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Comparison of the analysis of β-blockers by different techniques

E. Pujos, C. Cren-Olivé*, O. Paisse, M.M. Flament-Waton, M.F. Grenier-Loustalot

Service Central d'Analyse, USR 059, CNRS, 69360 Solaize, France

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ABSTRACT

In this work we have compared three analytical techniques (ELISA, GC–MS, and LC–MS) for the analysis of 16 β-blockers: acebutolol, alprenolol, atenolol, betaxolol, bisoprolol, carteolol, labetalol, metipranolol, metoprolol, nadolol, oxprenolol, pindolol, propranolol, sotalol, timolol, and bupranolol. Several sample-preparation methods were optimized for each technique and enabled compounds of interest to be extracted from small urine samples (1–2.5 mL). The results enabled us to assess the possibilities and the sensitivity of each technique for application to doping tests. ELISA, whose selectivity is very poor and sensitivity the lowest one, is, nevertheless, useful as a rapid screening method. GC/MS and LC/MS provide confirmation procedures with the identification and quantification of the β-blockers with good sensitivity, accuracy, precision. The LC–MS analytical procedure allows the determination of the target analytes in the lower ng/mL range (0.53–2.23 ng/mL). The methodology was applied to the analysis of β-blockers in different urines.

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1. Introduction

β-Blockers are, since January 1988, on the list of substances prohibited in athletic competitions by the World Anti-Doping Agency [1]. Designed primarily as drugs used for the management of cardiac arrhythmias and cardioprotection after myocardial infarction, under conditions of impaired cardiovascular system, the β-blockers also improve the heart's ability to relax and exhibit calming neurological effects decreasing anxiety, nervousness and stabilizing motor performance [2]. The improved psychomotor performance may be beneficial in sports requiring coordination, steady hands, precision and accuracy such as shooting, archery, golf, billiards, and gymnastics [2].

A common feature in the chemical structure of β-blockers is that there is at least one aromatic ring structure attached to a side alkyl chain possessing a secondary hydroxyl and amine functional group. They present a wide range of lipophilicity ($\log P$ ranging from −1.0 to 4.0), similar molecular weights (∼300) and pK_as (∼9.2) [3–5]. For the above reasons, this study investigated analysis of 16 β-blockers with varying lipophilicities ($\log P$ ranging from 0.16 to 3.48, Table 1), used in doping.

Different techniques have been used to determine β-blockers in urine, including methods based on spectrofluorimetry [6–8], ELISA [9,10], and chromatography [11–26]. Most of the chromatographic methods are based on gas chromatography/mass spectrometry coupling (GC/MS), requiring the derivatization of polar groups (aminopropanol chain) of compounds [11,14,17–19,21–23,25,26]. However, the GC–MS analysis of the most polar β-blockers appeared a real analytical, which has limited the use of this technique. This is why alternative methods have been developed in particular liquid chromatography/mass spectrometry coupling (LC/MS) [12,13,16,20,24].

In this context, the present work focused on the detection and quantification of 16 β-blockers presenting a wide range of lipophilicity ($\log P$ ranging from 0.16 to 3.48, Table 1): acebutolol, alprenolol, atenolol, betaxolol, bisoprolol, carteolol, labetalol, metipranolol, metoprolol, nadolol, oxprenolol, pindolol, propranolol, sotalol, timolol, and bupranolol. The work involved the development of β-blockers multi-residue analysis in urine by three different techniques ELISA, gas chromatography coupled with mass spectrometry, and liquid chromatography coupled with mass spectrometry, in order to compare the possibilities and limits of each for doping tests.

2. Experimental part

2.1. β-Blockers

The following β-blockers were used in this work: acebutolol hydrochloride (Specia Laboratory, Paris, France), alprenolol hydrochloride (Astra France, Nanterre, France), atenolol, betaxolol hydrochloride, bisoprolol hemifumarate, carteolol hydrochloride (Lipha-Santé, Lyon, France), labetalol hydrochloride (Sigma–Aldrich, St. Quentin Fallavier, France), metipranolol, metoprolol tartrate, nadolol, propranolol, sotalol hydrochloride

* Corresponding author at: Service Central d'Analyse, Chemin du Canal, Echangeur de Solaize, 69360 Solaize, France.
E-mail address: c.cren@sca.cnrs.fr (C. Cren-Olivé).

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Table 1
Physico-chemical characteristics of studied β-blockers.

Compounds studied	Molar mass (g/mol)	Log P[a]
Atenolol	266.34	0.16
Sotalol	272.37	0.24
Nadolol	309.41	0.81
Carteolol	292.38	1.42
Acebutolol	336.43	1.71
Pindolol	248.33	1.75
Timolol	316.43	1.83
Bisoprolol	325.45	1.87
Metoprolol	267.37	1.88
Oxprenolol	265.36	2.10
Betaxolol	307.44	2.81
Metipranolol	309.41	2.66
Bupranolol	271.79	3.07
Labetalol	328.41	3.09
Alprenolol	249.36	3.10
Propranolol	259.34	3.48

[a] Experimental values from SRC Database [3].

(Bristol-Myers Squibb Pharmaceuticals, Moreton, United Kingdom), oxprenolol hydrochloride, pindolol (Sandoz, Aubervilliers, France), timolol maleate (Merck Sharp & Dohme Chibret, Clermont-Ferrand, France), and bupranolol hydrochloride (Schwarz Pharma AG, Manheim, Germany). They were used with no prior purification.

