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Detection of main urinary metabolites of β_2-agonists clenbuterol, salbutamol and terbutaline by liquid chromatography high resolution mass spectrometry

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ABSTRACT

Clenbuterol, terbutaline and salbutamol are B_2-agonists drugs included in the list of banned substances of the World Anti Doping Agency (WADA) prohibited in and out of competition. In this article, the excretion of urinary metabolites of clenbuterol, terbutaline and salbutamol have been studied using liquid chromatography electrospray time-of-flight mass spectrometry (LC-TOFMS), after a single therapeutic dose administration in rats. Urine collected was processed with solid-phase extraction prior to LC-TOFMS analyses using electrospray in the positive ion mode and pseudo MS/MS experiments from in-source collision induced dissociation (CID) fragmentation (without precursor ion isolation). The strategy applied for the identification of metabolites was based on the search of typical biotransformations with their corresponding accurate mass shift and the use of common diagnostic fragment ions from the parent drugs. The approach was satisfactory applied, achieving the identification of 11 metabolites (5 from clenbuterol, 4 from salbutamol and 3 from terbutaline), 4 of them not previously reported in urine. Novel metabolites identified in rat urine included N-oxide-salbutamol, hydroxy-salbutamol, methoxy-salbutamol glucuronide and terbutaline N-oxide, which are all reported here for the first time.

1. Introduction

β-Agonists are widely used for the treatment of respiratory diseases. Amongst them, clenbuterol (CL) is the most effective from the class [1] and has been used in sports as an anabolic agent because of its stimulation of the central nervous system and as a growth-promoting agent, so it is included in the list of prohibited substances published by the World Anti-Doping Agency (WADA) [2]. Salbutamol, [2-(tert-butylamino)-1-(4-hydroxy-3-hydroxy methylphenyl) ethanol], also known as albuterol, is a relatively selective β_2-adrenergic bronchodilator commonly used as an aerosol for relieving the acute symptoms of asthma [3]. The list of prohibited substances in sports published by the World Antidoping Agency (WADA) specifies that the use of salbutamol is only permitted by inhalation [4,5]. The administration by the oral or parenteral route or the administration of very large inhaled doses is forbidden due to a strong adrenergic stimulatory effect and an anabolic-like effect [6,7]. Salbutamol is excreted in urine as a mixture of the unchanged drug and its conjugate metabolites, mainly sulphate, being the percentage of glucuronide of salbutamol in urine negligible [8–10]. Terbutaline (5-[2-(*tert*-butylamino)-1-hydroxyethyl]benzene-1,3-diol) is a selective fast-acting β2-adrenergic agonist. It has marketing authorization for use by injection, inhalation or oral dosage for the treatment of obstructive pulmonary diseases and as a short-term asthma treatment [11]. Terbutaline can be misused in sports and therefore appears on the prohibited list of the World Anti-doping Agency (WADA) for use in and out of competition [2]. Following oral administration, one-third to one-half of the dose is excreted in the urine, whereas after intravenous and subcutaneous administration more than 90% is eliminated by this route [12,13]. Metabolism studies of terbutaline in man revealed a sulphate conjugate as the main metabolite in urine beside the unchanged drug and a glucuronidated conjugate which was detected as minor component [11].

Gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–mass spectrometry (LC–MS) are the two techniques approved by the WADA in doping control [5]. Both the chromatographic retention time and the diagnostic mass spectrometric ions are required for the identification of a compound [14–16]. However, GC–MS may require additional derivatisation steps to enhance the volatility of analytes, which might produce

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interferences and consequently make more difficult quantitation. In contrast, LC–MS has become one of the standard techniques in doping control analysis over the last decade. Several analyzers, such as ion trap, triple quadrupole, time-of-flight or Orbitrap have been used for the urinary detection of several prohibited substances such as corticosteroids, diuretics, B_2-agonists or β-blockers [17–19]. Besides its importance for sensitive, specific, fast and comprehensive doping control analyses, LC–MS has proved particularly useful and versatile for the identification and characterization of metabolic products derived from prohibited substances. In particular, the increasing availability of high resolution/high accuracy mass spectrometry combined with MS/MS or MS^n experiments provide valuable information to help the assignment of structures to tentative metabolites [20,21]. In this sense, the discovery of new long-term metabolites can increase the retrospectivity of the analysis and therefore their inclusion in screening methods represents a valuable contribution for doping control laboratories.

