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Supported Liquid Membrane Extraction of Anabolic Androgenic Compounds in Biological Matrices and Detection by LC-ESI-MS

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ABSTRACT

A sample work-up and enrichment technique involving the use of supported liquid membrane (SLM) and detection by high performance liquid chromatography coupled to a mass spectrometer operating under positive ion electrospray mode (LC-PI-ESI-MS) has been developed for the determination of six anabolic androgenic compounds in different biomatrices; mainly urine, kidney, liver and milk. Anabolic androgenic compounds analysed included 17α-trenbolone, 17β-trenbolone, 19-nortestosterone, testosterone, 4-androstene-3,17-dione and testosterone benzoate. Several factors affecting the extraction efficiency during SLM enrichment, such as donor pH were studied. The detection limits (DL) were 0.08 μg L^{-1} for 4-androstene-3,17-dione, 0.7 ng L^{-1} 19-testosterone, 1.1 ng L^{-1} for testosterone, 0.1 ng L^{-1} for 17β-trenbolone, 1.6 ng L^{-1} for 17α-trenbolone and 0.03 μg L^{-1} for testosterone benzoate. Modification at C17 in the structures of 4-androstene-3,17-dione and testosterone benzoate affected their recoveries with SLM and explained their observed high detection limits.

KEYWORDS

Anabolic androgenic compounds, supported liquid membrane, liquid chromatography, electrospray ionization, mass spectrometry

1. Introduction

The use of supported liquid membrane (SLM)-assisted extraction as a sample preparation technique has proved to be an attractive alternative to many others due to its high selectivity and enrichment power.[1] In the recent past many publications have reported the effectiveness and the advantages of SLM in the extraction and selective enrichment of a number of ionizing organic pollutants in both natural and biological medium. Audunson first reported the use of supported liquid membranes in the determination of amines[2,3] and later the technique found applications in the enrichment of metals in natural waters.[4–6] The same technique has been used in the monitoring of benzimidazole anthelmintics, sulfonamides and macrolides in various biomatrices.[7–9] SLM has also been used successfully in the enrichment of herbicides in natural water samples.[10–12] In this work supported liquid membranes were used for the enrichment of anabolic androgenic compounds which are used in animal husbandry as growth promoters.[13–17] The same compounds are frequently abused in the sports industry because they have been shown to elevate the performance of athletes. Consequently, they have been banned in sports since the Olympic games in Montreal in 1976.[14,15,18–20] Their use in veterinary industry may lead to residues in meat and meat products, which could be harmful to the consumers. Some of the anabolic compounds are known carcinogens and prolonged ingestion of large doses could disturb the endocrine balance and hence lead to a large number of side-effects.[15] Breast enlargement[21] and precocious puberty[22] are examples of side-effects associated with these compounds. Concerns of consumer exposure to some of these residues have led many National and International health institutions, particularly in the European Union (EU) to ban their use as growth promoters.[17]

The residues of anabolic compounds in biological matrices such as animal tissue (meat), faeces, urine, animal feed, food products (milk, etc.) and plasma have been monitored by a wide variety of techniques. These include immunoassay techniques such as radioimmunoassay (RIA), enzyme immunoassay (EIA) and enzyme linked immunosorbent assay (ELISA).[23–25] Although these methods are sensitive and useful for screening purposes, they are limited to single-compound analysis and can sometimes lead to false positive results.[26] In addition to immunoassay, other methods such as thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) coupled with a variety of detection systems have been utilized for confirmatory purposes.[27–33] Trenbolone and diethylstilbestrol, for example, were analysed using HPLC with UV detection.[34–35] Trenbolone and testosterone have also been determined by HPLC with fluorescence detection.[36]

Gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) are the most commonly used techniques for the analysis of anabolic compounds.[37–44] However, both GC and GC-MS require derivatization prior to analysis due to the lack of volatility and the thermal instability of anabolic compounds.[45] Examples of GC-MS application include the monitoring of some of the anabolic hormones in muscles via pentafluoropropionyl derivatization and quantification of anabolic drugs in meat after conversion to trimethylsilyl esters by GC-EI-MS.[46,47]

Liquid chromatography on the other hand, is a more attractive method of monitoring anabolic compounds since derivatization is not a requirement.[45] However, the limitation of HPLC in the monitoring of anabolic compounds has been the lack of sensitiv-