2.2. Solvents and reagents

Ethyl acetate, diethyl ether and methanol, hydrochloric acid and ammonium formate, tert-butanol and methyl boronic acid (MBA) were obtained from Sigma–Aldrich (St. Quentin Fallavier, France). N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) was purchased from Merck (Fontenay-sous-Bois, France) and N-methyl-bis(trifluoroacetamide) MBTFA from Pierce (Brebières, France). $NaHCO_3$, K_2CO_3, Na_2SO_4, and sodium acetate were obtained from VWR International S.A.S. (Fontenay-sous-Bois, France), *Escherichia coli* β-glucuronidase was purchased from Boehringer Mannheim (Mannheim, Germany), and formic acid from Riedel de Haën (Seelze, Germany).

2.3. Urine samples

Four urine samples were analyzed in this study: (i) a mean standard urine which was prepared by collecting and pooling samples from men who were not taking any treatment ($n = 20$) (ii) and three urine samples from subjects treated with β-blockers: (A) with propranolol at a concentration of 20 mg/day, (B) with propanolol at a higher concentration (60 mg/day), and lastly (C) with sotalol at 160 mg/day.

2.4. ELISA technique

2.4.1. Material

The ELISA kit used for the analysis of β-blockers is commercialized for the analysis of bronchodilators (Neogen, Cat. No. 100319) (Lexington, USA). No other kit specific for β-blockers was, in fact, commercially available. ELISA plates were washed with a Wellwash 4Mk2 (ThermoLabsystems, Issy Les Moulineaux, France) automatic plate washer. Absorbance measurements were carried out with a Multiskan EX spectrophotometer (ThermoLabsystems, Issy Les Moulineaux, France).

2.4.2. Methods

2.4.2.1. Preparation of standard solutions of β-blockers. The compounds were dissolved in methanol at a concentration of 1 mg/mL, dilutions were first prepared in the same solvent, and then in urine (without exceeding a final methanol concentration of 5%). A mean standard urine was prepared by collecting and pooling samples from men who were not taking any treatment. This urine was first used as blank and was then spiked with the different standard solutions of β-blockers (without exceeding a final methanol concentration of 5%).

2.4.2.2. Kit procedure. The principle of this kit is based on a competitive-type ELISA. Since this kit is designed for screening bronchodilators, anti-terbutalin antibodies are bound to the solid support (plate). In these conditions, terbutalin reactivity is about 100%. β-Blockers react via cross-reaction; and their reactivity was evaluated.

The ELISA test was applied according to the supplier's specifications. Standard solutions or samples (20 μL) were added to each microplate well with 180 μL of the conjugate solution (terbutalin + peroxidase). Wells were incubated at room temperature in darkness for 45 min. After washing, 100 μL of substrate (TMB) was added to each well, and incubation for 30 min at room temperature was performed for color development. Acid solution (75 μL, 1N HCl) was finally added to stop the reaction. The optical density for each test well was determined at 450 nm. A blank urine and a positive control were included in each strip when analyzing samples.

2.4.2.3. Kit validation. The ELISA kit used is usually used for the analysis of bronchodilators. So, β-blockers only cross-react and it was necessary to select a standard to calibrate and quantify the reactivity of β-blockers. The calibration was performed using solutions of bupranolol.

2.4.2.4. Processing the results. The ratio (% maximal absorbance) was calculated for each sample as follows:

$$\text{Ratio}\,(\%) = \frac{A(\text{sample}) - A(\text{blank})}{A(\text{control}) - A(\text{blank})} \times 100$$

where A is the measured absorbance.

The calibration line was prepared by plotting this ratio *vs.* the bupranolol concentration (logarithmic abscissa) and carrying out a logarithmic regression. The equation of the line is: ratio = $b \log(\text{concentration}) + a$.

2.4.2.5. Reactivities. The percentage reactivity (cross-reaction) was calculated for each β-blockers. ED (50) (effective dose) is the β-blockers concentration furnishing a ratio of 50%. As bupranolol was chosen as the reference compound, the measured reactivities are relative to bupranolol in this study.

2.5. GC/MS technique

2.5.1. Material

2.5.1.1. Gas chromatography coupled with mass spectrometry (GC/MS). Analyses were carried out with an Agilent apparatus equipped with a 5973 mass detector, an HP 6890 GC gas chromatograph and an HP 7683 sample changer. Chromatographic separation was developed on an HP-5MS capillary column (JW Scientific, Courtabœuf, France) (30 m × 0.25 mm inner diameter; 0.25 μm film thickness). Injector temperature was 250 °C and 1 μL was injected in splitless pulsed mode.

Two types of derivatization of β-blockers were tested and required the development of two different chromatographic methods. The first method was developed for TMS derivatives. Helium pressure at the column head was adjusted to 25.73 psig for the entire analysis. The initial temperature of the oven was 160 °C (maintained for 1 min), followed by a gradient of 20 °C/min up to 290 °C (maintained for 2 min), and then 20 °C/min gradient up to 300 °C (maintained for 1 min). Detector temperature was

280 °C. The second method was developed for boronate derivatives. Helium pressure at the column head was adjusted to 22 psig for the entire analysis. The initial temperature of the oven was 110 °C (maintained for 1 min), followed by a gradient of 30 °C/min up to 230 °C, and then 20 °C/min gradient up to 270 °C, and lastly 30 °C/min up to 300 °C (maintained for 5 min). Detector temperature was 280 °C. Measurements in the GC–MS were performed in the single-ion monitoring (SIM) mode and in scan mode, the mass detector acquired masses from 200 to 500 amu.

2.5.2. Methods

2.5.2.1. Preparation of standard solutions. β-Blockers were dissolved in methanol at a concentration of 1 mg/mL. All dilutions were prepared in this solvent. A mixture of the 16 β-blockers was also prepared by adding the same volume of stock solution of each compound. The final concentration of each 16 β-blocker in the mixture was 62.5 μg/mL.

