

Screening of Corticosteroids in Urine by Positive Atmospheric Pressure Chemical Ionization LC/MS/MS

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Introduction

Corticosteroids are a class of components often abused and misused in sport. They are very potent drugs in the treatment of inflammations and asthma [1]. Corticosteroids can have an effect on the nervous system and can improve an athlete's ability to concentrate and perform in endurance and power events [2,3]. Moreover, corticosteroids can alleviate pain in general. To prevent their misuse related to euphoria and pain suppression, the anti-doping governing bodies are restricting the use of corticosteroids. Systemic use of corticosteroids is forbidden in all circumstances. However, when medically necessary, local and intra-articular injections or dermatological preparations are allowed under the approval of a therapeutic use exemption [4].

The samples collected for doping control are mainly urine samples because large sample volumes can be collected in a non-invasive way. Therefore, the abuse of corticosteroids is analyzed using urine samples.

A simple and sensitive LC/MS/MS method for the screening of 17 corticosteroids is described below. The method is able to detect corticosteroids from the doping control urine samples at 20 ng/mL – below the WADA minimum required performance level (MRPL), which is 30 ng/mL.

Instrumentation

- Varian ProStar™ 430 AutoSampler
- Varian ProStar™ 210 Solvent Delivery Modules
- Varian 1200L LC/MS equipped with Atmospheric Pressure Chemical Ionization (APCI) source
- Harvard Syringe Pump model 11

Materials and Reagents

- Standards of corticosteroids, from Sigma-Aldrich, USA
- Methanol, gradient grade for liquid chromatography, from Merck, Germany
- Water supplied by a Simplicity 185 ultrapure water system, from Millipore, Great Britain
- α -glucuronidase from E. Coli K12, from Roche Diagnostics, Germany
- All other chemicals are pro analysis or HPLC grade

Sample Preparation

The samples are prepared by a standard procedure for steroids. A 2 mL urine sample is transferred in a tube. A 40 μ L aliquot of a 10 ppm desoximetasone (internal standard) solution, 1 mL phosphate buffer 0.8M pH 7.0 and 25 μ L beta-glucuronidase are added, the mixture is vigorously vortexed and kept for 1 hour at 50 °C for enzymatic hydrolysis. (The enzymatic hydrolysis step is needed since the corticosteroids are mainly excreted in a conjugated form with the glucuronic acid). Adding 750 μ L of 20% buffer K_2CO_3 / $KHCO_3$ (1:1), which brings the pH around 9, stops the hydrolysis. Next, 5 mL of tertbutylmethylether is added, and the mixture shaken for 15 min. After centrifugation, the organic layer is transferred to another tube and evaporated to dryness. The remaining residue is dissolved in 100 μ L mobile phase (20:80, solvent A / solvent B). Then, 10 μ L is injected in LC/MS/MS [5].

HPLC Conditions

Column	ChromSep SS 100x2.0 mm with guard column ChromSep OmniSpher 3 C18 (Varian Part No. CP27839)
Solvent A	0.1% acetic acid : 5 mM ammonium acetate in water (v/v)
Solvent B	Methanol

LC Program	Time (min:sec)	%A	%B
	0:00	70	30
	0:30	70	30
	1:00	50	50
	16:00	30	70
	17:00	30	70
	17:06	70	30
	22:00	70	30

Flow	0.25 mL/min
Mixer	250 μ L
Injection Volume	10 μ L
Injection Solvent	20% solvent A / 80% solvent B

MS Parameters

Ionization Mode	APCI negative
Collision Gas	1.5 mTorr Argon
Housing	50 °C
API Drying Gas	12 psi at 150 °C
API Nebulizing Gas	58 psi at 400 °C
Auxiliary Gas	17 psi
Scan Time	1 - 1.7s
SIM Width	0.7 amu
Corona current	5 µA
Shield	600 V
Capillary	Tuned Values
Detector	1500 V

