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Screening for Corticosteroids in Urine with on-line Extraction and LC-MSMS Detection

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Introduction

A new method has been developed for routine analysis of corticosteroids in urine. This assay for corticosteroids and their metabolites is performed by on-line solid phase extraction, liquid chromatography separation and tandem mass spectrometry detection, so called XLC-MSMS technology – automated from sample list to MSMS results.

The glucocorticosteroids are on the IOC/WADA Prohibited List[1] and the doping control laboratories are obliged to analyse them in the samples taken during competition. They are prohibited when administered orally, rectally, intravenously or intramuscularly. Their use requires a Therapeutic Use Exemption approval. There is a list of glucocorticosteroids to be detected by IOC/WADA accredited laboratories: beclomethasone, betamethasone, budesonide, desonide, dexamethasone, fludrocortisone, flumethasone, flunisolide, fluticasone, fluocortolone, methylprednisolone, prednisolone, prednisone, triamcinolone.

There are several methods available to analyse this group of compounds using different techniques and instrumentation which have been published[4-5] and presented[6-9] during annual meetings in Cologne. Gas chromatography mass spectrometry has never become a good method for analysing corticosteroids since it requires derivatisation which is both time consuming and occasionally incomplete for these compounds[7]. To solve this problem liquid chromatography mass spectrometry LC-MS as well as LC-MSMS technique[4-6, 8-9] have been employed.

In this paper we present our way of dealing with the problem of analysing corticosteroids using the LC-MSMS technique with the implemented on-line solid phase extraction procedure to diminish the workload in the laboratory. At this stage the method is designed for screening of glucocorticosteroids only.

Experimental

20 µL of urine sample is extracted by solid phase extraction (SPE) using the Symbiosis Pharma instrument from Spark Holland interfaced to the Finnigan TSQ Quantum Discovery tandem mass spectrometer.

Confirmation of corticosteroids can be performed with the same extraction and chromatography system utilizing a modified mass spectrometry method to register at least two ion transitions for each compound (Tab. 1).

Sample pre-treatment

Urine samples are centrifuged after adding 50 ng/mL of methyltestosterone and D_4-cortisol (internal standards) and transferred to vials, which are placed in a cooled chamber of the automatic injector, a so called Reliance of Symbiosis Pharma instrument. For screening purposes an unhydrolyzed urine fraction is used. If necessary (e.g. confirmation of budesonide) the sample is hydrolyzed with β-glucuronidase from E.Coli at pH 7 using our standard procedure for androgenic anabolic steroids. After hydrolysis and centrifugation, the sample is applied directly to XLC-MSMS on-line solid phase extraction.

Symbiosis Pharma Spark Holland on – line extraction

20 µL of urine sample is injected in partial loopfill mode. Total sample consumption for one injection is 65 µL.

The cartridge, which is used for corticosteroid extraction is a Sorbent HySphere C2 8 µm silica based ethyl phase.

Sample Extraction Procedure

- Conditioning of Cartridge
    - Solvation 1 mL of methanol
    - Equilibration 1 mL of water
- Sample loading 0.5 mL of water
- Cartridge washing
    - 1 mL of 5% methanol
    - 1 mL of water
- Analyte Elution Mobile phase A/B (70:30)
    - Elution time 6 min.

As soon as the cartridge is eluted the next sample is prepared. As long as the SPE time is shorter than the time required for LC-MSMS, the total process time is determined by the LC-MSMS time only.

Chromatographic conditions

Column Phenomenex Luna 3u C18(2), 100 x 2.0 mm

Mobile phase: A: Water / Acetonitril (95:5), 1% Acetic Acid

B: Acetonotril / Water (95:5), 1% Acetic Acid

Flow: 0.3 mL/min.

Gradient: A/B (70:30) 1 min., A/B (70:30) to A/B (35:65) 9 min.,

A/B (35:65) 1 min., A/B (35:65) to A/B (70:30) 1 min., A/B (70:30) 4 min.