2.5.2.2. Preparation of doped urine. 2.5 mL urine from man (standard urine) was doped by adding 20 μL of the standard mixture of β-blockers. This urine was extracted and analyzed by GC/MS before and after spiking.

2.5.2.3. Processing urine samples for GC/MS analysis. The starting sample was 2.5 mL urine. The first step was an enzymatic hydrolysis. This step performed in mild conditions does not lead to β-blockers structure hydrolysis, contrary to chemical hydrolysis. Enzymatic hydrolysis was then conducted with 50 μL of *E. coli* β-glucuronidase in buffered medium (500 μL of 2 M acetate buffer, pH 5.2) for 3 h, at 55 °C. The sample is cooled and then, liquid/liquid extraction was performed with 2.5 mL of diethyl ether. The mixture was vortex mixed (30 s), and centrifuged at 2500 rpm for 5 min. The ether phase was recovered and the pH of the remaining aqueous phase was adjusted to 9.5 by adding 0.25 g of $NaHCO_3/K_2CO_3$ buffer (1/2, w/w). Then, 500 μL of tert-butanol and 2.5 mL of ether were added for a second extraction (vortex mixed for 30 s, then centrifugation for 5 min at 2500 rpm). The ether phases were pooled, dried over anhydrous sodium sulfate, and evaporated to dryness under nitrogen at 40 °C.

2.5.2.4. Study of the derivatization reaction. The first tested derivatization is the silylation/acetylation of the β-blockers. The dry residue was combined with 100 μL of MSTFA. The mixture was vortex mixed and then heated at 80 °C for 10 min. 30 μL of MBTFA was added. The mixture was heated at 80 °C for 10 min. The mixture is cooled and an aliquot of 1 μL was injected onto the GC–MS system. The derivatization of an alcohol function to a trimethylsilylated function (noted OTMS) increases molar mass by 72. The transformation of an amine function into a trifluoroacetate derivative (noted NTFA) increases molar mass by 96.

The second tested derivatization is the formation of boronates. 250 μL of methyl boronic acid (MBA at 20 mg/mL in ethyl acetate) was added to the dry residue and the mixture was heated at 60 °C for 10 min. The mixture is cooled, evaporated to dryness under nitrogen and then reconstituted in 150 μL of ethyl acetate. An aliquot of 1 μL was injected onto the GC–MS system. These conditions were optimized in order to obtain the total derivatization of β-blockers. The formation of a methyl boronate derivative increases molar mass by 24.

2.6. LC/MS technique

2.6.1. Material

2.6.1.1. Liquid chromatography coupled with mass spectrometry (LC/MS). Analyses were conducted with a Hewlett-Packard HP 1100 MSD (Agilent, Massy, France) equipped with a UV detector diode array and a mass detector with atmospheric pressure ionization (API) and electrospray ionization (ESI) systems. Chromatographic separation was on an Uptisphere HDD C18 column (100 mm × 2 mm inner diameter, 3 μm particle size). The mobile phase was prepared from an aqueous eluent (A) (10 mM ammonium format buffer in water, adjusted to pH 3.9 with formic acid) and an organic eluent (B) (methanol). 100 μL was injected. The flow-rate of the mobile phase was 0.3 mL/min. Oven temperature was maintained at 50 °C and an elution gradient was used. The methanol content was increased linearly from 17 to 40% (v/v) in 22 min, then up to 100% in 8 min. It was adjusted back to 17% (v/v) and held for 15 min. UV detection was performed at 260 nm. Measurements were performed in the single-ion monitoring (SIM) mode, and in scan mode, the mass detector acquired masses from 200 to 450 amu. The electrospray conditions of the mass spectrometer were as follows: nebulizer gas (nitrogen) at 60 psi, drying gas (nitrogen) at 13 mL/min, and 350 °C, capillary voltage at 4000 V and the voltage of fragmenter at 70 V.

2.6.2. Methods

2.6.2.1. Preparation of standard solutions. The β-blockers were dissolved in methanol at the concentration of 1 mg/mL (stock solutions) and all subsequent dilutions were prepared in this solvent. A standard mixture of β-blockers was prepared by adding the same volume of stock solution of each compound. The final concentration of each 16 β-blocker in the mixture was 62.5 μg/mL.

2.6.2.2. Preparation of doped urine. 2.5 mL of urine from man (standard urine) was spiked by adding 20 μL of the standard mixture of β-blockers. This urine was extracted and analyzed by LC/MS before and after spiking.

2.6.2.3. Processing urine samples for LC/MS analysis. The procedure was similar to that described for GC–MS analysis. After the hydrolysis step the sample can be directly analyzed by LC–MS or it can be submitted to liquid/liquid extraction as previously described, reconstituted in 200 μL of methanol and then analyzed by LC–MS.

3. Results and discussion

3.1. ELISA technique

3.1.1. Kit validation

The ELISA kit used for the analysis of β-blockers is commercialized for the analysis of bronchodilators. So, in one hand, false positive responses of the ELISA kit with clenbuterol, salbutamol and other substances can occur and the use of this ELISA test is restricted to screening method needing a second confirmation step.

In the other hand, all β-blockers cross-react and so it was necessary to select a standard to calibrate and quantify the reactivity of β-blockers. The calibration was performed, for each analysis, by assaying bupranolol standards. The range of linearity was between 1 and 100 ng/mL. Using the equation of the line (ratio = b log(concentration) + a), the relative standards deviation obtained on the same plate were: 2.71% for a and 0.41% for b. The differences were relatively low, showing good reproducibility with regard to the calibration lines.

3.1.2. Determination of reactivities and limits of detection

Cross-reactivities were determined for the β-blockers by analyzing male urines spiked with standard solutions of these different compounds. The limits of detection were estimated as the analyte concentration with a signal-to-noise ratio of 3 when analysing spiked urine of decreasing concentrations. The results (Table 2) show that the limits of detection for the different products could

Table 2
Limits of detection of β-blockers with the ELISA technique.

