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Multi-residue extraction and analysis method for prohibited substances in animal urine

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Abstract

As well as analysing human urine for sports drug testing, the Australian Sport Drug Testing Laboratory (ASDTL) also analyses animal urine samples for the presence of stanozolol, nandrolone, boldenone and their metabolites and methandriol. ASDTL undertakes these analysis as part of a contract with the National Residue Survey (NRS) providing results of testing for Androgenic substances in bovine, ovine and equine urines. The NRS is a vital part of the Australian system for managing the risk of chemical and environmental contaminants in Australian food products. A summary of ASDTL findings over the last five years for the NRS program is reviewed as well as presenting a new method using liquid chromatography tandem mass spectrometry (LC/MS/MS) which can analyse all the required compounds in a single test.

Introduction

The existing procedure for detecting these substances relies on a combination of two extraction and two analysis techniques. Methandriol requires solvent extraction, derivatisation and GC/MS analysis while nandrolone, boldenone, stanozolol and metabolites are detected at detection limits using LC/MS/MS. Methandriol cannot be detected at the required levels using the current LC/MS/MS. The metabolites are excreted mainly as glucuronides except for equine urine where sulphates are also excreted. A dual hydrolysis procedure is used to detect both conjugates.
The study proposed using AB Sciex Q-Trap® 5500 to detect all substances in a single analysis at required confirmation levels. Methandriol has a required confirmation level of 0.005 mg/kg, while the other analytes must be determined to the lower confirmation limit of 0.001 mg/kg.

Experimental

Sample Extraction

Sample extraction is as follows: to 4 mL urine, 40 µL deuterated internal standard mix, 1.5 mL 0.2M phosphate buffer and 50 µL β-glucuronidase is added and hydrolysed for 2 hours at 50 °C. Following a return to room temperature, samples undergo solid phase extraction (SPE) with Agilent C18 HF cartridges previously conditioned with 5 mL methanol and 5 mL water. Columns are washed with 2 x 3 mL water and eluted with 2 x 2 mL methanol. Eluate is evaporated to dryness then acid hydrolysed with 1 mL tetrahydrofuran (THF) and 2 µL 4.0M sulfuric acid for 1 hour at 50 °C. 250 µL of sodium carbonate (2M) is added and the THF evaporated. The residue is dissolved in water and undergoes a second SPE extraction using 3M Empore C18 cartridges conditioned with 2 mL methanol and 2 mL water. Columns are washed with 1 mL water, 0.5 mL 25% methanol in water and 1 mL hexane then eluted with 2 x 1 mL 5% methanol/95% ethyl acetate. 50 µL of methyltestosterone internal standard is added to each eluate and evaporated to dryness. Samples are reconsituted in 250 µL methanol, filtered and transferred to instrument vials.

Sample Analysis - UPLC-MS/MS

The UPLC-MS/MS system employed was a Waters Acquity Ultra Performance LC coupled to a AB Sciex Q-Trap® 5500 equipped with a Waters Acquity UPLC BEH C18 (100 mm x 1 mm, 1.7 µm) column. Mobile phases were 0.2% formic acid in water (A) and 0.2% formic acid in 90% acetonitrile (B). Analysis was in positive ion mode.

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Results and Discussion

Methandriol in particular needs to be considered as the detection limits of diols by electrospray ionisation are generally poor. Methandriol was spiked to a set of known blank animal urines at 2, 4, 6 and 8 ng/mL. The analysis was performed seven times at each concentration. Within assay variation was determined by the replicate analysis of samples within a run while the between assay variation was the analysis of spiked samples on different days. The mean concentration, standard deviation and the coefficient of variation (CV%) were calculated for each set of spikes and reproducibility was determined. The method used for quantification is an internal standard process, using isotope dilution with a deuterated compound to compensates for recovery. The surrogate deuterated internal standard mix added to each sample prior to hydrolysis are D3-nandrolone, D3-boldenone and D3-stanozolol. Methyltestosterone is added to each sample after extraction and prior to injection as a volumetric internal standard and is used to work out recovery for the surrogate internal standards. Since D3-methandriol is not available, D3-nandrolone was used in calculation for Methandriol.
Under the EU Directive2002/657/EC – Reproducibility CV for concentration at 100 µg/kg is 23% and should be as low as possible for concentrations lower than 100 µg/kg. The EU points system requires 4 points for LC/MS/MS technique which is the combination of selecting the precursor ion and two product ions, along with the ratio of the product ions for identification confirmation.