In this article, the excretion of urinary metabolites of β_2-agonists drugs clenbuterol, terbutaline and salbutamol have been studied in rats after a single dose administration using liquid chromatography time-of-flight mass spectrometry (LC-TOFMS). For identification of the metabolites, urine collected was processed with solid-phase extraction prior to LC-TOFMS analyses using electrospray in the positive ion mode and pseudo MS/MS experiments from in-source collision induced dissociation (CID) fragmentation (without precursor ion isolation). The strategy applied for the identification of metabolites was based on the search of typical biotransformations with their corresponding accurate mass shift and the use of common diagnostic fragment ions from the parent drugs.

2. Experimental

2.1. Chemicals and reagents

Clenbuterol and terbutaline analytical standards were purchased from Sigma–Aldrich (Madrid, Spain). Salbutamol analytical standard was obtained from Dr. Ehrenstorfer (Madrid, Spain). HPLC grade acetonitrile and methanol were acquired from Sigma-Aldrich (Madrid, Spain). Formic acid and ammonium formate were obtained from Fluka (Madrid, Spain). A Milli-Q-Plus ultra-pure water system from Millipore (Milford, MA, USA) was used throughout the study to obtain the HPLC water used.

2.2. Sample collection

The study was performed on adult male Wistar rats (250–300 g) (Charles River Laboratories, Barcelona, Spain). The animals were weighed and placed in individual metabolic cages 48 h prior to treatment to acclimatize them to this environment, maintained under standard conditions of light and temperature and allowed *ad libitum* access to food and water to the end of the experiment. All the procedures followed the Spanish guidelines on the use of animals for research (RD 1201/2005) [22] and were approved by the institutional Committee for Ethics. Groups of three rats were treated with clenbuterol (1 mg kg^{-1} body weight, intraperitoneal), salbutamol (12 mg kg^{-1} body weight, intraperitoneal), and terbutaline (11 mg kg^{-1} body weight, intraperitoneal). The vehicle solution for the three drugs was saline solution. After drug administration, urine was daily collected in graduate cylinders for 3 days (24, 48 and 72 h). The urine collected 24 h prior to treatment was used as control.

2.3. Sample treatment

2 mL of urine were diluted with 2 mL of formic acid/formate pH 2.6 buffer and then was passed through a polymeric Bond Elut PLEXA™ SPE cartridge from Agilent Technologies (Santa Clara, CA) and then washed with H_2O/methanol (95:5, v/v). The cartridges were previously conditioned with 4 mL of methanol/acetonitrile (50:50, v/v) and 4 mL of MilliQ water. The analytes were eluted from the cartridge with 4 mL of methanol/acetonitrile (1:1) and the extract was evaporated to near dryness with a TurboVap LV (Caliper LifeSciences, Hopkinton, MA) and then reconstituted with 0.5 mL of the initial mobile phase to achieve a preconcentration factor of 4:1. The extract was filtered through a 0.22 μm PTFE syringe filter and transferred to a 2-mL glass vial prior to LC–MS analyses.

2.4. Liquid chromatography electrospray time-of-flight mass spectrometry

The chromatographic separation was performed using a HPLC system (Agilent 1200 series, Agilent Technologies, Santa Clara, CA) equipped with a reversed-phase Zorbax XDB-C_{18} analytical column of 4.6 × 50 mm and 1.8 μm particle size (Agilent Technologies, Santa Clara, CA). Mobile phases A and B were water with 0.1% (v/v) formic acid and acetonitrile. The chromatographic method held the initial mobile phase composition (10% B) constant for 3 min (min), followed by a linear gradient to 100% B up to 15 min and kept for 3 min at 100%. The flow rate used was 0.5 mL min^{-1} and 20 μL of the urine extract were injected in each run. The outlet of the liquid chromatography system was connected to an Agilent 6220 accurate mass TOF (Agilent Technologies, Santa Clara, CA) equipped with an electrospray interface operated in the positive ionization mode, using the following operation parameters: capillary voltage: 4000 V; nebulizer pressure: 40 psig; drying gas flow rate: 9.0 L min^{-1}; gas temperature: 325 °C; skimmer voltage: 65 V; octapole 1 rf: 250 V; fragmentor voltage (in-source CID fragmentation): 160–350 V. LC–MS accurate mass spectra were recorded across the range *m/z* 50–1000. The instrument performed continuous accurate-mass calibration using a second sprayer with a reference solution containing purine (*m/z* 121.0509) and hexakis-(1H,1H,3H-tetrafluoropropoxy)phosphazine (HP-921) ($C_{18}H_{18}O_6N_3P_3F_{24}$, at *m/z* 922.009798). The instrument was operated in the 4-GHz high resolution mode, providing a typical resolution of *ca.* 10,000 at *m/z* 118 and *ca.* 18,000 at *m/z* 1522. The full-scan data were recorded with Agilent Mass Hunter Data Acquisition software (version B.04.00) and processed with Agilent Mass Hunter Qualitative Analysis software (version B.04.00).

