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Structural elucidation of seven prednisolone metabolites by LC-ESI-MS/MS using a PGC column

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1. Introduction

Several methods have been reported for the determination of prednisolone metabolites in human urine. Thoses methods were carried out using high-resolution gas chromatography (GC) [1] or based on high performance liquid chromatography coupled to UV detector [2] and tandem mass spectrometry (LC-MS/MS) for human and equine excretion urine [3]. Reversed-phase HPLC is usually the chromatographic mode of choice for bioanalysis in drug metabolism studies because of its ability to separate compounds with a broad range of polarities and its compatibility with aqueous based biological samples. However, reversed-phase HPLC is not universally suitable for drug metabolism studies since metabolites often have very similar polarity as the parent drug and therefore elute close to the parent drug. An attempt was made to increase the stereochemical differences between thoses metabolites using a porous graphitic carbon column (PGC) and positive electrospray tandem mass spectrometry. A good separation with baseline resolution of prednisolone, prednisone and six metabolites were obtained allowing thus their unambigous identification. This work presents a sensitive and specific LC-ESI-MS/MS method for rapid qualitative identification of prednisolone and its metabolites (phase I metabolites) in SPE extracted urine samples after ingesting of 50 mg of Prednisolone.

2. Materials and Methods

Prednisolone, Prednisone, Acetic acid and Ammonium acetate were purchased from Sigma (Germany). HPLC grade of water and methanol were obtained from Labscan (Ireland).

2.1. *Standard samples*

Solutions were prepared by dissolving 1 mg of Prednisolone and prednisone standards in 10 mL of methanol and diluting to the concentration of 5µg/mL.

2.2. *LC-MS/MS parameters*

Instrument: Agilent 1100 HPLC coupled to Quattro micro (Micromass)

Ionization mode: *ESI +* High voltage electrodes: *3500V*

Data acquisition mode: *MS and MS/MS*

Source temperature: *120ºC* Desolvation temperature: *400 ºC*

Desolvation gaz flow: *500L/h* Neubilisation gaz flow: *90 psi*.

Collision gaz at *2 10 -3 mbar* Column: *Hypercarb (2.1×100 mm, 5 µm) at 80 ºC*

Mobile phase: *Ammonium Formate-Methanol [5mM, pH =3.5] (10:90) at 0.3 mL/min.*

2.3. ***Administration and purification***

Following administration of a single 50 mg oral dose of prednisolone to a 24 years old male that volunteered for the study, urine were collected at different time-points up to 48 h. Four milliliters of urine was spiked with methyltestosterone (IS, 250 ng), the pH was adjusted to 7.0 (K_2CO_3 1M or acetic acid 1M) and diluted with potassium phosphate buffer (pH= 6.5, 0.2M, 1 mL). The samples were loaded into the extraction cartridges and eluted with 5 mL of MTBE after evaporation the dry residue was redissolved in 100 µL of mobile phase.

3. Results and Discussions

The total ion chromatograms of SPE extracted urine samples after ingesting of 50mg of Prednisolone was compared with that of blank urine sample to detect suspicious peaks. The corresponding retention-times, MS and MS/MS mass spectra were then used to obtain structural information. The LC-ES(+)–MS chromatograms showed the presence of seven peaks which appears at t_R = 5.1, 5.7, 6.2, 6.5, 7, 7.7 and 8.2 min. It is clear that the presence of thoses peaks in the region of 5 to 8 min suggest a close structural similarity with the parent drug. In fact, The obtained pseudo-molecular ions $[M+H]^+$ of all eluted peaks were at m/z 359, 361 (3 peaks) and 363 (4 peaks) respectively. LC–MS/MS ions chromatogram of the prednsiolone metabolites, illustrated in Figure 1, 2, 3 were obtained via fragmentation of protonated molecular ions that used for more precise structural identification of metabolites. The retention time, the MS and MS^2 spectra of pseudo-molecular ion at m/z 359 were the same as those of prednisone with characteristic product ions at *m*/*z* 311, 295 (Fig1). The product chromatogram of ions at m/z= 363 showed the presence of four resolved peak (A), (B), (C) and (D). The corresponding product ions spectra MS^2 of peak (A) and (C) showed the presence of fragment ion at m/z=307=$[M+H-4H_2O]^+$ as well as the presence of an intense ion at m/z=267 (Fig.2), seems to be relative to the reduction of 20 keto according to a previous study in man and equine urine [3]. This finding suggests that peak (A) and (C) should be the 20α and 20β-dihydroprednisolone. Whereas, the absence of hydroxyle function in C-11 seems to disfavor the spontaneous cleavage of water on the product ions spectra MS^2 of peak (B) and (D) similar to the observed MS^2 spectra of prednisone. In addition, the low intensity of

products ion due to the losses of $3H_2O$ (m/z= 307) might indicate the reduction of ring A at C-4 and C-3 (double bond and carbonyl respectively) leading to 3α, 5-tetrahydroprednisone and 3β, 5-tetrahydroprednisone. This result is in fair agreement with reported investigation [5] which indicated the preferential reduction of double bond at C-4 prior to that at C-1.

