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Identification by LC-ESI-MS/MS of new clomifene metabolites in urine

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1. Introduction

Clomifene is a synthetic anti-estrogen, structurally related to diethylstilbestrol, and is a first choice therapy to treat women with absent or irregular ovulation [1]. It is also used by male steroid abusers to bind the estrogens receptors for blocking unwanted effects of estrogens and to restores the natural production of testosterone. Recently X. de la Torre [2] has reported the presence of four glucoconjugate metabolites of enclomifene and six sulfoconjugate metabolites of Zuclomifene in urine by mean of liquid chromatography-mass spectrometry using electrospray ionization. However, the excretion investigation of a single dose of 50mg of clomifene (Serpafar®) in man and women using a triple quadripole LC-ESI-MS/MS shows the presence of new glucoconjugates metabolites in addition to those recently identified. Excretion studies were collected over one week and the identification of metabolites was based on the retention time, ES-MS and MS-MS mass spectra.

2. Materials and Methods

2.1. Sample preparation

To 2 mL of urine, 20µL of 17α-methyltestosterone (20µg/mL) as internal standard were added, 200 µL of phosphate buffer pH=7 and 25 µL of beta-glucuronidase from *E. coli* were added and hydrolysis was performed for 1 h at 55°C. The buffered solution was then alkalinized with 300 µL of carbonate buffer. The sample was treated with 5 ml of a tert-butylmethyl ether and thoroughly homogenized by shaking on a mechanical shaker for 10 min, followed by centrifugation at 2500 rpm for 5 min to allow the separation of the organic phase. The aqueous phase was discarded and the organic phase was then evaporated to dryness under a Nitrogen stream. The dry residue was dissolved in mobile phase.

2.2. Urine Samples

Excretion study was performed on one healthy female volunteer. One single dose of Serpafar® (50 mg of clomifene) was administrated. For 6 times a day all urine samples were collected.

2.3. LC-MS/MS parameters

Instrument: Agilent 1100 HPLC with Quattro micro (Micromass, UK)

Ionization mode: ES(+) Data acquisition mode: MRM

Methyltestosterone 303/107 Met1, Met2 and Met3 Met4, Met5 and Met6

394>72, 422>100 and 486>100 440>100, 452>100 and 424>72 respectively.

High voltage electrodes: 3500V Source temperature: 120ºC

Desolvation temperature: 350 ºC Desolvation gaz flow: 500L/h

Neubilisation gaz flow: 90 psi. Collision gaz maintained at 2 10 -3 mbar

Column: Zorbax RX C8 (2.1×150 mm, 5 µm) at 60 ºC.

Mobile phase: Acetonitrile (A)/Ammonium Formate (B) (5 mM pH = 3.5) at 0.4 mL/min flow rate. 0 to 4 min 60% (A), 4 to 10 min 90% (A), 10 to 15 min 90% (A).

3. Results and Discussion

The full scan mass spectrum of extracted urine samples after ingesting of 50 mg of clomifene was compared with that of blank urine sample to find out the probable metabolites. Then, these compounds were analyzed by LC-ESI (+)-MS/MS. Their retention-times, changes in observed mass (*M*) and MS/MS spectra were compared with the substructural 'template' of Clomifene standard to identify metabolites and elucidate their structures. Based on the method mentioned above, the parent drug and its main metabolites were found in human urine after ingesting of 50 mg of Clomifene. The LC-ES (+)–MS chromatograms showed the presence of six peaks appeared at t_R = 4.28 (m/z=486), 5.6 (m/z=440), 8.8 (m/z=394), 10.91 (m/z=422), 11.5 (m/z=424) and 12.01 min (m/z=452). In agreement with previous studies, the main metabolic reactions of clomifene were oxidation in both phenyl rings, hydroxylation in *para* position and methoxylation in 3-*meta* position [2-3]. The 4-hydroxyclomifene and 3-methoxy-4-hydroxyclomifene were identified in urine as glucoconjugate (Fig. 1A and B). However, the presence of 4-hydroxy-desethylclomifene and 3-methoxy-4-hydroxy-desethylclomifene with a characteristic product ion at m/z=72 (Fig. 1C and D) in glucoconjugate fraction, indicate that clomifene undergo N-desalkylation and oxidation reactions. Whereas, the dissociation of pseudo-molecular ion at m/z= 486 and m/z= 440 gave four product ions at m/z=486/422, 450/404, 100 and 72. The presence of the m/z =100 and 72 ions, which were produced by the partial losses of the side chain (Fig. 1E and F), indicate the similarity of these compounds to clomifene. In addition, the presence of fragment ions at m/z= 468 (respectively 422) and m/z= 450 (respectively 404) suggest a spontaneous losses of two hydroxyl groups. This finding indicates that these metabolites could be the N-oxide-α-