	Cross-reactivity (%)	LOD (ng/mL)
Bupranolol	100	1
Carteolol	69.6	1.4
Nadolol	28.1	3.6
Sotalol	15.3	6.5
Timolol	6.1	16.5
Metipranolol	4.5	22.3
Propranolol	4.3	23.4
Bisoprolol	2.9	34.7
Pindolol	2.7	36.4
Atenolol	2.1	46.9
Betaxolol	2.1	46.9
Alprenolol	1.9	53.3
Metoprolol	1.0	95.8
Acebutolol	0.5	192.5
Labetalol	0.5	205.7
Oxprenolol	0.2	575.4

Table 3
Analyses of urines with the ELISA technique.

Urine sample	ELISA detection	Treatment
Urine A	++	Propranolol, 20 mg/day
Urine B	+++	Propranolol, 60 mg/day
Urine C	++	Sotalol, 160 mg/day
Blank	–	None

be determined. Reactivities (and thus the limits of detection) are highly variable among the compounds because of their different structures. The highest reactivities were obtained with β-blockers presenting a tert-butyl group on their amine function. This can be explained by the fact that the used ELISA kit was designed to detect terbutalin (which also possesses this chemical group).

3.1.3. Application to analyses of urine

Three urine samples from subjects treated with β-blockers were collected. The first subject (A) was treated with propranolol at a concentration of 20 mg/day, the subject B was treated with the same molecule (propanolol) but at a higher concentration (60 mg/day), lastly, the subject C treated with sotalol at 160 mg/day. Urine samples from control (untreated) subjects and those treated with β-blockers were then analyzed.

The results obtained with the ELISA kit (Table 3) show that it is possible to discriminate subjects treated with β-blockers from untreated individuals. So, the ELISA test furnishes an assay of β-blockers present in urine and can be used as a technique for systematic and rapid detection. Nevertheless, additional chromatographic analysis is required to confirm the results and determine the nature of the detected β-blockers.

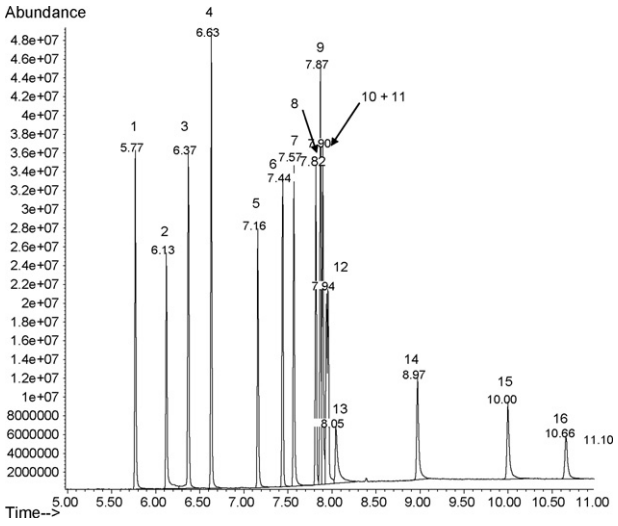

Fig. 1. Chromatogram in SIM mode of the boronate derivatives of the 16 β-blockers at 625 ng/mL analyzed by GC/MS (1 alprenolol, 2 oxprenolol, 3 bupranolol, 4 metoprolol, 5 propranol, 6 metipranolol, 7 pindolol, 8 bisoprolol, 9 betaxolol, 10 timolol, 11 sotalol, 12 nadolol, 13 atenolol, 14 carteolol, 15 acebutolol, and 16 labetalol).

3.2. GC/MS technique

3.2.1. Development of the analysis by GC/MS

Different derivatization reactions for the alcohol and the amine functions of β-blockers have been discussed [11,22,26]. Two different reactions were evaluated in this work: (i) the formation of cyclic methylboronate derivative [26] and (ii) the silylation/acetylation of the β-blockers leading to TMS–TFA derivative [22].

3.2.1.1. Formation of cyclic methylboronate derivative. A chromatographic method for separation of the 16 β-blockers was developed. The results were acceptable to the extent that only two compounds (timolol and sotalol) were coeluted (tr = 7.90 min); but their characteristic ions were different, enabling us to identify and quantify these two compounds without any doubt (Fig. 1 and Table 4). Each β-blocker was, indeed, identified by its retention time and the presence of three characteristic ions which must respect relative intensities of the standard in the range of ±10% (Table 4). The most abundant characteristic ion was then selected for quantification of each compound in SIM mode (Table 4). The fragment ion selected

Table 4
Identification and limits of quantification of β-blockers (boronate derivatives) by GC/MS.

Number	Compounds	Retention time (min)	Molecular mass (g/mol)	Characteristic ions	Ion selected for quantification (SIM)	LOQ (ng/mL)
1	Alprenolol MBA	5.77	273	273, 258, 138	258	6.0
2	Oxprenolol MBA	6.13	289	289, 274, 218	274	17.4
3	Bupranolol MBA	6.37	295	295, 280, 155	280	7.5
4	Metoprolol MBA	6.63	291	291, 276, 140	276	8.4
5	Propranolol MBA	7.16	283	283, 268, 128	283	23.3
6	Metipranolol MBA	7.44	333	333, 291, 140	291	8.4
7	Pindolol MBA	7.57	272	272, 257, 124	272	17.2
8	Bisoprolol MBA	7.82	349	349, 334, 230	230	15.8
9	Betaxolol MBA	7.87	331	331, 316, 140	316	19.5
10	Timolol MBA	7.90	340	340, 325, 138	138	15.0
11	Sotalol MBA	7.90	296	296, 281, 239	281	2565
12	Nadolol 2 MBA	7.94	357	357, 342, 217	342	17.1
13	Atenolol MBA	8.05	290	290, 275, 164	275	17.7
14	Carteolol MBA	8.97	316	316, 301, 138	301	27.6
15	Acebutolol MBA	10.00	360	299, 246, 124	124	39.6
16	Labetalol MBA	10.66	352	271, 229, 207	271	132

Table 5
Identification of TMS/TFA derivatives of β-blockers by GC/MS.

