

## **Effects of synthetic glucocorticoids administration on the urinary endogenous corticosteroids profile**

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### *Introduction*

Since 2004 glucocorticoids (GCS) are included in the WADA list and their use is prohibited, in competition, when administered systemically [1]. At present, apart from the WADA MRPL [2], no criteria have been fixed to discriminate the systemic from the topical (permitted) administration. It has been demonstrated that systemic administration of synthetic GCS leads to a suppression of endogenous steroids urinary levels [3]; whereas the administration of GCS that are also produced endogenously leads to a significant alteration of the tetrahydrocortisol/tetrahydro-11-deoxycortisol ratio [4]. Here we have developed and validated a LC-MS/MS method for the detection of several GCS in human urine, to investigate the effects of GCS administration on the urinary endogenous GCS profiles.

### *Materials and Methods*

#### Samples, Chemicals and Reagents

Urine samples were collected before (for at least three days) and after topical (TA) and/or systemic administration (SA) of three representative synthetic GCS: triamcinolone acetonide (TAC, Kenakort<sup>®</sup>), dexamethasone (DEX, Luxazone<sup>®</sup>, Decadron<sup>®</sup>) and betamethasone (BET, Bentelan<sup>®</sup>, Betamesol<sup>®</sup>). Each synthetic GCS was administered either topically (twice a day for 3 days at 8.00 am and at 4.00 pm) or systemically (once a day for 2 days at 8.00 am) to the same subject with a wash out period of three days. All urine samples were stabilized with sodium azide (1 mg/mL) and stored at -20 °C. All measured values were adjusted for a value of specific gravity of 1.020 according to the WADA guidelines.

11-deoxycortisol (S), tetrahydro-11-deoxycortisol (THS), cortisol (F), 6 $\beta$ -hydroxycortisol (6bOHF), 20 $\alpha$ -dehydrocortisol (20aDHF), tetrahydrocortisol (THF), 5 $\alpha$ -tetrahydrocortisol (5aTHF), cortisone (E), 20 $\alpha$ -dehydrocortisone (20aDHE), tetrahydrocortisone (THE), 11 $\beta$ -hydroxy-androsterone (11OHA), 11 $\beta$ -hydroxy-etiocholanolone (11OHE) were supplied by Sigma-Aldrich (MI, Italy). Deuterated androsterone (Ad4) was supplied by NMI (National Measurement Institute, Pymble, Australia). Deuterated Cortisol (Fd4) was purchase by

Medical isotopes. All chemicals were from Carlo Erba (MI Italy). The enzyme  $\beta$ -glucuronidase from *E. coli* was purchased from Roche (MI, Italy).

#### Analytical procedure

All experiments were performed using an Agilent 1200 Rapid Resolution Series HPLC. Liquid chromatography was performed using a Discovery C18 column (150 x 2.1 mm, 5  $\mu$ m). The solvents were: water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B). The gradient programme started at 10% B and increasing to 20% B in 3 min, then, to 60% B in 3 min, then after 3 min to 50% B in 1 min and at the end after 4 min to 60% B in 1 min. The column was flushed for 1 min at 100% B and finally re-equilibrated at 10% B for 4 min. The flow rate was set at 250  $\mu$ L/min using an oven temperature of 60 °C. Data were acquired using the API4000 instrument. The ion source was operated at 550 °C, the applied capillary voltage was 5500 V and SRM experiments were performed (see Table 1). The values of the urinary GCS concentration were calculated by the peak areas of the detected signals relative to the peak areas of the internal standards Fd4 (m/z 267/121) and Ad4 (m/z 295/267).

The samples were pre-treated according to a specific procedure presently followed by our laboratory. Briefly, 3 mL of urine were hydrolyzed using  $\beta$ -glucuronidase from *E. coli*, then a liquid/liquid extraction was carried out with *tert*-butylmethylether. The organic layer was evaporated and the residue was reconstituted in 50  $\mu$ L of mobile phase.

### *Results and Discussion*

#### LC-MS/MS method development and validation

Chromatographic conditions allowed an adequate separation of all compounds, a part from THF and 5aTHF. The ion suppression was in the range of 5-30%. The repeatability of RRTs (CV% < 1) and of relative ion abundances (CV% < 15) was adequate. Recoveries ranged from 75 to 85% with standard deviations  $\leq$  10%. The limits of detection (LODs) and quantitation (LOQs) were in the range 1-15 ng/mL and 5-30 ng/mL respectively (see Table 1). Good precision of peak areas (RSD) do not exceed 5% for intra-day assays and 10% for inter-day assay and linearity ( $r^2 > 0.992$ ) within the 5-1000 ng/mL range was obtained.

