Analytica Chimica Acta 761 (2013) 1–10

Contents lists available at SciVerse ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Combined data mining strategy for the systematic identification of sport drug metabolites in urine by liquid chromatography time-of-flight mass spectrometry

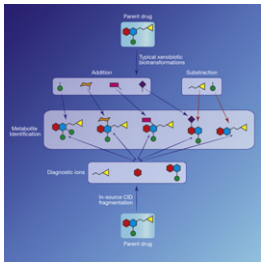
Juan C. Domínguez-Romero[a], Juan F. García-Reyes[a], Rubén Martínez-Romero[b], Paula Berton[c], Esther Martínez-Lara[b], María L. Del Moral-Leal[b], Antonio Molina-Díaz[a,*]

[a] Analytical Chemistry Research Group, Department of Physical and Analytical Chemistry, University of Jaén, 23071 Jaén, Spain
[b] Department of Experimental Biology, University of Jaén, 23071 Jaén, Spain
[c] Analytical Chemistry Research and Development Group (QUIANID), Instituto de Ciencias Básicas, Universidad Nacional de Cuyo, Padre Jorge Contreras 1300, Parque Gral. San Martín, C.P. M5502JMA Mendoza, Argentina

HIGHLIGHTS

- A strategy based on the use of two complementary data mining tools is proposed.
- Accurate *m/z* extraction of diagnostic ions and mass shifts from biotransformations.
- Nine sport drugs from different classes were studied after single doses to rats.
- Several non-previously reported metabolites were identified with the approach.
- 24 propranolol metabolites detected (15 non previously described in literature).

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 6 August 2012
Received in revised form
16 November 2012
Accepted 25 November 2012
Available online 5 December 2012

Keywords:
Liquid chromatography
High resolution mass spectrometry
Drug metabolites
Sport drug testing
Propranolol

ABSTRACT

The development of comprehensive methods able to tackle with the systematic identification of drug metabolites in an automated fashion is of great interest. In this article, a strategy based on the combined use of two complementary data mining tools is proposed for the screening and systematic detection and identification of urinary drug metabolites by liquid chromatography full-scan high resolution mass spectrometry. The proposed methodology is based on the use of accurate mass extraction of diagnostic ions (compound-dependent information) from in-source CID fragmentation without precursor ion isolation along with the use of automated mass extraction of accurate-mass shifts corresponding to typical biotransformations (non compound-dependent information) that xenobiotics usually undergo when metabolized. The combined strategy was evaluated using LC–TOFMS with a suite of nine sport drugs representative from different classes (propranolol, bumetanide, clenbuterol, ephedrine, finasteride, methoxyphenamine, methylephedrine, salbutamol and terbutaline), after single doses administered to rats. The metabolite identification coverage rate obtained with the systematic method (compared to existing literature) was satisfactory, and provided the identification of several non-previously reported metabolites. In addition, the combined information obtained helps to minimize the number of false positives. As an example, the systematic identification of urinary metabolites of propranolol enabled the identification of up to 24 metabolites, 15 of them non previously described in literature, which is a valuable indicator of the usefulness of the proposed systematic procedure.

* Corresponding author. Tel.: +34 953 212147; fax: +34 953 212940.
E-mail address: amolina@ujaen.es (A. Molina-Díaz).

0003-2670/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.aca.2012.11.049

1. Introduction

Drug metabolism is a complex process, involving multiple enzymatic pathways that result in a variety of metabolites with uneven concentrations [1]. The knowledge of the metabolism and excretion of doping agents is of particular interest in sport drug testing, where the information related on the time course in which a parent drug or its metabolites can be detected, is essential. 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. For these purposes, comprehensive methods able to tackle with the systematic identification of drug metabolites in an automated fashion are of great interest.

Liquid chromatography–mass spectrometry (LC–MS) has an extensive role in metabolism research. Many reviews in the literature highlight principles and usage of different MS instruments for metabolite identification [2,3]. In modern doping control laboratories, the use of LC–MS has become mandatory to meet the needs of fast, robust, sensitive, and specific detection methods in sport drug testing [4]. Several LC–MS methods based on ion trap, triple quadrupole, time-of-flight or Orbitrap analyzers have been used for the urinary detection of several prohibited substances such as corticosteroids, diuretics or beta blockers [5,6]. Concerning sport drugs metabolism, 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.

The use of full-scan high resolution mass spectrometry generates an enormous amount of data, offering the advantage of enabling simultaneous analysis of a virtually unlimited number of analytes with no method performance compromise when increasing the number of compounds included in the scope of the method. The features of this instrumentation map well against the requirements of sport drug metabolism studies. However, despite the development of high resolution mass spectrometry instrumentation has improved the quality of metabolite identification processes, data processing and interpretation still remains as the main bottleneck in metabolite identification [7]. Depending on the matrix, the huge amount of data can also cause problems in compound identification step, which may be hindered by matrix interferences.

For this reason, several data mining strategies have been proposed to perform objective searching/filtering of accurate-mass-based LC–MS data to facilitate metabolite detection [8,9]. Among them, *in-silico* tools for metabolite prediction/detection [10], the use of precursor ion and constant neutral loss scanning modes [11,12], isotope-pattern-filtering algorithms [13], mass defect filter (MDF) [14,15], and retention-time-shift-tolerant background subtraction algorithms [15–17] have been proposed. In this sense, the need of preliminary knowledge of the parent compound can be used as a valid criterion to classify these data mining tools. While for instance, constant neutral-loss experiments require no knowledge of the parent compound, because expected neutral losses from the analyte are traced (*e.g.* loss of 176 Da from a glucuronide conjugate), in the case of precursor ion scanning experiments, the operator needs to know the fragmentation pattern of the parent ion in advance to perform the experiment [18].

In this article, a strategy based on the combined use of two complementary data mining tools is proposed for the screening and systematic detection of urinary drug metabolites by liquid chromatography full-scan high resolution mass spectrometry. The proposed methodology is based on the use of accurate mass extraction of diagnostic ions (compound-dependent information) from in-source CID fragmentation experiments without precursor ion isolation, along with the use of automated mass extraction of accurate mass shifts corresponding to typical biotransformations (non compound-dependent information) that xenobiotics usually undergo when metabolized. The methodology can be considered as a comprehensive "top-down/bottom-up" approach, since identification of metabolites can be obtained from accurate mass shifts starting from the unaltered parent drug (top-down approach), while the use of diagnostic ions (those fragment ions common in a class of species) can also be used as markers for identifying metabolites (bottom-up approach) starting from the core/nuclei of the metabolite structure which may be common to that of the parent drug. The combined strategy was evaluated using LC–TOFMS with a suite of nine sport drugs representative from different classes, after single doses administered to rats.

2. Experimental

2.1. Chemicals and reagents

Propranolol, finasteride and salbutamol analytical standards were purchased from Dr. Ehrenstorfer, bumetanide, clenbuterol, ephedrine, methoxyphenamine, methylephedrine and terbutaline standards were acquired from Sigma–Aldrich. HPLC grade acetonitrile and methanol were acquired from Sigma–Aldrich. Formic acid and dimethyl sulfoxide were obtained from Fluka. A Milli-Q-Plus ultra-pure water system from Millipore (Milford, MA, USA) was used throughout the study to obtain the HPLC water used during the analyses. Bond Elut PLEXA SPE cartridges (200 mg, 6 mL) were purchased from Agilent Technologies (Santa Clara, CA) and a Supelco (Bellefonte, PA) Visiprep^{TM} SPE vacuum system was used for SPE experiments.

