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**Development and validation of an UHPLC-MS/MS method for  $\beta$ 2-Agonists in human urine and application to real samples**

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### Abstract

A fast analytical method for the simultaneous detection of 24  $\beta$ 2-agonists in human urine was developed and validated. The method covers the therapeutic drugs most commonly administered, but also potentially abused  $\beta$ 2-agonists. The procedure is based on enzymatic deconjugation with  $\beta$ -glucuronidase followed by SPE clean up using mixed-phase cartridges with both ion-exchange and lipophilic properties. Instrumental analysis conducted by UHPLC-MS/MS allowed high peak resolution and rapid chromatographic separation, with reduced time and costs. The method was fully validated according ISO 17025:2005 principles. The following parameters were determined for each analyte: specificity, selectivity, linearity, limit of detection, limit of quantification, precision, accuracy, matrix effect, recovery and carry-over. The method was tested on real samples obtained from patients subjected to clinical treatment under chronic or acute therapy with either formoterol, indacaterol, salbutamol, or salmeterol. The drugs were administered using pressurized metered dose inhalers. All  $\beta$ 2-agonists administered to the patients were detected in the real samples. The method proved adequate to accurately measure the concentration of these analytes in the real samples. The observed analytical data are discussed with reference to the administered dose and the duration of the therapy.

<b>Keywords</b>	$\beta$ 2-agonists; UHPLC; MS/MS; human urine; validation; asthma
<b>Taxonomy</b>	Determination of Drugs in Biological Samples, High-performance Liquid Chromatography With Sub-3-micron Particle
<b>Manuscript category</b>	Bioanalytical Applications
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<b>Corresponding Author's Institution</b>	CENTRO REGIONALE ANTIDOPING E DI TOSSICOLOGIA "A. BERTINARIA"
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<b>Suggested reviewers</b>	Fabio Gosetti, Xi Xia, Ru-Song Zhao

## Submission Files Included in this PDF

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Orbassano, November 20<sup>th</sup>, 2017

**Editorial Board**  
**Journal of Pharmaceutical and Biomedical Analysis**

Dear Editors,  
please find enclosed the revised version of the manuscript entitled "Development and validation of an UHPLC-MS/MS method for  $\beta$ 2-agonists detection in human urine and application to real samples".

The authors are: Cristina Bozzolino, Marta Leporati, Federica Gani, Cinzia Ferrero, Marco Vincenti.

Moderate revision and modification of the article in light of the reviewer comments was requested.

All the comments made by the Reviewers have been addressed and all the requested changes were made in the text. An itemized list of these responses and changes is reported in a dedicated file.

Thank you for reconsidering the paper for publication in Journal of Pharmaceutical and Biomedical Analysis.

Best regards.

Yours faithfully,

Marta Leporati

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## COMMENTS FROM THE EDITORS AND REVIEWERS

### REVIEWER 1

#### Comments to Author

This manuscript describes the validation UPLC-MS/MS method for the quantitation of 24  $\beta$ 2-agonists in urine. The authors selected a large group of  $\beta$ 2-agonists for its determination and quantitation in samples. In these regards, the work is a development in analytical methodology with potential applications in therapeutic monitoring and forensic toxicology.

The work is well written and easy to follow. The validation of the method is very complete and well performed. I am glad to see that the LOD and LOQ values were experimentally confirmed (line 149). Moreover, some interesting conclusions arise from the analysis of clinical samples.

#### Specific comments:

- The research group has experience in the determination and quantitation of  $\beta$ 2-agonists with GC-MS (ref. 23 in the text). The advantages of the present method, compared to the previous one, are justified by the observed improvement in sensitivity, which is of particular importance for some analytes. This must be due to the use of tandem MS, although this is not specified in the text. The use of LC instead of GC is justified by the growing implementation of LC for the study of  $\beta$ 2-agonists, since this technique does not require previous derivatization steps. Although these ideas are spread along the manuscripts, a deeper discussion of the advantages of this method compared to other commonly used methods would be very useful for the reader. This could be done in an additional section between current sections 3.2 and 3.3. In this section, specific points such as the time for sample preparation, use of reagents, sensitivity or selectivity could be discussed. **A new 3.3 section was included, as suggested.**
- The reasons for the selection of urine as sample for the determination of  $\beta$ 2-agonists is not stated in the manuscript. This could also be useful for readers. **A brief discussion on this topic was introduced in lines 67-71.**

#### Other minor comments:

- Title: I suggest to replace the term "real samples" by "clinical samples" in the title. **The change was made.**
- Graphical abstract: If the authors are determined to submit a graphical abstract, the file size should be proportional to 531 × 1328 pixels. Please, have a look at some examples here: <https://www.elsevier.com/authors/journal-authors/graphical-abstract> **The graphical abstract was reviewed.**
- Highlights: Please, highlight that the method has been validated according to ISO 17025:2005 principles. This is the main point in this work. **A new highlight was added.**

- Abbreviations: Avoid defining abbreviations that are standard in the journal: [https://www.elsevier.com/\\_\\_data/promis\\_misc/JPBA%20Abbreviations.pdf](https://www.elsevier.com/__data/promis_misc/JPBA%20Abbreviations.pdf) Many defining abbreviations were removed (lines 34, 68, 73, 74, 76, 77, 87, 153, 155, 162, 186, 187, 193, 293).
- Line 65. "These methods are..." The change was made.
- Line 162. I guess these individuals were not in any treatment with  $\beta$ 2-agonists. Please, specify what the term "blank" means. The text "not in therapy with  $\beta$ 2-agonists" was added.
- Line 186. "With respect to the RSD" The change was made.

## REVIEWER 2

The manuscript deals with the development of a development of an UHPLC-MS/MS method for beta2-agonists determination in human urine samples.

The paper is well written and organized. The validation procedure is precisely carried out and the topic of the paper is worthy of publication in the JPBA after some revisions of the text.

### Comments:

-Title: beta2-agonists were quantified in urine human samples and not only detected. I suggest to change the title in turn. The change was made.

- L134-136. I don't understand why the eluate was reconstituted with a mixture 95/5 metanol/water, instead of a mixture 95/5 water/methanol, i.e. the initial gradient composition of the mobile phase. This is enough strange. Yes, it was wrong. It was a typographical error. The right percentage was replaced.

- L141. Modify "BEH C18 column (2.1 mm x 100 mm, 1.8  $\mu$ m)" and specify the geometry of the pre-column too. The change was made.

-L146. delete "Sciex", since already afterward reported. The change was made.

- L 264. Did the authors use weighting factors for calibration plots? No, we did not.

- L359, 382 Add the standard deviation. Media value and standard deviation was added. In L359 also a typographical error was correct: as reported in Table 5 for patients under acute therapy with formoterol, all the detected concentrations were lower than 2.0 ng/mL and not 0.70 ng/mL.

- L 374, 379. Change "ppb" in appropriate concentration unit. The change was made.

- Table 1. Column 6. Change "target fragment" with "Quantifier fragment" for analogy with column 9. The change was made.

- Table 2. The linearity range reported in this table are not correct. The lowest value of the linearity range must be always the LOQ value! The change was made.

- Table 2. In this table, the LOD values are the same of LOQ values for 3 analytes (clencicloexerol, hydroxymethylclobuterol and salmeterol). Although the authors use the Hubaux-Vox method for the

calculation of LODs and LOQs, I ask them to justify these values, as the meaning of LOD and LOQ are different, and if LOD = LOQ an explanation in the text must be given. LOD and LOQ appeared to be the same for these analytes because of the approximation to a single significant digit. By adding a decimal digit, as in the new Table 2, different and more correct expression of LOD and LOQ is obtained.

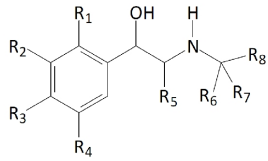
- Caption of figure 3. Please check the significant digits and add the standard deviations. Significant digits were checked and standard deviations were added.