Group	Number	β-Blockers	Retention time (min)	Molecular mass (g/mol)	Characteristic ions	Ion selected for quantification (SIM)
I, II, III	1	Bupranolol OTMS (IS)	4.93	343	328, 227, 86	328
I	2	Sotalol NTFA bis-OTMS + 18	5.22	530	515, 375, 362	362
I	3	Alprenolol NTFA OTMS	5.32	417	417 ($M^{+\bullet}$), 402, 284	284
I	4	Oxprenolol NTFA OTMS	5.73	433	433 ($M^{+\bullet}$), 418, 284	284
I, II, III	5	Bupranolol NTFA OTMS (IS)	6.00	439	368, 242, 167	242
I	6	Metoprolol NTFA OTMS	6.36	435	435 ($M^{+\bullet}$), 420, 284	284
I	7	Propranolol NTFA OTMS	6.85	427	427 ($M^{+\bullet}$), 412, 284	284
I	8	Metipranolol NTFA OTMS	7.11	477	477 ($M^{+\bullet}$), 435, 284	284
I	9	Bisoprolol NTFA OTMS	7.64	493	493 ($M^{+\bullet}$), 332, 284	284
I	10	Pindolol NTFA bisNOTMS	7.72	488	488 ($M^{+\bullet}$), 318, 284	284
I	11	Acebutolol NTFA bis-OTMS	8.47	576	576 ($M^{+\bullet}$), 561, 284	129
II	12	Timolol OTMS	6.64	388	373, 358, 272	373
II	13	Carteolol bis-OTMS	7.24	436	436 ($M^{+\bullet}$), 421, 235	235
II	14	Nadolol tri OTMS	7.47	525	525 ($M^{+\bullet}$), 510, 409	510
II	15	Timolol NTFA OTMS	7.58	484	484 ($M^{+\bullet}$), 413, 242	242
II	16	Betaxolol NTFA OTMS	7.70	475	475 ($M^{+\bullet}$), 460, 284	284
II	17	Carteolol NTFA bis-OTMS	8.21	532	532 ($M^{+\bullet}$), 517, 375	375
II	18	Nadolol NTFA tri OTMS	8.41	621	621 ($M^{+\bullet}$), 606, 474	474
III	19	Atenolol NTFA OTMS	7.67	416	416 ($M^{+\bullet}$), 326, 284	284
III	20	Labetalol NTFA OTMS	8.97	478	478 ($M^{+\bullet}$), 292, 221	292

for SIM quantification was, often, formed by the loss of a methyl group (M-15).

3.2.1.2. Silylation/acetylation of the β-blockers leading to TMS–TFA derivative. The derivatization of the bifunctional polar groups of their aminopropanol side-chain using MSTFA/MBTFA usually led to the formation of TMS–TFA derivative (Table 5). But for some β-blockers, other derivatives could be formed; notably TMS derivative only, in addition of the TMS–TFA derivative (Table 5). This was particularly the case for compounds presenting an amine function substituted by a tert-butyl group (bupranolol, carteolol, nadolol and timolol) that limits the formation of the TFA derivative because of its considerable steric hindrance. The multiplication of the number of analyzed compounds complicated the analysis. Indeed, even if there is no coelution, some compounds are eluted with very close retention time. For example, on the periods 7.58–7.72 min and 8.21–8.97 min of the chromatogram, 5 and 6 peaks appeared respectively (compounds 9, 10, 15, 16, 19 for the first period and 17, 18, 20 and its isomers for the second one). So we decided to divide the β-blockers of these two periods into three groups which were analyzed separately and successively. Group 1 was composed of bupranolol (IS (internal standard)), sotalol, alprenolol, oxprenolol, metoprolol, propranolol, metipranolol, bisoprolol, pindolol and acebutolol. Group 2 is constituted with bupranolol (IS), carteolol, nadolol, timolol and betaxolol. Group 3 was composed of bupranolol, atenolol and labetalol, and its stereoisomers and different derivatives. So an analysis is made of three successive injections (corresponding to the three groups of β-blockers) in SIM mode, in order to verify the presence or absence of the compounds of interest.

The three chromatograms (Figs. 2–4) illustrate the separation obtained for the three groups described above. Each derivative was identified by its retention time and the presence of three characteristic ions which must respect relative intensities of the standard in the range of ±10%. The most abundant characteristic ion was then selected to quantify the compound in SIM mode (Table 5). The frequently observed fragments were [M-CH_3] (loss of 15 amu) and [M-OTMS] (loss of 90 amu). The ion $m/z = 284$ corresponds to the isopropylaminopropanol chain and yields to the ion $m/z = 242$ through a loss of isopropyl and to the TMS fragment at $m/z = 73$.