Mass spectrometer

Finnigan TSQ Quantum Discovery tandem mass spectrometer with electrospray ionization ESI ion source is operated in the positive ionization mode with SRM monitoring and 0.20s dwell time. All compounds produce $[M+H]^+$ ions as listed in Table 1. ESI voltage used is 4800V, capillary temperature 350^{o}C and collision gas pressure 1.5 mTorr.

Results and discussion

The presented XLC-MSMS on-line solid phase extraction method is used in our laboratory for screening and confirmation of glucocorticosteroids (Tab.1, Fig.1).

The method has been accredited by the Swedish Accreditation Body (SWEDAC) according to the standard ISO 17025.

Table 1 – Screening and confirmation of glucocorticosteroids, mass spectrometry parameters, retention time (RT) and limit of detection (LOD)*

Compound	**Precursor ion**	**Product ion screening (confirmation)**	**Collision Energy**	**RT min**	**LOD ng/mL**
Beclomethasone	409	279 (279, 147, 171)	21	6.66	0.8
Betamethasone	393	279 (279, 147, 171)	20	6.18	0.2
Budesonide	431	147 (147, 173, 225)	41	8.88	0.9
Desonide	417	225 (147, 173, 225)	28	6.93	0.5

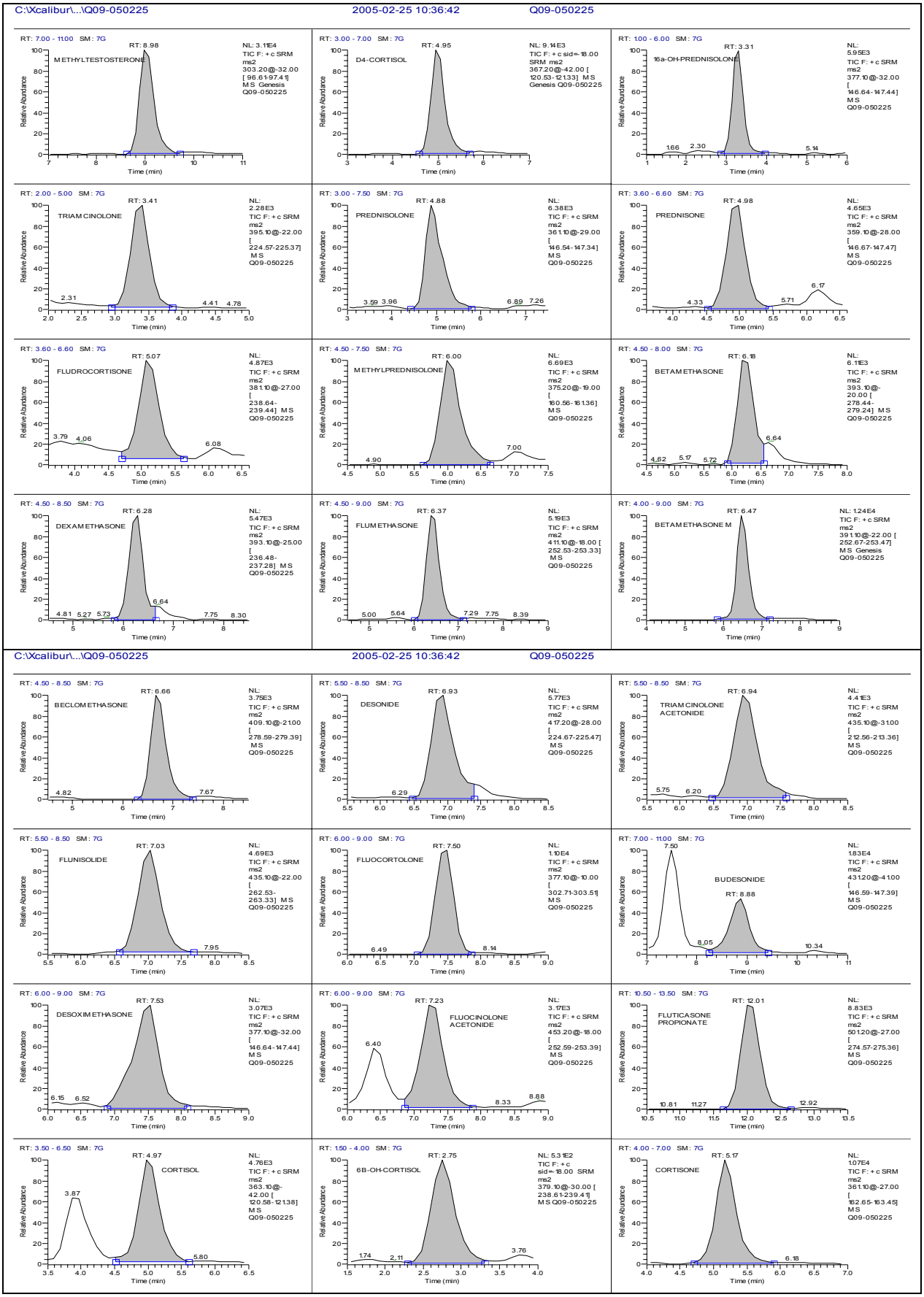
Compound	Precursor ion	Product ion screening (confirmation)	Collision Energy	RT min	LOD ng/mL