2.2. Samples and sample collection

The metabolism 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) and were approved by the institutional Committee for Ethics. The details of doses and sampling of the experiments are included in Table S1 (supplementary data). In the case of propranolol, rats were treated with (25 mg kg^{-1} (body weight, intraperitoneal)). The solution of propranolol was made in 15% DMSO in saline. After drug administration, urine was daily collected in graduate cylinders for 2 days (24, 48 and 72 h). The urine collected 24 h prior to treatment was used as control. Human urine was collected from a female volunteer treated with oral propranolol (40 mg twice a day) for more than 5 years.

2.3. Sample treatment

Urine samples were concentrated by a solid-phase extraction (SPE) procedure using Bond Elut PLEXA cartridges. The cartridges were preconditioned with 4 mL of MeOH/MeCN (1:1) and 4 mL of HPLC grade Milli-Q water. After the conditioning step, 2 mL of urine buffered with 2 mL of formic acid/formate pH 2.6 buffer were passed through the SPE cartridge. 4 mL of 5% MeOH in Milli-Q water was then added to rinse the cartridge prior to elution. The cartridges were dried under vacuum in order to remove the excess water and the analytes were finally eluted with 4 mL of MeOH/MeCN (1:1).

The extracts were evaporated until near dryness using a Turbo Vap LV from Zymark (Hopkinton, MA), with a water bath temperature of 37 °C and a N_2 pressure of 15 psi. The samples were then taken up with 0.5 mL of MeOH/water (10:90, v/v) to achieve a preconcentration of 4:1. The reconstituted extracts were passed through a 0.45 μm PTFE syringe filter and then transferred to a vial prior to LC–TOFMS analyses.

2.4. Liquid chromatography time-of-flight mass spectrometry

The separation of the analytes from the urine extract was carried out using a high-performance liquid chromatography (HPLC) system (consisting of vacuum degasser, auto sampler and a binary pump) (Agilent Infinity 1290, Agilent Technologies, Santa Clara, CA) equipped with a reversed-phase XDB-C_{18} analytical column of 4.6 mm × 50 mm and 1.8 μm particle size (Agilent Technologies, Santa Clara, CA). 20 μL of the extract was injected in each run. Mobile phases A and B were water with 0.1% formic acid and acetonitrile. The chromatographic method held the initial mobile phase composition (10% B) constant for 3 min, followed by a linear gradient to 100% B up to 15 min and kept for 3 min at 100% B. The flow rate used was 0.5 mL min^{-1}. The HPLC system was connected to a time-of-flight mass spectrometer Agilent 6220 accurate mass TOF (Agilent Technologies, Santa Clara, CA) equipped with an electrospray interface operating in both positive and negative ion 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): 190 V (range tested: from 160 to 350 V). Different fragmentor voltages can be established in the same experiment, so that complete fragmentation information may be obtained within a single LC–MS run. LC–MS accurate mass spectra were recorded across the range of m/z 50–1000 in positive ion mode and m/z 50–1100 in negative ion mode. The instrument performed an internal calibration using a second sprayer with a reference solution containing the reference masses $TFANH_4$ (ammonium trifluoroacetate, m/z 112.985587 in negative ion mode), purine (m/z 121.050873, in positive ion mode) and HP-0921 (m/z 922.009798 in positive ion mode and m/z 1033.988109 in negative). For this reason, a different mass range was used in negative ionization mode in order to collect the data from the two reference masses. The instrument was operated in the 4 GHz high resolution mode, providing a typical resolution of *ca.* 20,000 at m/z 922. 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).

2.5. Approach for systematic identification of drug metabolites using LC–TOFMS

Two strategies were combined: (a) the use of automated mass extraction of accurate-mass shifts corresponding to typical biotransformations (non compound-dependent information) that xenobiotics usually undergo when metabolized, so that selected moieties or functional groups are added or removed from the parent molecule, and (b) the use of accurate mass extraction of diagnostic ions (compound-dependent information) from in-source CID fragmentation experiments without precursor ion isolation. The maximum accurate-mass error tolerance for considering tentative metabolites was set to 5 ppm.

2.5.1. Use of automated mass extraction of accurate-mass shifts corresponding to typical biotransformations

It consists in searching potential metabolites based on the original drug by applying a set of typical biotransformations [19] which have an associated change in the molecular formula and its corresponding mass shift. The search for these potential metabolites in a sample is automatically performed with the database searching tool of Agilent MassHunter software. A predefined set of possible biotransformations were considered (Table S-2, supplementary data). A *csv*.-format excel file was created for each drug with its possible biotransformation including the molecular formula of the metabolites, its accurate mass and as name the corresponding biotransformation. This file is used by the automated accurate-mass extraction tool of the software. When a positive result was found, its extracted ion chromatogram and mass spectrum are automatically obtained from the raw data. An example of this approach is depicted in Fig. 1, where the results from a sample of rat urine after the treatment with propranolol are shown, including the identification of seven metabolites identified through this strategy.

2.5.2. Accurate mass extraction of diagnostic ions from in-source CID fragmentation of parent drug

Diagnostic ions are fragment ions whose presence and abundance are characteristic of a class of compounds and thereby may assist in the identification of any species belonging to this class. Diagnostic ions of the original molecule can be easily obtained by in-source CID fragmentation capability of LC–MS instrumentation. This strategy is based on the fact that many of the diagnostic ions are preserved in the metabolites. Metabolites exhibiting a structure similar to the parent molecule, usually display the same fragments. To implement this strategy, a database is created with the diagnostic ions of each compound and the automatic database searching tool of the MassHunter software is used. When one of these diagnostic ions is found, its chromatogram and mass spectrum is extracted automatically, the spectrum is analyzed to find the parent ion and a tentative molecular formula is automatically generated by the software, with mass error and an isotope pattern matching coefficient. Therefore, if one of these diagnostic ions is found with a retention time different from the original molecule, this could be used as a marker to tentatively detect and identify a new metabolite. By extracting the accurate mass corresponding to the tentatively identified metabolite ($[M\pm H]^{\pm}$ ion), candidate elemental composition and structure assignment for this detected metabolite can be accomplished. As an example, diagnostic ions from in-source CID fragmentation of propranolol are shown as electronic supplementary material (Figure S-1, supplementary data). An example of the proposed strategy is highlighted in Fig. 2, where the extracted ion chromatogram EIC of diagnostic ion m/z 115.0542 is shown, and reveals the presence of various chromatographic peaks, candidates to be metabolites. The accurate mass spectrum of each peak enables the confirmation of the presence of this diagnostic ion and also assists the tentative identification of the corresponding parent compound (metabolite).

3. Results and discussion

The proposed combined approach was tested using 10 sport drugs from different classes (propranolol, bumetanide, clenbuterol, ephedrine, finasteride, methoxyphenamine, methylephedrine, salbutamol and terbutaline). As a case study, the identification of propranolol metabolites is described in detail.