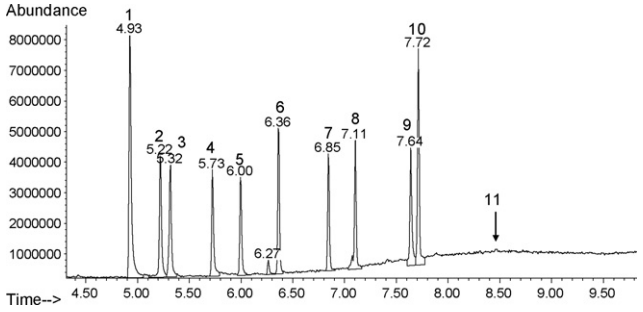

Fig. 2. Chromatogram in SIM mode of TMS/TFA derivatives of the 11 β-blockers (Group 1) at 625 ng/mL by GC/MS (1 bupranolol OTMS (IS), 2 sotalol NTFA bis-OTMS, 3 alprenolol NTFA OTMS, 4 oxprenolol NTFA OTMS, 5 bupranolol NTFA OTMS (IS), 6 metoprolol NTFA OTMS, 7 propranolol NTFA OTMS, 8 metipranolol NTFA OTMS, 9 bisoprolol NTFA OTMS, 10 pindolol NTFA bisNOTMS, and 11 acebutolol NTFA bis-OTMS).

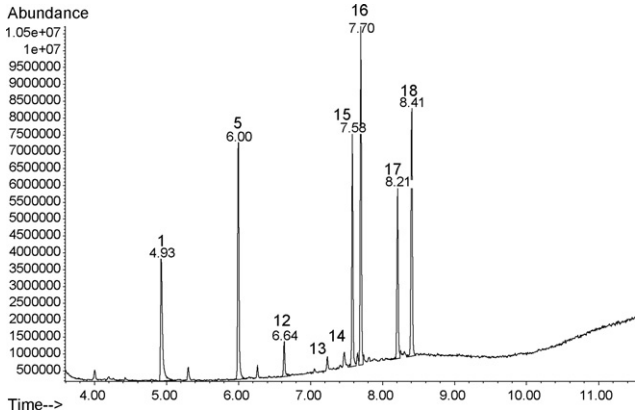

Fig. 3. Chromatogram in SIM mode of TMS/TFA derivatives of the 9 β-blockers (Group 2) at 625 ng/mL by GC/MS (1 bupranolol OTMS (IS), 5 bupranolol NTFA OTMS (IS), 12 timolol OTMS, 13 carteolol bis-OTMS, 14 nadolol tri OTMS, 15 timolol NTFA OTMS, 16 betaxolol NTFA OTMS, 17 carteolol NTFA bis-OTMS, and 18 nadolol NTFA tri OTMS).

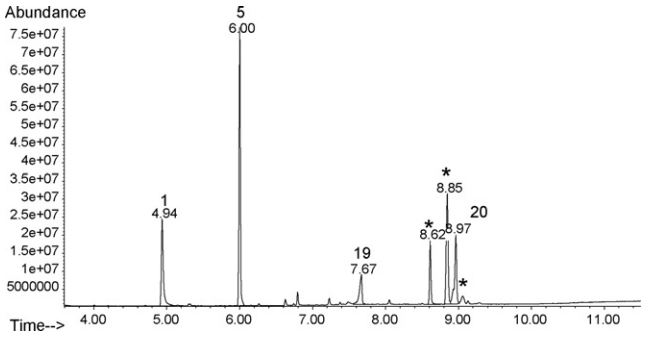

Fig. 4. Chromatogram in SIM mode of TMS/TFA derivatives of the 4 β-blockers (Group 3) at 625 ng/mL by GC/MS (1 bupranolol OTMS (IS), 5 bupranolol NTFA OTMS (IS), 19 atenolol NTFA OTMS, and 20 labetalol NTFA OTMS, *labetalol stereoisomers and different derivatives).

Table 6
Extraction recovery for the 16 β-blockers.

	Extraction recovery (%)	RSD (%) (n = 4)
Alprenolol	84	3.9
Oxprenolol	81	3.9
Bupranolol	76	3.6
Metoprolol	83	5.1
Propranolol	80	4.0
Metipranolol	79	3.4
Pindolol	75	4.4
Bisoprolol	84	4.1
Betaxolol	92	3.5
Timolol	80	3.0
Sotalol	90	4.3
Nadolol	43	2.8
Atenolol	8	4.3
Carteolol	76	5.8
Acebutolol	63	3.9
Labetalol	65	4.4

3.2.2. Method performance

The method performance was studied by the evaluation of sensitivity, recoveries, precision, linearity, and accuracy.

The sensitivity was evaluated by the determination of the LOQ. The limits of quantification for all the β-blockers were evaluated using a signal-to-noise ratio of 10 by injecting male urines spiked with standard solutions of these different compounds. The results (Table 4 for cyclic methylboronate derivative and Table 9 for TMS–TFA derivative) show that the limits of quantification are highly dependent on the nature of the β-blocker. Nevertheless, these compounds generally respond well in GC/MS and present relatively low instrumental limits of quantification: for the TMS–TFA derivatives, the LOQ ranged from 0.30 ng/mL for sotalol to 9.01 ng/mL for nadolol (except for atenolol (40.1 ng/L) and labetolol (102 ng/mL), Table 9). Besides, for the cyclic methylboronate derivatives, they ranged from 6.0 ng/mL for alprenolol to 39.6 ng/mL for acebutolol (except for labetolol (132 ng/mL) and sotalol (2.5 μg/mL), Table 4). Moreover, the sample preparation enables 2.5-fold concentration. So the majority of compounds could be analyzed with a single injection down to 2.5–16 ng/mL in real urine samples.

Analyte recoveries were determined in spiked urine by adding known and appropriate volumes of the working standard solution. Table 6 lists the results and the relative standard deviation (RSD). All analyte recoveries were very good with RSD inferior to 6%; except atenolol, as expected since it is the more polar compound.

The linearity was studied by injecting seven concentrations of the standard solutions of the target compounds in the range LOQ to 200 μg/mL. A good linearity was observed over the specified range with correlation coefficients varying between 0.9928 and 0.9989.