3. Results and discussion

3.1. Identification of clenbuterol and its urinary metabolites

The identification and confirmation of clenbuterol was performed by LC-TOFMS accurate mass measurements and retention time matching with a standard (8.1 min). For confirmation purposes and the subsequent metabolite search based on diagnostic ions, in-source collision induced dissociation (CID) fragmentation of clenbuterol was performed, being the main fragment ions detected with *m/z* 259.0763 (neutral loss of water), *m/z* 203.0137, *m/z* 168.0449, *m/z* 132.0682 and *m/z* 104.0499. Five clenbuterol urinary metabolites were identified in the rat urine samples (CL-M1–CL-M5) as summarized in Table 1. An extracted ion chromatogram (EIC) and the mass spectrum of each metabolite detected are shown in Fig. 1. These include the conjugation with sulphate (CL-M1) and glucuronic acid (CL-M3), the hydroxylation of clenbuterol (CL-M2), the oxidative cleavage of the side chain (CL-M4), and the oxidation of the amine group in the aromatic ring (CL-M5). All these findings are consistent with previous literature available on urinary metabolism of clenbuterol [23–28] (see Supplementary data, Table S1). All compounds were identified by accurate mass

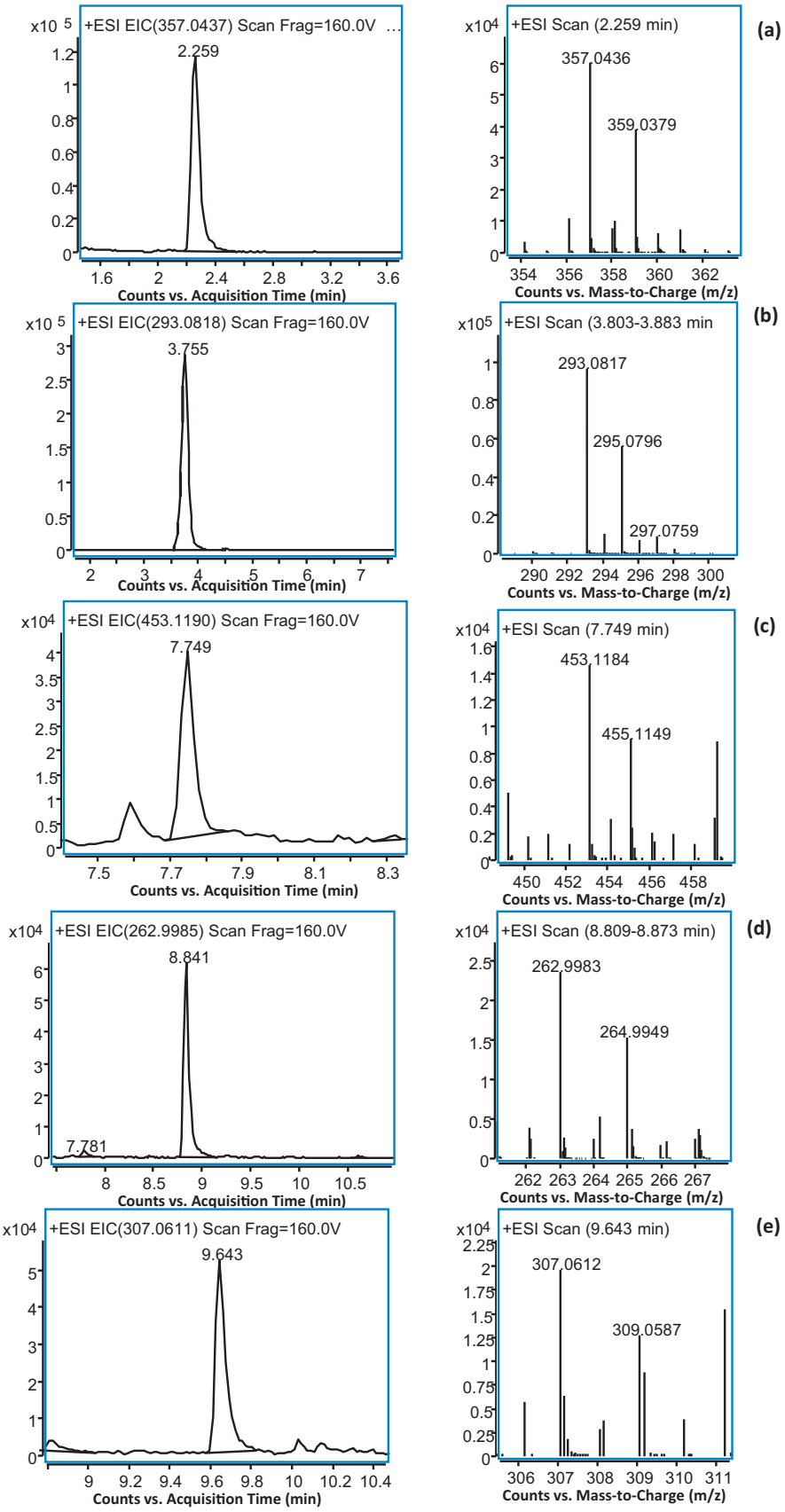

Fig. 1. Extracted ion chromatograms (EICs) (left) and accurate mass spectra (right) of clenbuterol identified metabolites: (a) CL-M1, (b) CL-M2, (c) CL-M3, (d) CL-M4, and (e) CL-M5.

Table 1
Summary of clenbuterol, salbutamol and terbutaline identified metabolites in rat urine with liquid chromatography time-of-flight mass spectrometry.

Metabolite	Retention time (min)	Experimental *m/z*	Theoretical *m/z*	Error		Proposed formula (M)
				mDa	ppm	
Clenbuterol	8.14	277.0874	277.0869	0.50	1.80	$C_{12}H_{18}Cl_2N_2O$
CL-M1	2.26	357.0436	357.0437	−0.08	−0.22	$C_{12}H_{18}Cl_2N_2O_4S$
CL-M2	3.76	293.0817	293.0818	−0.11	−0.38	$C_{12}H_{18}Cl_2N_2O_2$
CL-M3	7.75	453.1184	453.1190	−0.59	−1.29	$C_{18}H_{26}Cl_2N_2O_7$
CL-M4	8.84	262.9983	262.9985	−0.21	−0.66	$C_9H_8Cl_2N_2O_3$
CL-M5	9.64	307.0612	307.0611	0.09	0.08	$C_{12}H_{16}Cl_2N_2O_3$
Salbutamol	1.65	240.1598	240.1594	0.40	1.67	$C_{13}H_{21}NO_3$
S-M1	1.34	416.1918	416.1915	0.33	0.70	$C_{19}H_{29}NO_9$