3.1. Identification of propranolol and its metabolites in urine

Propranolol is a non-selective β-blocker included in the list of banned substances of the World Anti Doping Agency (WADA). Its use is prohibited in competition in some sports and also out of competition in archery and shooting [20,21]. The metabolism study of

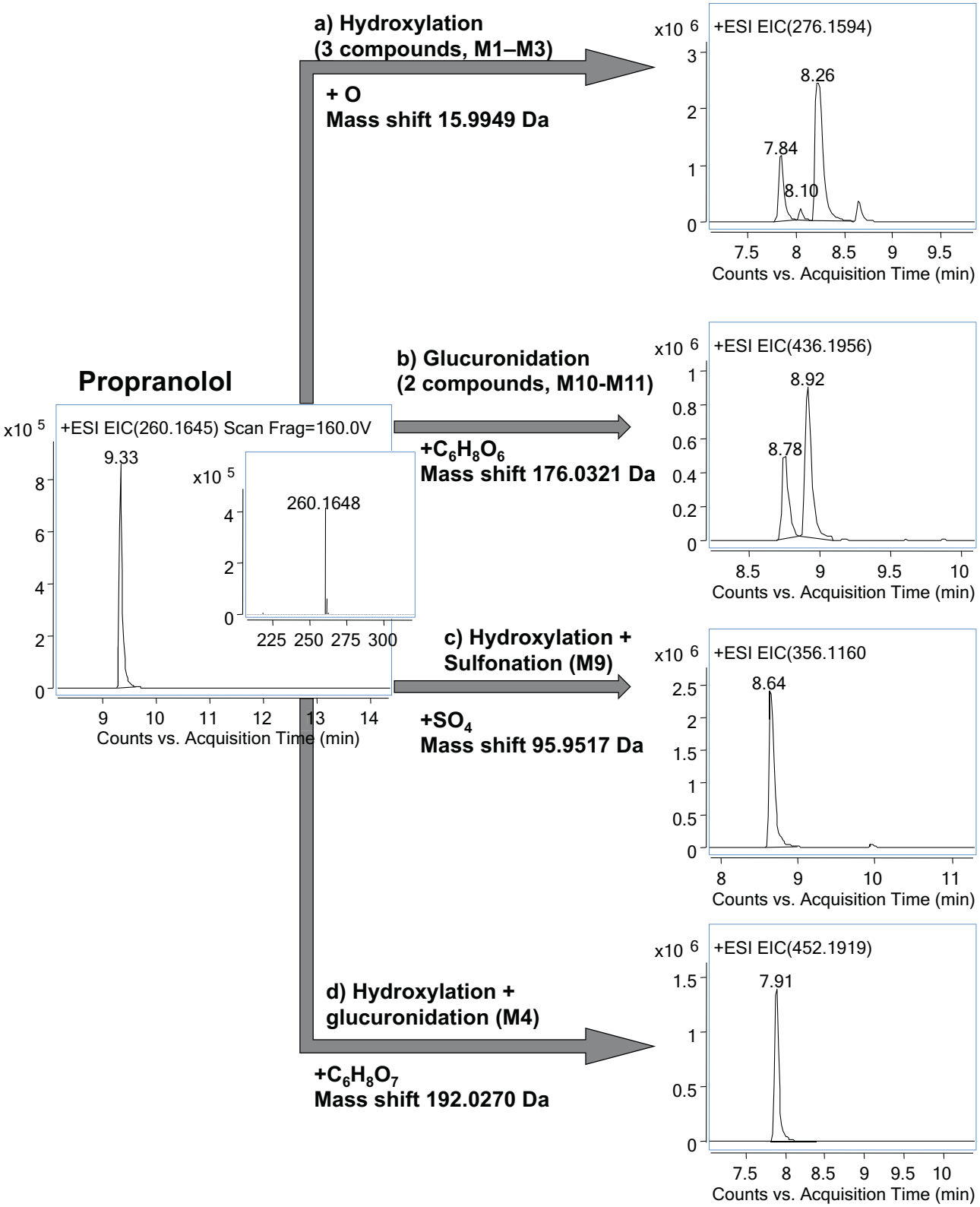


Fig. 1. Example of a typical biotransformations search in a rat urine sample after the treatment with propranolol is shown. The metabolites identified correspond to the hydroxylation of propranolol (a), its conjugation with glucuronic acid (b) and the conjugation with sulfate and glucuronic acid of the hydroxylated derivative (c and d).

propranolol was carried out using the proposed procedure and the results were compared to the metabolites reported in the existing literature. Detailed information on the metabolism and literature available is provided in electronic supplementary data.

The identification and confirmation of propranolol was performed by LC–TOFMS accurate mass measurements and retention time matching. For confirmation purposes and the subsequent metabolite search based on diagnostic ions, in-source collision

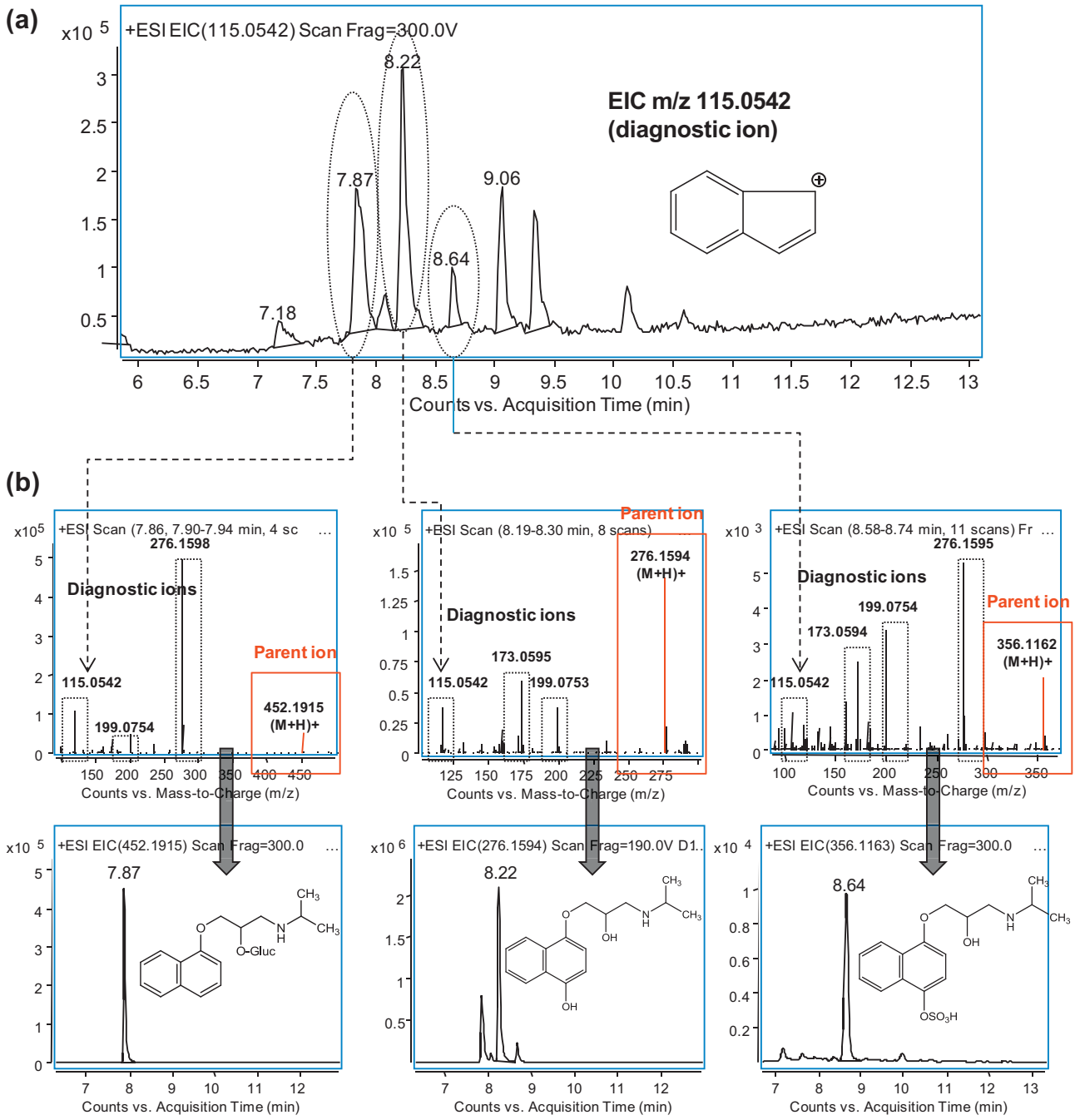


Fig. 2. Example of a diagnostic ion based search: (a) extracted ion chromatogram of diagnostic ion 115.0542. (b) Metabolites found with the diagnostic ion 115.0542: hydroxylation + sulfonation (*m*/*z* 356.1162), hydroxylation (*m*/*z* 276.1594) and glucuronidation (*m*/*z* 452.1915).