- Figures 2 and 3. Show both the quantifier and qualifier transitions for each chromatographic peak. The qualifier fragments were added in both figures. The text "In black is reported the quantifier fragment and in red the qualifier fragment." was added in the figure captions.

## Highlights

- A UHPLC-MS/MS method was developed for the detection of 24  $\beta_2$ -agonists in urine
- The method was fully validated according to UNI EN ISO IEC 17025:2005
- The suitability of the method was proved by real samples testing
- The  $\beta_2$ -agonists urinary concentration varies with administered dose and duration



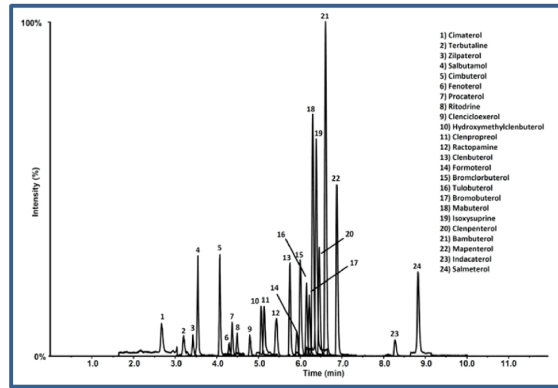


24  $\beta_2$ -agonists

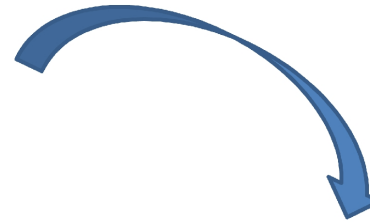
UHPLC-MS/MS



Human urine



Validated according to  
UNI EN ISO IEC 17025:2005



Salmeterol

Salbutamol

Formoterol

Indacaterol



Real samples from  
clinical treatments

1 DEVELOPMENT AND VALIDATION OF AN UHPLC-MS/MS METHOD FOR  $\beta_2$ -AGONISTS **DETECTION**  
2 **QUANTIFICATION** IN HUMAN URINE AND APPLICATION TO **REAL-CLINICAL** SAMPLES

3  
4 Cristina Bozzolino<sup>a</sup>, Marta Leporati<sup>b</sup>, Federica Gani<sup>c</sup>, Cinzia Ferrero<sup>d</sup>, Marco Vincenti<sup>a,b</sup>

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22  
23  
24  
25  
26 **KEYWORDS:**  $\beta_2$ -agonists; UHPLC; MS/MS; human urine; validation; asthma.

27

28

29 **ABSTRACT**

30 A fast analytical method for the simultaneous detection of 24  $\beta_2$ -agonists in human urine was  
31 developed and validated. The method covers the therapeutic drugs most commonly administered,  
32 but also potentially abused  $\beta_2$ -agonists. The procedure is based on enzymatic deconjugation with  
33  $\beta$ -glucuronidase followed by SPE clean up using mixed-phase cartridges with both ion-exchange  
34 and lipophilic properties. Instrumental analysis conducted by ~~ultra-high performance liquid~~  
35 ~~chromatography-tandem mass spectrometry (UHPLC-MS/MS)~~ allowed high peak resolution and  
36 rapid chromatographic separation, with reduced time and costs. The method was fully validated  
37 according ISO 17025:2005 principles. The following parameters were determined for each analyte:  
38 specificity, selectivity, linearity, limit of detection, limit of quantification, precision, accuracy,  
39 matrix effect, recovery and carry-over. The method was tested on real samples obtained from  
40 patients subjected to clinical treatment under chronic or acute therapy with either formoterol,  
41 indacaterol, salbutamol, or salmeterol. The drugs were administered using pressurized metered  
42 dose inhalers. All  $\beta_2$ -agonists administered to the patients were detected in the real samples. The  
43 method proved adequate to accurately measure the concentration of these analytes in the real  
44 samples. The observed analytical data are discussed with reference to the administered dose and  
45 the duration of the therapy.

46

## 47 1. Introduction

48  $\beta$ -adrenergic agonists, or  $\beta_2$ -agonists, are among the oldest and most commonly prescribed  
49 therapeutic agents for the treatment of asthma, a chronic inflammatory airway disorder.  $\beta_2$ -  
50 agonists are widely used also to treat chronic obstructive pulmonary disease (COPD) and  
51 numerous other respiratory diseases, either alone or in combination with bronchodilators and  
52 corticosteroids [1]. From the chemical point of view,  $\beta_2$ -agonists are phenyl  $\beta_2$ -ethanolamines  
53 bearing different substituents on the amino- nitrogen and the phenylic ring, with chemical  
54 structures closely related to endogenous catecholamines [2].

55 Because of their therapeutic stimulatory effect on the breath capacity and central nervous system,  
56  $\beta_2$ -agonists are occasionally abused by chronic asthmatic patients, possibly leading to severe  
57 intoxication. They are also misused in sport and animal husbandry as growth promoters, due to  
58 their side effects on protein synthesis and lipolysis, that depend on the dose and administration  
59 route, possibly resulting in an anabolic-like action [3]. For these reasons, the identification and  
60 quantification of  $\beta_2$ -agonists in various biological matrices is important, and requires selective and  
61 sensitive analytical methods.

62 Among the biological matrices, urine presents many advantages for drugs detection. Unlike blood,  
63 urine sampling is non-invasive and naturally produce a larger volume. In urine,  $\beta_2$ -agonists are  
64 typically excreted as either phase I or phase II metabolites, accompanied by a large percentage of  
65 the unmodified drug, depending on the dose and administration route. This allows to explore a  
66 wider temporal window after the last assumption, extending to a few days.

67 Laboratory methods for  $\beta_2$ -agonists are commonly based on a chromatographic technique coupled  
68 with ~~mass spectrometry~~ (MS), which undoubtedly offers great performances for complex mixture  
69 analysis, in terms of analytical sensitivity and specificity, detection limits and quantitation  
70 capabilities [4]. These methods are required for confirmation purposes in official zootechnics  
71 controls [5], but they can also be used as screening methods, since they generally provide the  
72 required sensitivity for a wide range of analytes, together with qualitative and quantitative  
73 information [6]. ~~Gas chromatography-MS~~ (GC-MS) methods had been initially reported for  $\beta_2$ -  
74 agonist analysis [7,8], but they were subsequently replaced by ~~LC-MS liquid chromatography-~~  
75 ~~mass spectrometry~~ methods (LC-MS) that require no derivatization steps, and allow higher  
76 sensitivity and specificity by means of ~~MS/MS tandem MS~~ conditions (MS/MS) [9,10].

77 Recent efforts were made to develop analytical methods based on ~~ultra-high performance liquid~~  
78 ~~chromatography~~ (UHPLC)-MS/MS to obtain high peak resolution, high sensitivity, rapid

79 chromatographic separation, and reduced analysis time and costs at the same time. To date, only  
80 few UHPLC methods have been described to detect simultaneously a large set for  $\beta_2$ -agonists.  
81 Multi-target methods were developed respectively for 18  $\beta_2$ -agonists detection in bovine urine  
82 [11], 20  $\beta_2$ -agonists in bovine hair [12], 16  $\beta_2$ -agonists in pig liver, kidney and muscle [13], and 11  
83  $\beta_2$ -agonists in human urine [14]. The latter method reported homogeneous LOD values of 0.1  
84 ng/mL for all targeted analytes, including salmeterol, whose actual concentration in urine after  
85 clinical administration is frequently below this 0.1 ng/mL limit [15].

86 To improve sensitivity toward  $\beta_2$ -agonists, several clean-up procedures have been proposed  
87 before instrumental detection. Most clean-up methods were based on ~~solid phase extraction (SPE)~~  
88 using different sorbents [16,17], but also other techniques such as matrix solid phase dispersion  
89 [18], immunoaffinity based techniques [19] and supercritical fluids extraction [20] have been  
90 reported. Lately, molecularly imprinted polymer technology [21,22] has also been experimentally  
91 applied to the clean-up of  $\beta_2$ -agonists.

