Application Note: 496

Screening in Equine Doping Control Analysis with Ultrahigh Resolution and Accurate Mass

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Key Words

- Exactive
- LC/MS
- Orbitrap Technology
- Forensic Toxicology
- ToxID Software

Introduction

Triple quadrupole or tandem mass analyzers have been used most frequently in the accurate identification, confirmation, and quantitation of prohibited compounds in a single analysis. In addition, ion trap and quadrupole time-of-flight mass analyzers have been useful for screening and confirming results. However, these technologies cannot address the main requirements of equine doping control analysis such as:

- Data re-interrogation
- Analyze and monitor a vast number of compounds
- Fast and easy method development, instrument operation, and data interpretation
- Efficient separation of analytes from interferences present in the matrix
- Highly confident identification of compounds

Here we present a screening approach that uses ultrahigh resolution (R = 50,000) and accurate mass in positive and negative mode for the screening of illicit substances in urine matrix using the Thermo Scientific Exactive benchtop mass spectrometer. More than 120 analytes are screened using this method. Confirmation is made using the exact mass of the analytes in positive and negative mode (if available) and the retention time.

Goal

To demonstrate a new approach using ultrahigh resolution (> 50,000) and accurate mass for the screening of illicit substances in a urine matrix using the Exactive™ mass spectrometer, a new high performance benchtop LC/MS instrument equipped with Thermo Scientific Orbitrap technology, for doping control analysis.

Figure 1. Thermo Scientific Exactive high performance benchtop LC/MS system

Experimental

Sample preparation

Solid phase extraction (SPE) was used for sample pre-treatment and clean up. The details of the procedure are described below.

- To 5 mL of urine add 25 µL of hydrocortisone d3 at 10 µg/mL
- Add 1 mL of phosphate buffer
- Add 50 µL of β glucuronidase and 50 µL of protease
- Incubate for 1 hour at 55 °C
- Centrifuge at 4,000 rpm for 30 minutes
- Transfer the supernatant to a tube
- Add 5 mL of water
- Condition the C18-HF cartridge with 3 mL of methanol and 3 mL of water
- Load the sample and wash the cartridge with 3 mL of water and 3 mL of hexane
- Elute with 3 mL of a mixture containing dichloromethane and ethanol
- Evaporate to dryness
- Reconstitute with 100 µL of a mixture containing water and acetonitrile (80/20)

Instrumentation Method

HPLC conditions

Chromatographic analyses were performed using Shimadzu binary pumps LC-20ADxr (Champs sur Marne, France). The chromatographic conditions were as follows:

Column:	Reversed-phase, silica-based C18 (3.5 µm, 150 x 2.1 mm) column
Flow rate:	0.3 mL/min
Injection volume:	10 µL
Mobile phase:	A: Water containing 0.1% formic acid
	B: Acetonitrile containing 0.1% formic acid

Gradient:

T(min)	A(%)	B(%)
0.0	80	20
5.0	80	20
20.0	50	50
25.0	0	100
25.2	80	20
30.0	80	20

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Mass Spectrometry conditions

MS analysis was carried out on an Exactive benchtop mass spectrometer with an electrospray ionization (ESI) source (Figure 1). The MS conditions were as follows:

Ion Polarity:	Polarity switching scan dependent experiment
Spray Voltage:	4500 V in positive mode and –3900 V in negative mode
Sheath gas pressure (N_2):	45 (arbitrary units)
Auxiliary gas pressure (N_2):	3 (arbitrary units)
Capillary temperature:	300 °C
Resolution:	50,000 (FWHM)
AGC Target Value	500,000

Results and Discussion

The screening method was set up for the identification and confirmation of more than 100 compounds, including anabolic agents, steroids, anesthetics, anti-inflammatory agents, and diuretics, as listed in Table 1

Acquisition was performed using the full MS scan mode with polarity switching and external calibration. All data were reprocessed using 5 ppm mass accuracy. Figure 2 shows the sensitivity obtained for a urine sample spiked with 4 compounds: dexamethasone, flumethasone, triamcinolone acetonide, and triamcinolone. The injected concentrations were 50 pg/mL for dexamethasone and flumethasone and 1 ng/ml for triamcinolone and triamcinolone acetonide. In the positive mode, the analytes were identified as protonated species and in the negative mode, as formate adducts. As data acquired was in full scan MS mode, re-interrogation of the data file, particularly for non-targeted or unknown compounds or metabolites, is easily made possible.

Thousands of real urine samples have been analyzed using this approach. Figure 3 shows an example of a real sample that has been analyzed using this method.

All data have been processed using Thermo Scientific ToxID software. ToxID™ software for Exactive processes data using the mass accuracy and retention time of the analytes. An example of the automatically generated report can be seen in Figure 4.

