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Rapid screening of anabolic steroids in horse urine with ultra-high-performance liquid chromatography/tandem mass spectrometry after chemical derivatisation

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ABSTRACT

Liquid chromatography/mass spectrometry (LC/MS) has been successfully applied to the detection of anabolic steroids in biological samples. However, the sensitive detection of saturated hydroxysteroids, such as androstanediols, by electrospray ionisation (ESI) is difficult because of their poor ability to ionise. In view of this, chemical derivatisation has been used to enhance the detection sensitivity of hydroxysteroids by LC/MS. This paper describes the development of a sensitive ultra-high-performance liquid chromatography/tandem mass spectrometry (UHPLC/MS/MS) method for the screening of anabolic steroids in horse urine by incorporating a chemical derivatisation step, using picolinic acid as the derivatisation reagent. The method involved solid-phase extraction (SPE) of both free and conjugated anabolic steroids in horse urine using a polymer-based SPE cartridge (Abs Elut Nexus). The conjugated steroids in the eluate were hydrolysed by methanolysis and the resulting extract was further cleaned up by liquid-liquid extraction. The resulting free steroids in the extract were derivatised with picolinic acid to form the corresponding picolinoyl esters and analysed by UHPLC/MS/MS in the positive ESI mode with selected-reaction-monitoring. Separation of the targeted steroids was performed on a C18 UHPLC column. The instrument turnaround time was 10.5 min inclusive of post-run equilibration. A total of thirty-three anabolic steroids (including 17β-estradiol, 5(10)-estrene-3β,17α-diol, 5α-estrane-3β,17α-diol, 17α-ethyl-5α-estran-3α,17β-diol, 17α-methyl-5α-androstan-3,17β-diols, androstanediols, nandrolone and testosterone) spiked in negative horse urine at the QC levels (ranging from 0.75 to 30 ng/mL) could be consistently detected. The intra-day and inter-day precisions (% RSD) for the peak area ratios were around 7–51% and around 1–72%, respectively. The intra-day and inter-day precisions (% RSD) for the relative retention times were both less than 1% for all analytes, except the inter-day precision for boldione at 1.2%. The extraction recoveries for all targets were not less than 48%. With exceptional separation achieved by the UHPLC system, matrix interferences were minimal at the expected retention times of the selected transitions. As detection was performed with an UHPLC system coupled to a fast-scanning triple quadrupole mass spectrometer, the method could easily be expanded to accommodate additional steroid targets. This method has been validated for recovery and precision, and could be used regularly for doping control testing of anabolic steroids in horse urine samples.

1. Introduction

According to the World Anti-Doping Agency (WADA) prohibited list [1] and Article 6 of the International Agreement on Breeding, Racing and Wagering published by the International Federation of Horseracing Authorities (IFHA) [2], anabolic steroids are prohibited substances in both human and equestrian sports. Gas chromatography/mass spectrometry (GC/MS) has been the gold standard for the detection of anabolic steroids in biological samples for many years [3–7], and still remains as a major technique for the doping control testing of anabolic steroids in many laboratories. In the past decade, there has been a shift in employing more and more liquid chromatography/mass spectrometry (LC/MS) methods for doping control testing in sports. This is mainly attributed to the rapid advancement of LC/MS in recent years, leading to better sensitivity, faster instrument turnaround time, and the ability to handle heat labile and large biomolecules. Although LC/MS has been applied successfully to doping control testing of many anabolic steroids in biological matrices [8–13], the technique does not provide adequate sensitivity for

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the detection of some steroids including important metabolites or markers for gender identification purpose. These problematic steroids are mainly saturated hydroxysteroids or oxosteroids, which exhibit low ionisation efficiency under electrospray ionisation (ESI). Chemical derivatisation has been reported to improve the ESI ionisation efficiency of saturated hydroxy and oxosteroids. For example, the introduction of a permanently charged moiety, such as 2-hydrazino-1-methylpyridine [14,15], into some oxosteroids was reported to enhance the LC/MS sensitivity by 70 to 1600-fold compared to the intact steroids. However, this approach is limited to mono-oxosteroids, because steroids possessing two or more carbonyl groups will form multiply-charged precursor ions. Similarly, the use of dansyl chloride to incorporate one or more dansyl moieties (with high proton-affinity) into some hydroxysteroids has shown a remarkable increase in the ionisation efficiency under ESI [16–18], but it was limited to only phenolic hydroxyl group. A different class of derivatisation reagents which have been shown to enhance the ESI ionisation efficiency for saturated hydroxysteroids was the pyridine-carboxylates [19]. Among the pyridine-carboxylate analogues studied, picolinic acid has been shown to give better results for some hydroxysteroids [20–24]. This paper describes an LC/MS screening method incorporating a picolinoyl derivatisation step, for the detection of over thirty anabolic steroids in horse urine. The method involved solid-phase extraction (SPE) of both free and conjugated anabolic steroids from horse urine using a polymer-based SPE cartridge (Abs Elut Nexus) [25]. The conjugated steroids (both glucuronide and sulfate conjugates) in the eluate were hydrolysed by methanolysis [26] and the resulting extract was further cleaned up by liquid–liquid extraction. The free steroids in the extract were derivatised with picolinic acid to form the corresponding picolinoyl esters, which were analysed by ultra-high-performance liquid chromatography/tandem mass spectrometry (UHPLC/MS/MS) in the positive ESI mode using selected-reaction-monitoring (SRM). The validation results showed that this LC/SRM method has sufficient sensitivity and robustness to be used as a regular qualitative screening method for detecting anabolic steroids in horse urine. The applicability of this method was demonstrated in the detection and confirmation of a horse urine sample reported to contain nandrolone and its major urinary metabolite 5α-estrane-3β,17α-diol, and in the identification of testicular steroid markers in an intact male horse urine sample.