92 Until recently, our laboratory used a fast-GC-MS method for the simultaneous determination of 15  
93  $\beta_2$ -agonists in human urine [23]. The need to improve further the sample throughput and general  
94 applicability made us develop a new sensitive and robust UHPLC-MS/MS method for the detection  
95 of 24  $\beta_2$ -agonists (Figure 1) in human urine, covering the therapeutic drugs most commonly  
96 administered, but also potentially abused  $\beta_2$ -agonists and “cocktails”, i.e. mixtures of  $\beta_2$ -agonists  
97 with or without other anabolic substances [24]. The present method was validated in agreement  
98 with the UNI EN ISO IEC 17025:2005 [25] principles and successfully applied to the therapeutic  
99 monitoring of patients with respiratory-related diseases, after their treatment with various  $\beta_2$ -  
100 agonists.

101

## 102 **2. Material and methods**

### 103 **2.1. Reagent and chemicals**

104 Bambuterol hydrochloride, bromchlorbuterol hydrochloride, brombuterol hydrochloride,  
105 cimaterol, cimbuterol, clenbuterol hydrochloride, clecicloexerol hydrochloride, clenpenterol  
106 hydrochloride, clenproperol, fenoterol hydrobromide, formoterol fumarate dihydrate,  
107 hydroxymethylclenbuterol, isoxsuprine hydrochloride, mabuterol hydrochloride, mapenterol  
108 hydrochloride, procatamol hydrochloride, ractopamine hydrochloride, ritodrine hydrochloride,  
109 salbutamol, terbutaline sulfate, tulobuterol hydrochloride, methanol, formic acid (LC-MS Ultra  
110 grade, ~98%), ammonium acetate,  $\beta$ -glucuronidase type II from helix pomatia, and ammonium

111 hydroxide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Indacaterol and formoterol-  
112 <sup>13</sup>C-d<sub>3</sub> were purchased from AlsaChim (Illkirch Graffenstaden, France). Salmeterol xinafoate was  
113 supplied by the European Directorate for the Quality of Medicines & HealthCare (, Strasbourg,  
114 France). Zilpaterol was obtained from Spectra 2000 srl (Rome, Italy). Clenbuterol-d<sub>6</sub> was supplied  
115 by the Netherlands National Institute for Public Health and the Environment (RIVM, Bilthoven,  
116 Netherlands). Salbutamol-d<sub>3</sub> was purchased from LGC (Teddington, Middlesex, United Kingdom),  
117 salmeterol-d<sub>3</sub> from CDN Isotope (Pointe-Claire, Quebec, Canada). Hydrochloric acid (HCl) 37% was  
118 purchased from Carlo Erba (Milan, Italy). Ultrapure water was obtained using a Milli-Q® UF  
119 apparatus (Millipore, Bedford, MA, USA).

120 All stock standard solutions were prepared in methanol at 1 mg/mL and stored at -20° C in the  
121 dark until use. Working solutions were prepared by dilution with methanol.

122 Four deuterated internal standards (salbutamol-d<sub>3</sub>, clenbuterol-d<sub>6</sub>, formoterol-<sup>13</sup>C-d<sub>3</sub>, and  
123 salmeterol-d<sub>3</sub>) were used for the quantitation procedure at equal concentration. Blank urine  
124 samples were collected from healthy volunteers (laboratory personnel), pooled, and used as the  
125 working matrix to develop and validate the analytical protocol.

126

## 127 **2.2. Sample preparation**

128 An aliquot (3 mL) of urine specimen was centrifuged at 3500 rpm for 5 min. Subsequently, 2.5 mL  
129 of supernatant was transferred into 30-mL glass tubes and 25 µL of the internal standard (IS)  
130 solution (concentration of 0.1 µg/mL) was added. A 0.2 M aqueous ammonium acetate solution at  
131 pH 5.0 was added (2.5 mL) into the samples, together with 10 µL of β-glucuronidase. Enzymatic  
132 deconjugation was carried out for 2 h at 37° C. The samples were allowed to cool down to room  
133 temperature and then loaded onto a SPE Strata-XC 33 µm, 60 mg x 3 mL cation exchange cartridge  
134 (Phenomenex, Castel Maggiore (BO), Italy), previously conditioned with 2 mL of methanol and 2  
135 mL of ultrapure water. After sample loading, the SPE cartridges were washed with 2 mL HCl 0.1 M  
136 and then 2 mL of methanol. The target analytes were eluted with 2 mL of methanol doped with 5%  
137 (v/v) of ammonium hydroxide. The eluate was evaporated to dryness under a gentle stream of  
138 nitrogen and at 50° C using a Techne Sample Concentrator (Barloworld Scientific, Stone, UK), and  
139 then reconstituted with 100 µL of a methanol-0.1% formic acid aqueous solution (95:25, v/v) and  
140 transferred into the analytical vial for UHPLC-MS/MS analysis.

141

## 142 **2.3. UHPLC-MS/MS analysis**

143 Chromatographic separations were performed on an Agilent Infinity 1290 UHPLC system (Agilent  
144 Technologies, Palo Alto, CA, USA), including a vacuum degasser, a binary pump, an autosampler  
145 and a column thermostat. The liquid chromatograph was equipped with a Waters (Milford, MA,  
146 USA) BEH C18 ~~column~~ (2.1 mm x 100 mm x 1.8-7  $\mu$ m) ~~column~~ maintained at 40°C and a ~~n~~  
147 ~~analogue~~ Waters (Milford, MA, USA) BEH C18 vanguard pre-column (2.1 mm x 5 mm x 1.7  $\mu$ m). The  
148 chromatographic run was carried out by a binary mobile phase of a 0.1% v/v aqueous formic acid  
149 solution and methanol, using the following program: isocratic with 5% methanol for 2 min; linear  
150 gradient from 5% to 80% in 8 min; isocratic with 80% methanol for 1 min; total run time 15 min.  
151 The injection volume was 1  $\mu$ L and the flow-rate was 0.4 mL min<sup>-1</sup>. The LC was interfaced to a  
152 ~~Sciex~~ QTRAP® 4500 triple-quadrupole mass spectrometer (Sciex, Ontario, Canada), operating in  
153 ~~electrospray ionization (ESI)~~ - positive ion mode. The other MS parameters were set as follows:  
154 curtain gas: 35 psi; nebulizer gas: 45 psi; heater gas: 40 psi; probe temperature: 550 °C; IS voltage:  
155 +3500 V. Ion acquisition was operated at unit mass resolution in the ~~selected reaction monitoring~~  
156 ~~(SRM)~~ mode, using the two transitions from the protonated molecular ion of each analyte to the  
157 fragment ions indicated in Table 1.

158

## 159 **2.4. Validation**

160 The analytical method was validated in accordance with the criteria and recommendations of UNI  
161 EN ISO/IEC 17025:2005 international standard [25]. The following parameters were investigated:  
162 specificity, selectivity, linearity range, ~~detection and quantification limits (LOD and LOQ)~~, intra-  
163 assay precision and accuracy. Carry-over, recovery and matrix effect were also investigated. Blank  
164 human urine samples obtained from healthy volunteers were used for the validation experiments  
165 following the analytical protocol described above.

166

### 167 *2.4.1. Specificity and Selectivity*

168 Ten blank urine samples from different individuals ~~not in therapy with  $\beta_2$ -agonists~~ were analysed.  
169 The occurrence of possible interferences from endogenous substances was tested by monitoring  
170 the SRM chromatograms characteristic for each investigated compound at the expected retention  
171 time interval. The signal-to-noise ratio (S/N) was measured on the least intense mass transition at  
172 the expected retention time. The noise was measured from the end of the peak till 0.05 min after  
173 it. A S/N<3 was considered satisfactory in order to verify the method specificity.