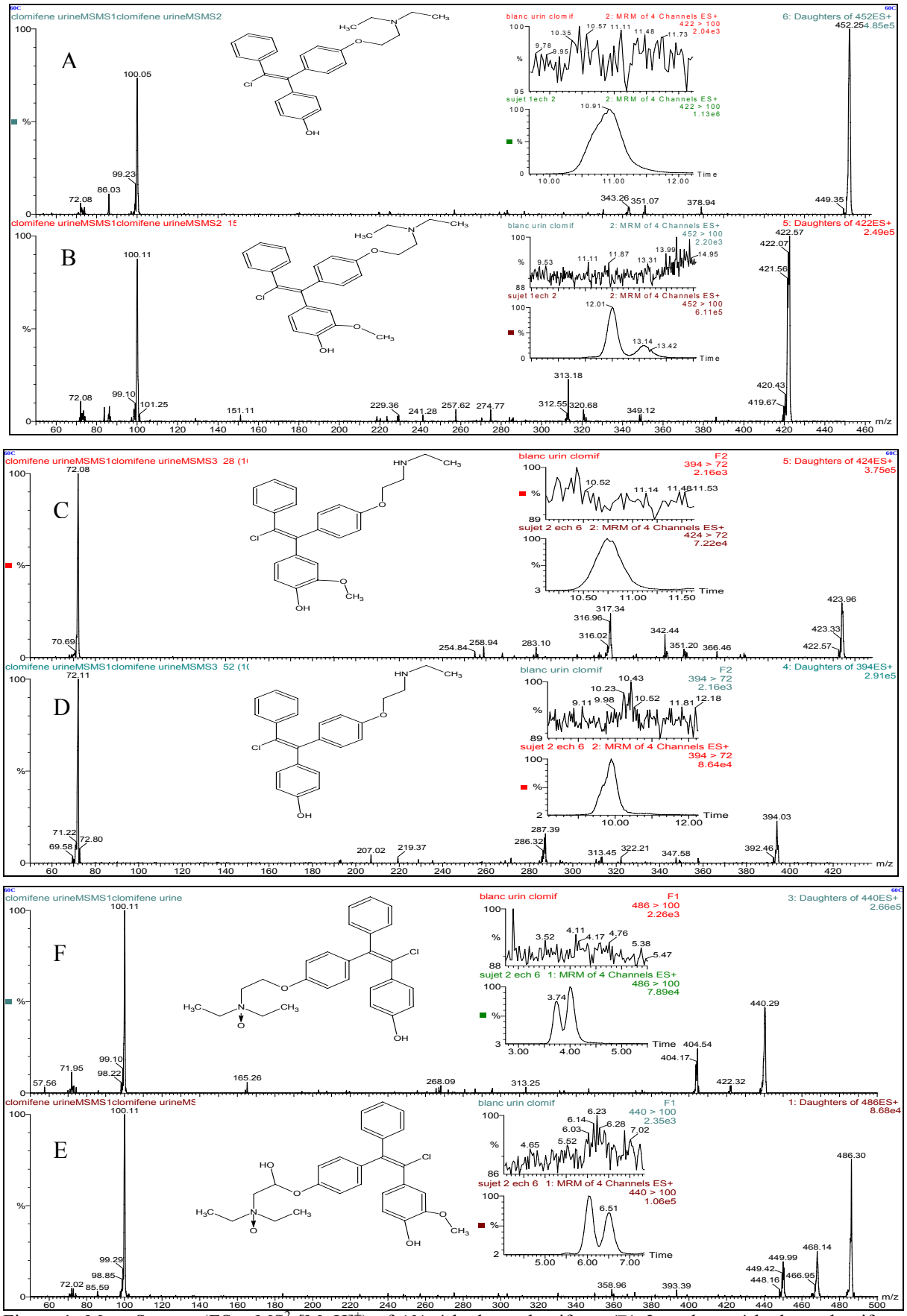

Figure 1: Mass Spectra (ES+, MS^2 $[M+H]^+$) of **(A)** 4-hydroxyclomifene, **(B)** 3-methoxy-4-hydroxyclomifene, **(C)** 4-hydroxy-desethylclomifene, **(D)** 3-methoxy-4-hydroxy-desethylclomifene, **(E)** N-oxide-α-hydroxy-3-methoxy-4-hydroxyclomifene and **(F)** the N-oxide-4-hydroxyclomifene.

hydroxy-3-methoxy-4-hydroxyclomifene and the N-oxide-4-hydroxyclomifene respectively in agreement with a previous study which showed the presence of α–hydroxy-tamoxifene, N-oxide and α-hydroxy-N-desmethyltamoxifen metabolite in the urine of patients on tamoxifen therapy [4]. Figure 2 illustrated the excretion study results. The maximum concentration of the two metabolites **(Met E)** N-oxide-α-hydroxy-3-methoxy-4-hydroxyclomifene and **(Met F)** the N-oxide-4-hydroxyclomifene has been detected in the urine sample collected 9 hours after administration; and they still exist all over the week.