2. Materials and methods

2.1. Materials

Androsterone, estrone and methandrostenolone were obtained from Alltech (Deerfield, IL, USA). Nandrolone was obtained from British Pharmacopeia (London, UK). 1α-Methyl-5α-androstan-3α-ol-17-one, 17α-ethyl-5α-estrane-3α,17β-diol, 1-testosterone, boldenone sulfate, d_3-testosterone sulfate, testosterone sulfate and tetrahydrogestrinone were obtained from National Measurement Institute (Sydney, Australia). 5α-Androstane-3β,17β-diol, bolasterone, dehydroepiandrosterone, deoxycortone, mestanolone, mesterolone and methandriol were obtained from Sigma–Aldrich (St. Louis, MO, USA). 17α-Methyl-5α-androstane-3α,17β-diol, 17α-methyl-5α-androstane-3β,17β-diol, 19-norandrosterone, 5(10)-estrene-3β,17α-diol, 5α-estrane-3β,17α-diol, androst-4-ene-3β,17β-diol, 5α-androstane-3β,17α-diol, boldione, clostebol, drostanolone, methenolone and normethandrolone were obtained from Steraloids (New Port, RI, USA). 17β-Estradiol was obtained from United States Pharmacopeia (Rockville, MD, USA). 9α-Fluoro-17,17-dimethyl-18-norandrostane-4,13-dien-11β-ol-3-one and 16α-hydroxyfurazabol were gift items from the Laboratory of Racing Chemistry (Tochigi, Japan). [16,16,17]-d_3-Androstane-3α,17β-diol was synthesised and characterised in-house.

Acetonitrile (LiChrosolv®), ammonium sulfate (Emsure®), chloroform (GR grade), diisopropyl ether (GR grade), ethyl acetate (GR grade), formic acid (Suprapur, 98–100%), *n*-hexane (GR grade), hydrochloric acid (HCl, 30%, Suprapur), methanol (LiChrosolv®) and sodium chloride (GR grade) were purchased from Merck (Darmstadt, Germany). Acetic anhydride and ammonium formate (extra pure, 98%) were purchased from International Laboratory Limited (San Bruno, CA, USA). 2-Methyl-6-nitrobenzoic anhydride (97%), 4-(dimethylamino)pyridine (ReagentPlus®, 99%), 2-picolinic acid (≥99%), pyridine (Chromasolv® Plus, ≥99.9%), sodium hydroxide (pellets, analytical grade) and triethylamine (puriss. p.a.) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Anhydrous methanolic hydrogen chloride used for methanolysis was prepared according to the procedures reported previously [26]. Abs Elut Nexus cartridges (60 mg, 3-mL) were purchased from Varian (Harbor City, CA, USA). Deionised water was generated from an in-house water purification system (Milli-Q, Molsheim, France).

2.2. Sample preparation, extraction and derivatisation

Horse urine (9 mL) was spiked with d_3-androstane-3α,17β-diol (270 ng) and d_3-testosterone sulfate (equivalent to 135 ng free d_3-testosterone) as the internal standards. Ammonium sulfate (1.5 g) was added and the mixture was vortexed for 0.5 min and centrifuged at around 1500 × *g* for 10 min. The supernatant (8 mL) was loaded onto an Abs Elut Nexus cartridge. The cartridge was washed with deionised water (3 mL) and *n*-hexane (3 mL), and then eluted with chloroform (2 mL) and followed by methanol/ethyl acetate (5:95, v/v, 3 mL). The combined eluate was evaporated to dryness at 60 °C under nitrogen. Anhydrous methanolic hydrogen chloride (1 M, 0.5 mL) was added and the solution was heated at 60 °C for 10 min. Diisopropyl ether (3.0 mL) was added and the mixture was transferred to a 15-mL graduated centrifuge tube containing NaOH/NaCl (1 M/0.15 M, 2 mL). The mixture was then vortexed for 0.5 min and centrifuged at around 1500 × *g* for 0.5 min. The organic layer was passed through a Pasteur pipette packed with cotton wool, and then evaporated to dryness at 60 °C under nitrogen.

The derivatising reagent was prepared freshly before use by mixing 2-methyl-6-nitrobenzoic anhydride (100 mg), 4-dimethylaminopyridine (30 mg), picolinic acid (80 mg), pyridine (1.5 mL) and triethylamine (200 μL). Picolinoyl derivatisation was achieved by incubating the dried sample extract with the derivatising reagent (170 μL) at 80 °C for 60 min. *n*-Hexane (1 mL) was added to the reaction mixture, vortexed for 0.5 min, and centrifuged at around 1500 × *g* for 0.5 min. The supernatant was evaporated to dryness at 60 °C under nitrogen and the dried residue was reconstituted in 30 μL of 50% acetonitrile in 5 mM ammonium formate buffer (pH 3.0) for analysis.

2.3. Instrumentation

UHPLC/MS/MS analyses were performed on a TSQ Quantum Ultra mass spectrometer (ThermoFisher, San Jose, CA, USA) equipped with a heated ESI source and connected to a Waters Acquity UPLC system (Waters Corporation, Milford, MA, USA). Solid-phase extraction was performed using a RapidTrace® SPE workstation (Zymark Corporation, Hopkinton, MA, USA).

2.4. UHPLC conditions

A reversed-phase Acquity UPLC® BEH C18 column (10 cm × 2.1 mm ID; 1.7 μm particle size, Waters Corporation, Milford, MA, USA) was used for the separation. The mobile phase

was composed of 5 mM ammonium formate in deionised water (pH 3.0) as solvent A and 0.1% formic acid in acetonitrile as solvent B. A gradient was run at 400 μL/min, with the initial composition being 40% solvent A and 60% solvent B (t = 0 min), and decreased to 35% solvent A from t = 0 min to t = 7.5 min using a gradient curve of 10. Solvent A was decreased linearly to 0% from t = 7.5 to 8 min, and then held for 1 min (until t = 9 min). The solvent composition was returned ballistically to the initial settings at t = 9.01 min, and stabilised until t = 10.5 min before the next injection. The injection volume was 5 μL.