Both intra- and inter-batch precisions were examined. For the intra-batch precision, a standard solution (500 ng/mL of each compound) was injected successively five times. The RSD was in the 2.2–7.5% range for all compounds. For the inter-batch experiment, three injections of the standard solution were performed on five different days distributed over one month. In this case the RSD were between 6.8 and 12.9%.

3.2.3. Application to the analysis of urine

The three urines previously analyzed with the ELISA method were extracted and analyzed by GC/MS. As a result of the high dose of β-blocker, the volume of the initial urine sample was reduced to 1 mL. Quantitative assays in both GC methods were carried out on the ions previously selected. The calibration curves of propranolol and sotalol were determined in the urine of an untreated male in order to assay these compounds in the urines of treated subjects.

Table 7 lists the GC–MS results obtained with the TMS–TFA derivatives and the cyclic methylboronate derivatives. The quantitative results are consistent for both methods: 1.52 μg/mL of propanol was found in urine A, 3.70 μg/mL in urine B and 165.4 μg/mL of sotalol in urine C. Moreover, an analysis in scan mode of urine B and C shows the metabolism of the two β-blockers. Hydroxypropanolol (MW 275, a bis-OTMS NTFA derivative of the 515 parent ion), was identified in urine B, as expected since it is the major propranolol metabolite described in the literature [27]. On the other hand, the elevated abundance of sotalol in the urine and the absence of metabolites confirm that sotalol is not metabolized in the human body [27].

The comparison of the two derivatization methods showed that the method based on the formation of TMS/TFA derivatives is more sensitive, but the background is much higher. This can be explained by the presence of a large number of derivatives. This background and the presence of different derivatives are prejudicial in the identification of a doping agent, and above all in the quantification of the β-blockers. On the other hand, the method based on the formation of boronates provides a high selectivity with a better separation and cleaner chromatograms. This method is easier to implement, faster and more robust.

3.3. LC/MS technique

3.3.1. Development and validation studies of the analysis by LC/MS

A chromatographic separation was developed. All the compounds were separated, enabling each molecule detected to be

Table 7
Analysis of urines of treated subjects by GC–MS and LC–MS.

	Concentration (μg/mL of urine)		
	GC–MS		LC–MS
	Method 1 (TMS derivatives)	Method 2 (boronate derivatives)	
Urine A	1.52 (propranolol)	1.64 (propranolol)	1.58 (propranolol)
Urine B	3.67 (propranolol)	3.70 (propranolol)	3.59 (propranolol)
Urine C	165.4 (sotalol)	163.8 (sotalol)	161.8 (sotalol)

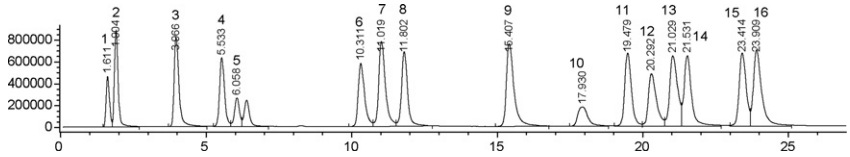

Fig. 5. Chromatograms in SIM mode of the 16 β-blockers at 150 ng/mL obtained by LC–MS (1 sotalol, 2 atenolol, 3 pindolol, 4 carteolol, 5 nadolol, 6 timolol, 7 metoprolol, 8 acebutolol, 9 oxprenolol, 10 labetalol, 11 bisoprolol, 12 propranolol, 13 metipranolol, 14 alprenolol, 15 betaxolol, and 16 bupranolol).

Table 8
Identification and limits of quantification of β-blockers by LC/MS.

Number	Compounds	Retention time (min)	Molar mass (g/mol)	Ion selected for quantification (SIM)	LOQ (ng/mL)
1	Sotalol	1.61	272	273	0.90
2	Atenolol	1.91	266	267	0.69
3	Pindolol	3.98	248	249	0.66
4	Carteolol	5.54	292	293	0.90
5	Nadolol	6.06	309	310	1.52
6	Timolol	10.32	316	317	1.13
7	Metoprolol	11.03	267	268	0.53
8	Acebutolol	11.81	336	337	0.74
9	Oxprenolol	15.42	265	266	0.95
10	Labetalol	17.91	328	329	2.23
11	Bisoprolol	19.49	325	326	0.72
12	Propranolol	20.31	259	260	1.73
13	Metipranolol	21.04	309	310	1.15
14	Alprenolol	21.53	249	250	1.57
15	Betaxolol	23.42	307	308	0.98
16	Bupranolol	23.92	271	272	0.77

easily quantified (Fig. 5). All the β-blockers were identified by their retention time and the presence of two characteristic ions which must respect relative intensities of the standard in the range of ±10%. One characteristic ion was selected for each compound in order to quantify it in SIM mode (Table 8). In all cases, it was the $[M+H]^+$ ion.

Statistical validation of the method was performed evaluating the limits of quantification (LOQ) as well as the accuracy, the precision and the linearity.

The limits of quantification were estimated as the analyte concentration with a signal-to-noise ratio of 10 when injecting male urines spiked with standard solutions (Table 8). In this case also, the limits are highly dependent on the nature of the compounds and in the range 0.53–2.23 ng/mL.

We carried out the study of precision, expressed as repeatability and accuracy, expressed as reproducibility using a standard solution (500 ng/mL of each compound). The accuracy was estimated by means of recovery experiments performing three injections of the standard solution on five different days distributed over one month. The precision of the method was determined in terms of relative standard deviation (RSD, %) from the recovery experiments ($n = 5$) of analyses performed in the same day. Results for repeatability show the good precision of the method with a RSD mean value of 3.5, 5.2 and 4.9 for the 1st day, 2nd day and 3rd day respectively. The results for reproducibility indicate the good robustness of the method with a RSD mean value of 3.5, 4.8 and 3.3 for the 1st day, 2nd day and 3rd day respectively while the reproducibility is better than 15% for all the compounds with a mean value of 3.9.

