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The potential use of complex derivatization procedures in comprehensive HPLC-MS/MS detection of anabolic steroids

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The use of two separate derivatization procedures with the formation of oxime (hydroxyl ammonium pretreatment) and picolinoyl (mixed anhydride method) derivates of anabolic steroids following HPLC-MS/MS analysis was proposed. The main product ions of obtained derivatives for 21 anabolic steroids were evaluated and fragmentation pathways were compared.

The analysis of MS/MS spectra for underivatized steroids versus oxime or picolinoyl derivatives showed that in case of analytes containing conjugated double bonds in sterane core all of the observed MS/MS spectra contained abundant product ions of diagnostic value. The implementation of derivatization procedures to such compounds is useful for upgrading sensitivity or selectivity of the evaluated method. On the other hand, MS/MS spectra of underivatized and oxime analytes without conjugated double bonds in sterane core produce spectra with large amounts of low abundant product ions. Picolinoyl derivatives formation leads to highly specific spectra with product ions of diagnostic value coupled with sensitive and selective analysis at the same time. The intra- and inter-group comparison analysis revealed that fragmentation pathways for underivatized steroids and correspondent oxime derivatives are similar.

The obtained oxime and picolinoyl derivatives provided 10–15 times higher ESI response in the HPLC-ESI-MS-selected reaction monitoring (SRM) when compared to those of underivatized molecules in positive HPLC-ESI-MS mode.

Due to the laborious sample preparation we suggest to use the performed strategy for confirmation analysis purposes, metabolic studies or while the identification of new steroids or steroid-like substances.

Keywords: anabolic steroids; liquid chromatography mass spectrometry; derivatization; oxime derivatives; picolinoyl derivatives

Introduction

Steroids promote anabolic pharmacological effects and thus can be used to influence athletic performance.[1] They are prohibited by the World Anti-Doping Agency (WADA) and are frequently detected in biological samples from athletes.[2]

Initially, the detection of steroids was mainly performed by radio-immunoassay (RIA) and gas chromatography (GC) analysis, but suffered from selectivity, sensitivity, and specificity issues. The implementation of gas chromatography coupled with mass spectrometry (GC-MS) techniques not only increases the number of observable steroid analytes but also improves the sensitivity and specificity of the method.[3] In general, derivatization step with the formation of perfluoroacyl-; O-methyloxime; trimethylsilyl or enol-trimethylsilyl derivatives before GS-MS analysis is used.[4]

In past 10 years, ionization of anabolic steroids by high pressure liquid chromatography mass-spectrometry (HPLC-MS) has been investigated extensively. Despite the good ionization efficiencies for steroids with a conjugated keto- groups, steroids containing only hydroxy- groups or non-conjugated keto- groups show poor ionization.[5–7] For this latter group of compounds, monitoring losses of water or adduct ions such as $[M+NH_4]^+$ can be alternative.[8–12]

Due to the problems described above, different types of chemical modifications (derivatization) of steroid's molecules are used prior to the HPLC-MS analysis.[5,11,13,14] Derivatization procedures are not essential in HPLC-MS, but their implementation provides better ionization conditions to assure proper sensitivity and selectivity of the evaluated methods. Unfortunately, nowadays there are no group derivatization reagents for both hydroxy- and keto- groups selective modification.[5,11]

Thus, the main aim of this work was to show the potential use of complex picolinoyl and oxime derivatives formation with following HPLC-MS/MS analysis not only to overcome the problem of ionization suppression and sensitivity limits but to cover the range of anabolic steroid that are problematic in free fraction (underivatized) HPLC-MS analysis also.[11,12]

Material and Methods

Chemical and reagents

Trenbolone; boldione; androstenedione; 1-androstenedione; boldenone (1-dehydrotestosterone), 1-testosterone; androst-1-ene-3α-ol-17-one (1-androstenedione metabolite); androstanedione; DHEA; androst-4-ene-3α,17β-diol; androsterone; 5-androstene-3β,17α-diol; methandienone; 17α-methyl testosterone; methyl-1-testosterone; epimethendiol; mestanolone; gestrinone and tetrahydrogestrinone were purchased from Steraloids (Newport, RI, USA). Testosterone; dihydrostestosterone

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(5α-DHT) and d_3-testosterone were obtained from Sigma (St Louis, MO, USA).

HPLC grade methanol was purchased from Merck KGaA (Darmstadt, Germany). Distilled water for HPLC-MS analysis and solutions was purified using the Milli-Q Elix system (Millipore, Milford, MA, USA).

Stock solutions were prepared by dissolving of solid reference material or diluting reference material available as solution in adequate amounts of methanol. Working solutions of each analyte were obtained by diluting adequate amounts of stock solutions in water. All stock and working solutions were stored at 4 °C.

Potassium hydroxide (p.a.), sodium hydroxide (p.a.), and sodium dihydrogen phosphate monohydrate (p.a.) were purchased from Merck (Darmstadt, Germany). β-Glucuronidase from *Escherichia coli* was supplied by Roche Diagnostics (Mannheim, Germany). Hydroxyl ammonium chloride, ammonium sulfate, triethylamine (TEA) and tetrahydrofuran (THF) dry were purchased from Panreac Quimica SA (Barcelona, Spain). Picolinic acid and 4-(dimethylamino)-pyridine (DMAP) were obtained from Fluka (Steinheim, Germany). 2-methyl-6-nitrobenzoic anhydride (2-MNA), tert.-butyl methyl ether (MTBE), formic acid and ammonium acetate were purchased from Sigma (St Louis, MO, USA). Potassium carbonate and sodium bicarbonate were purchased from Himmed (Moscow, Russia).

Instrumentation

A Thermo Finnigan TSQ Quantum AM triple stage quadrupole mass spectrometer (Thermo Electron Corp., San José, CA, USA) equipped with an ESI source and Surveyor autosampler plus and MS-pump plus was used in all experiments.

All separations were performed at 30 °C on Phenomenex Luna C18 column (150 × 2 mm i.d., 5 µm; Phenomenex, Torrance, CA, USA) connected to the HPLC guard cartridge system (10 × 2 mm i.d., 5 µm; Phenomenex, Torrance, CA, USA) at a flow rate of 200 µL/min. Methanol and 0.05% formic acid water solution with 20 mM ammonium acetate at pH 3.0 was selected as mobile phase. The gradient program was used; the percentage of organic solvent was changed linearly as follows: 0 min, 40%; 8 min, 90%; 9 min, 90%; 12 min, 40%; and 18 min, 40%.

The spray voltage of 4000 V was used in positive ionization mode. The collision gas was argon with the collision gas pressure of 1.5 mTorr. The ion source was operated at 245 °C. Total runtime was 18.0 min after the injection volume of 20 µL. Analytes were quantified in MRM mode with two MRM transitions monitoring. Data were collected and processed with the Xcalibur 2.0 SR2 software package (Thermo Electron Corp., San José, CA, USA).