2.5. MS conditions

Mass spectral data were acquired in multiple SRM mode in a single time segment, with 5 ms dwell time for each transition. The SRM transition, skimmer offset and collision energy for each target analyte were optimised by infusion of the corresponding reference material into the TSQ Quantum Ultra (Table 1). The source was operated in the positive heated ESI mode at 350 °C, with both the sheath gas and auxiliary gas pressure set at 50 arbitrary TSQ units. The capillary temperature was set at 350 °C. The spray voltage was set at 500 V and ion sweep gas was set at 2 arbitrary TSQ units with a curtain plate installed. Argon was used as the collision gas and the collision gas pressure in Q2 was set at 1.2 TSQ arbitrary units. The resolution for both precursor ions in Q1 and product ions in Q3 was 0.7 amu. Data processing was performed using the Xcalibur (Version 2.0.7) software.

2.6. Estimation of target analyte concentrations

For each batch of urine samples, a calibrator and a quality control (QC) sample each containing the target drugs spiked in negative horse urine were processed in parallel with the test samples. The spiked concentrations of individual targets in the QC sample (see Table 1) were one-fourth of the levels in the calibrator. [16,16,17]-d_3-Androstane-3α,17β-diol was used as the internal standard (IS) for all target analytes except for boldenone and testosterone. The total (free and conjugated) concentrations of the latter were estimated using d_3-testosterone (from d_3-testosterone sulfate) as the IS. An one-level calibration curve based on the peak area ratio (analyte/IS) was prepared for each analyte using the Xcalibur (Version 2.0.7) software. The estimated concentrations of the target analytes were calculated automatically by the software.

2.7. Method validation

The performance characteristics of this qualitative screening method, including sensitivity, selectivity, precision and extraction recovery, were established using negative horse urine samples and urine samples fortified with the target analytes. The applicability of this method was further assessed by analysing a horse urine sample reported to contain nandrolone and its major urinary metabolite 5α-estrane-3β,17α-diol, and a urine sample from an intact male horse.

3. Results and discussion

3.1. Chemical derivatisation of hydroxysteroids and UHPLC separation

The picolinic acid derivatisation procedure used in this study basically followed that reported by Yamashita et al. [19]. A picolinoyl moiety was introduced to the hydroxyl group on the steroid skeleton via simple pyridine-carboxylate esterification (Fig. 1). The derivatisation employed a mix anhydride approach using picolinic acid and 2-methyl-6-nitrobenzoic anhydride in the presence of 4-dimethylaminopyridine and triethylamine. The reagent mixture was incubated with the steroid extract at 80 °C for 60 min. The derivatised steroids were recovered from the reaction mixture using hexane, which was subsequently evaporated and reconstituted before analysis. Fig. 2 showed the positive ESI full scan and product-ion scan mass spectra of picolinated testosterone as an example.

Of the 34 anabolic steroids evaluated in this study (including the two IS), picolinoyl esters were formed for all except boldione and tetrahydrogestrinone. Since boldione possesses no hydroxyl group, it was not surprising that a picolinoyl moiety could not be incorporated to the steroid. In the case of tetrahydrogestrinone, the presence of ethyl groups at C17α and C19 positions seemed to have sterically hindered the derivatisation of the tertiary hydroxy group at C17β position. A similar phenomenon was also observed for 17α-ethyl-5α-estrane-3α,17β-diol (urinary metabolite of norethandrolone) where only the 3-hydroxyl group was derivatised. Picolinic acid derivatisation, however, appeared to work for the C17-hydroxyl groups of all C17-methyl steroids studied (namely 16α-hydroxyfurazabol, 17α-methyl-5α-androstane-3α,17β-diol, 17α-methyl-5α-androstane-3β,17β-diol, bolasterone, methandriol, mestanolone, methandrostenolone, and normethandrolone) probably because substituents at the β position to the hydroxy moiety exert a more pronounced steric effect than those at the α position. Mild steric hindrance could still be observed with the C17α-methyl group as both mono- and di-picolinated derivatives were detected in some dihydroxy-17α-methyl steroids (such as 17α-methyl-5α-androstane-3α,17β-diol). A rather interesting finding was that both mono- and di-picolinated derivatives were observed for two 3-oxo-17-ol steroids, namely mestanolone and mesterolone, with the di-picolinated derivatives being the more prominent species in both cases. The reason leading to the formation of di-picolinated derivatives in these two steroids was unclear, as the formation of enol-picolinated derivatives was not observed in other oxosteroids in this study.

Separation of the derivatised steroids was achieved using a C18 UHPLC column with a rather flat gradient program but high initial organic mobile phase content. Using a gradient curve of 10, the initial organic mobile phase was increased exponentially from 60% to 65% over a period of 7.5 min. The overall instrument turnaround time was 10.5 min inclusive of post-run equilibration. Almost all target steroids were completely resolved, except for the di-picolinated 5α-androstane-3β,17α-diol and 5α-androstane-3β,17β-diol which were separated with a retention time difference of less than 0.05 min. The chromatographic resolution of this pair of C3β,C17-diol isomers was poor, while another di-picolinated isomeric pair 17α-methyl-5α-androstane-3α,17β-diol and 17α-methyl-5α-androstane-3β,17β-diol was separated by about 0.4 min. This observation suggested that the stereochemistry of C3-hydroxyl group was likely to play a more prominent role than that of the C17-hydroxyl group in the retention properties of picolinated steroids.

3.2. Method sensitivity and selectivity

The concentrations of target analytes in the QC sample are shown in Table 1. All target analytes could be consistently detected at the QC levels, ranging from 0.75 to 30 ng/mL in urine. An estimate of the method sensitivity was performed by analysing the target analytes at different concentrations in spiked urine samples (equivalent to 10, 20 and 50% of the concentrations in the QC sample). The estimated limit of detection (LoD) of a particular target analyte as shown in Table 1 represents the lowest spiked concentration evaluated that gave a signal to noise ratio greater than 3 in the corresponding product-ion chromatogram. Thus, the

Table 1
MS parameters, spike concentrations of the quality control sample, estimated limits of detection, extraction recoveries and precision data for the target steroids.