induced dissociation (CID) fragmentation of propranolol was examined (Figure S-1, supplementary data). The metabolites identified in the rat urine sample (**M1–M4**, **M7–M11**, and **M14–M24**) are summarized in Table 1. These include: the hydroxylation of the ring in different positions (**M1–M3**); the glucuronic conjugates of the parent molecule (**M10–M11**); of the ring-hydroxylated metabolite (**M4**) and of the dihydroxylated derivatives (**M20–M24**); the sulfate conjugate of the ring-hydroxylated (**M7–M9**); the dihydroxylation of the rings (**M14–M15**); the N-desisopropylation of the propranolol (**M16**), the oxidation of the side chain to aliphatic acids (**M17–M18**) and α-naphthol sulfate (**M19**). All compounds were identified by accurate mass measurements of the intact molecules and diagnostic fragment ions with a mass error below 2 ppm in most cases as shown in Table 1.

Metabolites **M1**, **M2** and **M3** (*m*/*z* 276.1594) were found as ring-hydroxy derivatives of propranolol, with a mass shift of 15.9949 Da corresponding to the addition of an oxygen atom. They were confirmed with fragment ions *m*/*z* 199.0756, 173.0602 and 115.0542. According to elution order found in literature [22,23], the three isobaric species were assigned as 5′ (RT 7.84 min), 4′ (RT 8.10 min) and 7′ (RT 8.26 min) hydroxyl-derivatives of propranolol. **M4** detected at 7.92 min with *m*/*z* 452.1915 (with a mass shift of 192.0270 Da, addition of $C_6H_8O_6$) was identified as the glucuronide of the hydroxylated propranolol. In-source CID fragmentation of this metabolite showed characteristic fragments with *m*/*z* 276.1594, 199.0756, 173.0602 and 115.0542. **M7**, **M8** and **M9** metabolites with *m*/*z* of 356.1162 (mass shift of 95.9517 Da (addition of SO_4)) were identified as sulfate derivatives

Table 1
Identification and accurate mass measurements of propranolol metabolites found in rat urine and human urine.

Metabolite	Retention time	Experimental *m/z*	Theoretical *m/z*	Error		Proposed formula	DBE
				mDa	ppm		
Rat urine							
M1 – hydroxylation	7.84	276.1598	276.1594	0.37	1.35	$C_{16}H_{21}NO_3$	7
M2 – hydroxylation	8.10	276.1594	276.1594	0.02	0.09	$C_{16}H_{21}NO_3$	7
M3 – hydroxylation	8.26	276.1597	276.1594	0.28	1.01	$C_{16}H_{21}NO_3$	7
M4 – hydroxylation + glucuronidation	7.92	452.1919	452.1915	0.42	0.93	$C_{22}H_{29}NO_9$	9
M7 – hydroxylation + sulfonation	7.23	356.1166	356.1162	0.32	0.9	$C_{19}H_{21}NO_6S$	7
M8 – hydroxylation + sulfonation	7.68	356.1160	356.1162	0.19	0.52	$C_{19}H_{21}NO_6S$	7
M9 – hydroxylation + sulfonation	8.68	356.1158	356.1162	0.44	1.24	$C_{19}H_{21}NO_6S$	7
M10 – glucuronidation	8.78	436.1956	436.1966	0.96	2.21	$C_{22}H_{29}NO_8$	9
M11 – glucuronidation	8.92	436.1964	436.1966	0.19	0.43	$C_{22}H_{29}NO_8$	9
M14 – dihydroxylation	7.97	292.1545	292.1543	0.15	0.5	$C_{16}H_{21}NO_4$	7
M15 – dihydroxylation	8.41	292.1539	292.1543	0.41	1.39	$C_{16}H_{21}NO_4$	7
M16 – N-desisopropylation	8.77	218.1169	218.1176	0.63	2.9	$C_{13}H_{15}NO_2$	7
M17 – naphthoxylactic acid	10.58	231.0666	231.0663	0.34	1.45	$C_{13}H_{12}O_4$	8
M18 – naphthoxyacetic acid	11.64	201.0557	201.0558	0.03	0.13	$C_{12}H_{10}O_3$	8
M19 – α-naphthol sulfate	9.30	223.0071	223.0071	0.08	0.35	$C_{10}H_8O_4S$	7
M20 – dihydroxylation + glucuronidation	2.19	468.1868	468.1864	0.41	0.88	$C_{22}H_{29}NO_{10}$	9
M21 – dihydroxylation + glucuronidation	3.88	468.1867	468.1864	0.29	0.63	$C_{22}H_{29}NO_{10}$	9
M22 – dihydroxylation + glucuronidation	6.50	468.1868	468.1864	0.35	0.74	$C_{22}H_{29}NO_{10}$	9
M24 – dihydroxylation + glucuronidation	7.69	468.1865	468.1864	0.08	0.17	$C_{22}H_{29}NO_{10}$	9
Human urine							
M1 – hydroxylation	7.84	276.1591	276.1594	0.31	1.16	$C_{16}H_{21}NO_3$	7
M2 – hydroxylation	8.10	276.1589	276.1594	0.50	1.88	$C_{16}H_{21}NO_3$	7
M3 – hydroxylation	8.26	276.1598	276.1594	0.37	1.34	$C_{16}H_{21}NO_3$	7
M4 – hydroxylation + glucuronidation	7.92	452.1912	452.1915	0.31	0.69	$C_{22}H_{29}NO_9$	9
M5 – hydroxylation + glucuronidation	8.00	452.1912	452.1915	0.27	0.61	$C_{22}H_{29}NO_9$	9
M6 – hydroxylation + glucuronidation	9.30	452.1918	452.1915	0.29	0.64	$C_{22}H_{29}NO_9$	9
M7 – hydroxylation + sulfonation	7.23	356.1168	356.1162	0.52	1.46	$C_{19}H_{21}NO_6S$	7
M8 – hydroxylation + sulfonation	7.68	356.1162	356.1162	0.02	0.05	$C_{19}H_{21}NO_6S$	7
M9 – hydroxylation + sulfonation	8.68	356.1155	356.1162	0.73	2.06	$C_{19}H_{21}NO_6S$	7
M10 – glucuronidation	8.78	436.1973	436.1966	0.68	1.56	$C_{22}H_{29}NO_8$	9
M11 – glucuronidation	8.92	436.1965	436.1966	0.06	0.15	$C_{22}H_{29}NO_8$	9
M12 – dihydroxylation	7.52	292.1553	292.1543	0.94	3.24	$C_{16}H_{21}NO_4$	7
M13 – dihydroxylation	7.65	292.1546	292.1543	0.30	0.91	$C_{16}H_{21}NO_4$	7
M14 – dihydroxylation	7.97	292.1543	292.1543	0.04	0.14	$C_{16}H_{21}NO_4$	7
M15 – dihydroxylation	8.41	292.1547	292.1543	0.38	1.25	$C_{16}H_{21}NO_4$	7
M16 – N-desisopropylation	8.77	218.1169	218.1173	0.33	1.6	$C_{13}H_{15}NO_2$	7
M17 – naphthoxylactic acid	10.58	231.0669	231.0663	0.59	2.54	$C_{13}H_{12}O_4$	8
M18 – naphthoxyacetic acid	11.64	201.0557	201.0558	0.1	0.47	$C_{12}H_{10}O_3$	8
M23 – dihydroxylation + glucuronidation	7.12	468.1858	468.1864	0.65	1.39	$C_{22}H_{29}NO_{10}$	9