LC-MS/MS analysis of reference standards

Stock solutions of 1 mg/mL in methanol were prepared for each steroid and stored at 4 °C. Standard solutions containing all assayed steroids were prepared by methanol dilution at concentrations of 0.1; 0.5; 1; 5; 10; 50; 100; 1000; and 10 000 ng/mL. Standard solutions of steroids at 10 µg/mL were injected into HPLC-MS system. The product ion spectra of the respective $[M+H]^+$ were acquired in the full scan mode between the range of *m/z* 50–600 at ten different collision energies (5; 10; 15; 20; 25; 30; 35; 40; 50; and 60 eV). The peak width was set at 0.7 mDa and the scan rate at 0.5 s/scan. The product ion spectra of oxime and picolinoyl derivatives were obtained in the same conditions after the derivatization steps described below.

Sample preparation

One milliliter of urine was fortified with the internal standard solution (d_3-testosterone —5.0 ng/mL in urine). Than 0.5 mL of phosphate buffer (0.2 M, pH 7) was added, and an enzymatic hydrolysis was performed using 50 µL of β-glucuronidase from *E. Coli* at 50 °C for 60 min. After adjusting to pH 9 with solid buffer containing potassium carbonate and sodium bicarbonate (1 : 2 w/w), a liquid-liquid extraction was performed by adding 2 mL of MTBE and shaking for 2 min after addition of 5 g of ammonium sulfate. After centrifugation at 1200 *g* for 5 min, the organic layer was transferred into a clean glass tube and evaporated to dryness under nitrogen flow at room temperature. The underivatized steroids were analyzed by dissolving the solid residue in 100 µL of mobile phase solution (40% of methanol and 60% of 0.05% formic acid water solution with 20 mM ammonium acetate at pH 3.0).

Derivatization procedures

The organic extract obtained in previous steps was divided into two parts (2 ml of MTBE into 2 × 1 ml of MTBE) and evaporated. The following steps included separate formation of oxime and picolinoyl derivatives.

The oxime derivatives were obtained using previously described method of Liu *et al*.[13] In brief, 300 µL of 1.5 M hydroxyl amine solution (pH 10.0) was added to solid residue. After heating at 70 °C for 30 min 700 µL of 5% $NaHCO_3$ was added to chilled mixture. The extraction procedure was performed using 2 mL of MTBE and 5 g of ammonium sulfate as grain. After the extraction procedure sample was centrifuged at 3000 rpm for 5 min and supernatant was transferred to clean glass tube. The organic phase was evaporated under nitrogen flow, reconstituted in 100 µL of mobile phase solution and 20 µL of sample injected into HPLC-MS system.

The picolinoyl derivatives were obtained by mixed anhydride method.[14] 50 µL of THF, 100 µL of freshly prepared derivatization mixture (picolinic acid – 0.52 mmol; 2-methyl-6-nitrobenzoic anhydride – 0.52 mmol; and 4-dimethylaminopyridine – 0.37 mmol) and 20 µL of TEA were added to solid residue and stored for 30 min at room temperature. The extraction procedure and following procedures were the same as in case of oxime derivatives.

Method validation

The validation was carried out following Eurochem validation guidelines (Eurochem Working Group, 1998).[15] Blank urine (free of steroids) was divided to 51 samples. Each day (over the course of 3 days), 17 fortified samples were analyzed: 2 samples were not spiked, 3 samples were spiked at 0.5 ng/mL, 3 samples at 1 ng/mL, 3 samples at 5 ng/mL, 3 samples at 10 ng/ml and 3 samples at 100 ng/ml. The samples undergo extraction and derivatization procedures as described above.

The detection limit was defined as the lowest level at which a compound could be identified in all spiked samples with a signal-to-noise (S/N) ratio greater than 3 and retention time (RT) than differs not more than 0.2 min from the RT in the reference mixture.

Results and Discussion

Twenty-one anabolic steroids were selected and divided into six groups based upon the presence or absence of double bonds, 3-keto or 3-hydroxy groups and different subsistent at C-17 position

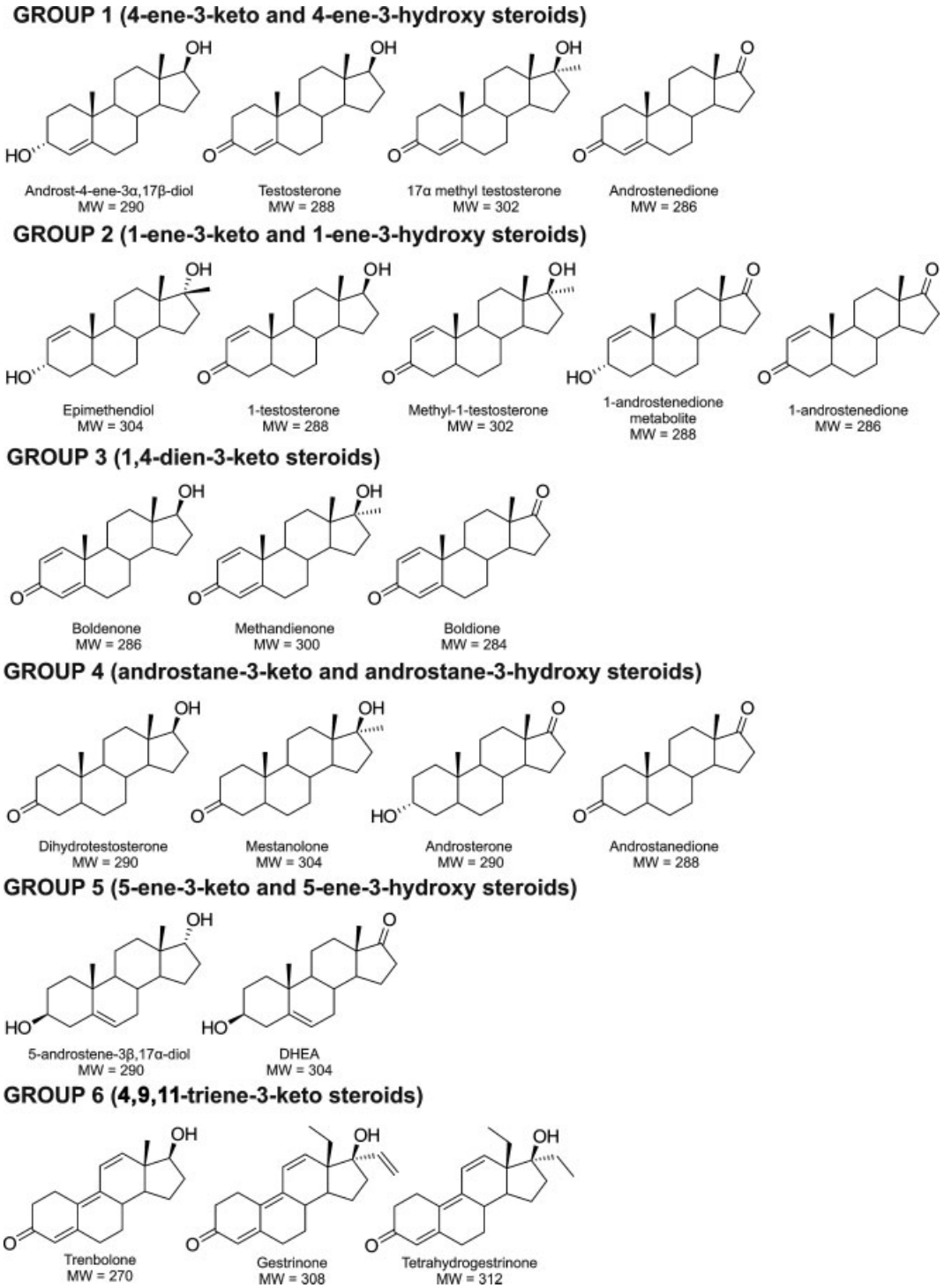

Figure 1. Molecular structures of analyzed steroids; MW = molecular weight.

in sterane core (Figure 1). The formed groups represented different variations in anabolic steroids chemical structure.