Dexamethasone	393	237 (237, 147, 279)	25	6.28	0.6
Fludrocortisone	381	239 (239, 91, 128)	27	5.07	1.5
Flumethasone	411	253 (253,121, 235)	18	6.37	1.5
Flunisolide	435	263 (171, 223, 263)	22	7.03	0.8
Fluocortolone	377	303 (303, 171, 121)	10	7.50	0.2
Methylprednisolone	375	161 (161, 185, 253)	19	6.00	2.8
Prednisolone	361	147 (147, 171, 121)	29	4.88	1.3
Prednisone	359	147 (147, 171, 237)	28	4.98	1.6
Triamcinolone	395	225 (225, 237, 147)	22	3.41	1.6
Triamcinolone acetonide	435	213 (213, 225, 275)	31	6.94	1.4
Fluocinolone acetonide	453	253 (253, 121, 279)	18	7.23	0.5
16α-OH-Prednisolone	377	147 (147, 173, 226)	32	3.31	1.9
Betamethasone M**	391	253 (253, 147, 237)	22	6.47	1.3
Desoximethasone	377	147 (171,147,128,91)	32	7.53	2.0
Fluticasone propionate ***	501	275 (275, 293, 205)	18	12.01	0.2
Cortisone	361	163 (163, 105, 121)	27	5.17	Endogenous
Cortisol	363	121 (121, 91, 145)	42	4.97	Endogenous
6β-OH-Cortisol	379	239	30	2.75	Endogenous
D4-Cortisol ISTD	367	121	42	4.95	Internal standard
Methyltestosterone ISTD	303	97	32	8.98	Internal standard

*LOD is appraised at a signal to noise ratio of 3:1 for all substances

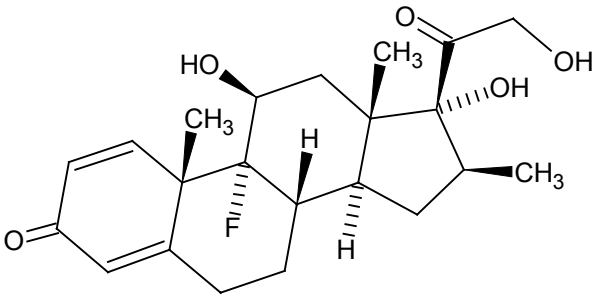
** Betamethasone M – (16β)-9-Fluoro-17,21-dihydroxy-16-methylpregna-1,4-diene-3,11,20-trione.