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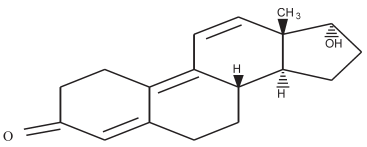
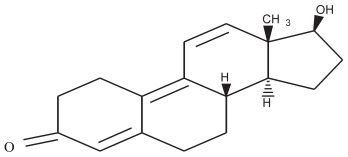
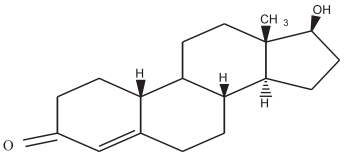
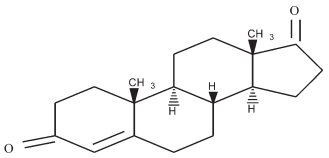
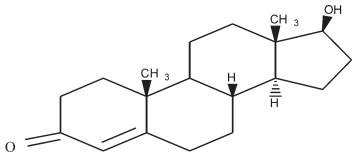
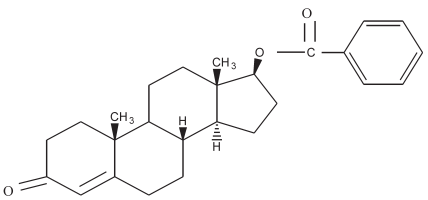
ity and selectivity of traditional LC detectors. The advent of LC-MS interfaces have, however, minimized the limitation of this approach. LC-MS methods can now offer sensitivity and selectivity similar to that of GC-MS without the need for prior derivatization. The capabilities of MS as a detector exceed all the conventional GC or LC detectors. The use of a MS detector in SIM mode (selected ion monitoring) not only adds to the selectivity but also enhances the sensitivity of the method. A sample preparation technique that has been widely reported for the clean-up and/or enrichment of anabolic androgenic steroid hormones involve the use of solid phase extraction (SPE). Most of SPE cartridges can, however, only be used once and the constant replacement of cartridges increases the cost of analysis. We report the use of supported liquid membranes in the enrichment of androgenic compounds in various biological matrices. Among the advantages of SLM are low organic solvent to feed volume ratio, low concentrations of extractants, high feed to strip ratio, low capital and operating costs, low energy requirements, a high degree of cleanup and the possibility to achieve high preconcentration factors[47–52] The theory of SLM extraction is well documented by Jönsson and Mathiasson.[50–52]

Table 1 Structures, molecular weights and CAS numbers for the six anabolic androgens compounds studied.

Compound	Structure	Mwt	CAS numbers
17α-Trenbolone		270.4	10161–34–9
17β-Trenbolone		270.4	10161–33–8
19-Nortestosterone		274.4	434–22-0
4-Androstene-3,17-dione		286.4	63–05–8
Testosterone		288.4	58–22–0
Testosterone benzoate		392.5	2088–71–3

2. Experimental

2.1. Materials and Reagents

The anabolic androgenic compounds used in this study (17α-trenbolone, 17β-trenbolone, 4-androstene-3,17-dione, 19-nortesterone, testosterone benzoate and testosterone) were all purchased from Sigma (St Louis, USA). Table 1 shows the structures, molecular weights and CAS numbers of the investigated compounds. HPLC grade methanol and diethyl ether were obtained from BDH Laboratory Supplies (Poole, UK). All organic solvents used in this work were filtered through a 0.45 μm organic membrane filter, type HVLP, Millipore (Dublin, Ireland). Ultra high purity water (UHP) was processed through a Millipore Quantum Ultrapure Ionex Gradient A10 purification system (Millipore, Molsheim-France). Aqueous solvents were further filtered through a 0.45 μm pore size cellulose nitrate membrane.

2.2. Preparation of Standard Solutions

One milligram of each of the anabolic compounds was dissolved in 1 mL of methanol to prepare an aqueous stock solution of 1000 mg L^{-1} which was diluted to prepare standard solutions of various concentrations ranging from 0.01 ng L^{-1} to 1 mg L^{-1}.