To overcome the ionization problems we supposed to compare ESI-MS/MS data of underivatized steroids[16–19] with the data obtained during oxime (for steroids with keto groups) and picolinoyl (for steroids with hydroxy group) derivatives fragmentation analysis. All diagnostic ions are presented in Table 1.

The analysis of MS/MS spectra for underivatized steroids versus oxime or picolinoyl derivatives showed that in case of analytes containing conjugated double bonds in sterane core all of the observed MS/MS spectra contained abundant product ions of diagnostic value (Figure 2). The implementation of derivatization procedures to such compounds is useful for upgrading sensitivity and selectivity of the evaluated method. On the other hand, MS/MS spectra of underivatized and oxime analytes without conjugated double bonds in sterane core produce spectra with large amounts of low abundant product ions.[16–18] Picolinoyl derivatives formation leads to highly specific spectra with product

Table 1. Optimized ESI- MS/MS conditions for native anabolic steroids, oxime and picolinoyl derivatives

			Underivatized				Oxime derivative				Picolinoyl derivative			
Groups	Compound name	MRPL (ng/ml)	RT (min)	Parent ion (m/z)	Product ions (m/z) (CE, eV)	LOD (ng/ml)	RT (min)	Parent ion (m/z)	Product ions (m/z) (CE, eV)	LOD (ng/ml)	RT (min)	Parent ion (m/z)	Product ions (m/z) (CE, eV)	LOD (ng/ml)
Group 1	Androst-4-ene-3α,17β-diol	**10**	10.1	291	274 (5)	>10	No oxime derivative formation (no keto groups)				11.6	396	273 (10)	0.5
					256 (10)								255 (15)	
					105 (45)								106 (25)	
					91 (40)								78 (45)	
	Testosterone	**10**	9.9	289	253 (15)	5	10.0	304	138 (35)	0.1	11.6	394	271 (15)	0.5
					201 (15)				124 (25)				253 (25)	
					109 (25)				112 (25)				147 (25)	
					97 (25)				78 (50)				124 (15)	
	17α-methyl testosterone	**2**	10.3	303	285 (10)	2	10.4	318	260 (25)	0.5	No picolinoyl derivative formation			
					227 (20)				138 (40)		(steric hindrance at C-17)			
					109 (30)				124 (35)					
					97 (30)				112 (35)					
	Androstenedione	**10**	9.4	287	269 (15)	10	9.7	317	138 (35)	0.5	No picolinoyl derivative formation			
					211 (20)				124 (35)		(no hydroxy groups)			
					109 (35)				112 (35)					
					97 (35)				77 (45)					

Table 1. (*Continued*)

Groups	Compound name	MRPL (ng/ml)	Underivatized RT (min)	Underivatized Parent ion (m/z)	Underivatized Product ions (m/z) (CE, eV)	Underivatized LOD (ng/ml)	Oxime derivative RT (min)	Oxime derivative Parent ion (m/z)	Oxime derivative Product ions (m/z) (CE, eV)	Oxime derivative LOD (ng/ml)	Picolinoyl derivative RT (min)	Picolinoyl derivative Parent ion (m/z)	Picolinoyl derivative Product ions (m/z) (CE, eV)	Picolinoyl derivative LOD (ng/ml)
Group 2	Epimethendiol	**10**	9.7	**305**	270 (10) 228 (20) 213 (25) 202 (20)	10	No oxime derivative formation (no keto groups)				No picolinoyl derivative formation (3-OH–limited derivatization) (steric hindrance at C-17)			
	1-testosterone	**10**	10.4	**289**	253 (15) 205 (15) 201 (15) 187 (20)	10	10.9	**304**	272 (25) 201 (25) 187 (25) 145 (35)	0.5	11.6	**394**	271 (15) 253 (15) 124 (15) 107 (35)	1
	Methyl-1-testosterone	**10**	10.9	**303**	267 (15) 227 (20) 201 (20) 159 (25)	10	11.0	**318**	260 (20) 201 (25) 159 (35) 145 (35)	1	No picolinoyl derivative formation (steric hindrance at C-17)			
	1-androstenedione metabolite	**10**	9.1	**289**	271 (5) 213 (15) 197 (15) 145 (25)	10	10.9	**304**	253 (15) 218 (20) 141 (25) 157 (20)	0.5	12.8	394	124 (5) 271 (10) 253 (20) 106 (35)	>10
	1-androstenedione	**10**	9.9	**287**	211 (10) 203 (20) 185 (20) 143 (20)	10	10.2	**317 dioxime**	285 (5) 218 (20) 134 (40) 77 (50)	1	No picolinoyl derivative formation (No hydroxy groups)			

Table 1. (*Continued*)

Groups	Compound name	MRPL (ng/ml)	Underivatized RT (min)	Underivatized Parent ion (m/z)	Underivatized Product ions (m/z) (CE, eV)	Underivatized LOD (ng/ml)	Oxime derivative RT (min)	Oxime derivative Parent ion (m/z)	Oxime derivative Product ions (m/z) (CE, eV)	Oxime derivative LOD (ng/ml)	Picolinoyl derivative RT (min)	Picolinoyl derivative Parent ion (m/z)	Picolinoyl derivative Product ions (m/z) (CE, eV)	Picolinoyl derivative LOD (ng/ml)
Group 3	Boldenone	**10**	9.2	**287**	269 (10) 121 (20) 135 (20) 77 (50)	2	8.4	**302**	285 (15) 136 (25) 120 (35) 106 (35)	0.5	10.6	**392**	269 (15) 149 (15) 135 (20) 121 (25)	1
	Methandienone	**2**	9.6	**301**	283 (10) 149 (20) 121 (20) 77 (50)	2	10.1	**316**	280 (20) 172 (25) 136 (30) 120 (35)	0.5	No picolinoyl derivative formation (steric hindrance at C-17)			
	Boldione	**10**	8.6	**285**	151 (15) 147 (15) 121 (15) 77 (40)	2	9.5	**315 dioxime**	281 (20) 264 (25) 136 (35) 120 (35)	0.5	No picolinoyl derivative formation (no hydroxy groups)			