*** Fluticasone propionate is used as indicator of properly working chromatography.

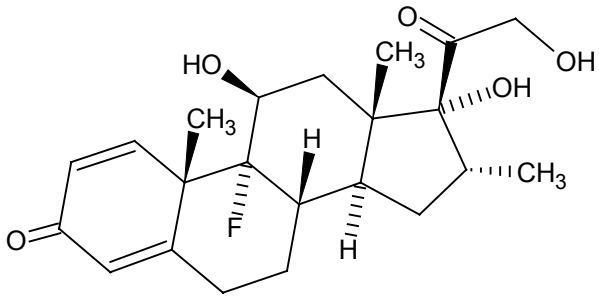
Fig.1 - Screening of glucocorticosteroids



When applying this method we use a negative control urine and two quality control samples (QC) which are analysed together with every batch of samples. The negative control urine samples are spiked with 30ng/mL of different corticosteroids. Two different QC samples are used to avoid coelution of compounds in each of them, for example betamethasone and dexamethasone are in different QC samples. Although bethamethasone and dexamethasone are only slightly separated in our screening method, they could be properly recognized. The fragment ions obtained from both stereoisomers are similar but their relative abundances are different, (Tab.1 and Tab.2). In a situation were both of them are present in the same sample, the chromatographic gradient is changed to allow separation. However, if only one of the compounds is present it is possible to prove its presence using difference in MSMS fragmentation (see Table 2).



Betamethasone
MW = 392



Dexamethasone
MW = 392

Table 2 - Sample positive for betamethasone

Betamethasone Method	Betamethasone		Dexamethasone	
$[M+H]^+$ 393	RT min.	Relative Abundance (% of base peak)	RT min.	Relative Abundance (% of base peak)
147	6.18	100	6.28	100
279		**93***		**48****
171		62		52

*See difference in relative abundance for transition m/z 393 - 279 which is 93% if betamethasone is present.

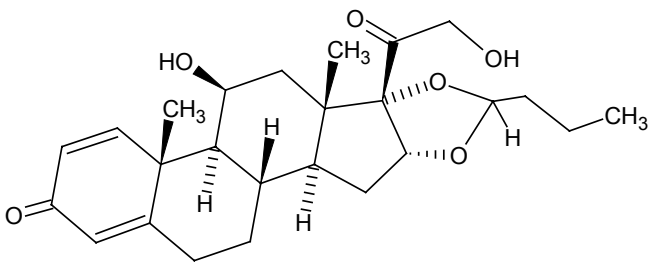
**The relative abundance is 48% for m/z 393 - 279 in this case because there is dexamethasone in the sample. To show this difference both betamethasone and dexamethasone were analysed with the confirmation method for betamethasone. For fragment ions used in respective methods (see Table 1).

Fluticasone can not be found using our screening method. Fluticasone is poorly absorbed and undergoes extensive first-pass metabolism. The only known metabolite in humans is a 17β-carboxylic acid metabolite. Less than 5% of a dose is excreted in urine as the metabolite with remainder being excreted in faeces as the parent drug (up to 75%) and the metabolite[2]. We included fluticasone propionate in our method (as the only available standard at the time of validation of the method) and decided to keep it as it appeared to be good indicator of properly working chromatography and SPE extraction as it elutes late in our system.

Confirmation of budesonide

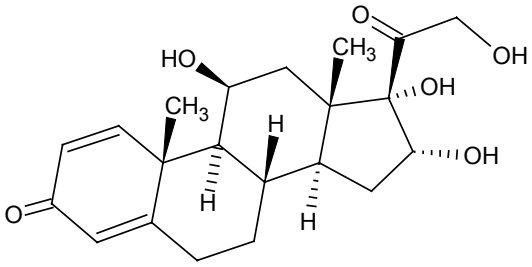
Budesonide is a mixture of two isomers, the content of the S-isomer in the mixture varies between 40 – 51% (Merck Index). It is available on the market as bidien, budeson, cortivent, micronyl, preferid, pulmicort, rhinocort, spirocort, symbicort. The metabolism of budesonide is well known and published[3].

