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Identification of Nandrolone and its Metabolite 5α-Estran-3β, 17α-Diol in Horse Urine after Chemical Derivatization by Liquid Chromatography Tandem Mass Spectrometry

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Abstract : Androgenic anabolic steroids (AASs) are synthetic derivatives of testosterone with a common structure containing cyclopentanoperhydrophenanthrene nucleus. Their use enhances the muscle building capacity and is beneficial during performance. The AASs are one of the most abused group of substances in horse doping. Liquid chromatography tandem mass spectrometry (LC/MS^n) has been successfully applied to the detection of anabolic steroids in biological samples. However, the saturated hydroxysteroids viz: nandrolone, 5α-estrane-3β, 17α-diol exhibit lower detection responses in electrospray ionisation (ESI) because of their poor ionisation efficiency. To overcome this limitation pre-column chemical derivatization has been introduced to enhance their detection responses in LC-ESI-MS^n analysis. The aim of present study was to develop a sensitive method for identification and confirmation of nandrolone and its metabolite in horse urine incorporating pre-column derivatization using picolinic acid. The method consists of extraction of targeted steroid conjugates by solid phase extraction (SPE). The eluted steroid conjugates were hydrolysed by methanolysis and free steroids were recovered with liquid-liquid extraction. The resulting steroids were derivatized to form picolinoyl esters and identification was done using LC-ESI-MS/MS in positive ionization mode. The picolinated steroid adduct enhanced the detection levels in comparison to underivatized steroids.

Keywords : Liquid chromatography tandem mass spectrometry, nandrolone, chemical derivatization, Horse urine

Introduction

Doping is contrary to the spirit of sports, be it human or animal. Doping in horse racing may occur by the use of performance enhancing or performance depleting drugs. Doping control in equine sports has always been highly challenging due to virtually unlimited scope of prohibited substances. According to article 6A of International Federation of Horseracing Authorities (IFHA),[1] any substance capable of causing an action or effect within a mammalian body system is prohibited. These prohibited substances include not only performance enhancing and impairing drugs, but also legitimate veterinary medications.

For many years gas chromatography mass spectrometry (GC-MS) has been the most preferred tool for detection of anabolic steroids in biological matrices but its utility is preferably limited to thermally stable compounds or small molecules.[2-3] The turnaround time (TAT) is also very high with most of the GC-MS methods. Rapid advancements in liquid chromatography tandem mass spectrometry (LC-MS^n) technique in recent years has overcome these limitations with shorter instrument TAT and ability to handle variety of biomolecules in complex matrices with better sensitivity.[4-10] In past two decades many of the LC-MS^n methods have been introduced for identification of androgenic anabolic steroids (AAS) using mass spectrometric techniques in conjunction with solid phase extraction (SPE).[11-20]

Although, LC-MS/MS has been successfully applied for the testing of anabolic steroids in biological specimens, still the technique is not very sensitive for detection of saturated hydroxysteroids or oxosteroids which exhibit low ionization efficiency towards electrospray ionization (ESI) resulting in sensitivity loss.[21-24] To overcome this limitation chemical derivatization is the most widely used and preferred choice of method to enhance the ionization efficiency of saturated hydroxysteroids or oxosteroids under ESI source.[25-27]

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Nandrolone is an anabolic steroid and its active metabolite is 5α-estrane-3β, 17α-diol in horses. Both the compounds belong to the class of saturated hydroxysteroids which are hard to detect on LC-ESI-MS/MS, as they exhibit low ionization efficiency in ESI mode which results in lower detection limits. As per IFHA and Association of Racing Commissioners International (ARCI) the threshold limit for free or conjugated 5α-estrane-3β, 17α-diol is 45 ng/mL and 1 ng/mL for nandrolone in hydrolysed urine.[28-29]