Table 1. (*Continued*)

Groups	Compound name	MRPL (ng/ml)	Underivatized RT (min)	Underivatized Parent ion (m/z)	Underivatized Product ions (m/z) (CE, eV)	Underivatized LOD (ng/ml)	Oxime derivative RT (min)	Oxime derivative Parent ion (m/z)	Oxime derivative Product ions (m/z) (CE, eV)	Oxime derivative LOD (ng/ml)	Picolinoyl derivative RT (min)	Picolinoyl derivative Parent ion (m/z)	Picolinoyl derivative Product ions (m/z) (CE, eV)	Picolinoyl derivative LOD (ng/ml)
Group 4	Dihydrotestosterone	**10**	9.9	**291**	273 (15) 215 (15) 199 (25) 145 (25)	10	10.4	**306**	255 (25) 201 (20) 173 (30) 159 (35)	0.5	12.1	**396**	273 (15) 255 (20) 237 (20) 124 (15)	2
	Mestanolone	**10**	8.0	**305**	229 (20) 213 (20) 187 (20) 159 (20)	10	10.8	**320**	269 (25) 229 (30) 213 (30) 173 (30)	1	No picolinoyl derivative formation (steric hindrance at C-17)			
	Androsterone	**10**	9.9	**291**	273 (10) 215 (20) 199 (20) 145 (20)	10	11.0	**306**	273 (15) 255 (20) 215 (20) 147 (25)	0.5	13.6	**396**	237 (10) 124 (15) 255 (20) 106 (35)	>10
	Androstanedione	**10**	9.9	**289**	253 (15) 215 (20) 199 (20) 145 (25)	>10	10.3	**318**	286 (20) 268 (20) 145 (25) 133 (25)	1	No picolinoyl derivative formation (no hydroxy groups)			

Table 1. (*Continued*)

Groups	Compound name	MRPL (ng/ml)	Underivatized RT (min)	Underivatized Parent ion (m/z)	Underivatized Product ions (m/z) (CE, eV)	Underivatized LOD (ng/ml)	Oxime derivative RT (min)	Oxime derivative Parent ion (m/z)	Oxime derivative Product ions (m/z) (CE, eV)	Oxime derivative LOD (ng/ml)	Picolinoyl derivative RT (min)	Picolinoyl derivative Parent ion (m/z)	Picolinoyl derivative Product ions (m/z) (CE, eV)	Picolinoyl derivative LOD (ng/ml)
Group 5	5-androstene-3β,17α-diol	**10**	10.3	**291**	273 (10)	>10			No oxime derivative formation		12.2	504	378 (10)	1
					255 (10)							dipicolinoyl	360 (15)	
					173 (20)								147 (20)	
					145 (25)								124 (15)	
	DHEA	**10**	10.4	**289**	271 (10)	10	10.6	**304**	286 (20)	0.5	11.2	**394**	271 (15)	1
					213 (15)				271 (10)				253 (20)	
					145 (30)				197 (30)				186 (25)	
					128 (50)				157 (35)				124 (10)	
Group 6	Trenbolone	**10**	9.0	**271**	253 (20)	1	9.1	**286**	269 (15)	0.5	8.4	**376**	253 (15)	1
					227 (20)				165 (50)				197 (20)	
					178 (40)				141 (50)				169 (35)	
					165 (50)				128 (50)				91 (45)	
	Gestrinone	**10**	9.6	**309**	262 (20)	1	9.8	**324**	278 (20)	0.1	No picolinoyl derivative formation (steric hindrance at C-17)			
					241 (20)				260 (25)					
					178 (50)				181 (45)					
					165 (50)				167 (50)					
	Tetrahydrogestrinone	**10**	10.9	**313**	266 (20)	1	11.0	**328**	264 (25)	0.1	No picolinoyl derivative formation (steric hindrance at C-17)			
					241 (25)				239 (25)					
					239 (15)				226 (25)					
					159 (25)				165 (40)					

ions of diagnostic value coupled with sensitive and selective analysis at the same time (Figure 2). The intra- and inter-group comparison analysis revealed that fragmentation pathways for underivatized steroids and correspondent oxime derivatives are similar.

As the main aim of this work was to show the potential role of derivatization procedures, chromatographic separation of all analytes was not optimized. Several analytes were overlapping, among that were 1-testosterone with 1-androstenedione metabolite and 17α-methyl testosterone with androstanedione in case of oxime derivatives (Figure 4). Testosterone and DHEA were overlapping in case of picolinoyl derivatives HPLC-MS/MS analysis and had the same retention time at 11.62 min (Figure 5). In all cases of poor chromatographic separation, we spiked overlapping analytes to blank urine separately to estimate appropriate limits of detection and retention times.

Group 1

4-ene-3-keto and 4-ene-3-hydroxy steroids

Four steroid agents (androst-4-ene-3α,17β-diol; testosterone; 17a-methyl testosterone and androstenedione) belonging to this group were selected for the analysis. Among listed substances only testosterone, 17α-methyl testosterone and androstenedione were covered to their oxime derivatives by treatment with hydroxyl amine solution. All of the oxime derivatives produced abundant molecular ions $[M+H]^+$ under positive ESI conditions. Keto-steroids derivatives with one or two oxime groups increased their molecular weight by 15 Da or 30 Da. The fragmentation analysis showed the common product ions for all represented oxime derivatives. Presumably the B-ring fragmentation at low and medium collision energy produced common for the group abundant ions with *m/z* 112; *m/z* 124 and *m/z* 138. The rise of collision energy up to 60 eV led to the formation of *m/z* 78 and *m/z* 67. Thus the presence of evaluated product ions in spectra can be the formal proof of 3-oxo-Δ^4-steroids structure.

More specific product ion spectra were obtained in case of picolinoyl derivatives analysis. Hydroxy-steroids derivatives increased their molecular weight by 105 Da. In spite of the fact that only androst-4-ene-3α,17β-diol and testosterone picolinoyl derivatives were found to be formed their fragmentation pathways were of diagnostic value. At lower and medium (10–20 eV) collision energy the losses of 123 Da and 141 Da were the common with the formation of *m/z* 271 and *m/z* 253 for testosterone and *m/z* 273 and *m/z* 255 for androst-4-ene-3α,17β-diol correspondingly. At higher collision energy non-specific pyridine-carboxylate moiety product ions at *m/z* 78, *m/z* 106, and *m/z* 124 were observed. Besides for androst-4-ene-3α,17β-diol the formation of two mono isomers with picolinoyl group at C-3 and C-17 with the similar fragmentation pathway was determined. We supposed to observe the formation of 17α-methyl testosterone picolinoyl derivative, but due to the presence of additional substitute at C-17, the generation of derivative was limited.

Since testosterone can form both derivatives (oxime and picolinoyl derivative), we considered to monitor the formation of oxime derivative while the signal-to-noise ratio of oxime derivative was approximately four times higher compared to picolinoyl products at the same levels of concentration (Figures 4 and 5).