2.3. Sample Treatment with Supported Liquid Membrane (SLM)

The SLM set up (built at the Department of Analytical Chemistry, Lund University, Lund, Sweden) shown in Fig. 1 was used for all experimental work. The liquid membrane was prepared by dissolving 5% tri-n-octylphosphine oxide (TOPO) in 1:1 di-n-hexyl ether/n-undecane. The liquid membrane support was a porous PTFE membrane, type FG Millipore (Bedford, Ireland) with an average pore size of 0.2 μm, a total thickness of 175 μm of which 115 mm is polyethylene backing and a porosity of 70%. After placement of the impregnated liquid membrane in the separator, both channels were flushed with ultra high purity (UHP) water to remove excess organic solvent from the surface of the membrane. Two peristaltic pumps (Minipuls 3, Gilson, Villiers-Le-Bel, France) were used to control the flow rates of the donor and acceptor phases independently. The tubes used for pumping solutions were acid-resistant (acid-flexible) (Elkay Products, Shrewsbury, MA, USA) with an internal diameter of 1.2 mm for the donor and 0.60 mm for the acceptor. The various parts of the flow system were connected with 0.5 mm internal diameter PTFE tubing and Alex screw fittings. Sample and buffer solutions in the donor stream were combined via a PTFE T-connection and then mixed in a coil (1.0 m × 0.5 mm i.d. coiled tubing) before entering the donor channel of the membrane device. The buffer for the donor stream was a mixture of disodium hydrogen phosphate and sodium dihydrogen phosphate adjusted to pH 6.0, while the acceptor (stripping) solution was 0.4 M sulphuric acid.

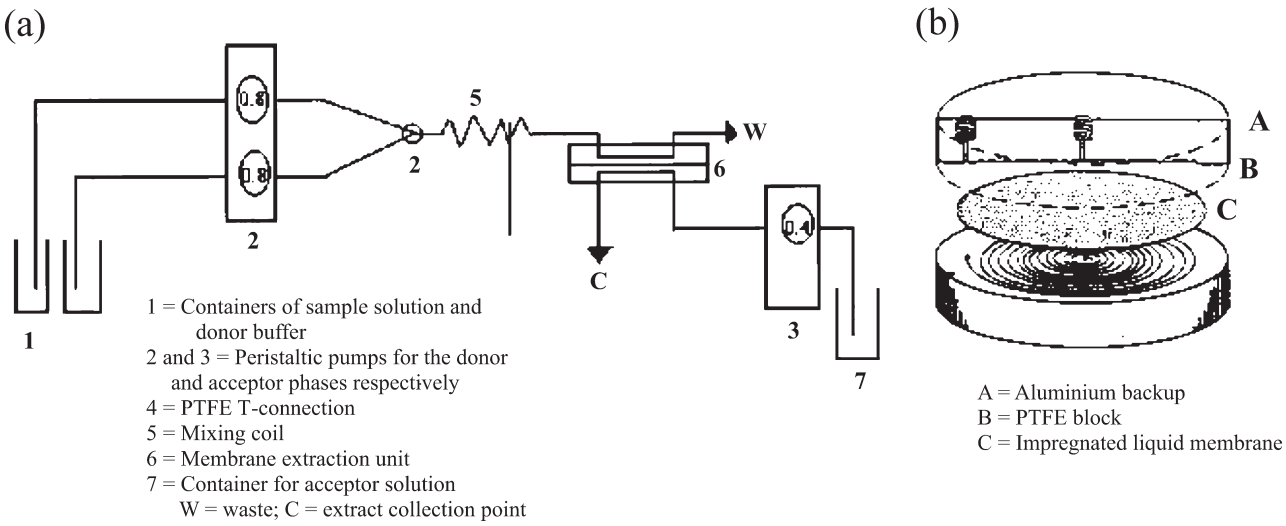


Figure 1 (**a**) Set-up of the flow system used for the liquid membrane; (**b**) membrane separator.

2.4. Preparation of Bovine Liver and Kidney Tissue Samples Spiked with Anabolic Androgens

Finely sliced liver (after removal of the gall bladder) and kidney carcasses (~20 mg) from the local abattoir were minced and spiked with known concentrations of mixtures of anabolic androgenic compounds. The concentration of the mixtures ranged from 1 ng L^{-1} to 1 mg L^{-1}. The spiked samples were homogenized using a blender. The anabolic androgenic compounds were then solvolysed in 1 mL ethyl acetate containing 2 μL of 0.5 M sulphuric acid at room temperature in a shaking incubator for 2 h. The organic phase containing the androgenic compounds was washed with UHP water and evaporated. The residues were dissolved in 1 mg of methanol-water (40:60, v/v) and then stored at 4°C until needed.

2.5. Preparation of Milk and Urine Samples Spiked with Anabolic Androgens

Aliquots of 10.0 mL of milk or urine were transferred into 25 mL centrifuge tubes and then spiked with similar concentrations of anabolic compounds as the liver and kidney tissues. The mixtures were then solvolysed in a similar manner as described for liver and kidney tissues.