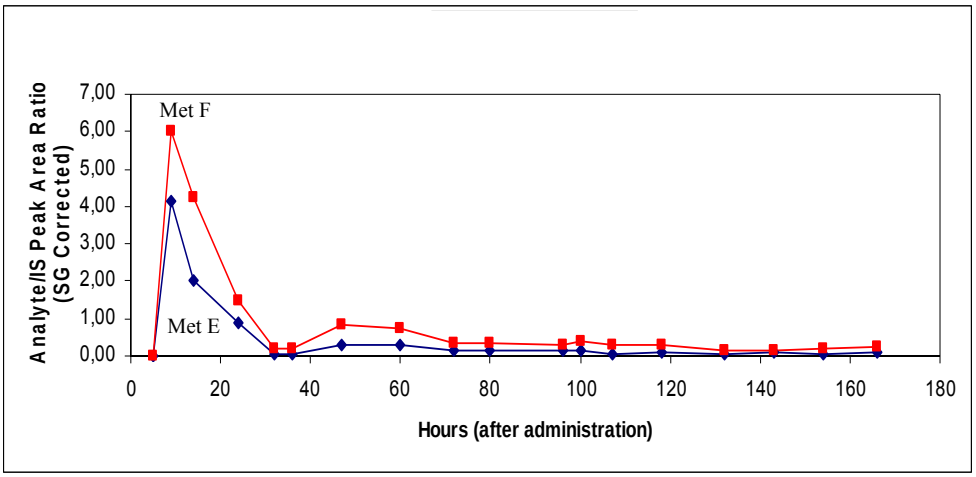

Figure 2: Excretion profiles of clomifene metabolites: **(Met.E)** and **(Met.F)**

4. Conclusion

Oral administration of clomifene resulted in detectable urinary elimination of six drug's metabolites; 4-hydroxyclomifene was identified as an important metabolite of clomifene. Other metabolites: 3-methoxy-4-hydroxy-clomifene, 4-hydroxy-desethylclomifene and 3-methoxy-4-hydroxy-desethylclomifene and the N-oxide-α-hydroxy-3-methoxy-4-hydroxy-clomifene, N-oxide-4-hydroxyclomifene are also detectable in human urine after oral administration of a single 50 mg dose of Serpafar®. The detection of an abuse even of a single therapeutical application of clomifene is possible over a long time period.

5. References

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