S-M2	1.40	446.2020	446.2021	−0.12	−0.16	$C_{20}H_{31}NO_{10}$
S-M3	1.46	256.1537	256.1543	−0.65	−2.49	$C_{13}H_{21}NO_4$
S-M4	3.67	254.1388	254.1387	0.14	0.46	$C_{13}H_{19}NO_4$
Terbutaline	1.72	226.1440	226.1438	0.20	0.88	$C_{12}H_{19}NO_3$
T-M1	1.34	402.1755	402.1759	−0.39	−0.89	$C_{18}H_{27}NO_9$
T-M2	3.12	242.1390	242.1387	0.32	1.31	$C_{12}H_{19}NO_4$

measurements of the intact molecules and diagnostic fragment ions when available. None of these compounds were detected in blank urine samples obtained from the same animals before the treatment with clenbuterol.

Metabolite CL-M1 was identified as the sulphate conjugate of clenbuterol. This biotransformation has an associated mass shift of +79.9568 Da, corresponding to the addition of SO_3 generating a derivative with *m/z* 357.0437, detected at 2.26 min. According to the literature [23], the conjugation with the sulphate group is produced in the primary amine group attached to the aromatic ring, as shown in the structure proposed in Fig. 2. In-source CID fragmentation experiments for this metabolite showed characteristic fragments at *m/z* 277.0869 (loss of sulphate), 259.0764, 203.0139, 168.0449, 132.0682 and 104.0499, all of them being common to clenbuterol in-source diagnostic fragments.

The addition of a hydroxyl group to clenbuterol yielded CL-M2 (RT 3.76 min) with *m/z* 293.0818 corresponding to a mass shift of +15.9949 Da (addition of oxygen). Besides the neutral loss of water (*m/z* 277), in-source CID fragmentation of CL-M2 showed fragment ions at *m/z* 219.0086 ($C_8H_9N_2OCl_2$, from subsequent loss of *tert*-butyl moiety) and *m/z* 202.0059 (odd electron fragment ion with $C_8H_8N_2Cl_2$, corresponding to the radical loss of remaining hydroxyl group), both ions consistent with electrospray data reported by Zalko et al. [27]. According to literature [23,24], this hydroxylation typically occurs in terminal primary amine group attached to the aromatic ring as shown in Fig. 2. Conjugation with glucuronic acid generated metabolite CL-M3, with *m/z* 453.119 and retention time 7.75 min, with an associated mass shift of +176.0321 Da (addition of $C_6H_8O_6$). Glucuronide conjugated typically are formed in the hydroxyl group [25], as shown in Fig. 2. CL-M4 (*m/z* 262.9985, RT