2.6. HPLC of Anabolic Compounds

Samples were separated using a Hewlett Packard Series 1100 HPLC consisting of a binary pump system, a photodiode array detector (DAD), a thermostated column compartment and a vacuum degasser coupled to a Thermo electron Finnigan LCQ^{DECA} ion trap mass spectrometer. A gradient elution mode was used for separation with the following mobile phase composition: A = 100% methanol; B = 85% 25 mM acetic acid in water + 15% methanol (see Table 2). 20 μL of sample were injected into a Waters XTerra microbore C_8 (150 mm × 2.1 mm × 3.5 μm) column. The separation was performed at a flow rate of 100 μL min^{-1} and monitored by ESI-MS.

2.7. The ESI-MS of Anabolic Compounds

The samples were introduced into the electrospray ionization source by direct infusion using a Harvard Apparatus 22 syringe pump (South Natick, Massachusetts, USA). 50 μL of dissolved sample were mixed with 200 μL of buffer and the resulting solution pumped at 3–5 μL min^{-1}. 25 mM acetic acid (1:1 v/v) in water/methanol was used as an ionization buffer. The spectra were recorded on a ThermoQuest LCQ Deca quadrupole ion trap mass spectrometer (San Jose, California, USA) and processed using ThermoQuest Xcalibur software (San Jose, California, USA).

Table 2 Gradient elution for anabolic androgenic compounds

Time (min)	Flow rate μL min^{-1}	A (%)	B (%)
0	100	50	50
10	100	30	70
20	100	65	35
30	100	100	0
45	100	100	0

A = 100% methanol; B = 85% 25 mM acetic acid in water + 15% methanol.

3. Results and Discussion

The SLM system in this work used a dynamic (flowing) donor and a stagnant acceptor solution. The most important parameter in this sample pre-treatment process is the extraction efficiency (% E), which is defined as the fraction of the analyte species extracted from the donor phase via the membrane phase to the acceptor side. %E is, however, highly dependent on other system parameters such as the partition coefficient of the analyte between aqueous donor phase and membrane, the donor flow rate, the pH in both the donor and acceptor channels, the trapping capacity in the acceptor and the concentration of the carrier (e.g. a chemical extractant such as TOPO was used in this experiment). In order to ensure the maximum recovery of the analyte all the above parameters were optimized.

3.1. Optimization of the SLM System

Selectivity in the SLM extraction process of anabolic androgenic compounds was achieved by adjusting the pH of the donor and acceptor channels in order to optimize the diffusion of the uncharged analyte molecules from the donor side across the membrane to the acceptor side. The variation of donor pH with percentage extraction efficiency was therefore investigated for all six anabolic androgenic compounds. Variation of % E with pH showed that the extraction efficiency increased with an increase in pH and reached a maximum between pH 4 and 6 (Fig. 2).

At pH values above and below the optimal values, the compounds are charged and the recovery is therefore diminished (Fig. 2) while in the intermediate pH range the compounds are

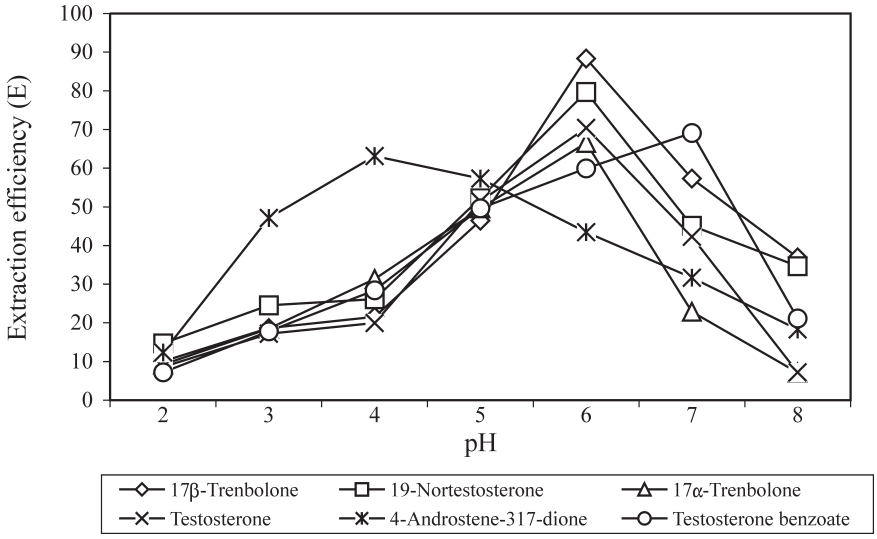


Figure 2 Effect of donor channel pH on the extraction efficiency of androgenic anabolic compounds spiked in urine. Liquid membrane composition: 5% TOPO in n-undecane/di-n-hexylether (1:1) donor channel flow rate = acceptor channel flow rate = 0.1 ml min^{-1}, acceptor = 0.4 M, analyte concentration = 0.1 $\mu g\ L^{-1}$.