of the (ring)-hydroxylated propranolol derivative. Similarly to the hydroxy derivatives (**M1**–**M3)**, the tentative elution order was established as follows: 5′-hydroxy sulfate (RT 7.23 min) **(M7)**, 4′-hydroxy sulfate (RT 7.68 min) **(M8)** and 7′-hydroxy sulfate (RT 8.68 min) **(M9)**. In-source CID fragmentation of **M7–M9** revealed the presence of fragments with *m/z* 276.1594, 199.0756, 173.0602 and 115.0542. Glucuronidation of propranolol (involved a mass shift of 176.0321 Da (addition of $C_6H_8O_6$)) yielded **M10** (RT 8.78 min) and **M11** (RT 8.92 min), with *m/z* 436.1966. Since the glucuronidation is only possible in two positions, the hydroxyl group and the amine group in the side chain, **M10** and **M11** correspond to the O-glucuronide and N-glucuronide derivatives of propranolol (elution order not set). Characteristic fragments of both metabolites were also found at *m/z* 260.1645 and 183.0804.

Hydroxylation at two positions of the rings yielded **M14** (RT 7.97 min) and **M15** (RT 8.41 min) metabolites, with *m/z* 292.1543 corresponding to a mass shift of 31.9898 Da (addition of O_2). In-source CID fragmentation of **M14** showed fragments ions at *m/z* 199.0756 and 173.0602. N-desisopropylation of propranolol led to **M16** with *m/z* 218.1176 (a mass shift of 42.0469 Da (loss of C_3H_7)), identified at a retention time of 8.77 min and showing a characteristic fragment at *m/z* 183.0804.

Side-chain oxidation of propranolol generated two metabolites, **M17**, identified as naphthoxylactic acid (*m/z* 231.0663, RT 10.58 min), and **M18**, identified as naphthoxyacetic acid (*m/z* 201.0558, RT 11.64 min). These metabolites were detected in negative ionization mode, and both exhibited a common fragment ion with *m/z* 143.0503, which corresponds to α-naphthol moiety. This fragment was also common to **M19**, detected at 9.30 min, which was found to be the sulfated conjugate of α-naphthol. Finally, metabolites **M20** (RT 2.19 min), **M21** (RT 3.88 min), **M22** (RT 6.50 min) and **M24** (RT 7.69 min), with *m/z* of 468.1864, corresponding to a mass shift of 208.0219 Da (addition of $C_6H_8O_8$), were identified as glucuronide conjugates of dihydroxylated propranolol derivatives. These four metabolites showed a similar CID fragmentation, generating two characteristic fragment ions with *m/z* 292.1543 and 215.0703. Additional dedicated CID-MS/MS experiments were performed with LC–QTOF-MS, and were not able to provide complementary fragmentation information to elucidate the relative positions of the hydroxyl groups and the glucuronic acid. This observation proofs that dedicated MS/MS experiments undertaken in collision cells after precursor ion isolation often do not provide additional fragmentation data compared to in-source CID fragmentation. This usually happens when the studied molecules easily undergo fragmentation even with low energy and only a few information can be extracted.

Identification of propranolol metabolites in human urine. The metabolites identified in human urine (**M1**–**M15**, **M17–M18** and **M23**) are also summarized in Table 1. The detected metabolites include the ring-hydroxylation (**M1**–**M3**), the glucuronidation or sulfonation of these hydroxy derivatives (**M4**–**M9**), a glucuronic

derivative of a double ring-hydroxylated derivative (**M23**), the glucuronic derivatives of propranolol (**M10**–**M11**), dihydroxylation (**M12**–**M15**), the N-desisopropylation of the parent molecule (**M16**), and the oxidation of the side chain to aliphatic acid (**M17**–**M18**). All the assignments were done with accurate mass measurements of these selected ions with relative mass errors below 2 ppm in most cases as shown in Table 1. As in rat urine, metabolites **M1**, **M2** and **M3** were identified at 7.84 min (5′-hydroxy), 8.10 min (4′-hydroxy) and 8.26 min (7′-hydroxy). Metabolites **M4**, **M5** and **M6** were identified as the glucuronide conjugated species of the ring-hydroxylated derivatives, with *m*/*z* 452.1915 (addition of $C_6H_8O_7$, mass shift of 192.0270 Da). Similarly to the hydroxyl-derivatives, the tentative elution order was tentatively set as 5′, 4′, and 7′: 7.92 min (5′-O-Gluc) **(M4)**, 8.00 min (4′-O-Gluc) **(M5)** and 9.30 min (7′-O-Gluc) **(M6)**. CID fragmentation experiments were performed for the metabolites, glucuronic derivatives showed as characteristic fragments *m*/*z* 276.1594 and 173.0602 for **M4** and *m*/*z* 276.1594, 199.0756, 173.0602 and 115.0542 for **M5–M6** was not concentrated enough to provide fragmentation information.

M7, **M8** and **M9** (sulfate derivative of the ring hydroxylated propranolol) and glucuronides from intact propranolol (**M10** and **M11**) were also identified in human urine. **M12**, **M13**, **M14** and **M15** (RT 7.52, 7.65, 7.97 and 8.41 min) were identified as the ring-dihydroxylated propranolol derivatives, with *m*/*z* 292.1543 (mass shift 31.9898 Da, addition of O_2). Only two of these metabolites were concentrated enough to be subjected to CID fragmentation. **M14** yielded fragment ions with 199.0756 and 173.0602, while **M13** exhibited an additional fragment with *m*/*z* 115.0542. Finally, as in rats, the oxidation of the side chain of propranolol generates two metabolites, **M17** (naphthoxylactic acid, RT 10.58, *m*/*z* 231.0663), and **M18** (naphthoxyacetic acid, RT 11.64, *m*/*z* 201.0558). These are the only metabolites detected in negative ionization mode. CID fragmentation of both compounds led to a fragment ion with *m*/*z* 143.0503, corresponding to α-naphthol. These two metabolites (**M17**, **M18**) and the sulfate conjugate of α-naphthol only identified in rat urine (**M19**) were identified based on previous literature [24–29,22,30–32].