Target analyte	Molecular mass	SRM parameters					Estimated LoD (ng/mL)	Extraction recovery (%)	Precision (% RSD)	
		Picolinoyl derivative		Skimmer offset (V)	Collision energy (eV)	QC sample (ng/mL)			RRT intraday (interday)	Peak area ratio intraday (interday)
		Precursor ion (m/z)	Product ion (m/z)							
d_3-Androstane-3α,17β-diol (I.S.)	295	506	260	10	20	–	–	–	–	–
16α-Hydroxyfurazabol	346	557	106	10	25	5	2.5	80	0.09 (0.28)	27 (62)
17α-Ethyl-5α-estrane-3α,17β-diol	306	412	271	10	15	5	0.5	48	0.19 (0.51)	51 (69)
17α-Methyl-5α-androstane-3α,17β-diol[b]	306	394	124	10	20	0.75	0.15	60	0.11 (0.23)	13 (33)
17α-Methyl-5α-androstane-3β,17β-diol[b]	306	394	124	10	20	1.5	0.3	63	0.16 (0.11)	16 (24)
17β-Estradiol	272	483	360	10	20	30	30	67	0.09 (0.29)	15 (7)
19-Norandrosterone	276	382	241	10	20	1.5	0.15	55	0.20 (0.29)	10 (4)
1-Testosterone	288	394	107	10	20	0.75	0.375	77	0.12 (0.25)	15 (60)
1α-Methyl-5α-androstan-3α-ol-17-one	304	410	269	10	15	2	0.2	65	0.14 (0.33)	7 (47)
5(10)-Estrene-3β,17α-diol	276	487	241	10	15	1	0.1	66	0.25 (0.08)	13 (27)
5α-Androstane-3β,17α-diol (5α-Androstane-3β,17β-diol[a])	292	503	124	10	15	1.5	0.15 (0.15)	71 (63)	0.01 (0.15)	14 (51)
5α-Estrane-3β,17α-diol	278	489	124	10	30	4	0.4	68	0.14 (0.25)	7 (33)
9α-Fluoro-17,17-dimethyl-18-nor-androstane-4,13-dien-11β-ol-3-one	318	424	281	10	25	1	0.2	54	0.20 (0.28)	18 (1)
Androst-4-ene-3β,17β-diol	290	501	255	5	17	20	2	80	0.22 (0.29)	9 (70)
Androsterone	290	396	124	10	20	0.75	0.15	61	0.24 (0.05)	16 (27)
Bolasterone[b]	316	299	97	10	25	1	0.5	68	0.26 (0.29)	13 (25)
Boldione	284	285[c]	121	10	20	20	10	68	0.08 (1.16)	18 (61)
Clostebol	322	428	269	10	20	3	0.6	63	0.09 (0.29)	18 (13)
Dehydroepiandrosterone	288	394	253	10	20	2	0.2	70	0.25 (0.02)	14 (52)
Deoxycortone	330	436	78	10	35	7.50	3.75	75	0.08 (0.60)	14 (72)
Drostanolone	304	410	203	10	20	1.5	0.75	50	0.16 (0.22)	8 (21)
Estrone	270	376	78	10	27	15	7.5	70	0.08 (0.11)	13 (49)
Mestanolone[b]	304	392	106	10	25	3	0.6	55	0.08 (0.11)	25 (24)
Mesterolone	304	515	106	10	25	1.5	0.75	53	0.13 (0.14)	21 (12)
Methandriol	304	515	269	10	25	4	2	70	0.22 (0.16)	15 (41)
Methandrostenolone[b]	300	283	121	10	15	0.75	0.375	80	0.45 (0.57)	19 (45)
Methenolone	302	408	121	10	20	3	0.6	66	0.26 (0.10)	14 (21)
Nandrolone	274	380	124	15	15	1.5	1.5	82	0.30 (0.10)	21 (52)
Normethandrolone	288	394	124	5	15	20	4	63	0.09 (0.99)	15 (23)
Tetrahydrogestrinone	312	313[c]	241	10	35	7.50	3.75	60	0.08 (0.09)	23 (53)
d_3-Testosterone (I.S.)	291	397	255	10	20	–	–	–	–	–
Boldenone[d]	286	392	135	10	20	1.5	1.5	74	0.33 (0.01)	8 (27)
Testosterone[d]	288	394	253	10	20	4	0.8	79	0.33 (0.40)	7 (25)

[a] 5α-Androstane-3β,17β-diol shares the same SRM transition as 5α-androstane-3β,17α-diol with a RT difference of less than 0.05 min.
[b] Loss of one picolinic acid moiety upon ionisation.
[c] Underivatised; precursor ion = $[M+H]^+$.
[d] Spiked as boldenone sulfate and testosterone sulfate; and using d_3-testosterone (from d_3-testosterone sulfate) as the internal standard.