neutral, resulting in better recoveries. Four of the six compounds investigated, testosterone, 17-αtrenbolone, 17-βtrenbolone and 19-nortestosterone were efficiently recovered at an optimal donor pH value of about 6.0. The other two, 4-androstene-3, 17-dione and testosterone benzoate, had their optimal extraction efficiencies at pH 4 and 7, respectively. The mixture was pumped to the SLM unit at a pH of 6.0 and therefore did not favour the efficient recovery of 4-androstene-3,17-dione and testosterone benzoate as compared to the other four compounds. This may explain the relative lower recoveries for testosterone benzoate and 4-androstene-3,17-dione compared to the rest of the compounds in the mixture. The reason for the peculiar behaviour of these two compounds in a mixture may be the replacement of the OH group at C17 with an aldehyde or an ester group. This OH group might be utilized in hydrogen bonding to the added chemical extractant (TOPO) hereby trapping the compounds. TOPO as component of the liquid membrane was found to improve the mass transfer of analytes through the liquid membrane.[47] TOPO is a known chemical extractant (carrier) which, when incorporated into the liquid membrane, facilitates the diffusion of analyte molecules through the membrane due to a reversible reaction between the analyte and the extractant molecule.[53] The two lone pairs on the oxygen atom of TOPO allow the formation of hydrogen-bonded adducts of various composition.[54] TOPO is also known to have a high solubility in organic solvents, such as the ones used as liquid membranes and at the same time is insoluble in water, a property which increases its selectivity.[55]

3.2. SLM Enrichment of Anabolic Androgens Spiked in Biological Matrices

Anabolic androgenic compounds were extracted using 5% TOPO in di-n-hexylether/n-undecane as liquid membrane. Figure 3 shows the result of extractions of 1 $\mu g\ L^{-1}$ samples of the

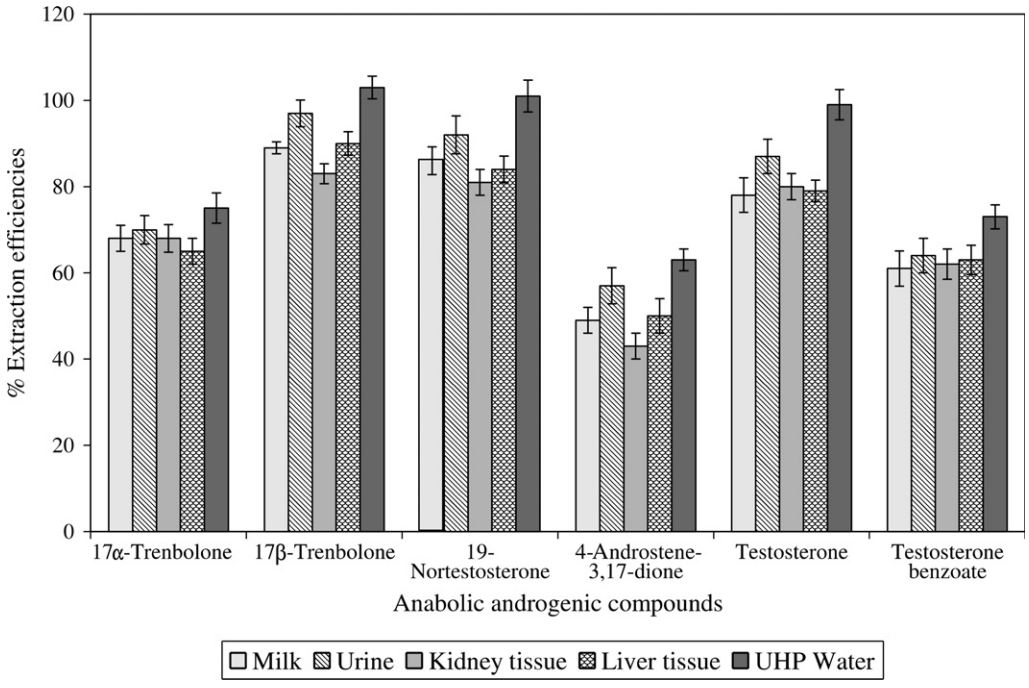


Figure 3 SLM/LC-ES-MS of 1 $\mu g\ L^{-1}$ anabolic androgenic compounds extracted from milk, urine, kidney, liver tissue and UHP water samples.

mixtures of six anabolic androgens in a variety of biological matrices, i.e. urine, milk, kidney and liver tissues and UHP water. The extraction efficiency increased with decreasing concentration of the compounds in milk. The observed lower extraction efficiency at higher concentrations was probably due to the saturation of the trapping solution in the acceptor side of the membrane. The extraction efficiency for 1 ng L^{-1} mixtures of the six compounds in milk was found to be 68, 74, 86, 89 and 61% for 17α-trenbolone, testosterone, 19-nortestosterone, 17β-trenbolone, and testosterone benzoate, respectively. 4-Androstene-3,17-dione was not quantifiable at this low concentration.