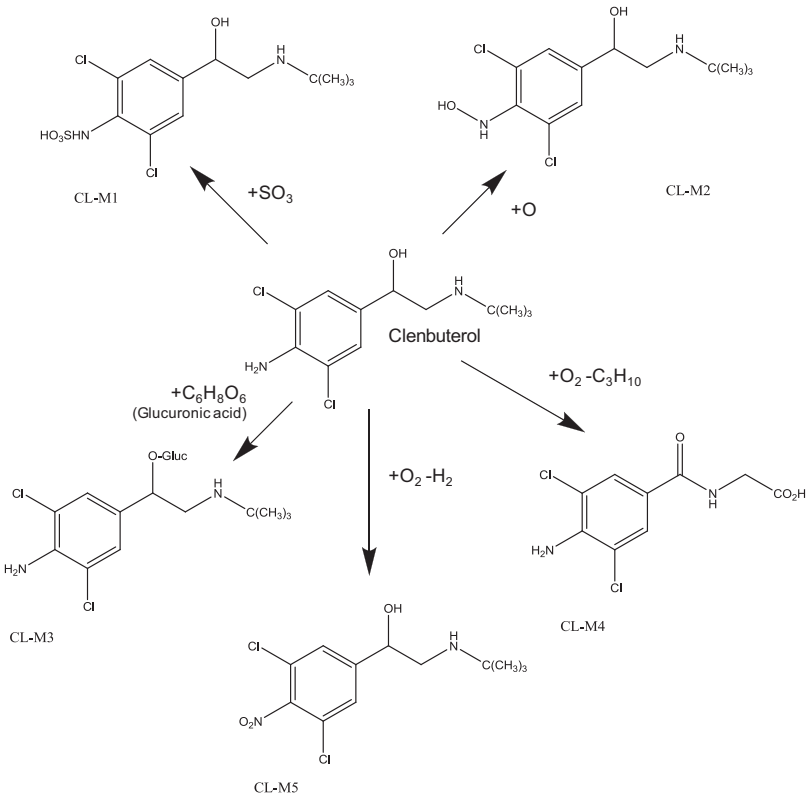

Fig. 2. Proposed structures of clenbuterol identified metabolites.