Finally, **M23** (*m*/*z* 468.1864), detected in human urine at 7.12 min, corresponds to the glucuronic conjugate of the ring-dihydroxylated derivative. This metabolite (involving an addition of $C_6H_8O_8$, and a mass shift of 208.0219 Da) also exhibited characteristic fragment ions with *m*/*z* 292.1543 and 215.0703. Fig. 3 shows the proposed structure for the 24 identified propranolol urinary metabolites. Only 9 of these detected metabolites were previously described in literature [24–29,22,30–32]. **M1**, **M2** and **M3** were previously described as 5′, 4′ and 7′ hydroxy derivatives of propranolol. Only one glucuronic derivative of the hydroxylated metabolite was previously described, while in this work 3 metabolites (**M4**–**M6)** have been identified, tentatively corresponding to the glucuronidation of the 3 hydroxylated metabolites (**M1**–**M3**). Similar results were also found in the case of sulfate derivatives of the hydroxylated metabolites; 3 metabolites have been identified, **M7–M9**, tentatively corresponding to the sulfonation of the three hydroxy derivatives (**M1**–**M3**) while in literature only one of them was described. **M10** and **M11**, identified as the two glucuronide derivative propranolol isomers, have not been described elsewhere. Neither, the four dihydroxylated metabolites (**M12**–**M15**) nor their glucuronic derivatives (**M20**–**M24**) have been previously reported. **M17** and **M18**, identified as naphthoxylactic and naphthoxyacetic acid, were previously described, although **M19** (α-naphthol sulfate) was not described in literature. In-source CID fragmentation of the detected metabolites provides structural information and confirmation in the identification. The spectral features of all the 24 identified metabolites are summarized in Table S-3 (supplementary data).

3.2. Implementation and evaluation of the proposed approach for multiclass sport drugs metabolite identification

The proposed approach was tested with different sport drugs corresponding to different classes such as diuretics, stimulants or anabolic steroids. Single dose experiments were performed in rats by triplicate. Details on the experiments are provided in Table S-1 (supplementary data). The total number of metabolites detected was compared with the reported metabolites in order to explore the metabolite identification coverage rate of the proposed approach. The results obtained are summarized in Table 2, and the details of the previous literature are included in Table S-4 (supplementary data). As it can be noted, the coverage rate was higher than 50% of metabolites in most cases. Besides, up to 28 putative new metabolites were identified in the present study. This reveals the usefulness of the proposed approach as a straightforward method to screen for metabolites on a systematic basis.

For instance, in the case of propanolol, 15 compounds were identified solely with mass shifts while 11 were identified using diagnostic ions produced in in-source CID experiments. But, more interestingly to the total number of metabolites identified, is the synergetic effect when using both tools. The combined use of diagnostic ion fragmentation and accurate-mass mass shifts clearly increases the metabolite identification coverage rate, but more importantly improve identification quality, because the number of false positives is drastically reduced. In the cases where a diagnostic ion is used to detect the metabolite candidate, accurate mass analyses of two ions (diagnostic ion and the intact molecule) along with isotope pattern information is enough to tentatively confirm the metabolite. Final characterization/standardization of the tentative species may involve synthesis, NMR, and additional dedicated CID MS/MS or even high resolution MS^n experiments to distinguish among isomers and to elucidate the relative positions of the functional groups from the different metabolites detected with the same *m*/*z* value.

With regards to the implementation of the proposed approach, the key issue is the mass spectrometer acquisition mode selection rather than specific software features. Table 3 summarizes information related to selected instrumentation suitable to implement the present approach. Not huge differences are found among different software packages available, mostly based on the use of scripts calculating mass shifts or alternatively the use of mass defect filtering (MDF) algorithms [14,15]. Straightforward scripts based on user-created excel spreadsheets can be easily implemented regardless the instrument manufacturer.

The key part is the ability to acquire simultaneously (or in consecutive runs) information from intact molecules and CID fragmentation without precursor isolation. The use of specific MS/MS experiments offer additional specificity, even considering precursor ion isolation is accomplished typically with mass-unit (low) resolution. However, the MS/MS data information that can be acquired in a run is limited by the acquisition time and compatibility with chromatographic peak shape requirement. Therefore, only a selected number of co-eluting ions (up to 10) may be isolated and fragmented within each acquisition cycle. If different collision energy is used to fine tune the fragmentation (two or three different values), the number of tentative species traced is much lower. On the other hand, the use of triggered data-dependent acquisition modes which isolates, for instance, the more abundant ions, is not particularly useful when dealing with complex matrices with abundant co-eluting species which are usually more intense than the targeted species. In this context, the need for different MS and MS/MS experiments is requested.

In contrast, the use of CID fragmentation without precursor isolation, offered by different vendors as shown in Table 3 provides much more advantages than disadvantages. The main drawback

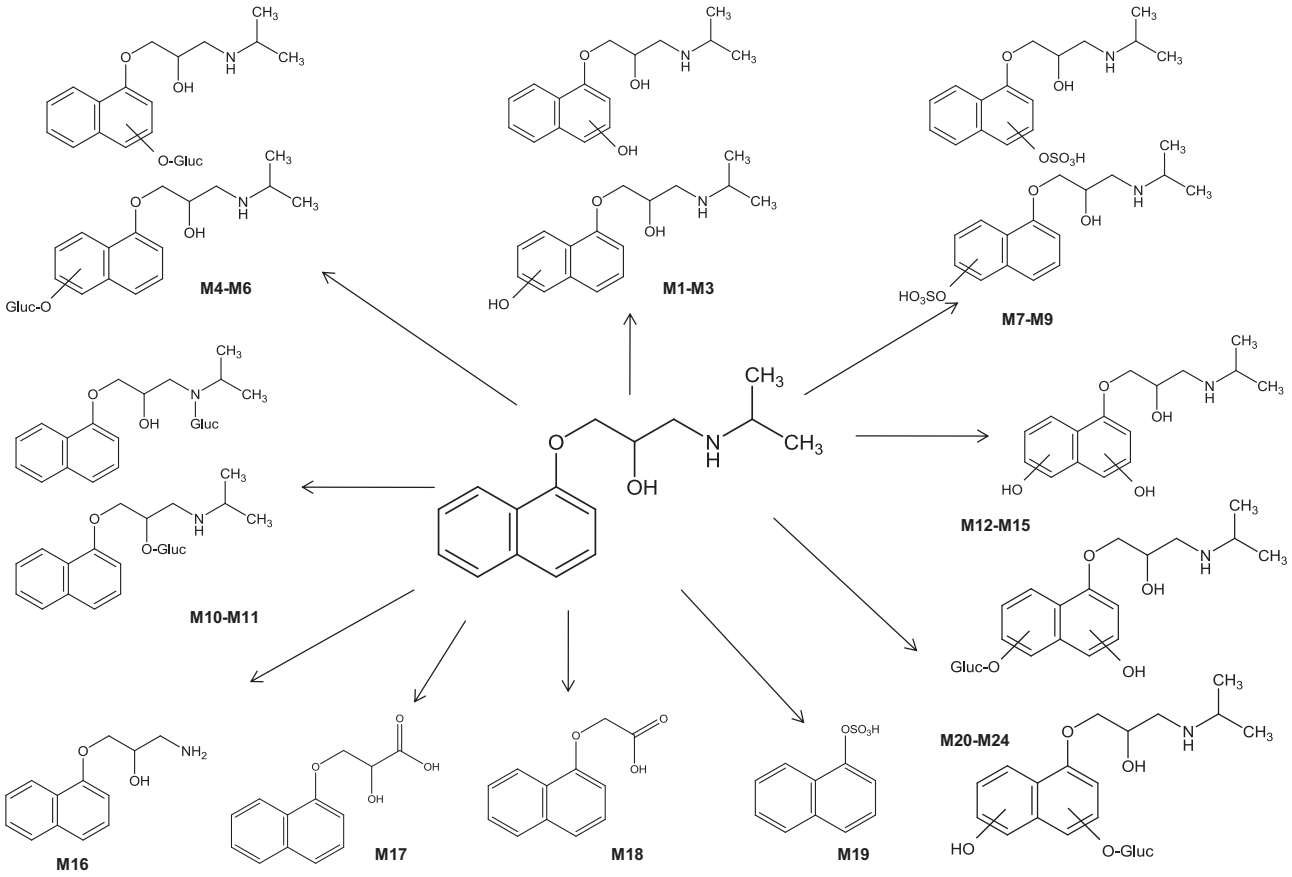


Fig. 3. Proposed structures of the 24 identified propranolol metabolites.