Table 3 ESI-MS/MS characteristic peaks of androgenic anabolic compounds under electrospray ionization (% relative abundance,% CID =% collision induced dissociation energy).

Anabolic compound	Molecular mass	Ion formed (mass)	Fragmentation pattern
Testosterone	288.4	289	$[M + H]^+$ (100,% CID = 0)
		311	$[M + Na]^+$ (<5,% CID = 0)
		271	$[(M + H)–H_2O]^+$ (100,% CID = 25)
		253	$[(M + H)–2H_2O]^+$ (65,% CID = 25)
Testosterone benzoate	392.5	393.5	$[M + H]^+$ (100,% CID = 0)
		416	$[M + Na]^+$ (<5,% CID = 0)
		159	$[[(M + H)–(C_6H_5CO + H_2O + CH_3)]^+$
		271	$[[(M + H)–(C_6H_5CO + H_2O)]^+$
		289	$[[(M + H)–C_6H_5CO]^+$
17α-Trenbolone	270.4	271	$[M + H]^+$
		293	$[M + Na]^+$
		253	$[(M + H)\ H_2O]^+$ (~5,% CID = 0)
17β-Trenbolone	270.4	271	$[M + H]^+$ (55,% CID = 0)
		293	$[M + Na]^+$ (100,% CID = 0)
		253	$[(M + H)–2H_2O]^+$
19-Nortestosterone	274.4	275.4	$[M + H]^+$ (100,% CID = 0)
		257.4	$[(M + H)–H_2O]^+$ (80,% CID = 30)
		239.4	$[(M + H)–2H_2O]^+$ (100,% CID = 30)
4-Androstene-3,17-dione	286.4	287.4	$[M + H]^+$ (100,% CID = 20)
		269.4	$[(M + H)–H_2O]^+$ (23,% CID = 20)
		251.4	$[(M + H)–2H_2O]^+$ (10,% CID = 20)
		309.8	$[M + Na]^+$

3.3. Electrospray Ionization-Mass Spectrometry (ESI-MS)

The initial part of the study was to establish whether the investigated compounds were ionizable under atmospheric pressure ionization (API) conditions and therefore ESI-MS active. Table 3 gives a summary of the fragmentation of the six compounds under ESI-MS and ESI-MS/MS conditions; the percentage relative abundance (% RA) and the percentage collision induced dissociation (% CID) energy used to generate the fragment is given in brackets. For all anabolic androgenic compounds the protonated parent ion $[M + H]^+$ was observed under electrospray ionization conditions. In the case of 17β-trenbolone both protonated and sodiated (sodium adduct) ions ($[M + H]^+$ and $[M + Na]^+$) were observed. Under ESI-MS/MS conditions 19 nortestosterone showed three distinct peaks at *m/z* 239, 257 and 275, which corresponded to $[(M + H)–2H_2O]^+$, $[(M + H)–H_2O]^+$ and $[M + H]^+$, respectively, and were observed by varying the percentage fragmentation energy from 20 to 35%. Testosterone was found not to ionize as well as the rest of the compounds in the mixture. It ionized better under APCI (atmospheric pressure chemical ionization) conditions. Since the majority of the compounds in this study were, however, monitored under ESI conditions we decided to monitor testosterone under the same conditions. The objective of the ESI-MS studies was to generate a mass spectral database for the individual compounds, which could be used for selected ion monitoring (SIM).

3.4. LC-MS separation and detection of anabolic androgens

All six anabolic compounds were separated on a Waters XTerra C_8 reversed-phase microbore column and detected with the electrospray ionization-quadrupole ion trap mass spectrometer (Fig. 4). The addition of acetic acid as a mobile phase additive not only improved the separation but was also useful to enhance ionization. Differences in polarity between these compounds were exploited in their separation on a gradient HPLC system.

Several experiments were carried in which the solvent composition was varied to determine the optimum separation method. Most solvent mixtures were unsuitable with respect to resolution and separation time. A gradient elution based on two solvent systems, A and B, where A = 100% methanol and B = 15% methanol + 85% 25 mM acetic acid (Table 2) was eventually found to give the best separation for the mixture of six anabolic androgenic compounds (Fig. 4).