174 Identification criteria for the analytes were established according to Decision 2002/657/EC [5]. For  
175 each analyte, one qualifying mass transition was monitored, in addition to the primary  
176 fragmentation (Table 1). Variations of relative peak intensities were tested at three concentration  
177 levels (concentration of first, third and fifth point of the calibration curves). Retention time ( $t_R$ )  
178 precision was also tested at the same concentrations.

179

#### 180 2.4.2. Linearity, LOD and LOQ

181 The linear calibration model was checked by analyzing (five replicates) blank urine samples spiked  
182 with the working solution at six concentration levels (see Table 2). The calibration was completed  
183 by internal standardization. The linear calibration parameters were evaluated using the least  
184 squares regression method. Determination coefficient ( $R^2$ ), slope, and intercept were calculated,  
185 and several significance tests were performed to evaluate linearity, including Lack-of-Fit test,  
186 ~~Analysis of Variance (ANOVA)~~ test, Mandel's test, evaluations of the ~~relative standard deviation of~~  
187 ~~the slope (RSD slope test)~~ and the residual plots, together with the analysis of the deviation from  
188 back-calculated concentrations.

189 The tests were passed when the calculated values ( $F_{exp}$ ) proved to lower than the corresponding  
190 critical value at  $\alpha=0.05$  significance level:  $F_{crit} = 2.776$  ( $n_1 = 4$  and  $n_2 = 24$  degrees of freedom) for  
191 the Lack-of-Fit test,  $F_{tab} = 3.842$  ( $n_1 = 1$  and  $n_2 = 28$  degrees of freedom) for the ANOVA test, and  
192  $F_{crit} = 2.62$  ( $n_1 = 5$  and  $n_2 = 24$  degrees of freedom) for the Mandel's test. With respect to the  
193 ~~RSDs~~ slope and back calculation tests, the adopted threshold values were 5.00% and 20%,  
194 respectively. The residual plot analysis turned out positive when a random pattern was observed,  
195 namely there was no recognizable trend of the residuals as a function of the concentrations.

196 The LOD values were estimated using the Hubaux and Vos approach [26]. Five independent  
197 calibration lines were prepared for all the target analytes and a significant level of 95% was  
198 selected at the corresponding number of degree of freedom. Then, LOD value was calculated by  
199 applying the Hubaux-Vos algorithms. LOQ was calculated as two times the LOD.

200 The calculated LOD and LOQ values were subsequently tested in experiments with blank urine  
201 samples spiked with analyte concentrations extremely close to the respective LOD and LOQ values,  
202 to confirm the estimation correctness.

203

#### 204 2.4.3. Precision and accuracy



205 For all analytes, intra-day precision (expressed as percent variation coefficient, CV%) and accuracy  
206 (expressed as bias%) were evaluated by analyzing ten blank urine samples spiked with the working  
207 solutions at low, medium and high calibration level (the same concentrations used for selectivity  
208 evaluation). Intra-assay precision was considered satisfactory when CV% values were below 25%  
209 for the low calibration level and below 15% for the other levels. Satisfactory accuracy was  
210 achieved when the experimentally determined average concentration lied within  $\pm 15\%$  from the  
211 expected value. All results are reported in Table 3.

212

#### 213 2.4.4. *Carry-over*

214 Carry-over was evaluated by injecting an alternate sequence of five blank urine samples spiked  
215 with all the analytes at concentrations higher than 5 ng/mL and five blank urine samples. To  
216 ensure the absence of any carry-over effect, S/N ratios had to be lower than 3 for each monitored  
217 transition.

218

#### 219 2.4.5. *Matrix effect and extraction recovery*

220 The matrix effect was evaluated as the percentage ratio between the area (mean value from five  
221 replicates) obtained by adding the analyte to the matrix extract and the one determined in a blank  
222 deionized water solution spiked with the analyte at the same concentration after the extraction  
223 step. The percentage difference highlighted matrix suppression (values below 100%) or  
224 enhancement (values above 100%) [27]. Matrix effect was estimated at the first level of the  
225 calibration curves.

226 The extraction recovery represents the percentage of analyte extracted after sample workup from  
227 a blank urine sample originally spiked with a known concentration of target analytes. It was  
228 calculated as the ratio between the analyte area determined in the extracted samples (5  
229 replicates) and the one determined in blank samples (5 replicates) in which the analyte was added  
230 after the extraction step. Extraction recoveries were estimated at the three concentration levels  
231 cited above.

232

### 233 2.5. *Real urine samples*

234 In order to verify the complete analytical procedure on authentic specimens, real urine samples  
235 were collected from 60 compliant patients, who were subjected to pharmacological treatment  
236 with  $\beta_2$ -agonists at San Luigi Gonzaga University Hospital. General information on the patients and

237 their treatment with  $\beta_2$ -agonists is listed in Tables 4 and 5. Briefly,  $\beta_2$ -agonists were administered  
238 using pressurized metered dose inhalers. The active ingredients were salbutamol (100  $\mu\text{g}/\text{puff}$ ),  
239 salbutamol/beclometasone (100  $\mu\text{g}/\text{puff}$ ), salmeterol/fluticasone (25 or 50  $\mu\text{g}/\text{puff}$ ),  
240 formoterol/budesonide (4.5 or 9  $\mu\text{g}/\text{puff}$ ), formoterol/beclometasone (6  $\mu\text{g}/\text{puff}$ ) and indacaterol  
241 (150  $\mu\text{g}/\text{puff}$ ). All analyses were routine therapeutic controls executed for clinical purposes.  
242 However, the patients provided written informed consent before attending the study, and an  
243 anonymous code was attributed to each subject participating to the present study to respect  
244 privacy regulations.

245

### 246 **3. Results and discussion**

#### 247 **3.1. Method development**

248 The optimized UHPLC-MS/MS method allowed the simultaneous determination of 24  $\beta_2$ -agonists  
249 in human urine. The whole chromatographic run, comprehensive of the time required for column  
250 re-equilibration before the following injection, was completed in less than 15 min. Retention times  
251 ranged between 2.70 min (cimaterol) and 8.84 min (salmeterol). Figure 2 shows the SRM  
252 chromatograms recorded from a blank urine spiked with all the analytes at 0.5 ng/mL.

253 The choice of the SPE procedure, that involved mixed-phase cartridges with both ion-exchange  
254 and lipophilic properties, was made after comparison of literature results. In particular, a very  
255 exhaustive comparative study of various sorbents types was made by Dos Ramos et al on bovine  
256 urine as the matrix, suggesting the use of mixed-phase sorbent as the one yielding the best results  
257 in  $\beta_2$ -agonists recovery [28]. The present results confirm that optimal clean-up is observed by  
258 using mixed-phase SPE cartridges.

259

#### 260 **3.2. Validation results**

##### 261 **3.2.1. Specificity and Selectivity**

262 The SRM chromatographic profiles obtained from blank urine samples collected from 10 untreated  
263 subjects did not show the presence of any significant signal ( $S/N < 3$ ) at the relative retention time  
264 typical of all the studied compounds and ISs, indicating that the method is selective and no  
265 interfering substance is present in the biological matrices.

266 The analytes were clearly identified in all the spiked samples, according to the criteria reported in  
267 the Decision 2002/657/EC, that were fully satisfied.

268

### 269 3.2.2. *Linearity, LOD and LOQ*

270 The linear matrix-matched calibration model was checked by analyzing five replicate blank urine  
271 samples spiked with the working solutions at six final concentrations. More in detail, two intervals  
272 were investigated for the analytes listed in Table 2, depending on the typical therapeutic dose of  
273 each  $\beta_2$ -agonist and its expected concentration in urine: 0.03–2.5 ng/mL (0.03, 0.1, 0.25, 0.5, 1.0  
274 and 2.5 ng/mL) or 0.15–5.0 ng/mL (0.15, 0.25, 0.5, 1.0, 2.5 and 5.0 ng/mL). Quantitative data  
275 resulting from area counts were corrected using the respective IS signal areas. All the linear  
276 calibration parameters turned out adequate and the results, reported in Table 2, confirm the  
277 appropriateness of the linear model. Further confirmation of the calibrations linearity was  
278 obtained from the analysis of the residual plots, since a random residuals pattern along the  
279 concentration range was observed for all the analytes.