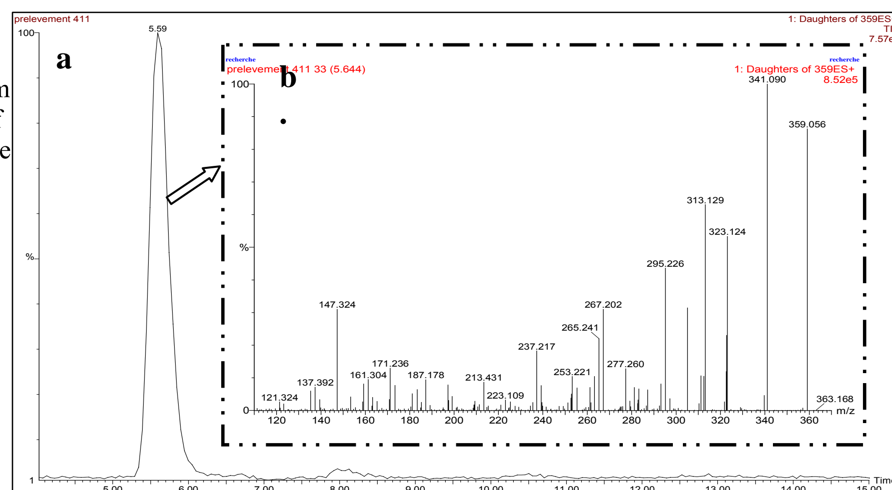
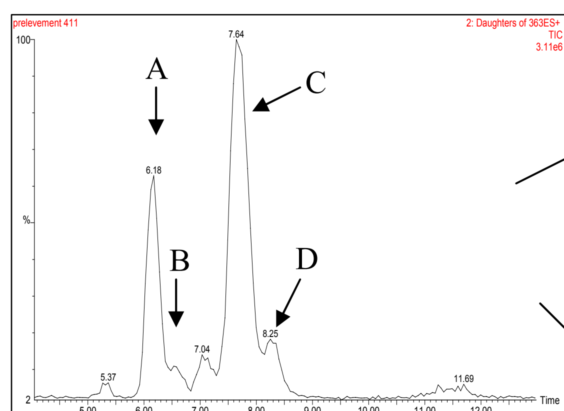


Fig 1: Product chromatogram (a) and mass spectrum (b) of $[M+H]^+$ =359 at cone voltage of 15V and collision energy of 20eV.



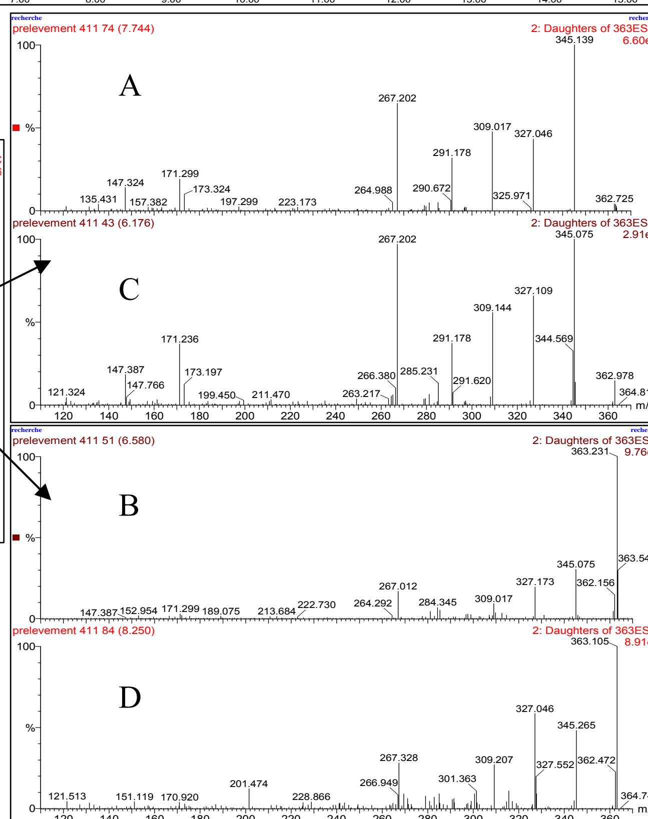


Fig 2: Product chromatogram (ES+, MS^2 $[M+H]^+$ =363) at cone voltage of 15V and collision energy of 20 eV, Mass spectra of (A) 20α, (C) 20β-dihydro-prednisolone, (B) 3α,5-tetahydro-prednisone and (D) 3β,5-tetrahydroprednisone.