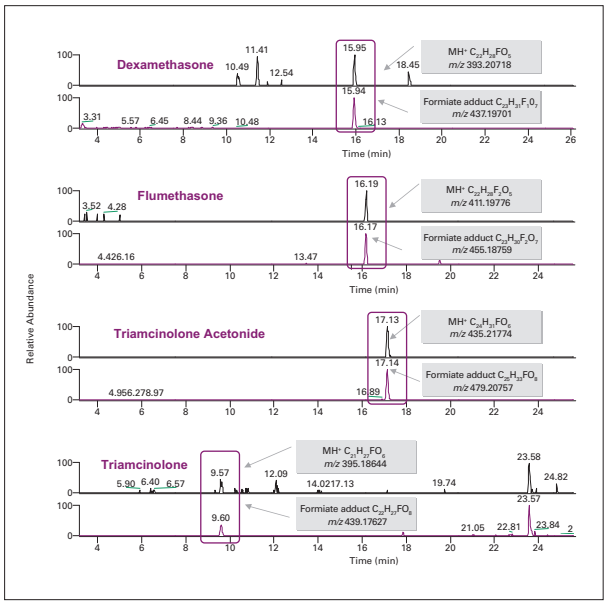


Figure 2: Extracted ion chromatograms for dexamethasone, flumethasone, triamcinolone acetonide, and triamcinolone in the positive and negative modes using 5 ppm mass accuracy

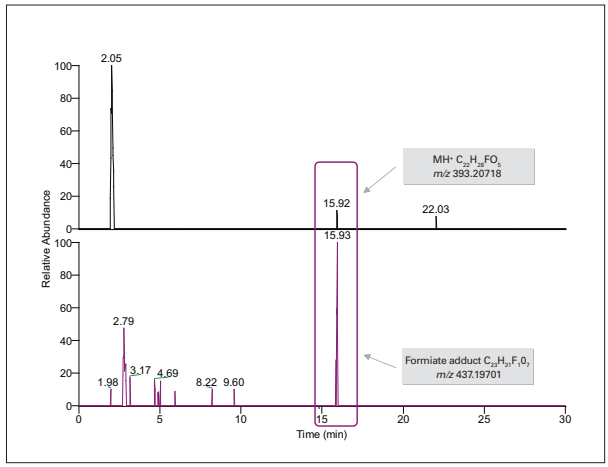
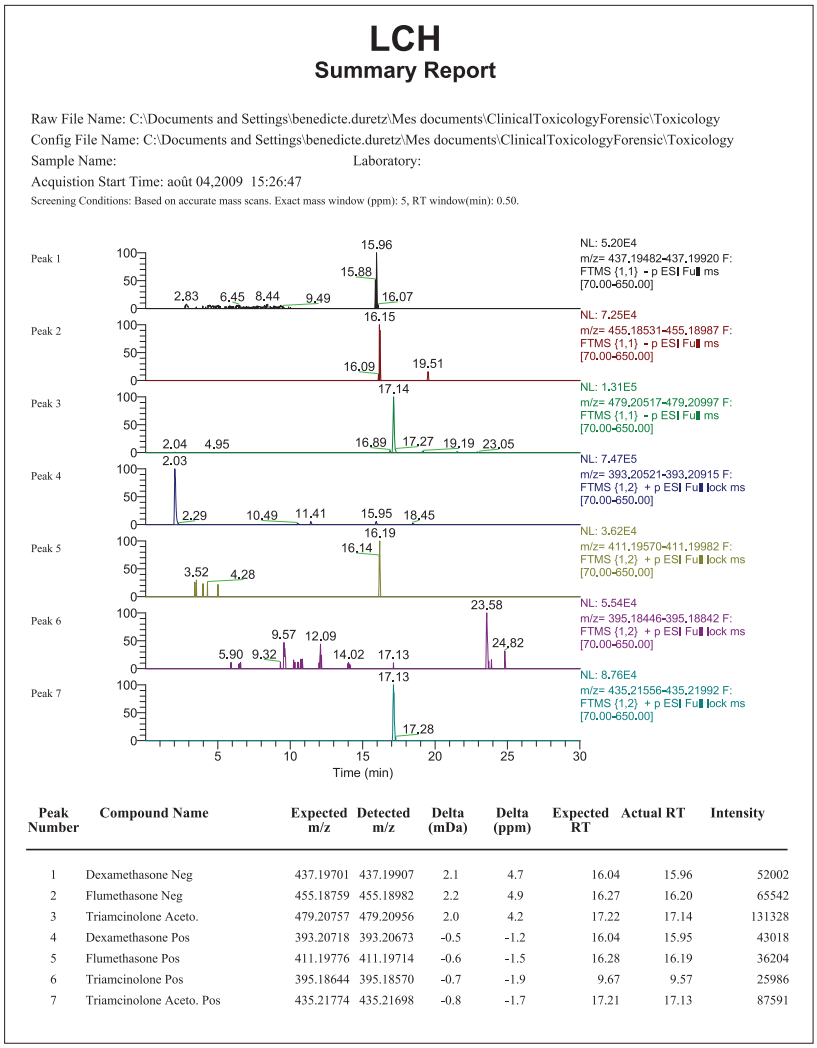


Figure 3: Dexamethasone identified in a real sample in positive and negative mode

Table 1: List of compounds monitored in the screening.