Group 2

1-ene-3-keto and 1-ene-3-hydroxy steroids

Five compounds belonging to this group were selected: epimethendiol; 1-testosterone; methyl-1-testosterone; 1-androstenedione metabolite and 1-androstenedione. During the derivatization procedures, four anabolic agents revealed to form oxime derivatives (1-testosterone; methyl-1-testosterone; 1-androstenedione metabolite and 1-androstenedione). 1-testosterone, methyl-1-testosterone, and 1-androstenedione metabolite showed the ability to form mono oxime derivatives products, while 1-androstenedione formed dioxime derivative at that. The product ion spectra at low and medium collision energy produced the water losses. All evaluated spectra contained the loss of one water molecule (18 Da) except two molecules for 1-androstenedione metabolite. At high collision energies, the fragmentation pathway for 1-testosterone methyl-1-testosterone and 1-androstenedione oxime derivatives were common with the exception of mass shift of 2 Da for 1-androstenedione. All spectra contained the losses of 187 Da; 173 Da; 159 Da; 145 Da; and 117 Da with the generation of corresponding abundant ions. The product ion spectra of 1-testosterone contained the specific loss of 103 Da.

The characteristic product ions for 1-testosterone and methyl-1-testosterone oxime derivatives were *m/z* 201; *m/z* 159; *m/z* 145; and *m/z* 131. Product ions at *m/z* 187 and *m/z* 173 were observed in case of 1-testosterone and methyl-1-testosterone oxime derivatives correspondingly.

The common neutral losses at high collision energy for 1-androstenedione and 1-androstenedione metabolite oxime derivatives were 193 Da and 176 Da. Product ions at *m/z* 285; *m/z* 245; *m/z* 218; *m/z* 141; *m/z* 131; and *m/z* 120 were corresponding to 1-androstenedione, while ions at *m/z* 218; *m/z* 141; *m/z* 128; *m/z* 115; and *m/z* 111 to 1-androstenedione metabolite. The characteristic mass shift of 99 Da was observed for 1-androstenedione with the formation of product ion at *m/z* 218.

The evaluated differences in fragmentation pathway for oxime derivatives allow us to use the specific mass shift knowledge for identifying new 1-ene steroids and their metabolites. Besides, at high collision energy, the minor common ions at *m/z* 105; *m/z* 91; and *m/z* 77 were observed for all analytes.

In spite of all our attempts, the picolinoyl derivative was obtained only for 1-testosterone and 1-androstenedione metabolite. We expected epimethendiol to form picolinoyl derivatives but none were found during the analysis. Besides, the limit of detection for 1-androstenedione metabolite picolinoyl derivative was higher than 10 ng/ml, so the appropriate HPLC-MS/MS analysis was unsuitable.

Similar to the 4-ene steroids, common losses (123 Da and 141 Da) for 1-testosterone at low and medium collision energy were observed. The most abundant ions were *m/z* 271 and *m/z* 253. The most abundant ions at high collision energy were *m/z* 107 and *m/z* 78. Since 1-testosterone can be monitored as oxime or picolinoyl derivative we considered to include 1-testosterone picolinoyl derivative in HPLC-MS/MS analysis based on signal to noise and specific spectral data evaluations (Figures 4 and 5).

Group 3

1,4-dien-3-keto steroids

Boldenone, methandienone, and boldione were the analytes of interest in this group. All the analytes were converted to corresponding oxime derivatives. While boldenone and

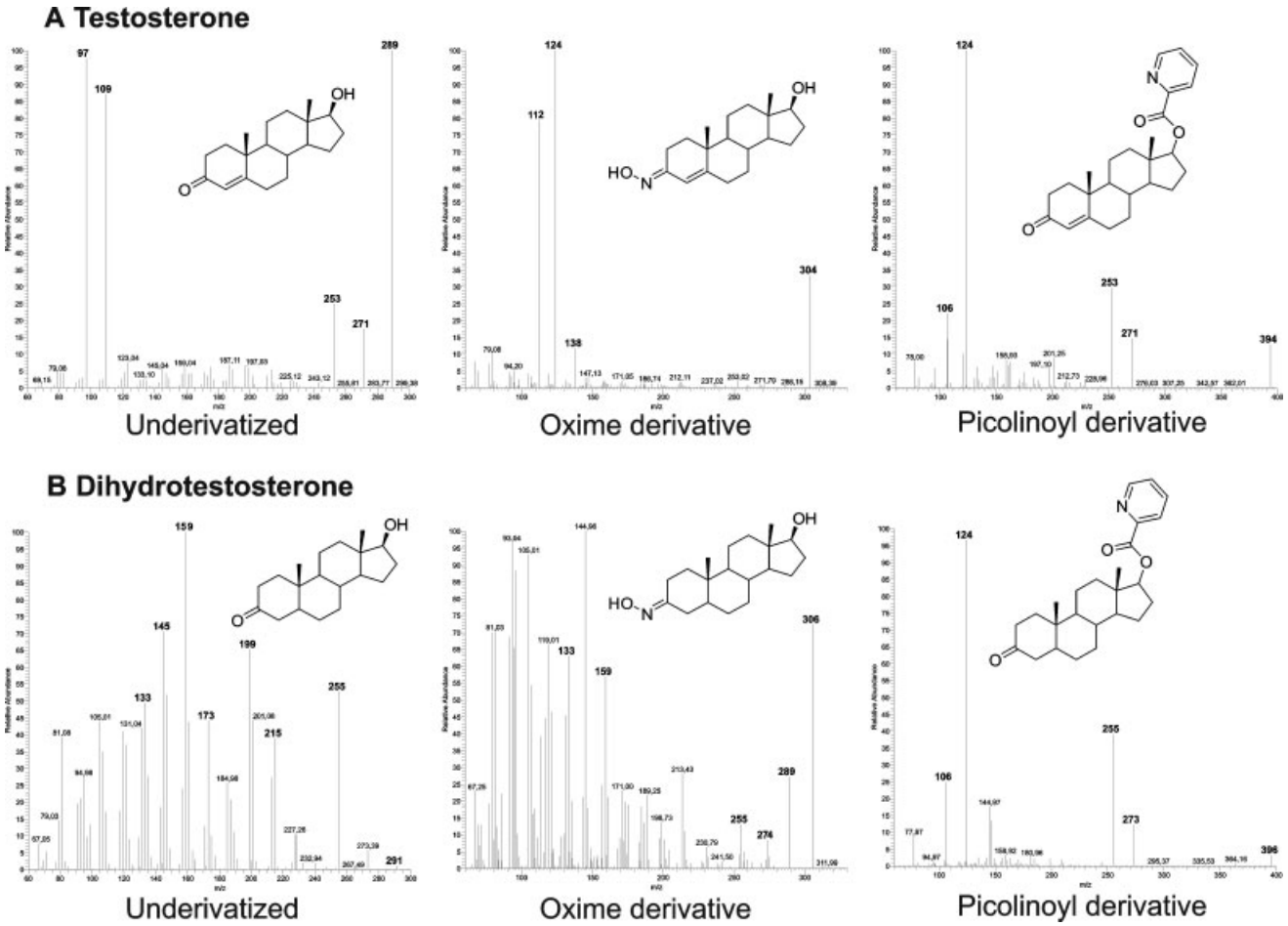

Figure 2. MS/MS spectra of underivatized steroids and their derivative products; A – testosterone; B – dihydrotestosterone.

methandienone were converted to mono oxime derivatives, boldione showed the ability to dioxime formation.