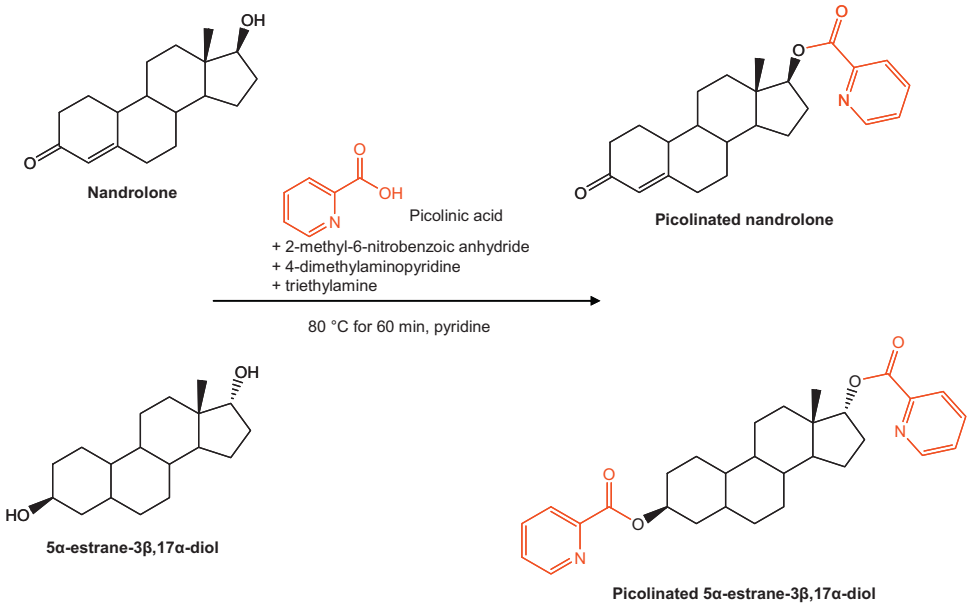

Fig. 1. The formation of picolinated nandrolone and 5α-estrane-3β,17α-diol.

actual LoDs could be lower. For 5α-androstane-3β,17β-diol, since this target shared the same SRM transition with 5α-androstane-3β,17α-diol and eluted within 0.05 min of each other, the estimated LoDs were investigated separately in different spiked urine samples. A total of 21 out of 32 analytes showed LoDs of less than or equal to 0.8 ng/mL, fifteen of which had LoDs at less than or equal to 0.5 ng/mL. Typical product-ion chromatograms for the analytes at the estimated LoDs are shown in Fig. 3. The sensitivity of this qualitative screening method is adequate as the expected physiological or post-administration levels of the target analytes in horse urine are in the range of low to mid ppb levels.

The method selectivity was assessed by analysing a total of 80 negative post-race horse urine samples. Interferences from different urine samples at the expected retention times of the target transitions were negligible. Detection was achieved by comparing the product-ion-chromatogram and the estimated concentration of a particular analyte from the sample in question with those from the negative samples and the QC samples. A suspicious sample will be subjected to follow-up analysis using multiple SRM.

3.3. Method precision

The intra-day and inter-day precision of the peak area ratios of analyte to IS and the relative retention times were evaluated by replicate analyses ($n = 8$) of spiked urine samples on 3 different days (over a 15-day period). The IS were d_3-androstane-3α,17β-diol (30 ng/mL) and d_3-testosterone sulfate (equivalent to 15 ng/mL free

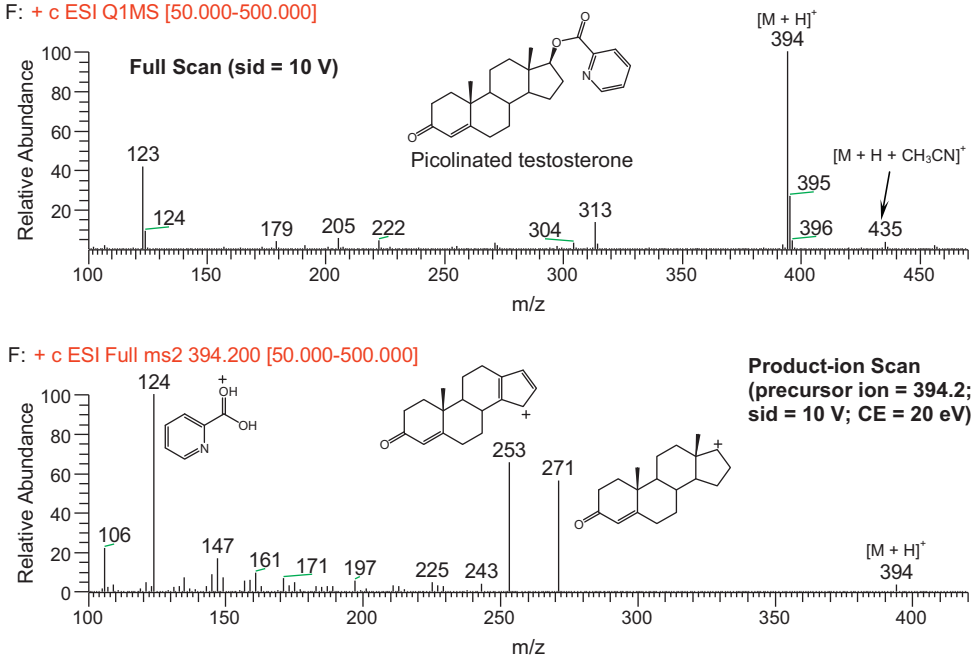

Fig. 2. Positive ESI full scan mass spectrum (upper panel) and product-ion scan mass spectrum (lower panel) of picolinated testosterone.