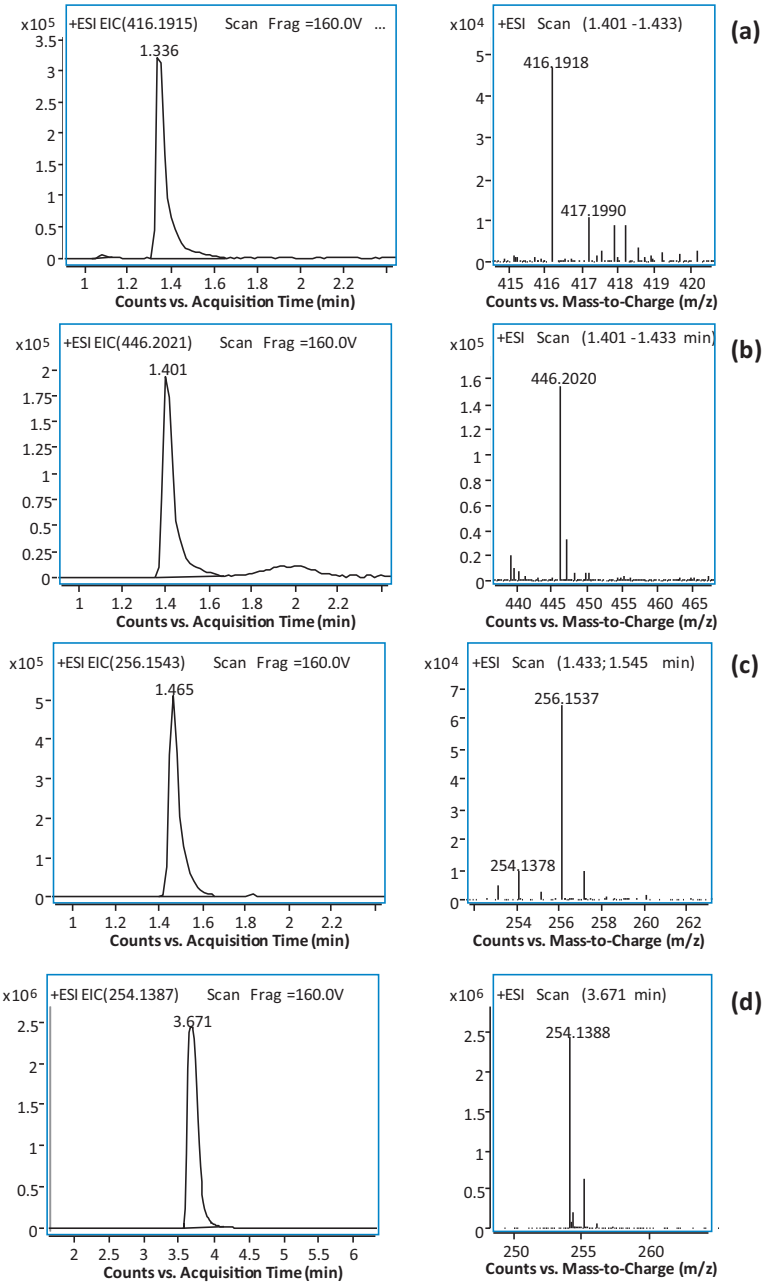

Fig. 3. EICs (left) and accurate mass spectra (right) of salbutamol identified metabolites: (a) S-M1, (b) S-M2, (c) S-M3, and (d) S-M4.

8.84 min) [4-amino-3,5-dichlorohippuric acid (ADHA)] is formed *via* a complex mechanism of oxidative cleavage of the side chain of clenbuterol molecule described in literature [23,28] (Fig. 2) which implies three intermediates ((4-amino-3,5-dichlororomandelic acid (ADMA), 4-amino-3,5-dichlororo benzoic acid (ADBA) and 2-(4-amino-3,5-dichlororophenyl)-2-oxoacetic acid (ADOA)) that were not detected, probably not retained on the solid-phase extraction cartridge. Fragmentation experiments were not possible to be performed due to the relatively low abundance of this metabolite. Finally, the oxidation of CL-M2 (hydroxylamine clenbuterol) led to CL-M5, the nitro-derivative of clenbuterol, with *m/z* 307.0611, detected at 9.64 min. Structure of this metabolite was previously reported [23,24,26].

According to the relative intensities of the metabolites detected (see Supplementary data, Fig. S1), the more abundant metabolite was CL-M2 (hydroxylamine-clenbuterol) followed by CL-M1 (sulphate conjugate). Therefore, in the case of clenbuterol, the metabolites detected were not particularly relevant in terms of relative intensity compared to the parent drugs. However, in some cases (CL-M1, CL-M3 and CL-M5) their presence over the course of time after administration is longer than the parent drug as shown in Fig. S1 (Supplementary data). These three metabolites were detected in urine after 48 h, this being interesting from the point of view of the retrospectivity of the test.

3.2. *Identification of salbutamol and its urinary metabolites*

In addition to the parent drug detected at 1.65 min (confirmed with standards), four salbutamol urinary metabolites were identified in the rat urine samples (S-M1–S-M4). They are summarized in Table 1 and their EICs with the corresponding mass spectra shown in Fig. 3. These include the conjugation glucuronic acid (S-M1), the

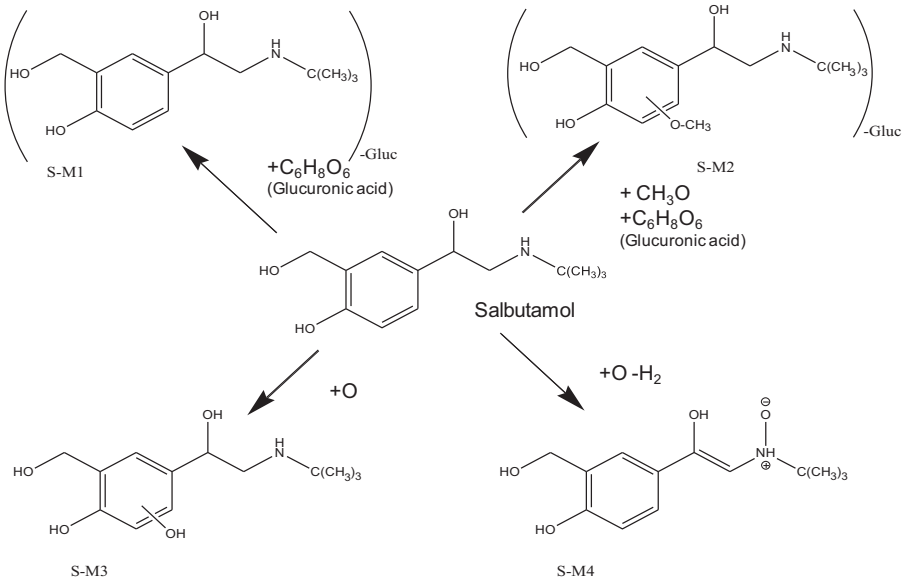

Fig. 4. Proposed structures of salbutamol identified metabolites.

combined addition of a methoxyl group and glucuronic acid (S-M2), the addition of a hydroxyl group (S-M3), and the formation of an unknown metabolite (S-M4) whose tentative structure is shown in Fig. 4. All compounds were identified by accurate mass measurements of the intact molecules and diagnostic fragment ions and none of these compounds were detected in blank urine samples obtained from the same animals before the treatment with salbutamol.