The product ion spectra for derivatives were formed by the water losses (18 Da and 36 Da). The fragmentation pathways for oxime derivatives were similar. At low and medium collision energy common specific for this group product ions at *m/z* 120, *m/z* 136, and *m/z* 159 were observed. Besides, the specific mass shifts of 60 Da and 144 Da were typical for the group with the generation of less abundant ions at *m/z* 242 and *m/z* 158 for boldenone; *m/z* 255 and *m/z* 171 for boldione; *m/z* 256 and *m/z* 172 for methandienone correspondingly. Increasing the collision energy up to 60 eV revealed the common sterane core ions at *m/z* 106, *m/z* 91, and *m/z* 77.

Boldenone was the only one analyte that formed picolinoyl derivative. The precursor ion scan revealed the most abundant ions at *m/z* 269, *m/z* 149, *m/z* 135, and *m/z* 121 using low and medium collision energy. Because the signal-to-noise ratio for boldenone picolinoyl derivative was three times higher than the corresponding oxime derivative it was included in HPLC-MS/MS analysis (Figures 4 and 5).

Group 4

androstane-3-keto and androstane-3-hydroxy steroids

Dihydrotestosterone, mestanolone, androsterone, and androstanedione were included in Group 4. All listed substances undergone to mono oxime derivatives conversion with the intensive production of $[M+H]^+$ molecular ions under positive ESI conditions. For androstanedione, we observed the formation of dioxime, but the amount of it was less than the corresponding mono oxime derivative.

The fragmentation analysis revealed non-specific mass shifts at low and high collision energies. At low and medium collision energies, we observed the presence of molecular ion or neutral losses of water molecules (18 Da and 36 Da). Increasing the collision energy produced non-specific product ions at m/z 145, m/z 133, and m/z 118 for all the oxime derivatives. For mestanolone and androsterone, common losses of 51 Da and 91 Da were evaluated and the intense of corresponding product ions were higher for androsterone at that. The characteristic losses for oxime derivatives were the same as for underivatized steroids: (−92 Da−15 Da) and (−76 Da−15 Da).

Dihydrotestosterone and androsterone were the substances with the ability to form picolinoyl derivatives. Since mestanolone has the methyl group at C-17 the conversion to corresponding picolinoyl derivative was not observed. No water losses in product ion spectra of dihydrotestosterone picolinoyl derivative were found. The most abundant product ions for derivative were at *m/z* 255; *m/z* 237; *m/z* 213; *m/z* 197; *m/z* 145; and *m/z* 124.

Since dihydrotestosterone and androsterone can be converted to both derivatives, we supposed to include both of derivatives in HPLC-MS/MS analysis due to the better signal-to-noise ratio for

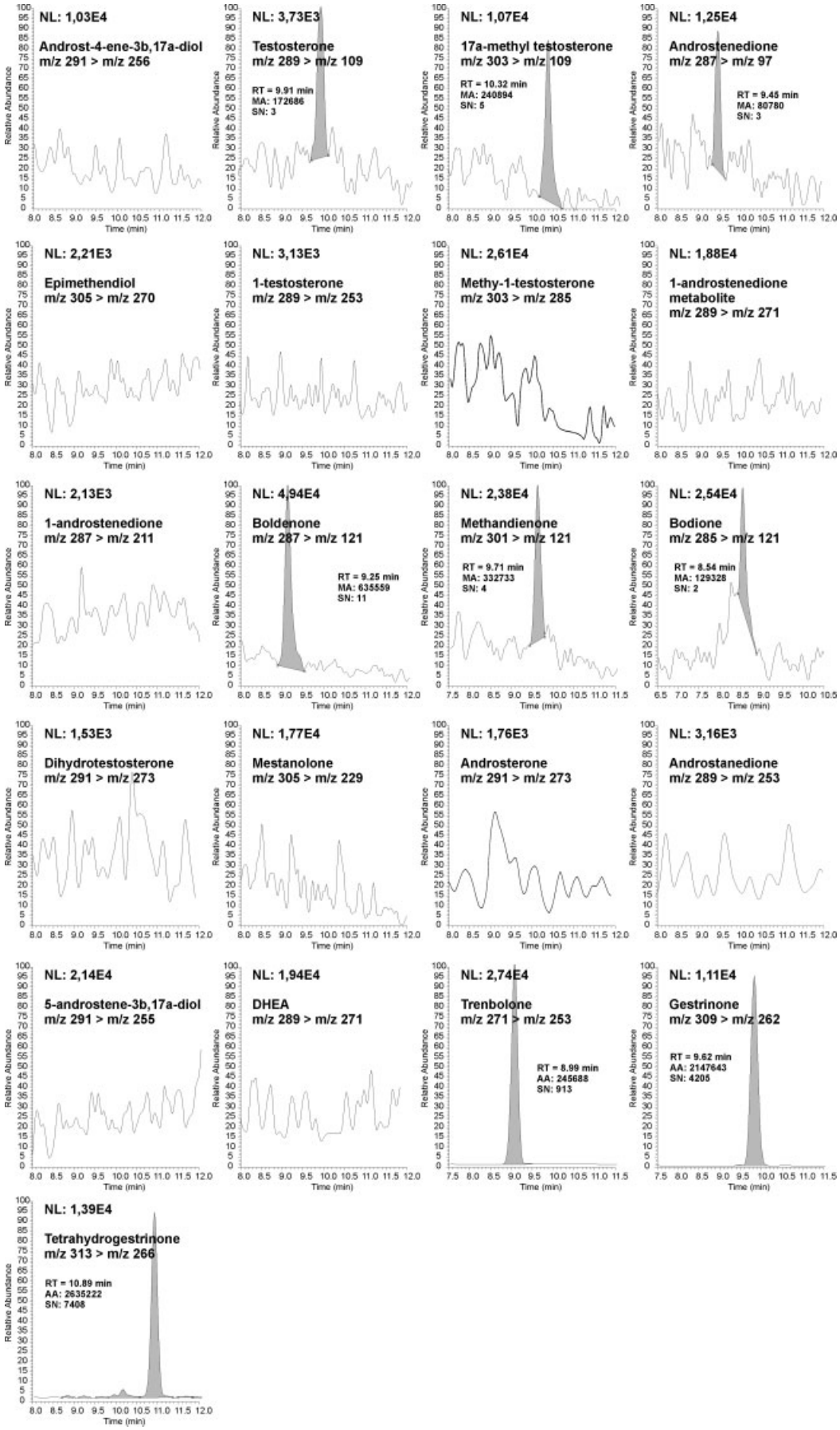

Figure 3. Selected ion chromatograms of underivatized steroids, spikes to blank urine at 1 ng/ml.