280 LODs were calculated by the Hubaux-Vos technique [26] and the experimental verification tests  
281 confirmed the correct estimation of LOD and LOQ. The Hubaux-Vos method is founded on rigorous  
282 statistical basis and should be preferred with respect to the LOD and LOQ estimation made from  
283 the S/N values of blank and spiked samples or the average signal and its standard deviation. In  
284 particular, the latter methods do not define the baseline position and amplitude from which the  
285 S/N value should be calculated, introducing a potential factor of bias related to the operator's  
286 discretion. The LODs and LOQs values reported in Table 2 proved to be at least one order of  
287 magnitude lower than those recorded in our previous fast-GC-MS method [23]. This increase in  
288 sensitivity turned out to be particularly important for some analytes, i.e. salmeterol, commonly  
289 excreted at low concentration after administration.

290

### 291 3.2.3. *Precision and accuracy*

292 Intra-day data on precision and accuracy are reported in Table 3. The results show satisfactory  
293 repeatability, as the ~~percent variation coefficient~~ (CV%) is lower than 15% for all the spiked  
294 analytes at low, medium and high concentrations, with only five exceptions (16% for cimaterol,  
295 19% for ritodrine, 16% for ractopamine, 17% for mapenterol and 18% for salmeterol at the lowest  
296 calibration level). The accuracy is also satisfactory, as the percent bias (bias%) ranges from -4.7%  
297 to +15%. Overall, all the experimental figures-of-merit satisfy the predetermined criteria (see  
298 paragraph 2.4.3).

299

### 300 3.2.4. *Carry-over*

301 The background chromatographic profiles of the main SRM transitions for each analyte, monitored  
302 during the analysis of blank urine injected after highly spiked samples, did not show the presence  
303 of any significant signal (i.e. the S/N value was always <3) at the retention times expected for the  
304 tested analytes, with the notable exception of procaterol that showed some carry-over effect in  
305 two runs out of five. It is recommended to inject a blank sample after the injection of the last point  
306 of the calibration curve and in general after any sample with high procaterol concentration.

### 308 3.2.5. *Matrix effect and recovery*

309 Recovery and matrix effect values are given in Table 3. In general, the results show satisfactory  
310 recovery values. The average extraction recovery is 100%: the minimum observed value is 80% for  
311 mapenterol at 0.03 ng/mL, while the maximum value equals 116% for fenoterol at 0.15 ng/mL.  
312 The average matrix effect is estimated around +1.2%: the highest negative effect is -33% for  
313 cimaterol, while the largest positive effect is +23% for clenbuterol. In conclusion, good extraction  
314 recoveries combined with acceptable matrix effect allowed the correct determination of all the  
315 target analytes.

### 317 **3.3. Method improvement**

318 The present UHPLS-MS/MS method was developed to be used in the daily laboratory activity in place of the  
319 fast-GC-MS procedure previously employed [23]. The former method provided reasonable sensitivity and  
320 adequate instrumental processing time to meet the requirements (in terms of LOQ and sample throughput)  
321 of most routine determinations. However, it needed to be updated to include the newest  $\beta_2$ -adrenergic  
322 receptor agonists, among which indacaterol, zilpaterol, isoxsuprine, and mabuterol, occasionally adopted in  
323 the clinical and veterinary practice or potentially abused. Moreover, the GC-MS method required a strong  
324 derivatization step under strictly controlled conditions, to reduce the polarity of most targeted analytes.  
325 For example, the derivatization with trimethylsilyltrifluoroacetamide introduced three TMS groups in  
326 salbutamol and formoterol and four TMS groups in salmeterol.

327 The new method is equally fast, does not require previous derivatization steps, and is validated for as many  
328 as 24 target  $\beta_2$ -agonists, namely all those potentially requested to our laboratory for both clinical and  
329 doping purposes. The combination of electrospray ionization with tandem mass spectrometry, together  
330 with the addition of SPE purification of the extract, considerably improved the sensitivity with respect to  
331 the previous method [23]. On average, LOD values were decreased by 1-2 orders of magnitude for all the  
332 analytes included in both methods. Moreover, a few specificity problems observed in the GC-MS method  
333 had been eliminated. For example, the signal obtained from low formoterol concentrations in real samples

334 was barely detectable in the GC-SIM profile as a shoulder at the right side of an interfering peak, making  
335 the quantification highly problematic. In the present method, the use of SPE purification and MS/MS  
336 detection removed all interferences from SRM profiles, enhancing both specificity and sensitivity.

337

#### 338 3.3.3.4. *Real samples results*

339 All four  $\beta_2$ -agonists (formoterol, indacaterol, salbutamol and salmeterol) administered to the  
340 patients were detected in the real samples. A representative SRM profile for each analyte is  
341 reported in Figure 3, showing optimal chromatographic profiles at both high and low  
342 concentrations. The therapeutic, chronological, and analytical data for real clinical samples are  
343 reported in Table 4 (chronic therapy) and Table 5 (acute therapy).

##### 344 3.3.1.3.4.1. *Indacaterol*

345 Indacaterol is a novel, long-acting inhaled  $\beta_2$ -adrenergic receptor agonist intended for long-term,  
346 single daily dose, maintenance treatment in patients with COPD [29]. The long-lasting  
347 pharmacological activity of indacaterol is due to the presence of a long, lipophilic side-chain in the  
348 chemical structure that binds to an exo-site on adrenergic receptors [30]. The urine of a 66 years  
349 old woman was collected 24 hours after the last assumption of 150  $\mu\text{g}$  of indacaterol and two  
350 weeks long therapy. The patient had been in therapy with salbutamol for 16 years, as needed, but  
351 with poor control of the disease. Then, salbutamol was substituted with indacaterol, which  
352 required single daily dose. The drug was found in urine at the concentration of 3.93 ng/mL (see  
353 Figure 3, line C). Salbutamol was still detected at the concentration of 42.1 ng/mL, 18 days after its  
354 replacement (reported below).

355

##### 356 3.3.2.3.4.2. *Salmeterol*

357 Salmeterol is a long-acting selective  $\beta_2$ -agonist used to control asthma in combination with inhaled  
358 steroid therapy. In contrast, salmeterol could be used as a monotherapy in COPD. Its  
359 concentration in urine after administration is very low, inasmuch as it is readily metabolized to  $\alpha$ -  
360 hydroxysalmeterol [31]. The urine samples from twelve patients in chronic therapy with variable  
361 doses and collecting intervals after administration were analyzed. The results, reported in Table 4,  
362 show that higher average salmeterol concentration is detected from patients with longer  
363 therapeutic periods (more than 6 months), possibly because of some accumulation effect. For  
364 example, different salmeterol concentrations were detected from patients 012, 039 and 043, i.e.  
365 women of a similar age under the same therapy conditions and sampling interval. The

366 concentration obviously depends also on the delay with which urine has been collected after the  
367 last administration.

368

#### 369 3.3.3.3.4.3. *Formoterol*

370 Formoterol is a potent long-acting  $\beta$ -agonist, typically applied by means of a metered dose inhaler.  
371 It shows rapid action offset, which is exploited in asthma - in association with inhaled steroid - not  
372 only for disease control but also as a reliever. In COPD, formoterol can be used in monotherapy. At  
373 high doses, it may also act as an anabolic agent, increase the heart rate, and produce excitement.  
374 Hence, formoterol might potentially be misused in sports for its stimulatory effect and possible  
375 anabolic action, although most studies on the effects of inhaled  $\beta$ -agonists did not show any  
376 improvement in the elite athletes performance [32].