3.5. Detection Limits (DLs)

The DLs were calculated as the analyte concentration giving a signal equal to the blank signal (yB) plus three standard deviations (for $n = 5$) of the blank (sB), that is, yB + 3sB. Most of the anabolic compounds studied in this work are controlled substances for which no MRL values exist. This means that they are banned by the EU and therefore a new analytical method should be sensitive enough to monitor the presence of very low concentrations in a variety of matrices. The detection limits obtained in this work were comparable or better than those reported for other methods (Table 4).

3.6. Precision and Accuracy

Bovine urine was spiked with anabolic androgens to give samples with concentrations ranging from 1 ng L^{-1} to 1 mg L^{-1}. Five replicates were prepared and enriched using SLM and detected with LC-ESI-MS. Table 5 shows the results of 19-nortestosterone spiked in urine. Precision was determined as the percentage relative standard deviation (% RSD) which for this compound (19-nortestosterone) ranged from 0.96 to 4.64. The other compounds gave also acceptable values of % RSD. Accuracy was determined as the percentage ratio of the amount recovered to the amount spiked and was found to range from 92–97%.

3.7. Linearity

The linearity of the SLM extraction method was investigated experimentally by injecting standard solutions of different concentrations ranging from 0.1 ng L^{-1} to 1 mg L^{-1} into water or another matrix and then recovering the standard using SLM and LC-MS. The obtained correlation coefficients (which were in the

