INVESTIGATION OF CORTICOSTEROIDS PROFILES IN BOVINE URINE PART A: DEVELOPMENT OF A METHOD FOR CORTISOL, PREDNISOLONE AND THEIR METABOLITES DETERMINATION

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Introduction

Prednisolone is a synthetic corticosteroid with anti-inflammatory and gluconeogenetic activities. Corticosteroids are used for the treatment of inflammatory diseases: for this reason, Commission Regulation 2010/37/EC established maximum residue levels (MRLs) for corticosteroids in liver and muscle; in urine no MRLs have been set therefore their presence at any concentration is not allowed.

In recent years the prednisolone was frequently observed at residue level (concentrations < 5 μg/l) in bovine urine, especially in samples collected at the slaughterhouse, but also in farming; in all cases, no therapeutic use was declared in advance by farm veterinarians. Recent studies have confirmed the hypothesis that prednisolone can be formed in vivo by endogenous cortisol in animals subjected to conditions of acute (e.g.: transport and slaughter), or chronic (e.g.: chronic diseases, inflammatory, ill-treatment) stress. In 2012 the Italian Ministry of Health has set at 5 μg/l the limit value for not compliant results; this provision should be temporary, pending on the identification of biomarkers reliable of exogenous origin of prednisolone. Thus, the interest for pattern recognition of corticosteroid metabolites profiles has been increased; the study of metabolites could be a promising approach that can be used to detect the abuse of these substances, in order to distinguish treated from non-treated animals.

The aim of this study was the development of a accurate and robust LC-MS/MS procedure for simultaneous quantitative determination of 22 molecules (Table 1) correlated to prednisolone and cortisol metabolism in bovine urine; this method will be used to map the metabolites profile and investigate the alteration of endogenous pattern in case of stress or pharmacological treatment and therefore to obtain a powerful instrument to detect illicit operations.

Standards

I.D.	ANALYTE	I.D.	ANALYTE	I.D.	ANALYTE
1	cortisol	2	cortisone	3	prednisolone
5	6β-hydroxycortisol	6	6β-hydroxycortisone	4	prednisone
9	5β-dhydrocortisol	17	5β-dihydroocortisone	7	6β-hydroxyprednisolone
10	3α, 5α-tetrahydrocortisol (allo-tetrahydrocortisol)	18	3α, 5α-tetrahydrocortisone (allo-tetrahydrocortisone)	26	20(S)-dihydroprednisolone (20α-hydroxyprednisolone)
11	3α, 5β-tetrahydrocortisol	19	3α, 5β-tetrahydrocortisone	27	20(R)-dihydroprednisolone (20β-hydroxyprednisolone)
12	20(R)-dihydrocortisol (20β-hydroxycortisol)	20	20(S)-dihydrocortisone (20α-hydroxycortisone)	33	20β-hydroxyprednisone
13	20(S)-dihydrocortisol (20α-hydroxycortisol)	21	20(R)-dihydrocortisone (20β-hydroxycortisone)	38	prednisolone-d6
22	α-cortolone			40	cortisone-d2
23	β-cortolone			49	6β-hydroxycortisol-d4

Table 1: 22 molecules + I.S.

Sample Preparation

- **Urine sample (5 mL) + 100 µL of a 100 ng/mL I.S.**
- **+ 10 ml of 0.15 M ammonium acetate buffer (pH 4.8)**
- **Enzymatic hydrolysis (2 h at 50°C) with 50 µL of Helix Pomatia glucuronidasi/arylsulfatase**
- **SPE cartridge (60 mg / 3 mL - Oasis HLB Waters)**
- **evaporation to dryness under a stream of nitrogen at 40°C**
- **Residue dissolution with 400 µL of a 50:50 (v/v) methanol-water**
- **Defatting with 2 x 1 mL of petroleum ether (40-60°C)**

LC-MS/MS Conditions

LC-MS/MS analysis was carried out by an HPLC Accela TM system coupled to a Triple quadrupole mass spectrometer TSQ Vantage EMR equipped with a H-ESI II operating in negative multiple reaction monitoring (MRM) mode. Chromatographic separation was performed on a Waters Xbridge BEH Phenyl® (150 x 3.0 mm i.d., 2,5 µm) analytical column equipped with a guard column. The LC eluents were: acetonitrile (A) and acetic acid 0,1% (B). The injection volume was 20 µL. The separation was obtained using a flow rate of 0.2 ml min^{-1}, during an overall run time of 40 min, with the gradient program reported in table 2. Column and tray temperatures were respectively set at 40°C and 15°C. The electrospray ionization was used to obtain the precursor ions [M+CH3COO]- and at least three product ions were monitored for each analyte.

TIME	A%	B%
0,0	10	90
24,0	53	47
24,5	70	30
25,0	80	20
30,0	80	20
35,0	10	90
40,0	10	90

Table 2: gradient program

Results and Discussion

This method showed to fit for the identification and quantification of corticosteroids cortisol, cortisone, prednisolone, prednisone and their major metabolites in bovine urine (Figure 1); therefore, it could be a potential way to solve the question about the endogenous vs. exogenous origin of prednisolone in positive animals. Further studies are needed to investigate with the developed method, using multivariate statistics, the alteration of endogenous pattern in case of stress or pharmacological treatment. The presence of certain metabolites or the ratio between different metabolites in urine could be a useful tool to discriminate between endogenous and administered prednisolone. This work is in progress.

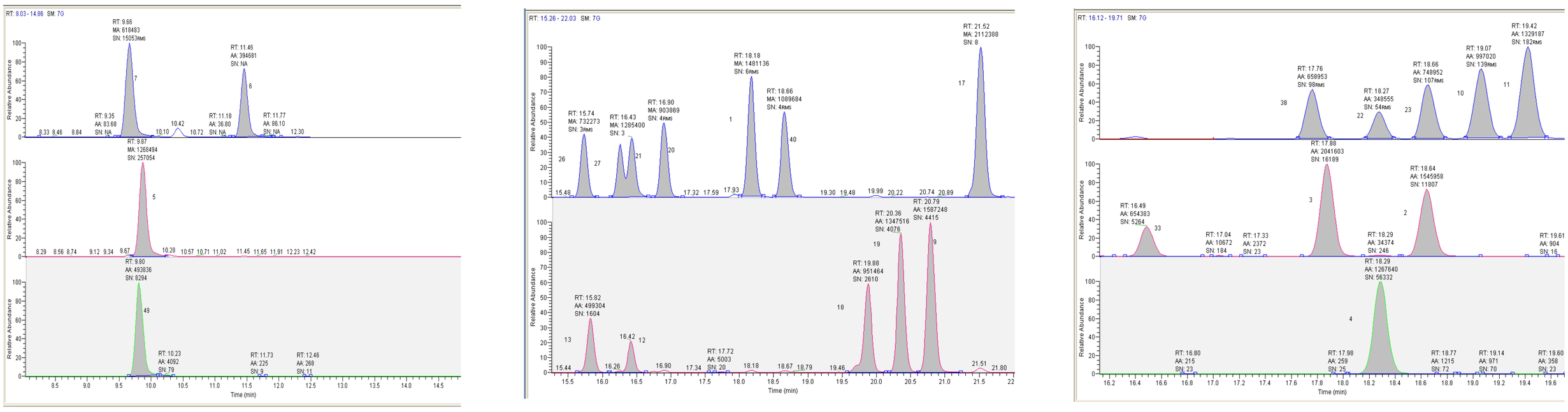

Figure 1. LC-MS/MS chromatograms of a urine spiked at 2 μg/l with all the analytes and ISTDs