The linearity was studied by injecting seven concentrations of the standard solutions of the target compounds in the range LOQ to 200 μg/mL. A good linearity was observed over the specified range with correlation coefficients higher than 0.998 for all the compounds.

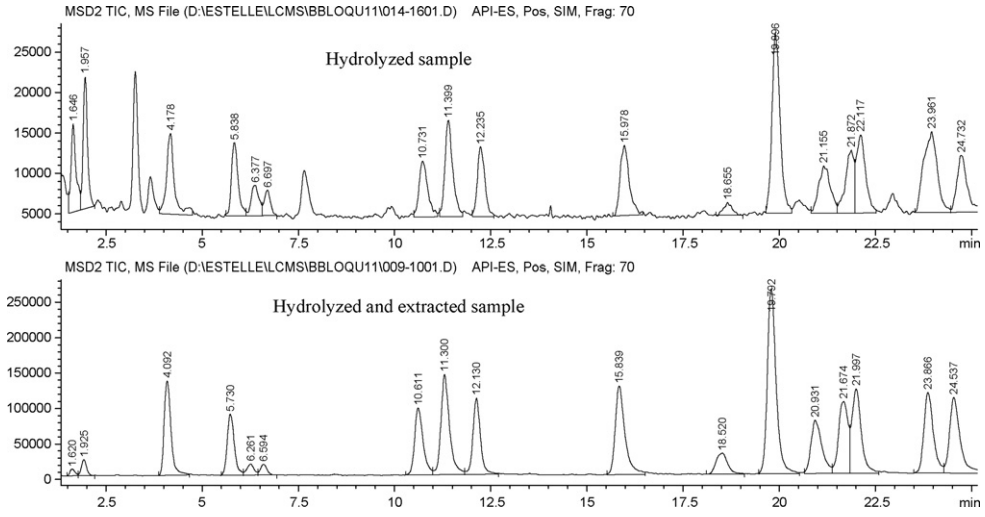

Fig. 6. LC/MS analysis of a doped urine at 500 ng/mL on hydrolyzed sample and hydrolyzed and extracted sample (cf. Fig. 6 for numbers).

Table 9
Comparison of chromatographic methods for the analysis of β-blockers.

		ELISA	GC/MS (TMS/TFA derivatives)	GC/MS (boronate derivatives)	LC/MS
Advantages	Preparation	Low	–	–	Possibility of injection after hydrolysis without extraction
	Analysis	Short	High sensitivity	High specificity short	Best sensitivity
Disadvantages	Preparation	–	Long	Long	–
	Analysis	Non-specific	Long (three injections)	Poor sotalol response Long	Long

Compounds	LOD (ng/mL)	LOQ (ng/mL)	LOQ (ng/mL)	LOQ (ng/mL)
Acebutolol	192.5	1.55	39.6	0.74
Alprenolol	53.3	3.02	6.0	1.57
Atenolol	46.9	40.1	17.7	0.69
Betaxolol	46.9	0.40	19.5	0.98
Bisoprolol	34.7	8.51	15.8	0.72
Bupranolol	1	0.51	7.5	0.77
Carteolol	1.4	5.74	27.6	0.90
Labetalol	205.7	102	132	2.23
Metipranolol	22.3	4.51	8.4	1.15
Metoprolol	95.8	1.20	8.4	0.53
Nadolol	3.6	9.01	17.1	1.52
Oxprenolol	575.4	6.58	17.4	0.95
Pindolol	36.4	2.83	17.2	0.66
Propranolol	23.4	3.44	23.3	1.73
Sotalol	6.5	0.30	2565	0.90
Timolol	16.5	0.37	15.0	1.13

3.3.2. Application to the analysis of urines

3.3.2.1. Analysis of doped urine. This analysis was used to compare the chromatograms from samples simply hydrolyzed and those both hydrolyzed and extracted. Fig. 6 illustrates the chromatograms of an urine doped with β-blockers and extracted. The results show that extraction furnishes a much cleaner chromatogram. This method was thus used to analyze the urine of untreated subjects and those receiving β-blockers. Nevertheless, analysis of samples hydrolyzed but not extracted is possible for screening, since it provides good detection and identification of compounds and sample preparation is more rapid.

3.3.2.2. Analysis of urines. Three urines from subjects treated with β-blocker, previously analyzed by ELISA and GC/MS, were prepared for LC/MS analysis. The quantitative results, listed in Table 7, are consistent with those obtained with GC/MS.

4. Conclusion

The analytical procedures described provide a simple comparison of the analysis of 16 β-blockers by different techniques: ELISA, GC–MS and LC–MS. The three methods are compared in Table 9. ELISA can be used as a screening method. Its sensitivity is lower than the sensitivity obtained with the chromatographic methods, but it can furnish a good discrimination of positive samples (since there is no interference from the urine matrix). Concerning the chromatographic methods, GC/MS and LC/MS provide identification and quantification of the β-blockers with good sensitivity, accuracy, precision. One of GC/MS drawbacks is a time-consuming sample preparation. Moreover, the poor gas chromatographic properties of several polar β-blockers derivatives can limit the use of GC–MS for doping analysis of these compounds. LC/MS results are promising since this technique enables compounds to be analyzed directly after sample hydrolysis. Furthermore, it offers, in these conditions, the best sensitivity. The subject of ongoing work is to extend the optimized methods to other matrices (e.g. environmental ones).

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