The classical routine LC-MS/MS method employed in the laboratory for analysis of anabolic steroids include SPE, hydrolysis of steroid conjugates using methanolysis followed by injection on LC-MS/MS. However, LC–MS applicability on the detection of AAS has limitations in relation to the limited ionization that a number of AAS present in soft ion sources such as ESI.[30-31] The two compounds did not exhibit good sensitivity with routine LC-MS/MS method employed in author's laboratory leading to poor detection limits. Derivatization using picolinic acid has shown prominent results for some hydroxysteroids in terms of enhanced detection levels and thus chosen for derivatization in this study.[26,32-34] Hence, the purpose of this study was to develop a new confirmatory method for the identification of nandrolone and 5α-estran-3β, 17α-diol using pre-column chemical derivatization to achieve desired limit of detection (LOD).

Experimental

Chemicals and reagents

Reference material for 5α-estrane-3β, 17α-diol was gifted from German Sport University, Institute of Biochemistry (Cologne, Germany). Nandrolone, nandrolone sulfate and d_3- nandrolone sulfate were obtained from National Measurement Institute (Australia).

Ammonium sulfate (Emsure®), formic acid (Emplura®), potassium dihydrogen phosphate (GR grade), di-Sodium hydrogen phosphate (GR grade), sodium hydroxide (pellets, GR grade), diethyl ether (GR grade), acetyl chloride (GR grade), di-isopropyl ether (GR grade) and ethanol (Emsure) were obtained from Merck (Dermstadt, Germany). Methanol (LC/MS reagent) was procured from J T Baker (USA).

2-methyl-6-nitrobenzoic anhydride (97%), 4-dimethylamin -opyridine (Reagent Plus®, 99%), 2-Picolinic acid (≥ 99%), pyridine (Chromasolv® plus, ≥ 99.9%) and triethylamine (puriss) were procured from Sigma-Aldrich (St. Louis, MO, USA). OASIS HLB cartridges for solid phase extraction were obtained from Waters (India) Pvt Ltd. Anhydrous methanolic hydrogen chloride (1 M) used for methanolysis was prepared by dropwise addition of acetyl chloride (7 mL) to anhydrous methanol (100 mL) while continuously stirring and cooling. Deionized water was prepared from an in-house Milli-Q laboratory plant (Millipore, Bedford, USA).

Preparation of derivatizing reagent

Derivatizing mixture was prepared freshly by mixing well 2-methyl-6-nitrobenzoic anhydride (100 mg), 4-dimethylamin-opyridine (30 mg), 2-picolinic acid (80 mg), pyridine (1.5 mL), triethylamine (200 μL) till dissolved and then kept in refrigerator at 2-8°C.

Urine sample preparation and derivatization protocol

To 6.5 mL of horse urine sample approximately 1.3 g of ammonium sulfate was added and vortexed for 0.5 min. Ammonium sulfate was added to eliminate unwanted urine impurities which may cause blockage of SPE cartridge and badly affect the proper elution of the sample. Samples were then centrifuged for 10 min at 3500 rpm and supernatant (6 mL) was transferred in a fresh test tube. To this supernatant, 60 μL of internal standard (IS) d_3-nandrolone sulfate (2-μg/mL) was added, followed by dilution with 2 mL of potassium phosphate buffer (0.1 M, pH 6-6.5). Samples were centrifuged for 10 min at 3500 rpm and loaded on to OASIS HLB cartridge pre conditioned with methanol (2 mL), water (2 mL) and potassium phosphate buffer (2 mL). Washing was performed with water (3 mL), 5% aqueous methanol (1 mL), potassium phosphate buffer (2 mL) and elution of target analytes was achieved using 3 mL of methanol. The methanol eluate was dried under nitrogen stream at 60°C. Hydrolysis of conjugates was performed with methanolysis by adding methanolic hydrogen chloride (0.5 mL, 1 M) to the dried residue followed by incubation at 60°C for 10 min. Samples were then cooled down at room temperature, and added, diethyl ether and di- isopropyl ether (5 mL, 3:2 (v/v)), Sodium hydroxide (2 mL, 1 M) and vortexed for 0.5 min and centrifuged at 2500 rpm for 3 min. Organic layer was separated in a fresh glass tube and evaporated to dryness under nitrogen stream at 50°C. The derivatizing reagent (170 μL) was added to the dried residue, vortexed and incubated at 80°C for 60 min. The mixture was brought to room temperature and derivatized products were recovered by adding 1 mL of n-hexane followed by centrifugation. Supernatant hexane layer was separated in to a fresh tube and dried under nitrogen evaporator at 60°C. The dried residue was then reconstituted in 100 μL mobile phase mixture (50% methanol in 0.1% aqueous formic acid), vortexed and transferred into conical plastic vial and injected into LC-MS/MS.