Index	Compounds	Index	Compounds	Index	Compounds
1	20 Beta dihydrocortisol	42	Diazoxide	83	Naftidrofuryl
2	4 Methylamino antypirine	43	Dichlorisone	84	Niketamide
3	5' Hydroxy Omeprazole	44	Diphenydramine	85	Nimesulide
4	Acepromazine	45	Diphylline	86	Nordazepam
5	Acide ethacrynic	46	Etamiphylline	87	Omeprazole
6	Althiazide	47	Etophylline (Etofylline)	88	Oxazepam
7	Ambroxol	48	Fenspiride	89	Oxyphenbutazone
8	Amcinonide	49	Fludrocortisone	90	Paramethasone
9	Amitryptylline	50	Flufenamic acid	91	Pentoxyphylline
10	Antipyrine (phenazone)	51	Flumethasone	92	Petidine (meperidine)
11	Beclomethasone	52	Flunisolid	93	Phenobarbital
12	Bendroflumethiazide	53	Flunixin	94	Phenylbutazone
13	Benzocaine	54	Fluocinolone acetonide	95	Phenytoin
14	Benzoylecgonine	55	Fluocinonide	96	Piroxicam
15	Benzydamine	56	Fluorometholone	97	Prednisolone
16	Betamethasone	57	Fluoroprednisolone	98	Prednisone
17	Budesonide	58	Flurandrenolide	99	Probenicid
18	Buflomedil	59	Fluticasone propionate	100	Procaine
19	Bumetanide	60	Furosemide	101	Prolintane
20	Bupivacaine	61	Guaifenesin	102	Promazine
21	Butorphanol	62	Halcinonide	103	Pyrilamine
22	Caffeine	63	Hydrochlorothiazide	104	Ranitidine
23	Capsaicine	64	Hydroflumethiazide	105	Sildenafil
24	Carbetapentane	65	Hydroxy Lidocaine	106	Sildenafil hydroxy
25	Chlorothiazide	66	Hydroxy Meloxicam	107	Sulindac
26	Chlorpheniramine	67	Hydroxy Piroxicam	108	Tenoxicam
27	Chlorpromazine	68	Hydroxy Tenoxicam	109	Tetracaine
28	Chlorthalidone	69	OH-Triamcinolone Aceto.	110	Tetrahydrogestrinone
29	Cimetidine	70	Imipramine	111	Tetramisole
30	Clenbuterol	71	Indapamide	112	Theobromine
31	Clobetasol	72	Isoflupredone	113	Theophylline
32	Cortisol	73	Ketamine	114	Timolol
33	Cortisol d3	74	Ketoprofen	115	Tixocortol pivalate
34	Cortivazol	75	Ketorolac	116	Tramadol
35	Cyclothiazide	76	Lidocaine	117	Triamcinolone
36	Dantrolene	77	Meloxicam	118	Triamcinolone acetonide
37	Dantrolene hydroxy	78	Mepivacaine	119	Triamcinolone hexacetonide
38	Desonide	79	Meprednisone	120	Trichlormethiazide
39	Desoximethasone	80	Methyl phenidate	121	Tripelennamine
40	Dexamethasone	81	Metocarbamol	122	Xipamide
41	Diazepam	82	Morphine	123	Xylazine



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Summary Report

Raw File Name: C:\Documents and Settings\benedicte.duretz\Mes documents\ClinicalToxicologyForensic\Toxicology
Config File Name: C:\Documents and Settings\benedicte.duretz\Mes documents\ClinicalToxicologyForensic\Toxicology
Sample Name: Laboratory:
Acquistion Start Time: août 04,2009 15:26:47
Screening Conditions: Based on accurate mass scans. Exact mass window (ppm): 5, RT window(min): 0.50.

Peak Number	Compound Name	Expected m/z	Detected m/z	Delta (mDa)	Delta (ppm)	Expected RT	Actual RT	Intensity
1	Dexamethasone Neg	437.19701	437.19907	2.1	4.7	16.04	15.96	52002
2	Flumethasone Neg	455.18759	455.18982	2.2	4.9	16.27	16.20	65542
3	Triamcinolone Aceto.	479.20757	479.20956	2.0	4.2	17.22	17.14	131328
4	Dexamethasone Pos	393.20718	393.20673	-0.5	-1.2	16.04	15.95	43018
5	Flumethasone Pos	411.19776	411.19714	-0.6	-1.5	16.28	16.19	36204
6	Triamcinolone Pos	395.18644	395.18570	-0.7	-1.9	9.67	9.57	25986
7	Triamcinolone Aceto. Pos	435.21774	435.21698	-0.8	-1.7	17.21	17.13	87591

Figure 4: ToxID report – short summary style

Conclusion

The Exactive high performance LC/MS demonstrates high resolving power (up to 100,000) and precise mass accuracy for easy, routine analysis and data re-interrogation of urine samples for illicit substances in equine doping control analysis.

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