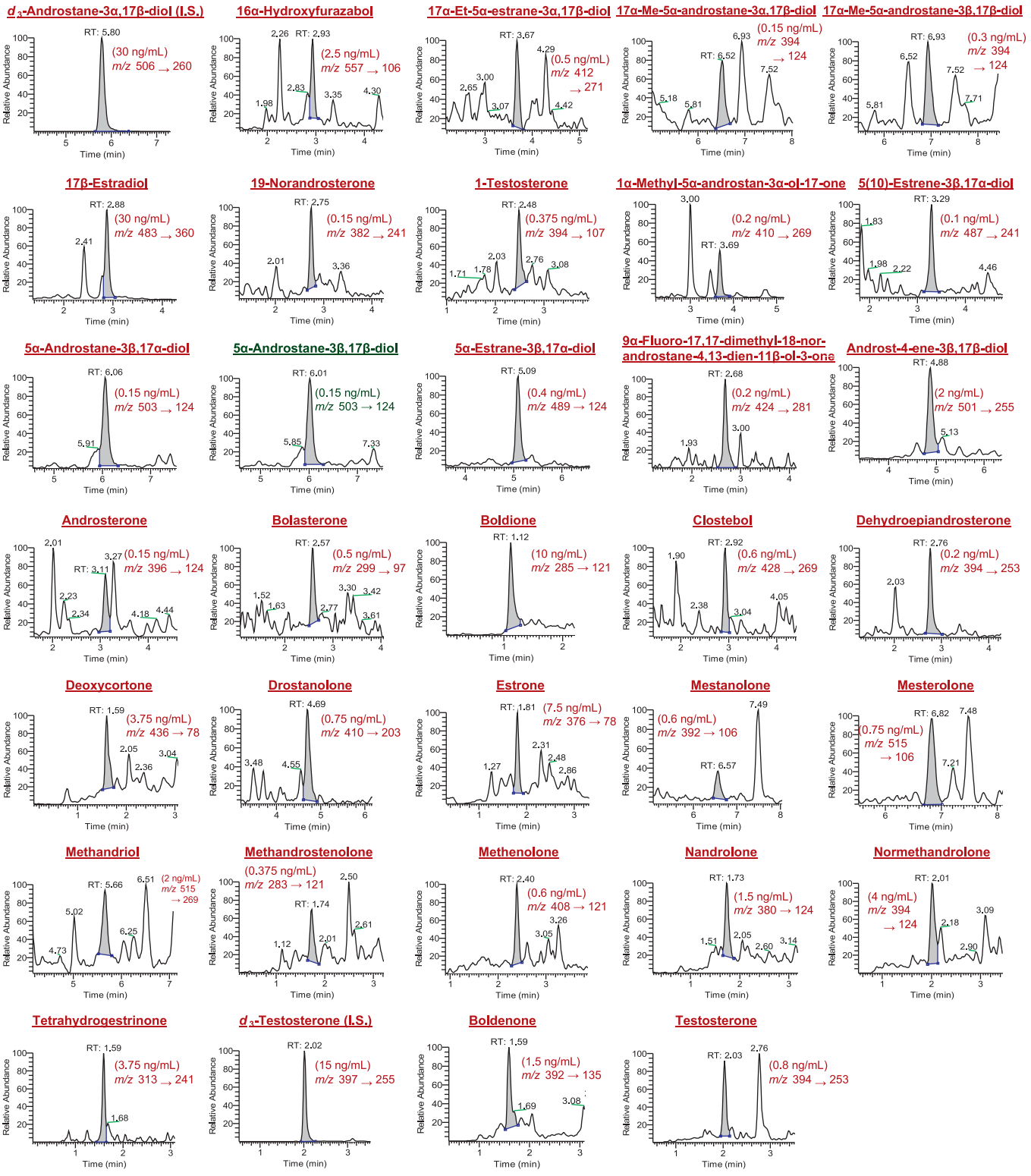

Fig. 3. Product-ion chromatograms of the target steroids at the estimated limit of detection.

d_3-testosterone). The efficiency of the deconjugation step was monitored by using the internal standard d_3-testosterone sulfate and the analytes boldenone sulfate and testosterone sulfate. The drug concentrations were the same as those in the QC samples (Table 1). For 5α-androstane-3β,17α-diol and 5α-androstane-3β,17β-diol, since they shared the same SRM transition and having a retention time difference of less than 0.05 min, only the method precision for 5α-androstane-3β,17α-diol was studied. The precision data are summarised in Table 1. The intra-day precisions (% RSD) for peak area ratios were around 7% to 51%, whereas those from the inter-day study were around 1% to 72%. The intra-day and inter-day precisions (% RSD) for relative retention times were both less than 1% for all analytes, except the inter-day precision for boldione at around 1.2%. The relatively large % RSD for boldione was probably

associated with its early elution under the UHPLC program. In all, these data indicate that the method has acceptable precision to be used as a qualitative screening method on a regular basis.

3.4. Extraction recovery

The extraction recovery of an analyte was determined by normalising the analyte/IS area ratio obtained from a spiked urine sample (at the QC level) to that obtained from a blank urine extract spiked with the same amount of the analyte. In both cases, the internal standards (d_3-androstane-3α,17β-diol and d_3-testosterone sulfate) were added to each sample immediately after SPE. Since 5α-androstane-3β,17α-diol and 5α-androstane-3β,17β-diol shared the same SRM transitions and a similar retention time, they were spiked in different urine samples and studied separately. The results are summarised in Table 1. All target analytes demonstrated recoveries of at least 48%, with 26 of them showing recoveries of not less than 60%.

3.5. Method applicability

The applicability of the method to detect target analytes in real samples was demonstrated by analysing a horse urine sample that had been reported to contain nandrolone and its major metabolite 5α-estrane-3β,17α-diol. Both nandrolone and 5α-estrane-3β,17α-diol were successfully detected using this screening method. Confirmation of the respective steroid in the sample was achieved by running the sample again under identical UHPLC conditions but monitoring four SRM transitions for each analyte. Fig. 4a and b shows that the retention times and relative abundances