Instrumentation

UHPLC Conditions

The chromatography was performed using Dionex Ultimate-3000 ultra-high performance liquid chromatography (UHPLC) system. Separation of analytes was performed using an Inertsil C-18 ODS-3 column (3.0 μm, 50 mm × 4.6 mm) from GL Sciences Inc (Tokyo, Japan). Mobile phase used for HPLC separation, composed of 0.1%

formic acid in deionised water as solvent A and methanol as solvent B. Separation of compounds was achieved by running a gradient flow at 700 μL/min, with the initial composition being 100% solvent A (t = 0 min), reducing to 0% solvent A at t = 7 min and then hold for 1 min (until t = 8 min). The solvent A composition increased to 100% at t = 9 min and then stabilized at 100% A until t = 11 min. Injection volume used for acquisition was 10 μL.

Mass Spectrometric (MS) conditions

Mass spectrometry was performed on Thermo TSQ-Quantiva mass spectrometer equipped with heated electro spray ionization (HESI) source. Mass spectral data was acquired in selected reaction monitoring (SRM) mode with 40 ms dwell time for each transition. SRM transitions, RF potential and collision energy (CE) for targeted analytes were optimized by direct infusion of the corresponding derivatized reference material solution in to the mass spectrometer. The optimized source parameters for target analytes were; vaporizer temperature 400°C, ion transfer capillary temperature 350°C and ion spray voltage of 4000 V in positive ionization mode, sheath gas (nitrogen, 50 arbitrary), auxiliary gas (nitrogen, 20 arbitrary) and sweep gas (nitrogen, 3 arbitrary). For collision induced dissociation (CID) experiments in CID cell, argon (1.5 mTorr) was used as collision gas. The UHPLC and MS/MS were controlled using the LC-quan software (version 3.0). The compound dependent parameters of the two target compounds are depicted in Table 1.

Validation

The negative horse urine sample used for the validation study and was prepared by pooling negative urine samples of gelding horses. Quantitative method validation was performed as per ISO:IEC 17025:2005 standard, by evaluating; linearity, extraction recovery, specificity, precision, limit of detection (LOD), limit of quantitation (LOQ) and method uncertainty by analyzing a minimum six batches of negative horse urine samples fortified with the target analytes. Calibrators and quality control (QC) samples spiked with the target analytes in blank horse urine sample were processed in parallel with the test samples. The LOD and LOQ were assigned as the lowest concentration where S/N ratios were found S/N ≥ 3 and S/N ≥ 10 for LOD and LOQ respectively. Applicability of this method was further investigated by analyzing routine horse urine samples received from various racing clubs in India showed presence of nandrolone and 5α-estrane-3β, 17α-diol below the reporting limits. The compound dependent parameters of the two target compounds are depicted in table-1. For every batch of horse urine samples, cleaning of curtain plate with 50% aqueous methanol was performed before sample analysis and the preparation of fresh mobile phases and maintenance of the ESI source was performed daily.

Results

A method for quantification of nandrolone and 5α-estrane-3β, 17α-diol using chemical derivatization (picolinoyl derivatives) followed by detection on LC-MS/MS was successfully developed. The identification was based on the compound's chromatographic and mass spectrometric properties.