377 Urine samples from patients who used formoterol in both acute and chronic therapy were  
378 analyzed. In chronic patients, the detected urinary concentrations of formoterol were scattered,  
379 varying in the range between fractions and units of ppb, with two notable exception of higher  
380 levels (patients 030 and 046). According to the data reported in literature, formoterol is excreted  
381 in urine either as phase I (O-demethylation of the methoxyphenyl group and deformylation) or  
382 phase II metabolites (glucuronidation mainly at the phenolic position, but a benzyl glucuronide is  
383 also formed) [33], accompanied by a large percentage of unmodified drug [34]. No differences  
384 were observed between patients in therapy from more or less than sixth months and no clear  
385 dependence from the dosage was detected.

386 In patients under acute therapy, all the detected concentrations were lower than 0.702.0 ng/mL  
387 (0.50±0.07 ng/mL). Urine samples collected too early (patient 015) or too late (patients 001-003-  
388 049) with respect to the administration lead to low or undetectable drug levels, although in  
389 patient 036 formoterol was still detected 48 hour after administration.

390

#### 391 3.3.4.3.4.4. *Salbutamol*

392 Salbutamol is a widely prescribed  $\beta_2$ -agonist for relieving bronchospasm in patients with asthma  
393 and COPD [35]. The list of prohibited substances in sports published by the WADA specifies that  
394 the use of salbutamol is only permitted by inhalation. Administration by the oral or parenteral  
395 route or the administration of very large inhaled doses are forbidden due to an strong adrenergic  
396 stimulatory effect and an anabolic-like effect. In contrast, administration of therapeutic inhaled  
397 doses have no ergogenic effect [36].

398 The urine samples of 10 patients to whom salbutamol was administered in acute therapy were  
399 analyzed (see Table 5). Although relatively high concentrations of salbutamol were always  
400 measured, it is evident that the urinary concentration of salbutamol decreased from hundreds to  
401 tens of ~~ppb-ng/mL~~ after 12 or more hours from the assumption (patients 026 and 027). The single  
402 recorded exception (patient 008) showing low salbutamol urinary concentration, actually received  
403 a small dosage of salbutamol. The persistence and possible accumulation of the drug in the body  
404 was supported by the analysis of an urine sample from a patient chronically treated with  
405 salbutamol (50+50 µg/day) for the last 16 years: 18 days after the therapy suspension, salbutamol  
406 was still present at the relatively high concentration of 42 ~~ppb-ng/mL~~ (Patient 006 in Table 4). Less  
407 pronounced effect was recorded for a 38-years old male subject, who was in therapy since one  
408 year with 200 µg/day salbutamol: after 3 hours from administration, its urinary concentration was  
409  $57 \pm 7$  ng/mL.

#### 411 4. Conclusions

412 The UHPLC-MS/MS method developed and validated for the simultaneous quantitative  
413 determination of 24 β-agonists in human urine proved adequate to measure the real  
414 concentration of these analytes in real samples of patients with asthma or COPD. The method  
415 proved simple, accurate and highly sensitive, allowing the simultaneous detection of all  
416 compounds within a short run time. In comparison with the method we previously used [23], more  
417 analytes (24 instead of 15) and increased sensitivity (at least one order of magnitude) were gained.  
418 The results on real samples allowed us to directly verify that salbutamol and salmeterol tend to  
419 accumulate in the body, when the therapy is administered for long periods of time, even if the  
420 excretion appears to vary significantly from one patient to another. Moreover, very low  
421 concentrations were detected after formoterol assumption, even when the drug was administered  
422 in high dosage to control acute asthma episodes.

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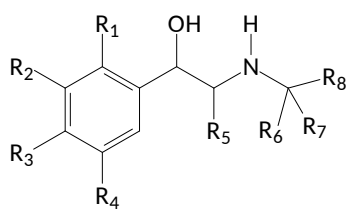
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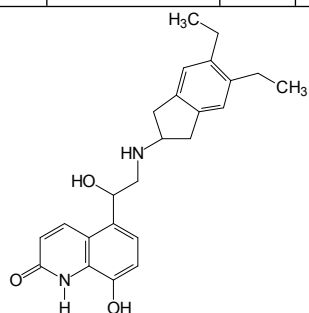
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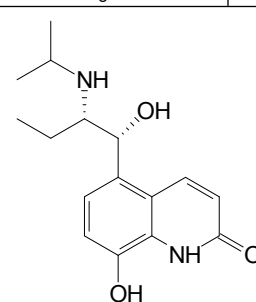


$\beta_2$ -agonist compounds	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>	R <sub>5</sub>	R <sub>6</sub>	R <sub>7</sub>	R <sub>8</sub>
Bambuterol	H	N(CH <sub>3</sub> ) <sub>2</sub> COO	H	N(CH <sub>3</sub> ) <sub>2</sub> COO	H	CH <sub>3</sub>	CH <sub>3</sub>	CH <sub>3</sub>
Bromchlorbuterol	H	Cl	NH <sub>2</sub>	Br	H	CH <sub>3</sub>	CH <sub>3</sub>	CH <sub>3</sub>
Bromobuterol	H	Br	NH <sub>2</sub>	Br	H	CH <sub>3</sub>	CH <sub>3</sub>	CH <sub>3</sub>
Cimaterol	H	C≡N	NH <sub>2</sub>	H	H	H	CH <sub>3</sub>	CH <sub>3</sub>
Cimbuterol	H	C≡N	NH <sub>2</sub>	H	H	CH <sub>3</sub>	CH <sub>3</sub>	CH <sub>3</sub>
Clenbuterol	H	Cl	NH <sub>2</sub>	Cl	H	CH <sub>3</sub>	CH <sub>3</sub>	CH <sub>3</sub>
Clencyclohexerol	H	Cl	NH <sub>2</sub>	Cl	H	H		
Clenpenterol	H	Cl	NH <sub>2</sub>	Cl	H	C <sub>2</sub> H <sub>5</sub>	CH <sub>3</sub>	CH <sub>3</sub>
Clenproperol	H	Cl	NH <sub>2</sub>	Cl	H	H	CH <sub>3</sub>	CH <sub>3</sub>
Fenoterol	H	OH	H	OH	H	CH <sub>3</sub>		H
Formoterol	H	NH-COH	OH	H	H	CH <sub>3</sub>		H
Hydroxymethylclenbuterol	H	Cl	NH <sub>2</sub>	Cl	H	CH <sub>2</sub> OH	CH <sub>3</sub>	CH <sub>3</sub>
Isoxsuprine	H	H	OH	H	CH <sub>3</sub>	CH <sub>3</sub>		H
Mabuterol	H	CF <sub>3</sub>	NH <sub>2</sub>	Cl	H	CH <sub>3</sub>	CH <sub>3</sub>	CH <sub>3</sub>
Mapenterol	H	CF <sub>3</sub>	NH <sub>2</sub>	Cl	H	C <sub>2</sub> H <sub>5</sub>	CH <sub>3</sub>	CH <sub>3</sub>
Ractopamine	H	H	OH	H	H	CH <sub>3</sub>		H
Ritodrine	H	H	OH	H	CH <sub>3</sub>	H		H
Salbutamol	H	CH <sub>2</sub> OH	OH	H	H	CH <sub>3</sub>	CH <sub>3</sub>	CH <sub>3</sub>
Salmeterol	H	CH <sub>2</sub> OH	OH	H	H	H	-(CH <sub>2</sub> ) <sub>6</sub> O(CH <sub>2</sub> ) <sub>4</sub> Ph	H
Terbutaline	H	OH	H	OH	H	CH <sub>3</sub>	CH <sub>3</sub>	CH <sub>3</sub>
Tulobuterol	Cl	H	H	H	H	CH <sub>3</sub>	CH <sub>3</sub>	CH <sub>3</sub>