#### Endogenous and synthetic glucocorticoids profile

Figures 1-3A show the urinary profiles of 11OHE, 11OHA and their ratio before and after either SA or TA of BET. We can notice that only after SA the urinary levels of 11OHE and 11OHA decrease significantly, while the ratio 11OHE/11OHA increased significantly.

Figures 1-3B show the urinary profiles of F, E and their ratio before and after either SA or TA of BET. We can notice that only after SA the urinary levels of F and E decrease significantly, while for the ratio F/E no significant variations were recorded. Figures 1-3C show the urinary profiles of THF+5aTHF, THE and their ratio before and after either SA or TA of BET. We can notice that only after SA the urinary levels of THF+5aTHF and THE decrease significantly, while for the THF+5aTHF/THE ratio no significant variations were recorded. Figures 1-3D show the urinary profiles of S, F and their ratio before and after either SA or TA of BET. We can notice that only after SA the urinary levels of S, F and their ratio were altered significantly.

The maximum urinary concentration value of the synthetic GCS were higher than 30 ng/mL after oral administration ( $\cong$  60 ng/mL, DEX and BET), lower than 30 ng/mL after administration of opftalmic preparations and trans-dermal injection ( $\cong$  20 ng/mL, DEX;  $\cong$  15 ng/mL, TAC) and lower than the limit of detection after cream administration ( $<$  1 ng/mL).

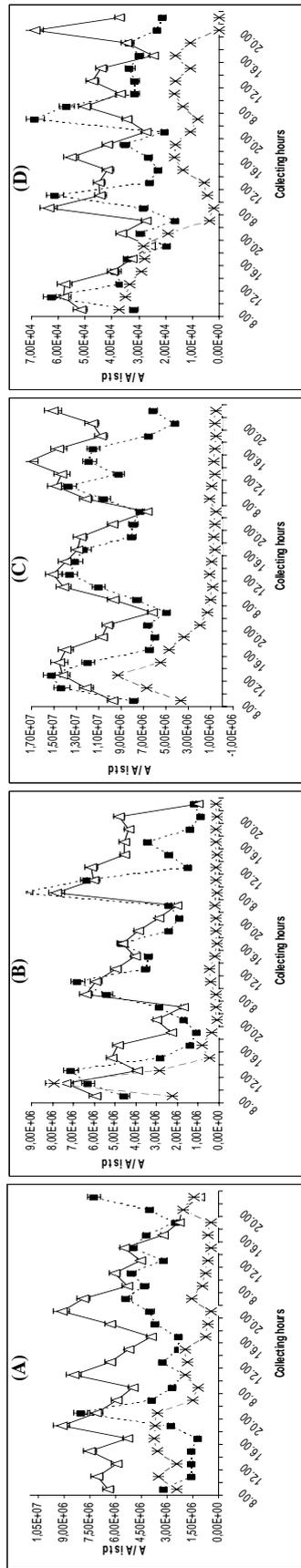
In summary this preliminary study indicate that only after SA of GCS significant variation from the baseline profile was recorded for endogenous GCS and for several ratios. Nonetheless additional experiments are needed to verify the effects on the endogenous GCS urinary profile of other synthetic GCS, including synthetic F and E, of different dosages and administration routes in order to confirm our data and to establish threshold values.