QC sample was spiked at QC levels contains nandrolone at 5 ng/mL and 5α-estrane-3β, 17α-diol at 20 ng/mL and the QC sample was injected at the beginning and end of the analytical sequence to verify that the analytical process was in control. No significant change in sensitivity was observed between the two QC sample injections throughout this study, indicating that the method is robust for routine and confirmatory use. The deconjugation of steroids was ensured by monitoring the IS d_3-testosterone sulfate and the analytes nandrolone sulfate and 5α-estrane-3β, 17α-diol. The hydrolysis yield was found more than 85% for target steroids. The full product ion scan spectrum of target steroids is depicted in Figures 1 and 2.

The calibration curves were plotted with calibrators of six different concentrations in range of 0.5-20 ng/mL (0.5, 1, 2, 5, 10, 20) for nandrolone and 1-30 ng/mL (1, 2, 5, 10, 20, 30) for 5α-estrane-3β, 17α-diol. The weighed (1/X)

Table 1. Compound dependent parameters for the detection of nandrolone and 5α-estrane-3β, 17α-diol.

Compound Name	Molecular mass	Polarity	Q1 mass	Q3 masses	RF Lens (V)	CE (V)
Picolinated nandrolone	274	+	380	124 257 239 147	69	16 16 19 23
Picolinated 5α-estrane-3β, 17α-diol	278	+	489	124 243 147 121	108	14 16 31 19
Picolinated d_3-nandrolone	277	+	383	260	97	16

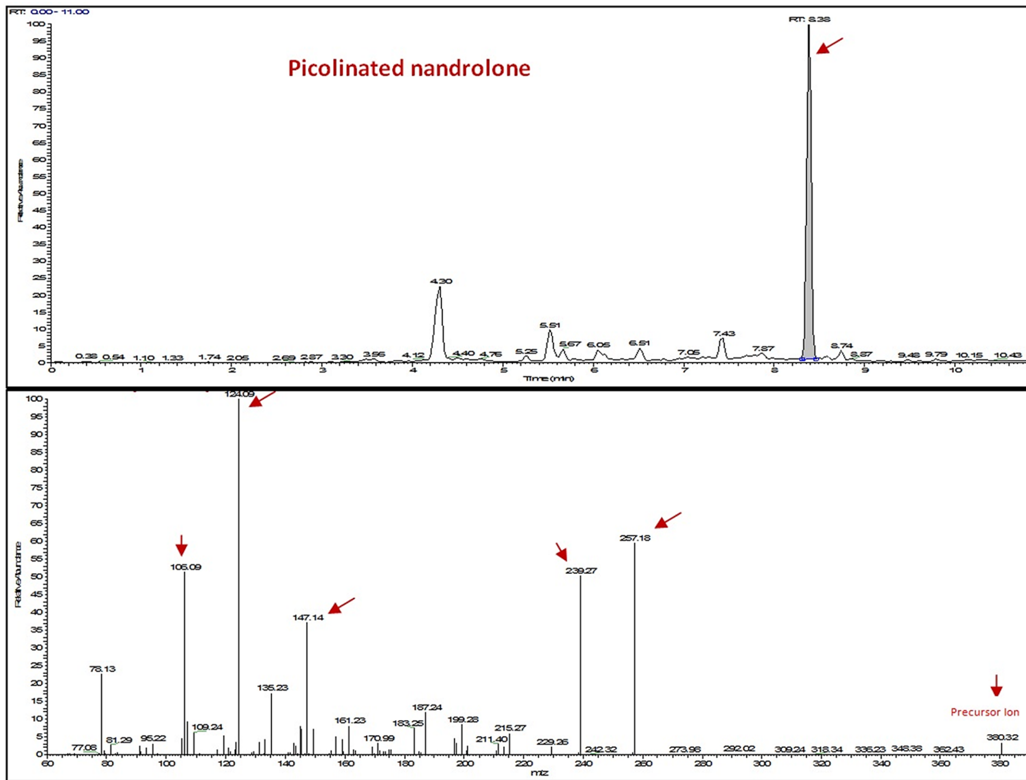

Figure 1. Product ion scan spectrum of picolinated nandrolone in spiked sample.