of this acquisition mode is the loss of specificity compared to a dedicated precursor ion isolation MS/MS experiment. In contrast, the last generation of instruments enables fast switching of fragmentation conditions from soft (producing intact (de)protonated species) to medium-hard fragmentation yielding most relevant diagnostic ions. Therefore, the entire data can be eventually collected within a unique run. Different solutions are proposed from the typical in-source CID fragmentation undertaken in the ion transportation region at low vacuum [33] used in this work with LC–TOFMS, which enable the acquisition of up to four different fragmentation conditions in the same run, to the use of a dedicated high-energy dissociation cell (HCD) in stand-alone Orbitrap mass spectrometers [34,35] or a typical Q-TOFMS MS/MS experiment, but without isolation in dedicated cell at high vacuum with auxiliary gasses (MS^E mode from Waters [36,37]). The versatility of the experiment (amount of information acquired) is achieved however at the expense of sensitivity in TOF instruments and resolving power in Orbitraps, because sensitivity and resolution decreases with increasing acquisition rates in TOF and Orbitraps respectively [34,35], although the introduction of newer and faster HRMS instruments partly compensates this issue.

4. Concluding remarks

The proposed approach based on CID fragmentation without precursor ion isolation combined with accurate mass shifts has been evaluated using LC–TOFMS with a suite of nine sport drugs representative from different classes, after single doses administered to rats. Metabolite identification coverage rate obtained with the systematic method (compared to existing literature) was

Table 2
Evaluation of the proposed systematic approach for multiclass sport drugs metabolite identification in terms of metabolite coverage with regards to previously reported literature.

Compound	Number of detected urinary metabolites	Number of previously described urinary metabolites	% Coverage[a]	Number of non-described urinary metabolites detected
Propranolol	24	13	69 (9/13)	15
Bumetanide	6	6	100	0
Clenbuterol	5	7	57	1
Ephedrine	6	9	33	3
Finasteride	9	10	60	3
Methoxyphenamine	5	5	60	2
Methylephedrine	5	5	80	1
Salbutamol	3	2	50	2
Terbutaline	2	2	50	1

[a] Coverage percentage was calculated excluding the compounds non-previously reported species detected for the first time.

Table 3
Implementation of the proposed approach based on CID fragmentation (without precursor ion isolation) and high resolution mass spectrometry (mass shifts from biotransformations), using different manufacturers [34–42].

Instrument/manufacturer	Features, acquisition modes and performance: advantages and disadvantages	Specific available software
Exactive Orbitrap™/Thermo	(+) Resolution up to *ca.* 100,000 (160,000 in Q-Exactive). The mass resolution is related to sampling/acquisition time. A single-stage Exactive™ instrument permits a resolution of 17,500 FWHM (12 Hz); 100,000 (1 Hz): a compromise between mass resolution and the number of spectra recorded per time unit should be adopted. Only a weak loss is observed in the dependency of sensitivity with acquisition time. (+) If the so-called high energy dissociation cell (HCD) is included, in full-scan mode, data acquisition software permits to rapidly alternate between two different HCD voltage settings, thus generating low- and high collision energy mass spectra during the same run [34,35]. (+) No interfering neutral molecules are present in the HCD, yielding more reliable fragmentations than in in-source CID experiment (without a dedicated collision cell). (+) Enhanced mass accuracy when measuring fragment ions. In Q-TOF MS/MS experiments, when the reference mass is lost, there is a decrease in mass accuracy. (−) The experiment (pseudo MS/MS without precursor ion isolation) in HCD cell is not as selective as dedicated MS/MS (with precursor ion isolation), so eventually the fragment ion information from two coeluting species could be misinterpreted. (−) When dealing with complex matrices, C-trap capacity may eventually be overloaded with matrix background ions, being precision and sensitivity affected [38].	MassFrontier, Metworks (product-ion filter, neutral loss filter [39])
TOF (Bruker, Agilent)	(+) Resolution up to *ca.* 50,000 (depending of manufacturer and reference *m*/*z* used). Resolution is not affected by acquisition time (but by the flight-path and electronic issues), although sensitivity relies on higher number of accumulated individual scans/transients. (+) When using full-scan and CID fragmentation without precursor ion isolation, all the ions are fragmented without any loss of information. (−) In-source CID experiment accomplished in ion transportation section (without a dedicated collision cell) may be less effective for analytes difficult to fragment.	Bruker metabolite tools and Meteor Software [40]
Q-TOF (Bruker, Agilent, Applied Biosystems, *etc.*)	(+) Resolution up to *ca.* 50,000 (depending of manufacturer and reference *m*/*z* used). Resolution is not affected by acquisition time (but by the flight-path and electronic issues), although sensitivity relies on higher number of accumulated individual scans/transients. (+) Dedicated fragmentation in a collision cell with precursor ion isolation is more selective. (−) Reduced number of acquired MS/MS spectra of enough quality per acquisition point. (−) Data dependent acquisition (or information dependent acquisition) mode (the instrument alternates between MS (full-scan and MS/MS mode) by selecting precursor ions in the quadrupole mass filter based on defined selection criteria applied to the mass spectral data) may be useless in complex matrices when targeting species at (ultra)trace levels. This technique has the inherent disadvantage of dedicating analysis time exclusively to a single precursor ion, while all other ions sampled from the ion source into the mass spectrometer escape detection. As the most commonly used criterion for selecting a precursor ion is its prevalence in the mass spectrum, the chance of selecting a drug molecule ion among a complex background is low.	Mass Profiler Professional and Molecular Feature Extraction (data mining tools) (MassHunter) (Agilent)
TOF/QTOF (Waters) featuring MS^E	(+) MS^E is based on the use of full-scan acquisition and CID fragmentation without precursor ion isolation, so that all the ions are fragmented without any loss of information. A dedicated collision cell (without precursor ion selection) enables effective fragmentation at reproducible conditions, so that exact-masses of precursor and fragment ion are obtained from every detectable component in the sample. Under appropriate conditions, the result is one spectrum containing the intact molecular ion, whereas the high-collision energy spectrum displays fragment ion information [36,37]. (+) Dedicated fragmentation in a collision cell is more effective and reproducible than in-source CID. (+) Enhanced mass accuracy when measuring fragment ions when using MS^E. In dedicated Q-TOF MS/MS experiments, when the reference mass is lost, there is a decrease in mass accuracy. (−) The experiment (pseudo MS/MS without precursor ion isolation) in HCD cell is not as selective as dedicated MS/MS (with precursor ion isolation), so eventually the fragment ion information from two coeluting species could be misinterpreted. (−) Data dependent acquisition may be less useful when working with low concentrated species.	MetaboLynx [41] and Mass Lynx [42] (prediction of metabolites/mass defect filtering (MDF)) MassFragment (tool for the structural assignment of product ions from known structures)
Triple Quadrupole (most vendors) or QTRAP (Applied Biosystems) (operated in precursor ion scan mode)	(+) Precursor ion scan (PIS) experiment is a smart approach highly useful for selected cases, *e.g.* to detect a family of compounds [6,18], although a previous knowledge on the species of interest prior to acquisition method implementation is required. (−) Do not feature high resolution/accurate mass measurements. (−) In precursor scanning experiments one needs to know the fragmentation pattern of the parent ion in advance in order to find the appropriate diagnostic ion. (−) Loss of sensitivity while using scanning modes such as precursor ion scan.	