**Table1.** Analytical parameters, LOD and LOQ

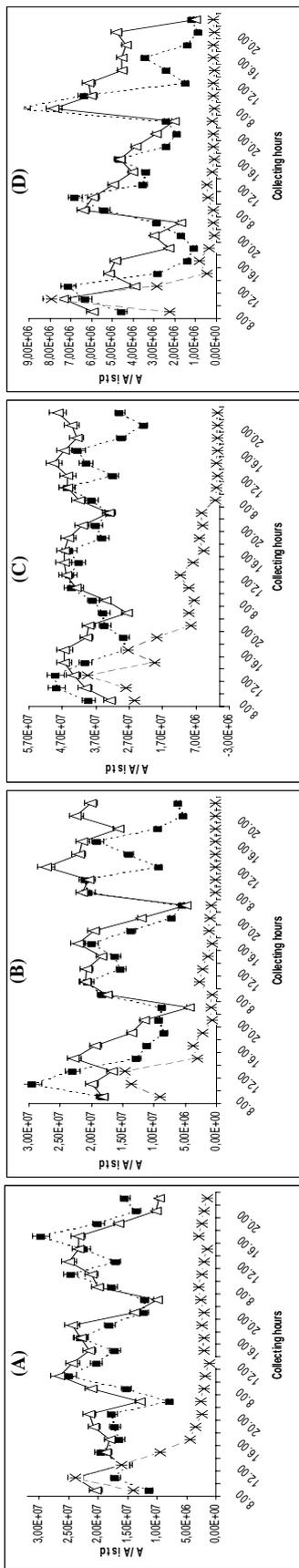
Compounds	SRM (m/z)	RT (min)	Polarity/CE (eV)	LOD/LOQ (ng/mL)
<i>6bOH</i>	379/283; 379/313	1.85	Positive/20	15/30
<i>20aDHF</i>	366/270; 366/287	10.22	Positive/20	10/15
<i>20aDHE</i>	363/163; 363/267	10.52	Positive/20	10/15
<i>F</i>	363/121; 363/327	11.14	Positive/20	1/5
<i>E</i>	361/163; 361/343	11.37	Positive/20	1/5
<i>THF+5aTHF</i>	331/313; 331/295	11.92	Positive/20	15/30
<i>THE</i>	345/121; 345/301	12.25	Positive/20	15/50
<i>S</i>	347/97; 347/109	12.80	Positive/20	5/10
<i>11bOHE/11bOHA</i>	271/253; 271/229	13.35/13.65	Positive/20	5/10
<i>THS</i>	315/279; 315/255	15.18	Positive/20	5/10

#### References

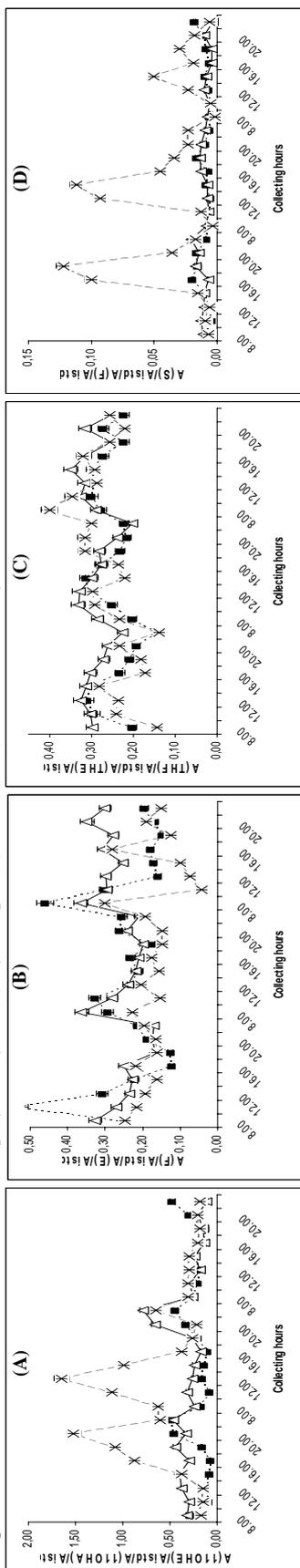
1. World Anti-Doping Agency. The 2010 Prohibited List. International Standard, Montreal (2010) [http://www.wada-ama.org/rtecontent/document/2010\\_List\\_En.pdf](http://www.wada-ama.org/rtecontent/document/2010_List_En.pdf)
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3. Mazzarino M, Rossi F, Giacomelli L, Botrè F. (2006) Effect of the systemic vs. inhalatory administration of synthetic glucocorticoids on the urinary steroid profile as studied by Gas Chromatography – Mass Spectrometry. *Anal. Chim. Acta* **559**, 30-36.
4. Meklat N, Tabet J-C, de Ceaurriz J. (2009) Urine ratio of tetrahydrocortisol to tetrahydrodeoxycortisol to screen for the systemic administration of cortisone and hydrocortisone *Forensic Science International* **185**, 13–17



**Figure 1:** 11OHE (A); F (B); THF+5aTHF (C); S (D) profiles before ( $\Delta$ ), after topic ( $\blacksquare$ ) and oral ( $\ast$ ) administration of BET



**Figure 2:** 11OHA (A); E (B); THE (C); F (D) profiles before ( $\Delta$ ), after topic ( $\blacksquare$ ) and oral ( $\ast$ ) administration of BET



**Figure 3:** 11OHE/11OHA (A); F/E (B); THF+5aTHF (C); THE (D) profiles before ( $\Delta$ ) and after topic ( $\blacksquare$ ) and oral ( $\ast$ ) administration of BET