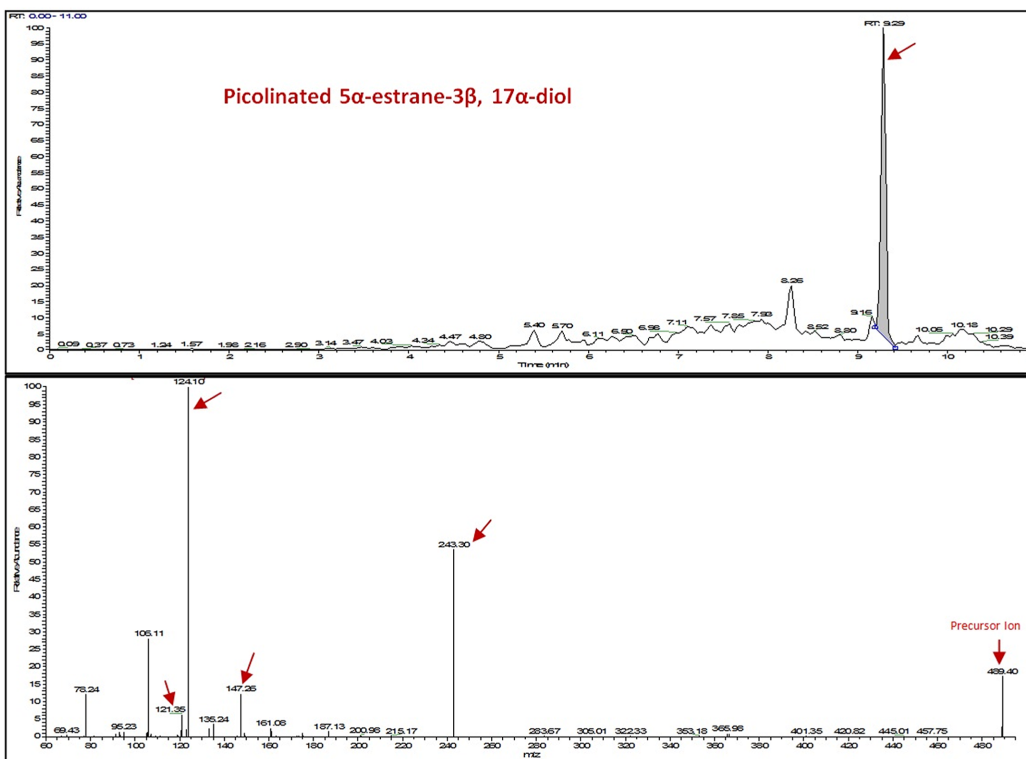

Figure 2. Product ion scan spectrum of picolinated 5α-estrane-3β, 17α-diol in spiked sample.

