 3-10 NL / 11 cm (Serva Electrophoresis GmbH, Germany). The dried reduced and alkylated sample was reconstituted in 192 µl rehydration buffer (7M urea, 2M thiourea (Sigma-Aldrich, USA), 2% CHAPS (VWR International, Belgium) in 20mM Tris-HCl (pH 9.0) (Sigma-Aldrich, USA)), followed by the addition of DTT (final concentration 20 mM) and ampholyte Servalyt® 3-10 (final concentration 2%) (Serva Electrophoresis GmbH, Germany). The 11 cm IPG strip (3-10 NL) was rehydrated in a reswelling tray overnight (~ 12-15h), covered in paraffin oil (2-3 ml) (Merck KGaA, Germany). The next day the IEF was performed on the Multiphor II (Amersham Biosciences, Sweden) in five steps at 20°C: step 1: 0 to 150 V within 1 h, step 2: 150 to 300 V within 1 h, step 3: 300 to 1000V within 1 h, step 4: 1000 to 3500 V within 2 h, step 5: constant at 3500V until around 15 kVh were reached. Current limit was 50 µA.

3.4. *The second dimension, SDS-PAGE*

After the focusing, IPG strips were reduced for 15 min (50 mg DTT, 1.8 g urea in 5 ml equilibration buffer (Serva Electrophoresis GmbH, Germany)) and alkylated for another 15 min (125 mg IAA, 1.8 g urea in 5 ml equilibration buffer). The second dimension was run on a horizontal system HPE FlatTop Tower, using the 2D HPE™ Double Gel NF 12.5%, 250 x 110 x 0.65 (Serva Electrophoresis GmbH, Germany) as described by the manufacturer, at 15^{0}C through 4 steps (step 1: 100 V, 7 mA, 1W, 30 min; step 2: 200 V, 13 mA, 3W, 30 min; step 3: 300 V, 20 mA, 5W, 10min; IPG strip was removed; step 4: 1000V, 40 mA, 25W, 150 min).

3.5. *Fluorescent staining*

Serva Purple (strike concentrate) (Serva Electrophoresis GmbH, Germany) was used to fluorescently stain the 2D gels according to the manufacturer's instructions. The 2D gel was scanned on the laser scanner (Typhoon™ FLA 9500, GE Healthcare Bio-Sciences, Sweden), at 532 nm excitation wavelength and at 610 nm emission wavelength.

3.6. *Deglycosylation and desialylation*

The cleavage of N-linked sugars and the terminal sialic acid residues was done through enzymatic treatment with PNGase F (peptide-N^4-(acetyl-β-glucosaminyl), from *Flavobacterium meningosepticum* and expressed in *E. coli* (Roche Diagnostics GmbH, Germany) and (α(2-->3,6,8,9) neuraminidase, from *Arthrobacter ureafaciens* (Sigma-Aldrich, USA) as described (Stubiger et al. 2005). Briefly, dried sample aliquots containing 847.5 ng (Menopur) and 497.5 ng (Meriofert) of TPA were reconstituted in 50 µL of respective buffers, Na_2HPO_4 (20 mM, pH 7.2) for PNGase F treatment and $NaHCO_3$ (10 mM, pH 8.0) for Neuraminidase treatment. Prior to treatment, proteins were reduced and alkylated as described above. Samples were incubated overnight (~18h) at 37^{0}C on a thermomixer (350 rpm).

3.7. *Protein identification*

Protein identification after in-gel digestion, MS and MS/MS analysis were performed as previously described (Marchetti-Deschmann et al. 2009). Bands and spots of interest were excised with a clean scalpel and silver stained gel particles were destained using 100mM $Na_2S_2O_3x5H_2O$ and 30mM $K_4[Fe(CN)_6]$ (1:1, v/v). After multiple washing and buffer exchange steps according to (Marchetti-Deschmann et al. 2009), samples were reduced with DTT (10 mM DTT in 100 mM NH_4HCO_3 (pH 8.5)) and alkylated with IAA (54 mM IAA in 100 mM NH_4HCO_3 (pH 8.5)). Afterwards, proteins were digested by adding approximately 10 µl digestion solution (dependent on the volume of the gel pieces) containing 1 ng/µl trypsin/lys C mix (Promega, USA) in digestion buffer (95% NH_4HCO_3 (50mM, pH 8.5) and 5% ACN). The proteins were digested overnight at 37 °C on a thermomixer (800 rpm). Generated peptides were desalted and concentrated by the ZipTip C_{18} pipette tips (Merck KGaA, Germany) and subjected to MALDI-TOF/RTOF instrument (UltrafleXtreme™, Bruker Daltonics, Germany) equipped with a Nd:YAG laser at 355 nm, by using the direct elution method with α-cyano-4-hydroxycinnamic acid (CHCA, 3mg/ml in 60:40 ACN:TFA 0.1%) (Sigma-Aldrich, USA) as matrix. Spectra were recorded in positive ion reflectron mode and externally calibrated (peptide calibration mix 4, Proteomix, 500-3500 Da, LaserBio Labs, France). Identification was performed using the SwissProt database (May 2019, 560292 sequences, 201357942 residues) via Mascot on an in-house server with the following search parameters for MS and MS/MS: homo sapiens (human) (taxonomy), trypsin (enzyme), maximum two missed cleavages, carbamidomethyl (C) as global modification and acetylation (N-Term), deamidation (NQ) and oxidation (M,) as variable modifications, peptide mass tolerance ±0.2 Da and ±0.5 Da fragment mass tolerance, monoisotopic $[M + H]^+$.

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Authors Contributions: LM: Conceptualization, Investigation, Methodology, Formal Analysis, Writing – Original Draft. VD: Investigation, Writing – Review & Editing. MN: Methodology, Writing – Review & Editing. CRN: Conceptualization, Methodology, Writing – Review & Editing. DN: Conceptualization, Funding acquisition, Supervision, Methodology, Validation, Writing – Review & Editing. MMD: Conceptualization, Funding acquisition, Supervision, Methodology, Validation, Writing – Review & Editing.

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