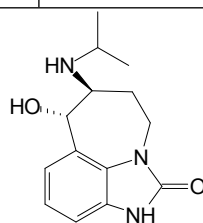
Indacaterol

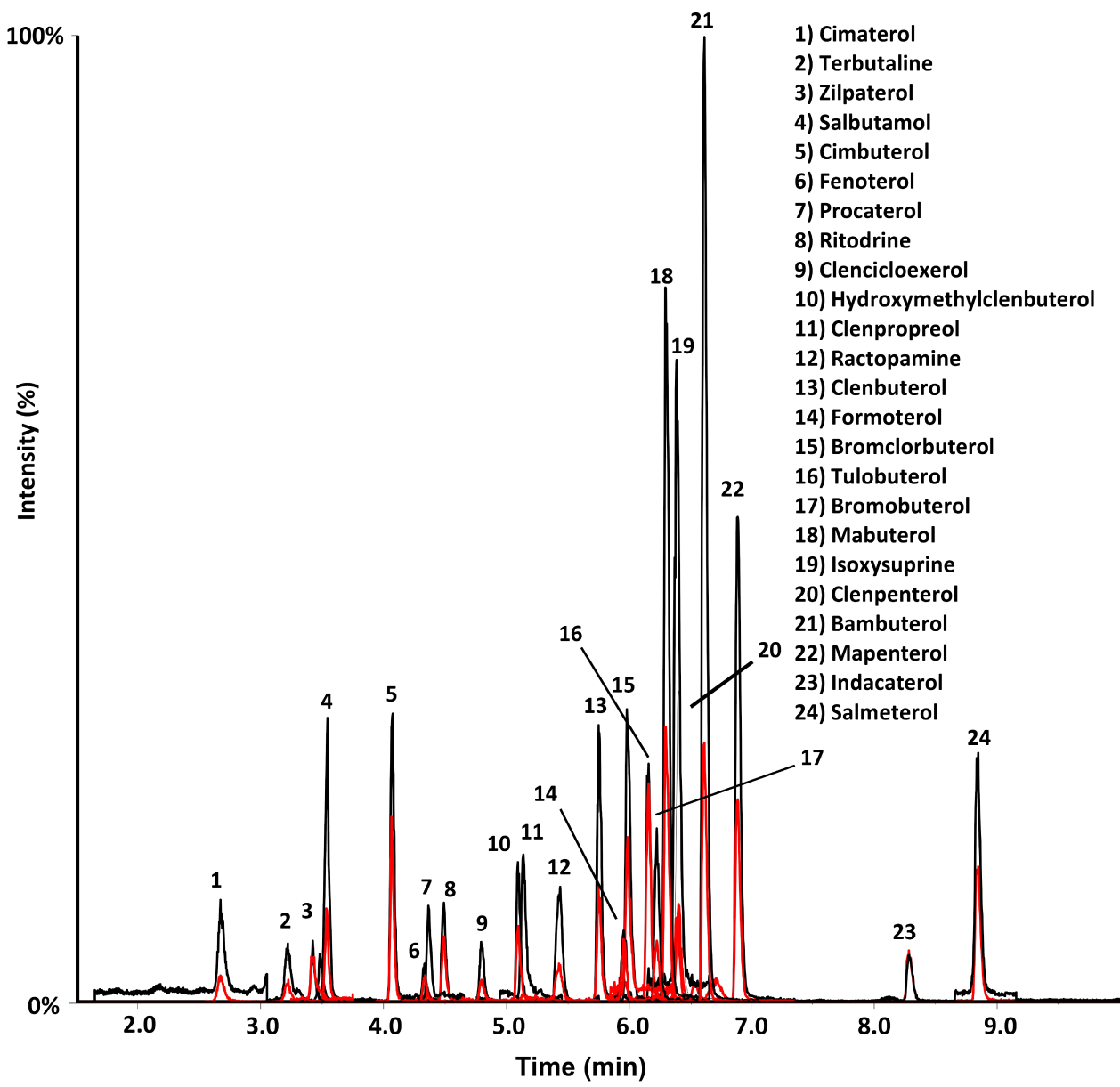


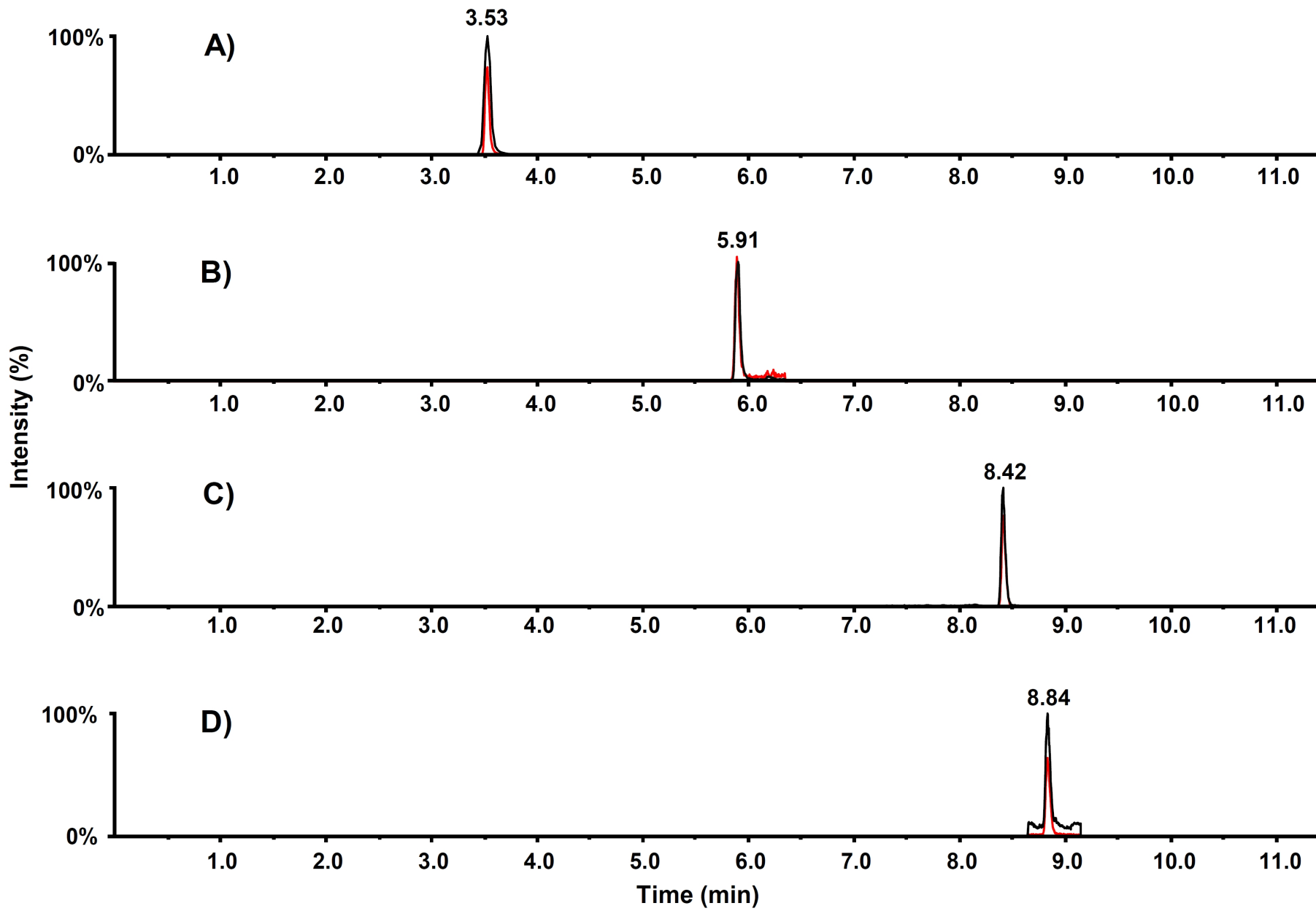
Procaterol



Zilpaterol







## Figure captions.

Figure 1. Chemical structures of  $\beta_2$ -agonists considered in this study.

Figure 2. SRM chromatograms of a blank urine sample spiked at 0.500 ng/mL for all the analytes. In black is reported the quantifier fragment and in red the qualifier fragment.