The conjugation of salbutamol with glucuronic acid yielded metabolite S-M1, with *m/z* 416.1915 (mass shift of 176.0321 Da, addition of $C_6H_8O_6$) detected at of 1.34 min. The conjugation with glucuronic acid and sulphate were the only previously reported in literature metabolites [29–36], but sulphate conjugate was not detected in these experiments. Perhaps, due to the sample preparation used based on solid-phase extraction we could not recover such a polar species, which were not properly retained on the cartridge with the conditions used. Glucuronide formation was almost negligible, this being consistent with recent data provided in human urine [10]. Because of this low abundance, in-source CID fragmentation experiments conducted were not useful to elucidate the position of the glucuronide moiety.

S-M2 is formed *via* the addition of a methoxyl group to the salbutamol glucuronide, with *m/z* 446.2021 and retention time of 1.40 min. This metabolite has an associated mass shift of +206.0427 (addition of $C_7H_{10}O_7$). In source CID fragmentation experiments generated as characteristic fragment ions with m/z 432.1864 (which is consistent with the loss of the methyl group of the methoxyl), 196.0968 and 178.0800.

The hydroxylation of salbutamol led to S-M3 (*m/z* 256.1543, RT 1.46 min). This biotransformation has an associated mass shift of

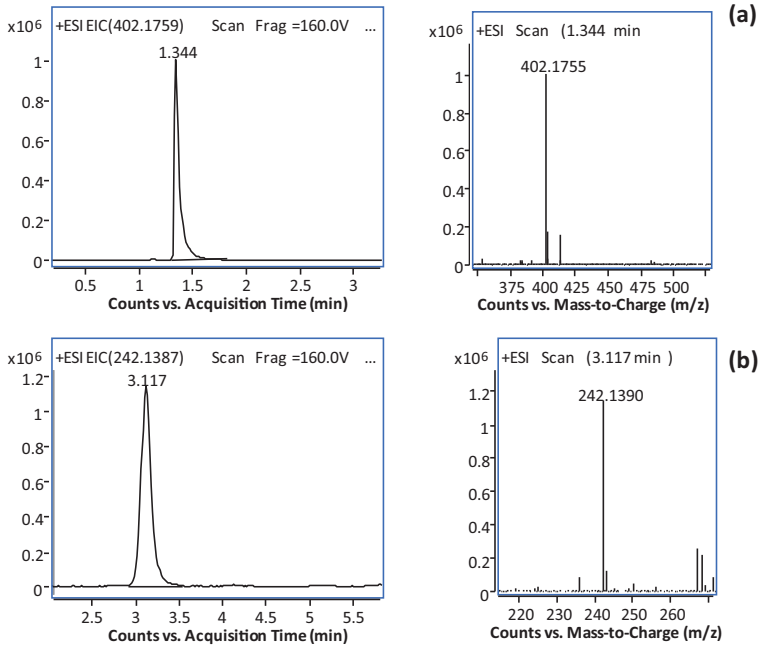

Fig. 5. EICs (left) and accurate mass spectra (right) of terbutaline identified metabolites: (a) T-M1 and (b) T-M2.

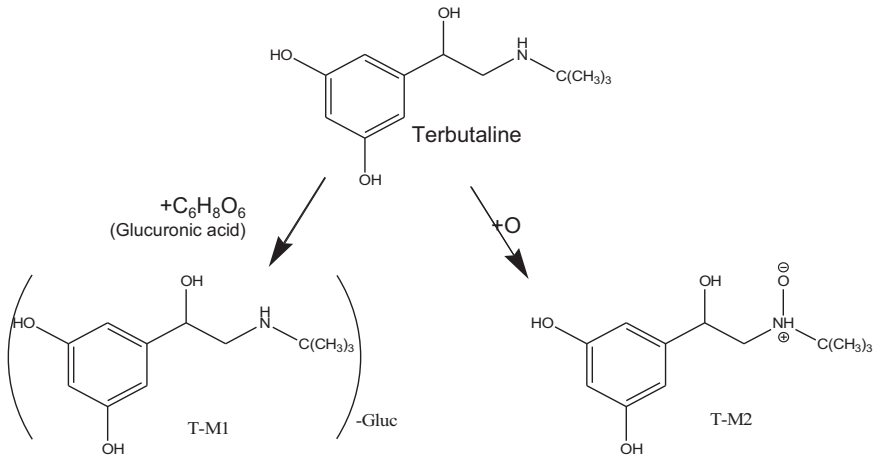

Fig. 6. Proposed structures of terbutaline identified metabolites.