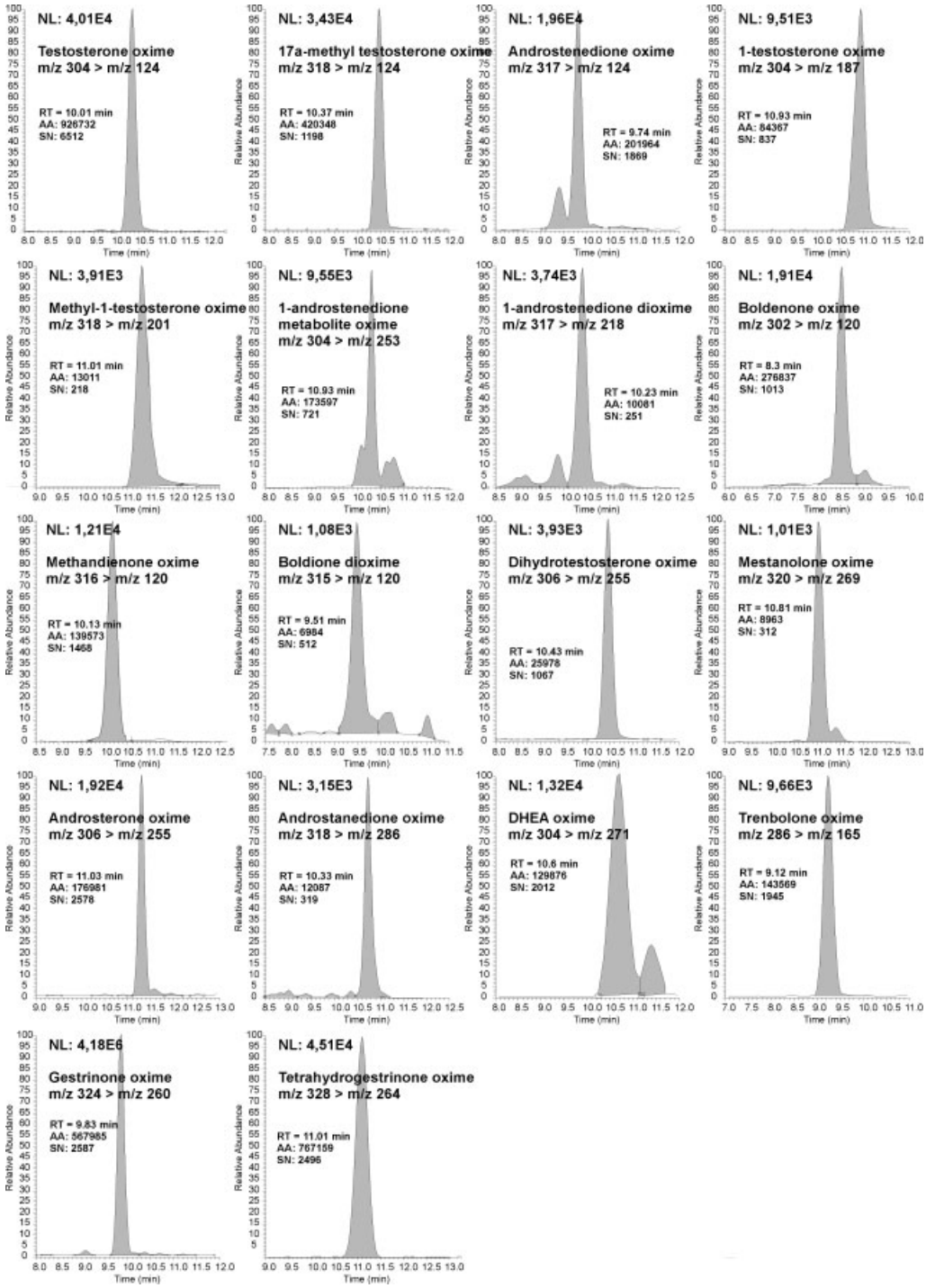

Figure 4. Selected ion chromatograms of oxime, spikes to blank urine at 1 ng/ml.

oxime and specific spectral data shifts monitoring for picolinoyl derivatives (Figures 4 and 5).

Group 5

5-ene-3-keto and 5-ene-3-hydroxy steroids

Among two listed substances in Group 5 only DHEA was converted to the corresponding oxime derivative. Product ions at *m/z* 286 and *m/z* 271 related to the water losses were obtained at low collision energy. At high and medium collision energies (30–60 eV) the corresponding abundant ions at *m/z* 197; *m/z* 171; *m/z* 157; and *m/z* 131 were observed.

During the derivatization procedures, both of the represented substances had undergone the picolinoyl conversion. DHEA was converted to mono picolinoyl derivative while 5-androstene-3β, 17α-diol showed an ability to both mono and dipicolinoyl production with the prevalence to dipicolinoyl derivative. Common mass shifts of 123 Da and 141 Da were observed at low and medium collision energies with corresponding ions generation at *m/z* 271 and *m/z* 253 for DHEA picolinoyl derivative and *m/z* 378