The product chromatogram of ions at m/z= 361 showed the presence of 3 resolved peak (E), (F) and (G). The retention time, the MS and MS^2 spectra of pseudo-molecular ion at m/z 361 of peak (E) were the same as those of prednisolone with a specific loss of waters. However, peak (F) and (G) showed a characteristic product ion at *m/z* 316, 297 which were similar to the dissociation of C-11 keto compound (prednisone) [4]. Therefore, the presence of product ions at m/z=307=$[M+H-3H_2O]^+$ due to the elimination of $3H_2O$ as well as the fragment ion at

m/z= 265 sems to be relative to the reduction of 20 keto giving thus 20α and 20β-dihydroprednisone in accordance with previous studies [1].

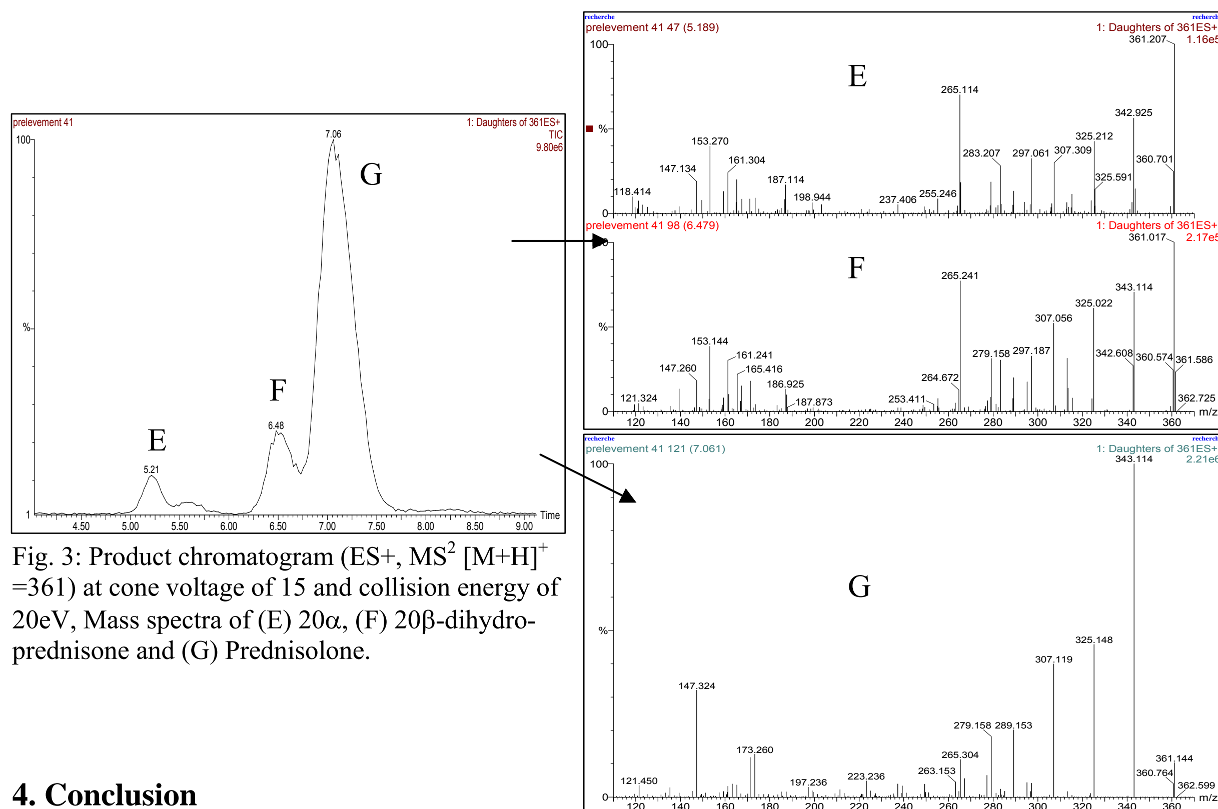


Fig. 3: Product chromatogram (ES+, MS^2 $[M+H]^+$ =361) at cone voltage of 15 and collision energy of 20eV, Mass spectra of (E) 20α, (F) 20β-dihydro-prednisone and (G) Prednisolone.

4. Conclusion

The identification of metabolites were based on the retention time, ES(+)-MS and ES(+)-MS/MS mass spectra. Using LC-ES(+)-MS/MS it was possible to identify 20α and 20β-dihydroprednisolone, 20α and 20β-dihydroprednisone, 3α and 3β, 5-tetrahydro-prednisone for 36 hours after oral administration of 50 mg of prednisolone. Based on this, prednisone, 20α and 20β-dihydroprednisolone, 20α and 20β-dihydroprednisone were selected for confirmation purposes in Tunisia anti-doping laboratory if the concentration of parent compound exceeds 30ng/mL.

5. References

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