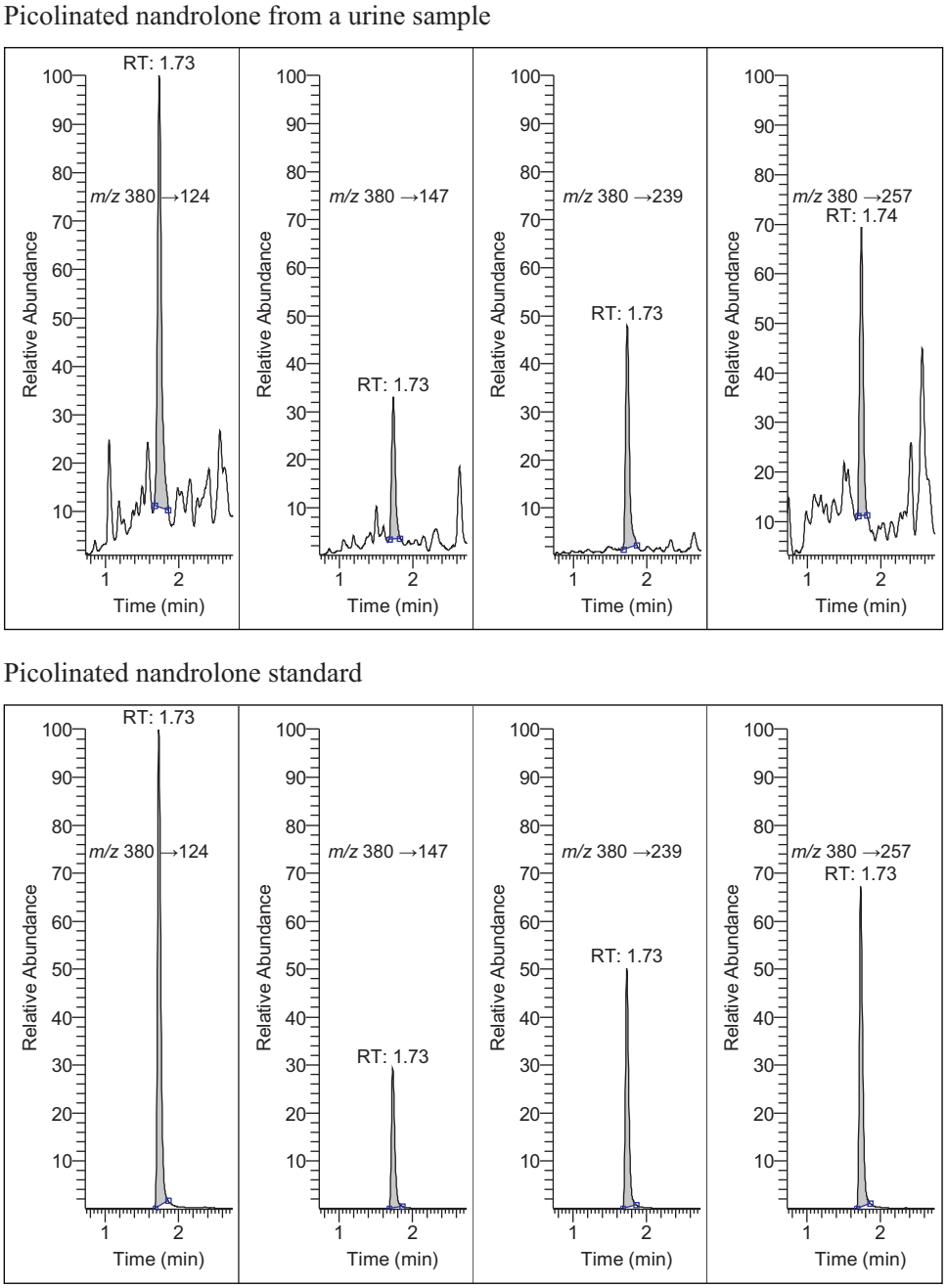

Fig. 4. Product-ion chromatograms of picolinated: (a) nandrolone and (b) 5α-estrane-3β,17α-diol obtained from a horse urine sample (upper panels) and the corresponding reference standards (lower panels).