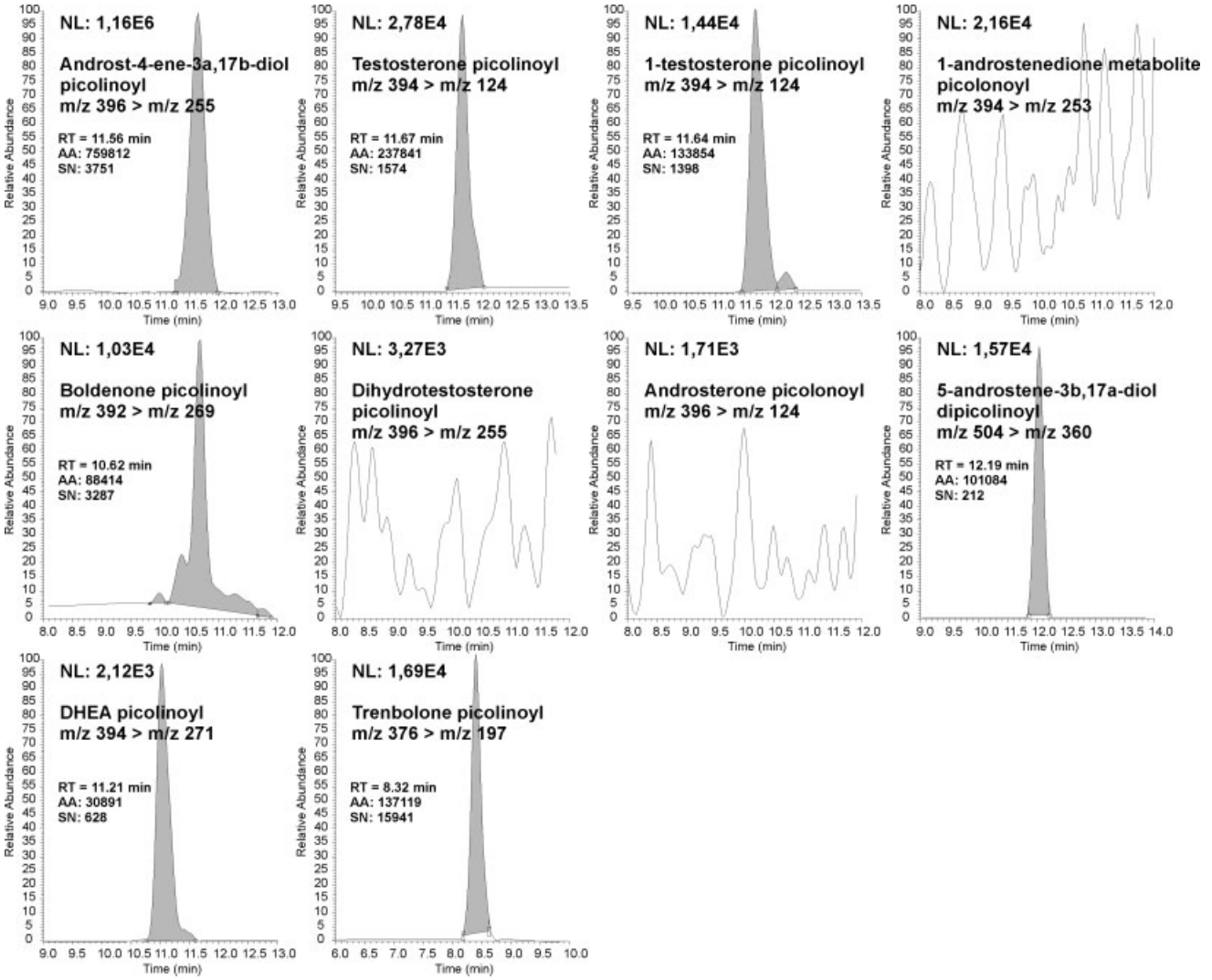

Figure 5. Selected ion chromatograms of picolinoyl derivatives, spikes to blank urine at 1 ng/ml.

and *m/z* 360 for 5-androstene-3β, 17α-diol dipicolinoyl derivative. Increase of the collision energy produced specific abundant ions for DHEA derivative at *m/z* 186, *m/z* 175, and *m/z* 161. For 5-androstene-3β, 17α-diol derivative these ions were at 255 *m/z*; *m/z* 159 and *m/z* 133. Product ions at *m/z* 124 and *m/z* 147 were observed for both derivatives.

Since DHEA can form both derivatives, we observed a one-and-a-half times higher signal-to-noise ratio for DHEA oxime derivative than for picolinoyl at the same concentrations level (Figures 4 and 5).

Group 6

4,9,11-triene-3-keto steroids

All substances represented in Group 6 formed oxime derivatives. At low and medium collision energies, ions corresponding to water losses (18 Da and 36 Da) and neutral 64 Da losses were observed. At higher collision energy, all product ion spectra included ions at m/z 181, m/z 167, m/z 155, and m/z 141.

Due to the absence of functional groups at C-17 position, only trenbolone was converted to picolinoyl derivative. The fragmentation analysis of derivative at low and medium collision energies revealed the formation of abundant protonated molecular ion $[M+H]^+$ and a loss of 123 Da with the generation of abundant product ion at *m/z* 253. At higher collision energies, ions at *m/z* 197; *m/z* 169; *m/z* 155; *m/z* 141; and *m/z* 129 were observed.

The signal-to-noise ratio of trenbolone picolinoyl derivative was eight times higher than for trenbolone oxime derivative, so it was included into final HPLC-MS/MS analysis (Figures 4 and 5).

Conclusion

Two different derivatization procedures for anabolic steroids were evaluated and compared. Due to the laborious sample preparation, we suggest to use the performed strategy for confirmation analysis purposes, metabolic studies, or for the identification of new steroids or steroid-like substances. The use of derivatization procedures made it possible to determine anabolic steroids at the minimum required performance levels and avoid the reduction of ionization process. However, the method presented in this work did not allow the formation of suitable derivatives for all represented substances at the same time, so the use of these approaches as screening methods for anabolic steroids is still limited.

Among 21 compounds under investigation, only 9 compounds were analyzed in underivatized form at 1 ng/ml concentration spiked to blank urine (Figure 3). Analytes with 3-keto group and/or 17-keto group underwent oximes formation successfully (mono oximes or dioximes), while the processes for picolinoyl formation are not still clear. For example, the presence hydroxy group and different subsistents at C-17 together limits the process of picolinoyl formation. Besides, abnormalities from the typical derivatives formation for epimethendiol and androsterone were observed.

Compounds with hydroxy group in the C-3 position, together with androstanedione, showed poor sensitivity for detection in human urine using HPLC-ESI-MS/MS. The highest sensitivity was obtained in case of 4-ene-3-keto oxime derivatives. The analysis of oxime derivatives revealed that the sensitivity between groups were the following: Group 1 > Group 6 > Group 3 ~ Group 5 ~ Group 4 ~ Group 2. The lowest sensitivity for oxime derivatives was observed for 1-ene-3(17)-keto steroids and androstane-3(17)-keto steroids (Figures 3–5). The limit of detection for oxime derivatives was between 0.1 and 1 ng/ml of human urine.

Picolinoyl derivatives of hydroxy steroids revealed the highest sensitivity for analytes with hydroxy group at C-17. Therefore, picolinoyl derivatives of 17-hydroxy steroids were more favourable than picolinoyl derivatives of 3-hydroxy steroids in this respect. The analysis of picolinoyl derivatives showed that the sensitivity between groups were the following: Group 1 > Group 6 > Group 2 > Group 3 > Group 4 ~ Group 5 (Figures 3–5). The limit of detection for picolinoyl derivatives was between 0.5 and 2 ng/ml in human urine.

The obtained oxime and picolinoyl derivatives provided 10–15 times higher ESI response in the HPLC-ESI-MS/MS-selected reaction monitoring (SRM) when compared to those of underivatized molecules in positive HPLC-ESI-MS/MS mode. Therefore, we succeeded in the development of a highly sensitive multi-analyte LC-ESI-MS/MS method that, in combination with analysis of underivatized steroids, is capable of detecting all these compounds at the WADA minimum required performance limit (MRPL). Moreover, for most compounds detected with LC-ESI-MS/MS the decision limit and the detection capability was 10–100 times below this MRPL value.

Metabolic pathways for novel anabolic steroids are not always clear or even studied as well as the metabolic profiles for long-living metabolites, which are essential to analyze. The implementation of derivatization methodology let us partially to overcome these problems and widen the applicability of LC-MS methods. Moreover, the implementation of evaluated strategy to other steroids or steroid-like substances is currently being assessed in our laboratory.

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