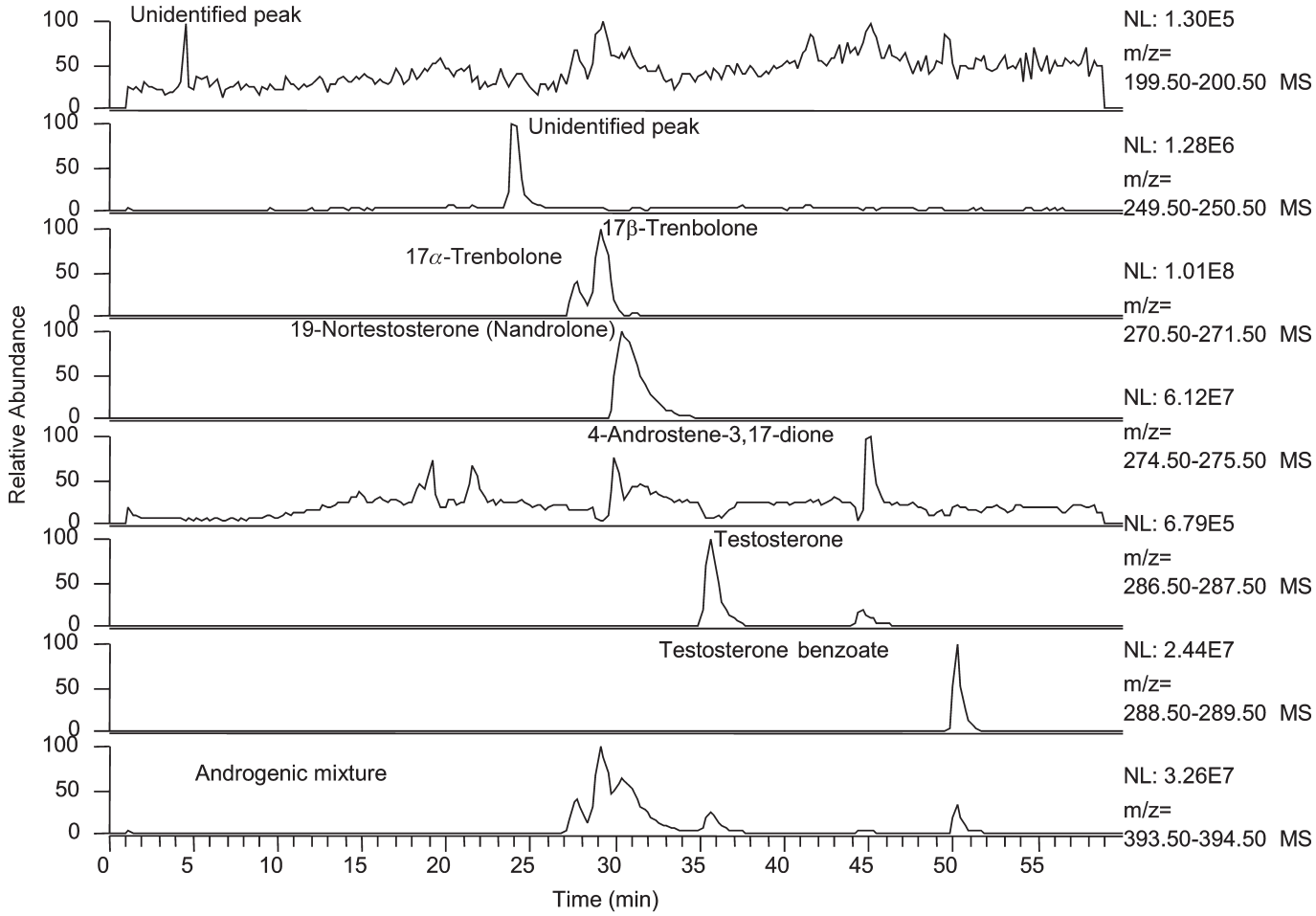


Figure 4 SIM-LC-ESI-MS chromatogram of anabolic androgens compounds mixture of 0.1 mg L^{-1} concentrations extracted from urine.

Table 4 Detection limits for androgenic anabolic steroid hormones.

Compound	Detection limits (DLs)					
	Urine		Liver and kidney tissues		Milk	
	This work	Literature	This work	Literature	This work	Literature
4-Androstene-3,17-dione	0.08 μg L^{-1}	0.5 μg L^{-1} (60) *	0.44 μg L^{-1}	0.02–0.1 mug kg^{-1} (57); 2 μg L^{-1} (61)	3.16 μg L^{-1}	1 μg L^{-1} (61)
19-Nortestosterone	0.70 ng L^{-1}		1.60 ng L^{-1}		4.10 ng L^{-1}	
Testosterone	1.10 ng L^{-1}	0.5 μg L^{-1} (60)	2.71 ng L^{-1}	0.02–0.1 mug kg^{-1} (57); 0.1–0.4 mug kg^{-1} (58); 2 μg L^{-1} (61)	7.30 ng L^{-1}	1 μg L^{-1} (61)
17β-Trenbolone	0.10 ng L^{-1}	0.3 μg L^{-1} (56)	1.30 ng L^{-1}	0.02–0.1 mug kg^{-1} (59); 2 μg L^{-1} (61)	4.15 ng L^{-1}	1 μg L^{-1} (61)
17α-Trenbolone	1.60 ng L^{-1}		3.62 ng L^{-1}	0.02–0.1 mug kg^{-1} (59); 2 μg L^{-1} (61)	9.25 ng L^{-1}	
Testosterone benzoate	0.03 μg L^{-1}		0.22 μg L^{-1}		5.50 μg L^{-1}	

* Values in brackets refers to references cited.

order of 0.99) and *y*-intercepts of the linear regression curve in a response *versus* concentration plot showed acceptable linearity.

4. Conclusions

This work has demonstrated the potential of combining SLM and LC-MS as a monitoring technique for veterinary anabolic androgenic compounds. A mixture of anabolic androgenic compounds was successfully separated using a Waters XTerra C_8 microbore column and gradient elution. The presence of buffers was crucial in achieving a good resolution and a reduction in analysis time. The addition of 25 mM acetic acid was found to be the most effective in achieving good separations of anabolic androgenic compounds.

The presented method (SLM-LC-ESI-MS) offers the possibility of monitoring the anabolic androgenic compounds directly without derivatization as necessary in the case of GC-MS. The application of the new method was demonstrated by analysing anabolic androgenic compounds in various biological matrices including urine, milk, kidney and liver tissue.

The ESI-MS spectra provided valuable information for selected ion monitoring (SIM). It was observed that most of the anabolic compounds in this study, had a high affinity for hydrogen and sodium, thereby forming quasimolecular ions of the corresponding species, i.e. $[M + H]^+$ and $[M + Na]^+$, respectively. Other compound characteristic fragments were also identified.

Table 5 Method precision and accuracy study for 19-nortestosterone in bovine urine matrix.

Concentration of 19-nortestosterone added to bovine urine matrix	Precision, measured as RSD; for $n = 5$	% ratio of the amount found to the amount added; for $n = 5$. This gives a measure for accuracy
1 mg L^{-1}	0.96	93.6
0.1 mg L^{-1}	1.02	93.8
0.01 mg L^{-1}	1.12	95.1
1 μg L^{-1}	1.28	95.7
0.1 μg L^{-1}	1.32	96.3
0.01 μg L^{-1}	1.94	96.8
1 ng L^{-1}	4.64	97.2

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