satisfactory and the approach enabled the identification of several non-previously reported metabolites on a systematic basis. The study with propranolol enabled the identification of up to 24 metabolites, 15 of them not been previously described in literature, which is a valuable indicator of the usefulness of the proposed systematic procedure. The methodology can be considered as a comprehensive "top-down/bottom-up" approach since identification of metabolites can be obtained from accurate mass shifts starting from the unaltered parent drug (top-down), while the use of diagnostic ions (those fragment ions common in a class of species) can also be used as markers for identifying metabolites (bottom-up) starting from the core/nuclei of the metabolite structure which may be common to that of the parent drug. The proposed methodology can be easily implemented with most of high resolution mass spectrometers available in the market. Differences will rely on whether CID fragmentation is performed during ion transportation (at relatively high pressure) or in dedicated collision cells, in both cases without precursor ion isolation.

Acknowledgments

The authors acknowledge funding from Junta de Andalucía (Research Project Ref. FQM-2614 and Research Group FQM-323). J.C.D.-R. also acknowledges a research contract from Junta de Andalucía (Project ref. FQM-2614). Pachy Orriach is also acknowledged for artwork preparation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aca.2012.11.049.

References

[1] N.J. Clarke, D. Rindgen, W.A. Korfmacher, K.A. Cox, Anal. Chem. 73 (2001) 431A–439A.
[2] B. Prasad, A. Garg, H. Takwani, S. Singh, Trends Anal. Chem. 30 (2011) 360–387.
[3] S. Ma, S.K. Chowdhury, Anal. Chem. 83 (2011) 5028–5036.
[4] M. Thevis, A. Thomas, W. Schänzer, Anal. Bioanal. Chem. 401 (2011) 405–420.
[5] M. Thevis, W. Schänzer, Mass Spectrom. Rev. 26 (2007) 79–107.
[6] O.J. Pozo, D. Van Eenoo, K. Deventer, T. Delbeke, Trends Anal. Chem. 27 (2008) 657–671.
[7] M. Zhu, H. Zhang, W.G. Humphreys, J. Biol. Chem. 286 (2011) 25419–25425.
[8] R.J.B. Peters, A.A.M. Stolker, H.G.J. Mol, A. Lommen, E. Lyris, Y. Angelis, A. Vonaparti, M. Stamou, C. Georgakopoulos, M.W.F. Nielen, Trends Anal. Chem. 29 (2010) 1250–1268.
[9] A. Lommen, Anal. Chem. 81 (2009) 3079–3086.
[10] R.J.B. Peters, M.C. van Engelen, M.E. Touber, C. Georgakopoulus, M.W.F. Nielen, Rapid Commun. Mass Spectrom. 23 (2009) 2329–2337.
[11] O.J. Pozo, W. Van Thuyne, K. Deventer, P. Van Eenoo, F.T. Delbeke, J. Mass Spectrom. 43 (2008) 394–408.
[12] R. Kostiainen, T. Kotiaho, T. Kuuranne, S. Auriola, J. Mass Spectrom. 38 (2003) 357–372.
[13] P. Zhu, W. Tong, K. Alton, S. Chowdhury, Anal. Chem. 81 (2009) 5910–5917.
[14] H. Zhang, D. Zhang, K. Ray, J. Mass Spectrom. 38 (2003) 1110–1112.
[15] H. Zhang, L. Ma, K. He, M.M. Zhu, J. Mass Spectrom. 43 (2008) 1191–1200.
[16] H. Zhang, Y. Yang, J. Mass Spectrom. 43 (2008) 1181–1190.
[17] P. Zhu, W. Ding, W. Tong, A. Ghosal, K. Alton, S. Chowdhury, Rapid Commun. Mass Spectrom. 23 (2009) 1563–1572.
[18] O.J. Pozo, K. Deventer, P. Van Eenoo, F.T. Delbeke, Anal. Chem. 80 (2008) 1709–1720.
[19] E. Liotta, R. Gottardo, A. Bertaso, A. Polettini, J. Mass. Spectrom. 45 (2010) 261–271.
[20] WADA technical document – TD2003IDCR. www.wada-ama.org
[21] The 2012 prohibited list. www.wada-ama.org
[22] H.A. Semple, F. Xia, J. Chromatogr. B 655 (1994) 293–299.
[23] C.R. Otey, G. Bandara, J. Lalonde, K. Takahashi, Biotechnol. Bioeng. 93 (2006) 494–499.
[24] G.L. Tindell, T. Walle, T.E. Gaffney, Life Sci. 11 (1972) 1029–1036.
[25] T. Walle, T.E. Gaffney, J. Pharmacol. Exp. Therap. 182 (1972) 83–92.
[26] V.T. Vu, F.P. Abramsom, Drug. Metab. Dispos. 8 (1980) 300–304.
[27] J.F. Pritchard, D.W. Schneck, W.J. Racz, A.H. Hayes, Clin. Biochem. 11 (1978) 121–125.
[28] V.L. Herring, J.A. Johnson, J. Chromatogr. 612 (1993) 215–221.
[29] J.F. Pritchard, D.W. Schneck, A.H. Hayes, J. Chromatogr. 162 (1979) 47–58.
[30] D.E. Easterling, T. Walle, E.C. Conradi, T.E. Gaffney, J. Chromatogr. 162 (1979) 439–445.
[31] S. Qureshi, H.S. Buttar, J. Chromatogr. 431 (1988) 465–470.
[32] E.C. Kwong, D.D. Shen, J. Chromatogr. (1987) 365–379.
[33] L. Abranko, J.F. García-Reyes, A. Molina-Díaz, J. Mass Spectrom. 46 (2011) 478–488.
[34] P. Eichorn, S. Pérez, D. Barceló, Compr. Anal. Chem. 58 (2012) 217–272.
[35] A. Kaufmann, Compr. Anal. Chem. 58 (2012) 169–215.
[36] An overview of the principles of MS^E, the engine that drives MS performance, Waters, 2011 (Publication ref. 720004036EN).
[37] F. Hernández, L. Bijlsma, J.V. Sancho, E. Diaz, M. Ibañez, Anal. Chim. Acta 684 (2011) 96–106.
[38] A. Kaufmann, M. Widmer, K. Maden, S. Walker, M. Widmer, Rapid Commun. Mass Spectrom. 24 (2010) 2162.
[39] Q. Ruan, S. Peterman, M.A. Szewc, L. Ma, D. Cui, W.G. Humphreys, M. Zhu, J. Mass Spectrom. 43 (2008) 251–261.
[40] A. Pelander, E. Tyrkko, I. Ojanpera, Rapid Commun. Mass Spectrom. 23 (2009) 506–514.
[41] T. Rousu, J. Herttuainen, A. Tolonen, Rapid Commun. Mass Spectrom. 24 (2010) 939–957.
[42] R.J. Mortishire-Smith, J.M. Castro-Pérez, K. Yu, J.P. Shockcor, J.K. Goshawk, M.J. Hartshorn, A. Hill, Rapid Commun. nine Mass Spectrom. 23 (2009) 939–948.