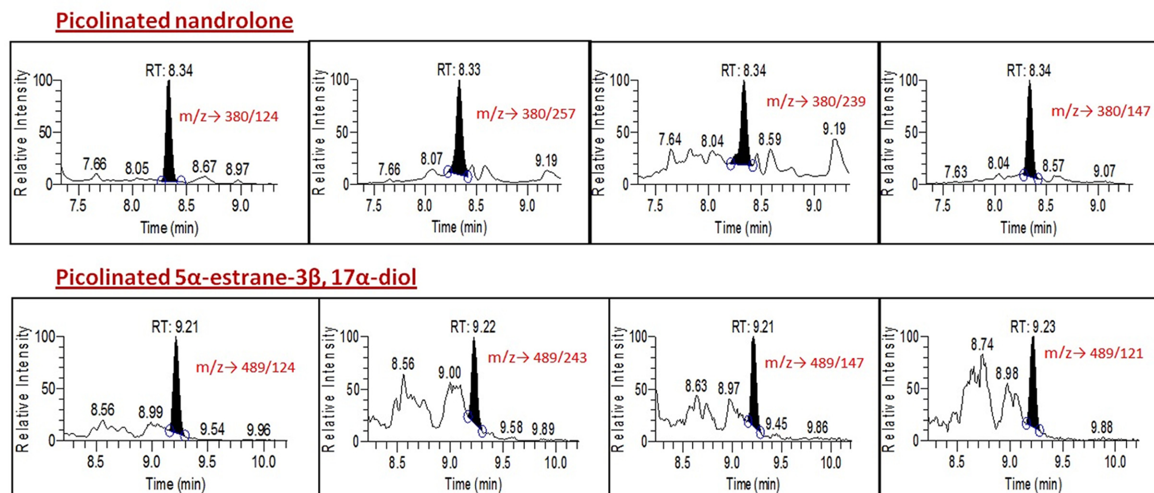

Figure 3. Product ion chromatograms of target steroids in spiked sample.

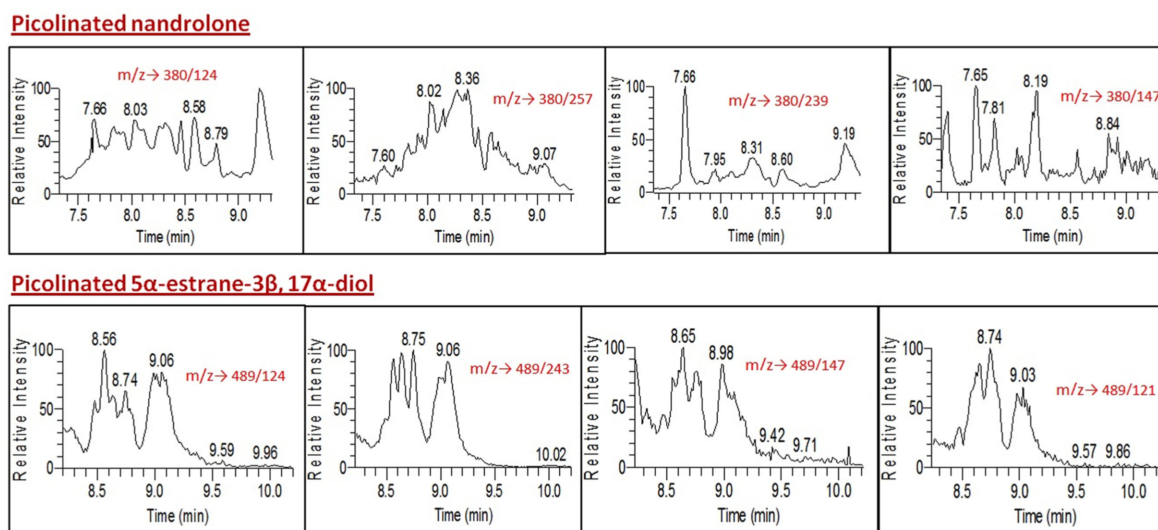

Figure 4. Product ion chromatograms of target steroids in negative horse urine sample.

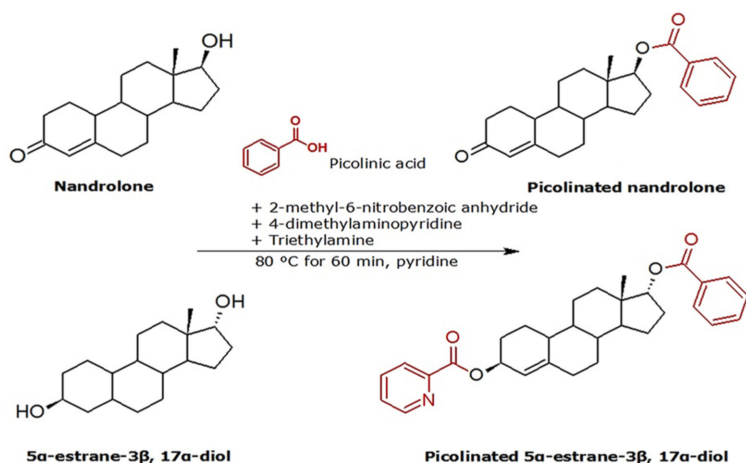

Figure 5. Derivatization reaction of picolinated nandrolone & 5α-estrane-3β, 17α-diol.