For screening purposes 16α-OH-prednisolone, which is a metabolite of budesonide is used[3]. It was observed in the unconjugated fraction (Fig. 2). Small amounts of budesonide could be found in conjugated fraction (Fig. 3).



Budesonide

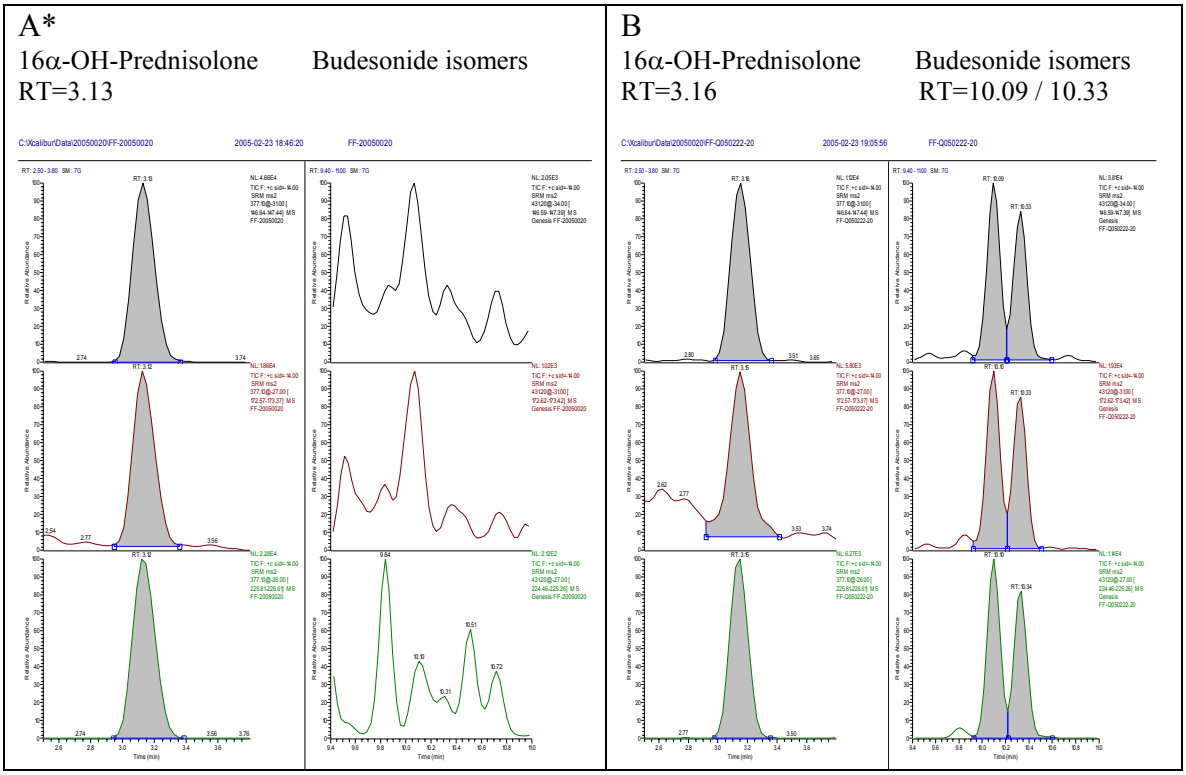
MW = 430



16α-OH-Prednisolone

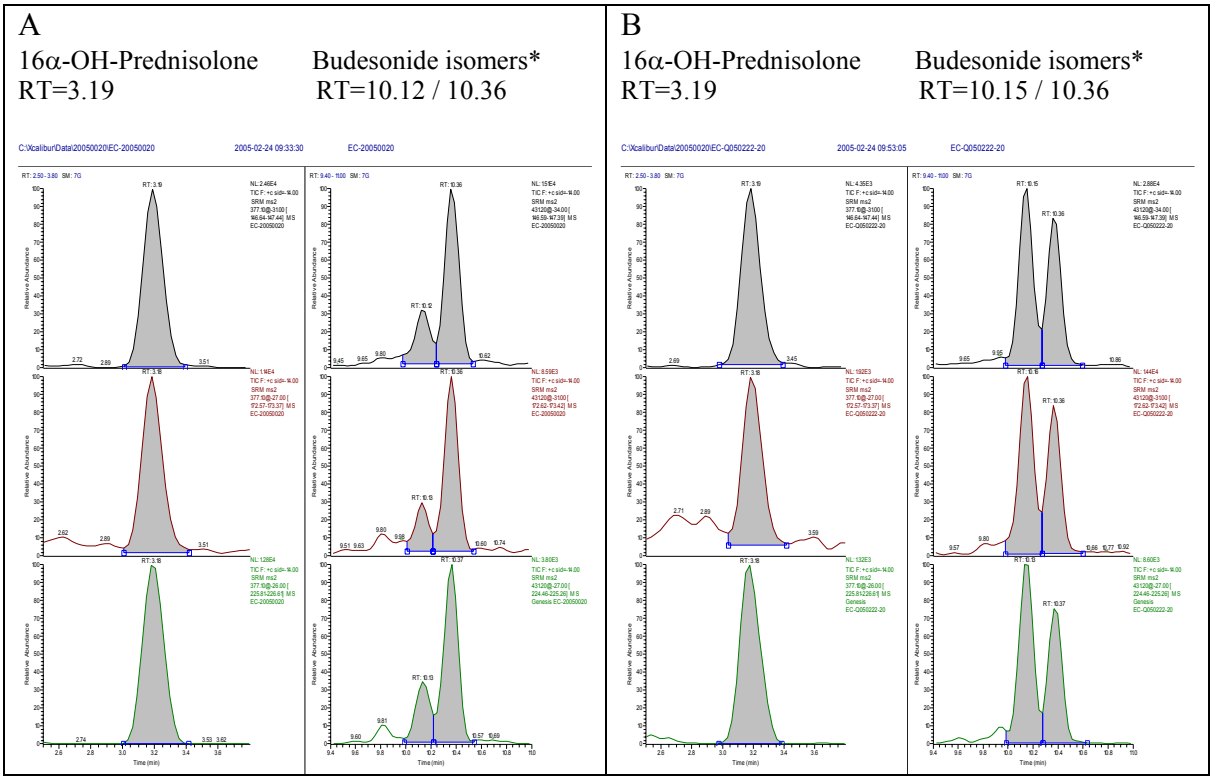
MW = 376

Fig. 2 - Unconjugated fraction: sample, (A), quality control at MRPL level 30 ng/mL, (B)



*Only 16α-OH-prednisolone is found in unconjugated fraction

Fig. 3 - Conjugated fraction after E.Coli hydrolysis: sample, (A), quality control at MRPL level 30 ng/mL, (B)



*Different composition of budesonide isomers found in the sample (A) than that from spiked urine (B)

Conclusions

- Screening method for Corticosteroids and their metabolites in urine based on XLC-ESI-MSMS was developed;
- Conjugated fraction after hydrolysis with β-glucuronidase from E.Coli (or Helix Pomatia) could be analysed with the same XLC-ESI-MSMS system;
- The method can be extended for more compounds e. g. new corticosteroids, unconjugated steroids, …;
- Confirmation of corticosteroids is performed with the same XLC-system and ESI-MSMS methods modified for respective compounds;
- All compounds analysed produce at least two transitions, which is sufficient for confirmation purposes;
- If coeluting compounds are present in the sample (e.g. betamethasone and dexamethasone) the LC gradient is modified.
- There is a drawback in the on-line extraction system in comparison to the off-line extraction. The limiting factor is the elution of the compounds from the cartridge, which is done with mobile phase.

References

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