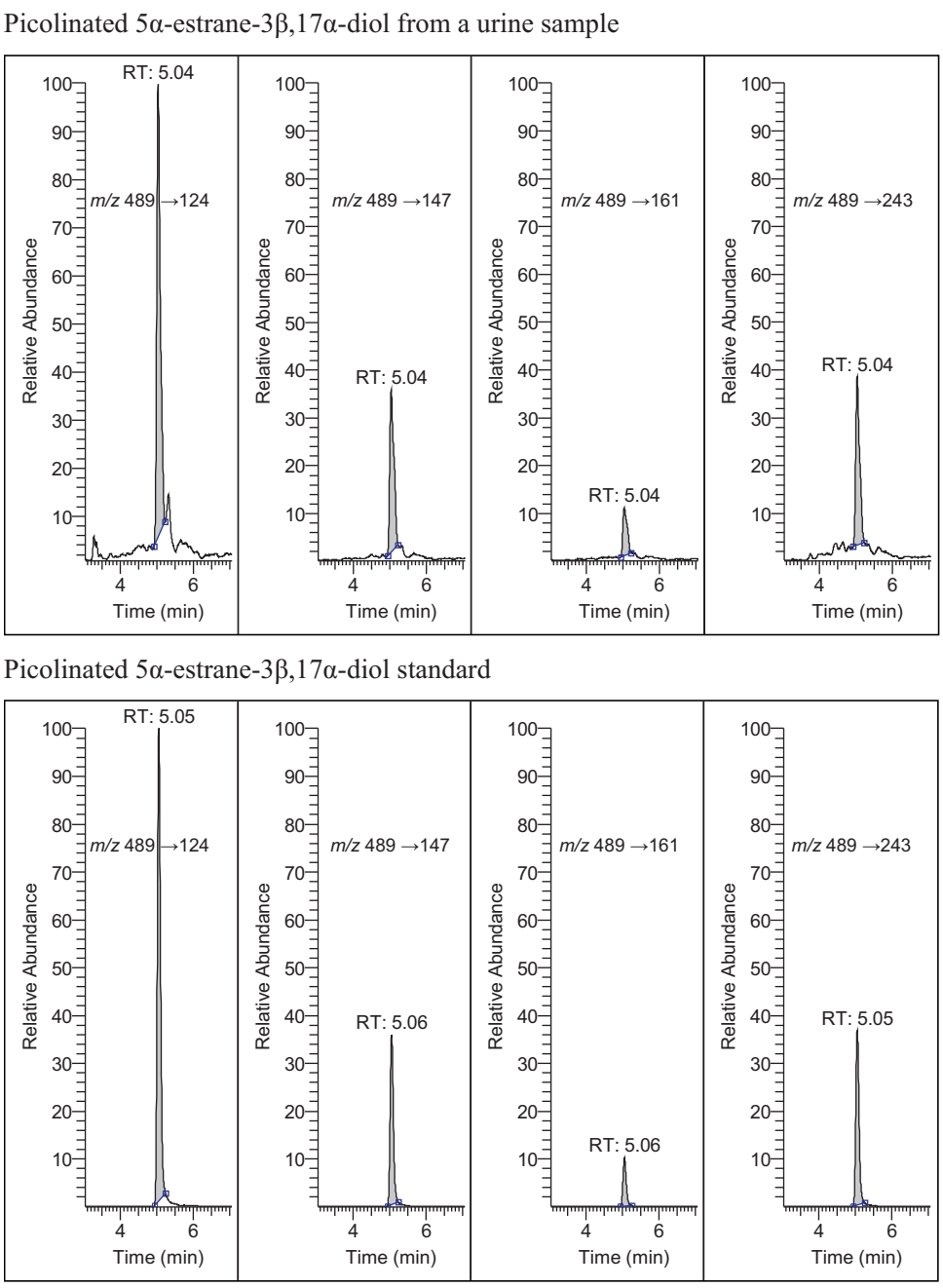

Fig. 4. (*Continued*).

in the product-ion chromatograms obtained from the sample matched well with those from the corresponding reference standards, with the results meeting the criteria stipulated in the AORC Guidelines for the Minimum Criteria for Identification by Chromatography and Mass Spectrometry [27]. Based on the screening method, the concentrations of nandrolone and 5α-estrane-3β,17α-diol in the sample were estimated to be 6 ng/mL and 17 ng/mL, respectively.

For the control of anabolic steroids in equestrian sports, it is essential to establish the true gender of the horse providing the sample in order to permit proper interpretation of test results. For example, the finding of steroids such as testosterone, nandrolone, and boldenone in urine from a presumed gelding may not constitute a violation if the horse in question is actually a rig (cryptorchid). Since rigs possess one or two hidden testicles, their urinary steroid profiles are very similar to those of entire male horses. Six urinary steroids (namely 5(10)-estrene-3β,17α-diol, estrone, estradiol, nandrolone, testosterone and 4-estrene-3,17-dione) have been identified as good indicators of testicular function [28]. Among these testicular steroids, 5(10)-estrene-3β,17α-diol was one of the most unique markers for cryptorchidism. While 5(10)-estrene-3β,17α-diol can be detected easily by GC/MS as the trimethylsilyl or pentafluoropropionyl derivative, the intact steroid exhibits very poor sensitivity when analysed by LC/MS under ESI mode. Fig. 5 shows the product-ion chromatograms for 5 testicular steroid markers (*i.e.* those amenable to picolinic acid derivatisation) obtained from urine samples collected from respectively an intact male horse and a castrated horse (gelding) using the procedure described in this study. The data showed that 5(10)-estrene-3β,17α-diol, and the other 4 testicular steroid markers, could be detected easily as their picolinated derivatives in urine from the intact male horse.

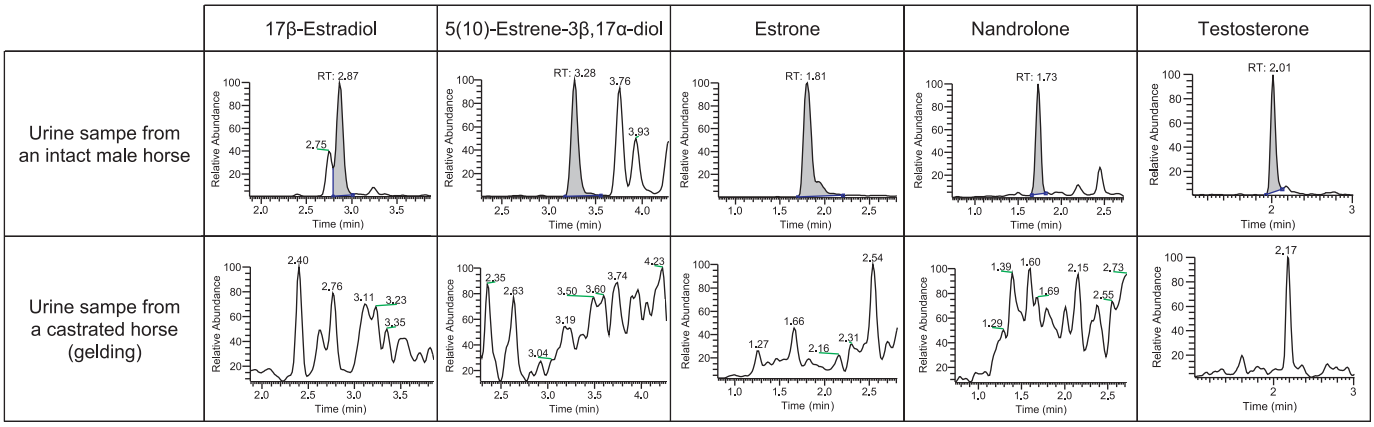

Fig. 5. Product-ion chromatograms of 5 steroid markers in urine samples from an intact male horse and a castrated horse (gelding).

4. Conclusion

A sensitive UHPLC/MS/MS method has been developed for the simultaneous screening of 32 steroids (mostly hydroxysteroids) in horse urine by monitoring their picolinated derivatives. The method had adequate intra-day and inter-day precisions to be used as a qualitative screening method on a regular basis. Matrix interference was minimal at the expected retention times of the target transitions. The applicability of the method was demonstrated by analysing a horse urine sample reported to contain nandrolone and its major metabolite 5α-estrane-3β,17α-diol. The method was also capable of detecting 5 diagnostic steroid markers useful for gender identification and result interpretation. The coupling of UHPLC with a fast scanning triple-quadrupole mass spectrometer would allow this method to be easily expanded to accommodate additional target analytes.

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