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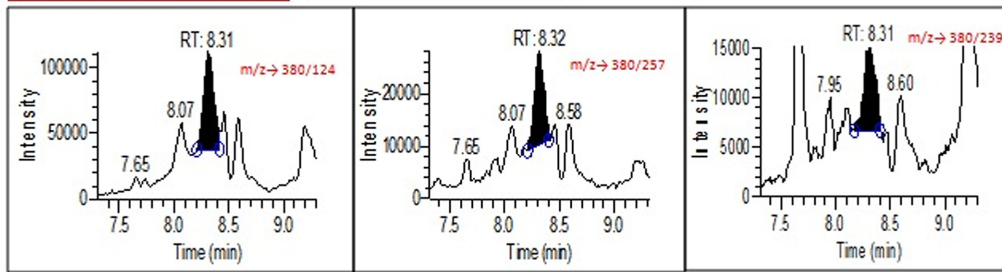
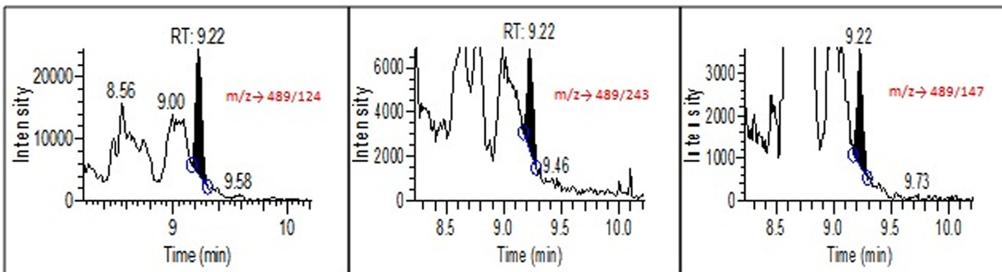
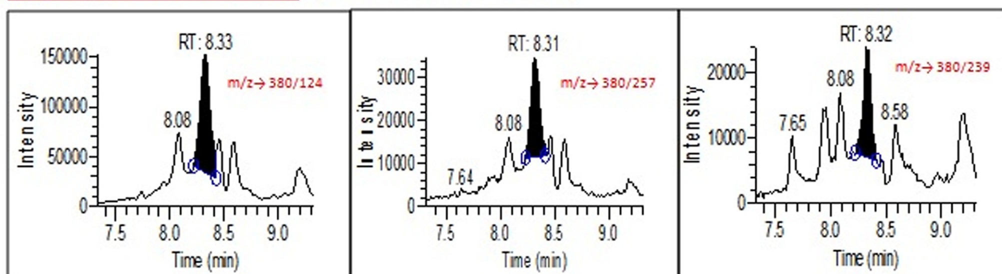
(a) LOD Chromatograms: **(Picolinated nandrolone)**



(Picolinated 5α-estrane-3β, 17α-diol)



(b) LOQ Chromatograms: **(Picolinated nandrolone)**



(Picolinated 5α-estrane-3β, 17α-diol)

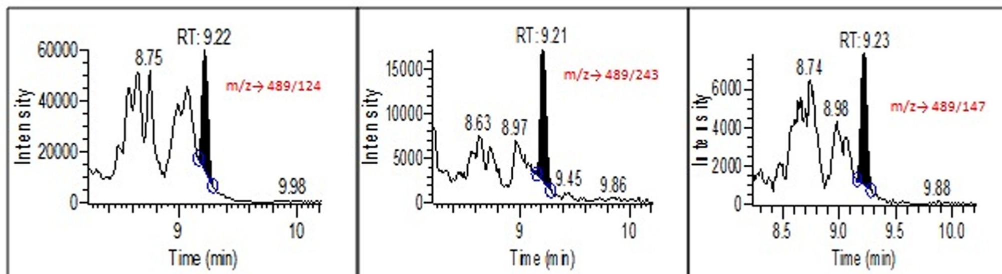


Figure 6. Comparison of the LOD Chromatograms (a) and LOQ Chromatograms (b).