Scan parameters

No.	RT (min)	Corticosteroid	Capillary (V)	Precursor Ion	Product Ion	CE (V)
1	5.5	Triamcinolone	-30	453.2	345 363	23 12
2	7.3	Prednisone	-30	417.2	327 357	18 8
3	7.6	Cortisone	-25	419.2	329	16
4	8.7	Prednisolone	-20	419.2	329 295	16 36.5
5	8.7	Hydrocortisone	-25	421.2	331	19
6	10.4	Flumethasone	-40	469.2	379 305	19 41
7	10.8	Betamethasone + Dexamethasone	-40	451.2	361 307	19 33
8	11.1	Triamcinolone acetonide	-25	493.2	375 413	14 22
9	11.1	Fludrocortisone acetate	-35	481.2	349 341	25.5 21
10	11.2	Metilprednisolone	-25	433.2	343 309	17.5 37
11	11.3	Beclomethasone	-55	467.2	377 341	14 21.5
12	11.8	Flunisolide	-55	493.2	375 357	14 21
13	12.0	Fluorometolone	-40	435.2	59 355	12 16
14	12.1	Flurandrenolide	-35	495.2	377 359	14 20
ISTD	12.6	Desoximetasone	-10	435.2	355	16
15	15.5	Fluocinolone acetonide acetate	-55	553.2	375 355	18 24
16	15.6	Budesonide	-50	489.2	357 339	13.5 19
17	17.9	Fluticasone propionate	-50	559.2	413 433	22.5 15.5

The scan method is divided in 3 segments of acquisition:

- 0 - 9.5 min 7 transitions Scan time 1s
- 9.5 - 15 min 17 transitions Scan time 1.7s
- 15 - 20 min 6 transitions Scan time 1s

Results and Discussion

In order to develop the MS parameters, 10 ppm solutions of each corticosteroid were prepared in a 20% buffer A / 80% methanol mixture. The mixture was meant to mimic the mobile phase that would elute with the compound of interest in an actual LC/MS analysis. The 10 ppm solutions were directly injected in the APCI with a syringe pump at a 50 µL/min rate. First, the most appropriate precursor ion was selected from the parent scan, and the capillary voltage was optimized for its highest abundance. Second, the product ions were selected and the collision energies optimized by the MS/MS breakdown automatic procedure.

Two MS/MS product ions, instead of one, are used to monitor each corticosteroid in order to better eliminate the false positives. Only cortisone and hydrocortisone, which are endogenous corticosteroids, are monitored with one ion. Three ions were not used in order not to increase the scan time. The confirmation of the positive sample can be done by a confirmation method specific for the suspected corticosteroid monitoring at least three of its MS/MS transitions.

Particular care was taken to separate the prednisolone from cortisone. The two corticosteroids have the same molecular masses, and cortisone gives an abundant peak on the transition (-) 419.2>329 of prednisolone. The triamcinolone acetonide-flunisolide and fluorometholone-desoximethasone pairs also share their transitions, but are separated by their retention times. The epimers betamethasone and dexamethasone have similar retention times and mass spectra, and they could not be separated in the LC/MS conditions described.

Figure 1 (page 3) shows the LC/MS analysis of a blank urine sample spiked with 20 ng/mL of each corticosteroid (except cortisone and hydrocortisone, which are endogenous) and 200 ng/mL internal standard. There are no matrix interferences, and the abundances and signal/noise ratios are satisfactory for all compounds of interest even with a standard gain of the detector. In order to increase the reliability of the result, the confirmation of a positive sample can be done with the detector set on high gain.