+15.9949 corresponding to the addition of an oxygen atom. In-source CID fragmentation of this metabolite showed characteristic fragment ions with *m/z* 238.1838, 182.0812 and 164.0706.

Finally, a new metabolite S-M4 was identified with *m/z* 254.1387 and retention time 3.67 min. This metabolite has a molecular formula of $C_{13}H_{20}NO_4$. This metabolite is formed *via* a dehydrogenation and an addition of an oxygen atom. The addition of oxygen could be produced *via* hydroxylation or the formation of the N-oxide, in this case, the retention time of the derivative is higher than the retention time of salbutamol which is consistent with the formation of the N-oxide. Moreover, in-source CID fragmentation of this compound yielded fragment ions with *m/z* 180.0655 (which is consistent with the loss of the *tert*-butyl group and the O in the N-oxide), 162.0554 and 105.0452, the proposed structure of this compound together with the rest of metabolites are shown in Fig. 4. Therefore, up to three non-previously described metabolites were detected (hydroxyl-salbutamol, methoxy-salbutamol glucuronide conjugate and N-oxide salbutamol). From the point of view of the retrospectivity, the identified metabolites were detected only after 24 h like the parent drugs (Fig. S2, Supplementary data).

3.3. Identification of terbutaline and its urinary metabolites

In-source CID fragmentation of terbutaline (confirmed with standards and detected at 1.72 min) yielded fragment ions with *m/z* 152.0706 and 107.0491, consistent with previous literature [11]. Terbutaline urinary metabolites identified in the rat urine samples (T-M1 and T-M2) are summarized in Table 1, and the EICs chromatograms and the mass spectra shown in Fig. 5.

Terbutaline metabolites previously described in literature were only the sulphate and glucuronic conjugates [37–40]. Similarly to the case of salbutamol, because of the additional polarity of salbutamol and terbutaline compared to clenbuterol, terbutaline sulphate conjugate could not be detected probably because it was not recovered from the solid-phase extraction step. This highlights the importance of the choice of sample preparation approach for this type of studies. Not only detection step matters but also the preparation of the interrogated extract is essential.

In contrast, terbutaline glucuronide was identified (T-M1), with *m/z* 402.1759 (corresponding to a mass shift of 176.0321 Da due to the addition of glucuronic acid) at 1.34 min. In-source CID fragmentation experiments generated a characteristic fragment ion with *m/z* 328.1027, which was not useful for the elucidation of the glucuronide moiety position. The addition of O yielded metabolite T-M2 with *m/z* 242.1387 (corresponding to a mass shift of +15.9949 Da), detected at 3.12 min. The addition of an oxygen atom can be attributed to the formation of a hydroxyl-derivative or the N-oxide. In this case, the formation of terbutaline N-oxide is more likely due to the retention time of the metabolite is higher than the retention time of terbutaline, which is consistent with the formation of the N-oxide instead of the hydroxylation which is usually accompanied with a shift towards shorter retention times. The structures of terbutaline identified metabolites are shown in Fig. 6. In this case, as shown in Fig. S3 (supplementary information) neither the relative abundance with respect to the parent drug nor the permanency in urine makes these metabolites of particular interest from the point of view of sport drug testing.

4. Concluding remarks

The excretion of urinary metabolites of clenbuterol, terbutaline and salbutamol has been studied using LC-TOFMS. The strategy applied for the identification of metabolites was based on LC-TOFMS analyses using pseudo MS/MS experiments from in-source CID fragmentation (without precursor ion isolation) to search for common diagnostic fragment ions from the parent drugs, together with the search of typical biotransformations with their corresponding accurate mass shifts. The approach was satisfactory applied, achieving the identification of 11 metabolites (5 from clenbuterol, 4 from salbutamol and 3 from terbutaline), 4 of them not previously reported in urine. Novel metabolites detected in rat urine included N-oxide-salbutamol, hydroxy-salbutamol, methoxy-salbutamol glucuronide and terbutaline N-oxide. In the case of selected clenbuterol metabolites (CL-M1, CL-M3 and CL-M5), they lasted longer than the parent drug, which may be interesting for testing purposes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2013.02.008.

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