Table 2. Validation results of the targeted compounds in spiked horse urine.

Compound Name	*t*R (Min.)	QC level (ng/mL)	Recovery (%)	Precision (% RSD)				LOD (ng/mL)	LOQ (ng/mL)	Uncertainty U (%)
				RRT		Peak area ratio				
				Intraday	Interday	Intraday	Interday			
Nandrolone	8.34	5	77.1	0.18	0.27	8	19	0.7	1	3.48
5α-estrane-3β, 17α-diol	9.22	20	55.2	0.24	0.33	5	14	3.5	8	4.59

least-squares regression lines were found greater than 0.998 for the two steroids, showing excellent linearity of the calibration curves.

Extraction recoveries were calculated by comparing the LC-MS/MS responses of six replicates (test sample) of nandrolone and 5α-estrane-3β, 17α-diol spiked at one fourth of QC sample concentration (Figure 3). The analysis of twenty independent horse urine samples allowed evaluation of specificity of the method. Interference from different horse urine matrices at the expected retention times of the target transitions was not observed (Figure 4). The intra-day and inter-day precision was expressed in terms of relative standard deviation (% RSD) by comparing the peak area ratios of target analyte to IS and the relative retention times were estimated by replicate analysis ($n = 6$ batches) of spiked urine samples. Derivatization yield was calculated by comparing the chromatographic peak areas of the derivatizied and native (underivatizied) steroids and was found $> 95\%$ for target steroids. Reaction of formation of picolinated nandrolone and 5α-estrane-3β, 17α-diol upon derivatization with picolinic acid is shown in Figure 5.

LOD and LOD were calculated by signal to noise (S/N) ratio method and the S/N ratio was obtained by comparing the signal of target analytes with adjacent background noise and was found greater than 3 for estimated LOD and greater than 10 for estimated LOQ (Figure 6).

The IS was monitored in each sample to detect variance in LC performance, sensitivity or *t*R variations. Results of method validation study are summarized in Table 2.

Discussion

The chemical derivatization is done to modify analytes functionality in order to enable better chromatographic separation and enhanced sensitivity.[26-27] The derivatization was achieved with a mix anhydride approach using picolinic acid and 2-methyl-6-nitrobenzoic anhydride in the presence of 4-dimethylaminopyridine and triethylamine resulted in the selective esterification of C17- hydroxyl group of nandrolone and esterification of C3 and C17- hydroxyl group of 5α-estrane-3β, 17α-diol via pyridine-carboxylate esterification. The picolinoyl derivatized products of 34 anabolic steroids have been reported by Wong *et al.*[26] by a rapid screening method in horse urine. However, the present study employed a different extraction procedure for nandrolone and 5α-estrane-3β, 17α-diol which improved sensitivity leading to improved LOD compared to routine screening procedure in use in the lab. The new developed method has improved the turnaround time with 12 min of run time as compared to employed GC-MS method with 23 min of runtime. The sensitivity in terms of LOD has increased by 3 folds for nandrolone and by 5 folds for 5α-estrane-3β, 17α-diol in comparison of routine GC-MS method. The picolinated derivatives of target steroids showed an enhanced sensitivity for nandrolone (20 folds) and for 5α-estrane-3β, 17α-diol (10 folds) in comparison of native (underivatizied) steroids. The developed method could be used successfully for confirmation of routine samples which showed nandrolone and its metabolite below reporting levels. The picolinoyl derivatization would be very useful to enhance the detection levels of wide variety of hydroxysteroids which offers inclusion of more AAS and glucocorticosteroids in existing scope.

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