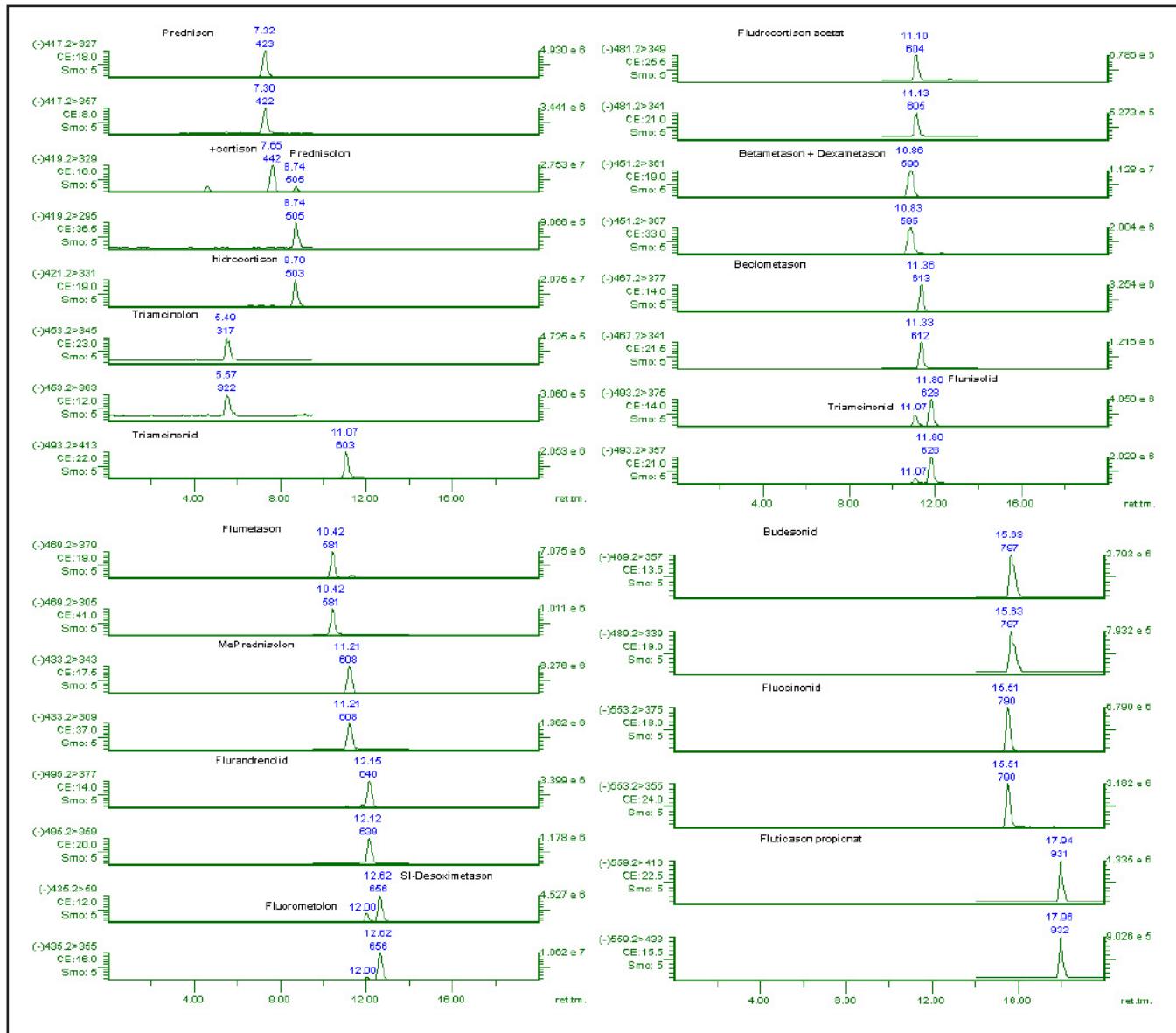


Figure 1. Analysis of a blank urine sample spiked with 20 ng/mL corticosteroids

Conclusion

The LC/MS/MS method described in this application note is simple and sensitive. In one run this method can screen for 17 corticosteroids and easily detect them below the WADA's MRPL, 30 ng/mL. The Varian 1200L system proved to be an essential tool for a doping control laboratory.

References

1. Hardmann, J.G.; Limbird, E.J., *The Pharmacological Basis of Therapeutics* (9th edn). New York, 1996
2. Polettini, A.; Marrubini Boulard, G.; Montagna, M.J.; *J. Chromatogr. B* 1998; 713:339
3. Cummiskey, J.; *Glucocorticosteroids in Doping in Sport, Concerted Action in the Fight Against Doping in Sport (CAF-DIS)*, Dublin, 2002.
4. 2005 WADA Prohibited List
5. Deventer, K.; Delbeke, F.T., *Rapid Commun. Mass Spectrom.* 2003; 17:2107-2114

These data represent typical results.

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