		Methandriol 287.1>159				Methandriol 287.1>213			
		Between Assay Variation		Within Assay Variation		Between Assay Variation		Within Assay Variation	
	No. of analysis / replicates	Mean	CV%	Mean	CV%	Mean	CV%	Mean	CV%
Spk 2ng/mL	7	1.96	19.7	1.63	17.5	1.75	20.4	1.66	12.3
Spk 4ng/mL	7	3.93	14.2	3.92	10	4.04	11.9	4.37	12.8
Spk 6ng/mL	7	6.1	12.2	5.65	4.6	6.28	13.8	6.13	13.9
Spk 8ng/mL	7	7.97	6.3	8.3	6.8	7.84	5.7	8.33	10.9

Table 1. Validation data for methandriol

There are no Maximum Residue Limits (MRL) set for any of the compounds and any detection is considered a contravention. The NRS Level of Reporting (LOR) is 0.005 mg/L for methandriol but from the validation data collected it could be confirmed at 0.002 mg/L and an extra ion was confidently used in the confirmation for positive findings (269.1>213).

		Methandriol 269.1>213			
		Between Assay Variation		Within Assay Variation	
	No. of analysis / replicates	Mean	CV%	Mean	CV%
Spk 2ng/mL	7	2.27	17.6	1.8	18.1
Spk 4ng/mL	7	4.35	9.3	4.42	14.3
Spk 6ng/mL	7	6.02	9.9	5.83	9.4
Spk 8ng/mL	7	7.78	3.8	8.21	7

Table 2. Validation for an extra methandriol transition used for confirmation

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PERIOD	CATTLE		SHEEP		HORSE	
	No.of samples tested	No.of residues	No.of samples tested	No.of residues	No.of samples tested	No.of residues
2010 - 2011						
19-Nortestosterone (17-alpha)	318	4	321	64	10	1
19-Nortestosterone (17-beta)	318	0	321	0	10	2
Boldenone (17-alpha)	318	2	321	0	10	0
Boldenone (17-beta)	318	0	321	0	10	0
Methandriol	318	0	321	0	10	0
Stanozolol	318	0	321	0	10	0
Stanozolol (16-hydroxy)	318	0	321	0	10	0
2009 - 2010						
19-Nortestosterone (17-alpha)	320	2	315	49	10	0
19-Nortestosterone (17-beta)	320	0	315	0	10	0
Boldenone (17-alpha)	320	0	315	0	10	0
Boldenone (17-beta)	320	0	315	0	10	0
Methandriol	320	0	315	0	10	0
Stanozolol	320	0	315	0	10	0
Stanozolol (16-hydroxy)	320	0	315	0	10	0
2008 - 2009						
19-Nortestosterone (17-alpha)	320	6	315	56	10	0
19-Nortestosterone (17-beta)	320	0	315	0	10	0
Boldenone (17-alpha)	320	0	315	0	10	0
Boldenone (17-beta)	320	0	315	0	10	0
Methandriol	320	0	315	0	10	0
Stanozolol	320	0	315	0	10	0
Stanozolol (16-hydroxy)	320	0	315	0	10	0
2007 - 2008						
19-Nortestosterone (17-alpha)	321	7	170	16	10	0
19-Nortestosterone (17-beta)	321	0	170	0	10	0
Boldenone (17-alpha)	321	1	170	0	10	0
Boldenone (17-beta)	321	0	170	0	10	0
Methandriol	321	0	170	0	10	0
Stanozolol	321	0	170	0	10	0
Stanozolol (16-hydroxy)	321	0	170	0	10	0
2006 - 2007						
19-Nortestosterone (17-alpha)	321	3	156	9	10	2
19-Nortestosterone (17-beta)	321	0	156	0	10	3
Boldenone (17-alpha)	321	0	156	1	10	0
Boldenone (17-beta)	321	0	156	0	10	1
Methandriol	321	0	156	0	10	0
Stanozolol	321	0	156	0	10	0
Stanozolol (16-hydroxy)	321	0	156	0	10	0

Table 3. Summary results for androgenic substances in animal urine

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Conclusions

- Given the UPLC-MS/MS sensitivity, all required analytes can be detected in one analysis.
- Historically 19-nortestosterone (17α) is found more frequently in sheep urine and 19-nortestosterone (17β) in equine urine. At detected levels, residues are more likely from endogenous sex hormone production.
- A few occurrences of boldenone and/or its metabolites have been detected. Investigations revealed no evidence of boldenone-containing product use on properties, so the cause of residues was undetermined.
- NRS programs encourage good agricultural practices and confirm Australia's clean food producer status. Australia and EU testing for banned substances focus on anlaysing urine, faeces or hair for 'on farm' animals; bile, blood, eyes and liver for slaughtered animals, while US testing is done on the edible product (muscle, fat, liver or kidney).

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