Figure 3. SRM chromatogram of four real samples positive to A) salbutamol at 171±21 ng/mL (patient 003), B) formoterol at 0.650.646±0.090 ng/mL (patient 002), C) indacaterol at 3.93±0.32 ng/mL (patient 006) and D) salmeterol at 0.374±0.048 ng/mL (patient 010). In black is reported the quantifier fragment and in red the qualifier fragment.





**Table 2. Calibration interval, squared correlation coefficient, LODs and LOQs values, Lack of Fit's, ANOVA, Mandel's, RDS slope and Back calculation test results for all analytes.**

Analyte	Experimentally tested Linearity range (ng/mL)	Correlation coefficient (R <sup>2</sup> )	LOD (ng/mL)	LOQ (ng/mL)	Lack of fit's test (F <sub>exp</sub> )	ANOVA (F <sub>exp</sub> )	Mandel's test (F <sub>exp</sub> )	RSD slope test (%)	Back calculation test (%)
Cimaterol	0.15-5.0	0.9983	0.0329	0.0586	0.24	0.494	1.34	2.98	6.07
Terbutaline	0.15-5.0	0.9969	0.0439	0.0878	0.67	0.707	1.47	2.85	10.1
Zilpaterol	0.15-5.0	0.9989	0.023	0.0546	0.16	0.415	1.23	2.63	3.90
Salbutamol	0.15-5.0	0.9998	0.011	0.022	0.04	0.285	1.45	2.00	1.88
Cimbuterol	0.03-2.5	0.9994	0.0109	0.0218	0.07	0.502	1.31	3.35	5.73
Fenoterol	0.15-5.0	0.9959	0.045	0.089	0.34	0.673	1.85	4.46	7.03
Procaterol	0.15-5.0	0.9994	0.0218	0.04035	0.08	0.664	1.40	3.72	5.25
Ritodrine	0.15-5.0	0.9955	0.0547	0.094	0.58	0.467	2.48	3.03	13.3
Clencicloexerol	0.15-5.0	0.9999	0.0071	0.014	0.03	0.127	1.04	1.11	2.84
Hydroxymethylclenbuterol	0.15-5.0	0.9999	0.0071	0.014	0.04	0.302	1.26	1.47	1.83
Clenproperol	0.15-5.0	0.9993	0.0218	0.0364	0.16	0.376	2.31	1.98	4.37
Ractopamine	0.15-5.0	0.9995	0.0216	0.031	0.07	0.794	1.30	3.89	1.89
Clenbuterol	0.15-5.0	0.9990	0.022	0.044	0.10	0.926	1.32	4.87	5.18
Formoterol	0.15-5.0	0.9998	0.010	0.021	0.06	0.081	1.09	0.84	2.38
Bromchlorbuterol	0.15-5.0	0.9993	0.0182	0.00436	0.09	0.510	1.59	3.01	5.47
Tulobuterol	0.03-2.5	0.9990	0.011	0.022	0.14	0.593	1.20	3.23	6.73
Bromobuterol	0.03-2.5	0.9979	0.0216	0.032	0.34	0.886	1.02	3.69	11.4
Mabuterol	0.15-5.0	0.9998	0.011	0.021	0.03	0.474	1.00	3.17	2.45
Isoxsuprine	0.15-5.0	0.9990	0.022	0.043	0.16	0.334	1.99	2.26	3.26
Clenpenterol	0.15-5.0	0.9990	0.022	0.045	0.16	0.443	1.97	2.64	3.99
Bambuterol	0.03-2.5	0.9992	0.010	0.020	0.18	0.373	2.17	1.99	6.46
Mapenterol	0.03-2.5	0.9977	0.0217	0.034	0.34	0.465	2.00	2.80	9.56
Indacaterol	0.15-5.0	0.9990	0.023	0.0465	0.18	0.572	1.73	2.87	2.85
Salmeterol	0.03-2.5	0.9998	0.0051	0.010	0.05	0.235	1.61	1.58	4.88

<sup>a</sup>Calibration levels (ng/mL) = 0.15 - 0.25 - 0.5 - 1.0 - 2.5 - 5.0

<sup>b</sup>Calibration levels (ng/mL) = 0.03 - 0.1 - 0.25 - 0.5 - 1.0 - 2.5

Lack of fit's test - F<sub>crit</sub> = 2.776 (n<sub>1</sub> = 4 and n<sub>2</sub> = 24 degrees of freedom)

ANOVA - F<sub>tab</sub> = 3.842 (n<sub>1</sub> = 1 and n<sub>2</sub> = 28 degrees of freedom)

Mandel's test - F<sub>crit</sub> = 2.62 (n<sub>1</sub> = 5 and n<sub>2</sub> = 24 degrees of freedom)

RSD slope test - %RSD threshold = 5.00%

Back calculation test - % threshold = 20%

**Table 3. Intra-day precision (CV%), accuracy (bias%), matrix effect and extraction recovery for all the analytes tested.**

Analyte	Level I			Level II			Level III			Matrix effect (%)	Recovery (%)		
	Conc. (ng/mL)	Precision (CV%)	Accuracy (bias%)	Conc. (ng/mL)	Precision (CV%)	Accuracy (bias%)	Conc. (ng/mL)	Precision (CV%)	Accuracy (bias%)	Level I	Level I	Level II	Level III
Cimaterol	0.15	16	+5.5	0.50	9.7	+0.1	2.5	5.1	+1.5	-33	108	100	101
Terbutaline	0.15	12	+1.3	0.50	7.3	-1.2	2.5	2.5	+1.6	+0.05	97	100	99
Zilpaterol	0.15	9.4	+1.2	0.50	8.3	+1.6	2.5	4.8	+2.0	-1.7	107	110	96
Salbutamol	0.15	7.1	-4.7	0.50	4.5	-1.4	2.5	4.0	+2.3	+5.7	91	94	100
Cimbuterol	0.03	7.5	+6.7	0.25	4.5	+3.5	1.0	4.5	+2.4	+5.7	97	95	97
Fenoterol	0.15	12	+8.4	0.50	5.2	-1.8	2.5	3.7	+2.4	-2.7	116	103	103
Procaterol	0.15	13	+3.2	0.50	3.7	-3.0	2.5	1.1	+0.4	+4.2	98	88	102
Ritodrine	0.15	19	+7.4	0.50	4.8	-0.9	2.5	3.4	+0.6	-19.3	95	102	97
Clencicloexerol	0.15	5.0	+4.6	0.50	4.3	+0.6	2.5	3.3	+0.3	-8.0	113	111	95
Hydroxymethylclenbuterol	0.15	11	-0.9	0.50	3.4	-3.1	2.5	0.3	+0.02	-1.9	98	107	96
Clenproperol	0.15	11	-0.7	0.50	7.0	+2.6	2.5	0.8	+0.2	+12	89	100	96
Ractopamine	0.15	16	+15	0.50	8.8	+1.8	2.5	1.6	-0.6	-1.2	106	98	100
Clenbuterol	0.15	9.9	+9.8	0.50	8.4	+1.0	2.5	7.1	+1.8	+23	98	99	98
Formoterol	0.15	10	+2.8	0.50	6.0	+1.1	2.5	2.4	-0.5	+17	102	105	98
Bromchlorbuterol	0.15	8.0	-0.2	0.50	1.5	+0.3	2.5	2.8	+0.8	+2.6	99	85	100
Tulobuterol	0.03	11	+10	0.25	3.2	-1.0	1.0	5.0	-0.4	+9.2	110	89	98
Bromobuterol	0.03	2.3	+3.4	0.25	3.4	+0.3	1.0	2.6	+0.5	-3.7	102	92	105
Mabuterol	0.15	10	+14	0.50	7.2	+0.1	2.5	7.3	-2.5	+16	101	102	99
Isoxsuprine	0.15	11	+1.8	0.50	2.5	-0.4	2.5	0.6	-0.1	-2.5	99	96	101
Clenpenterol	0.15	1.9	+0.4	0.50	3.8	-0.6	2.5	3.2	+1.3	-4.2	102	96	98
Bambuterol	0.03	3.5	+6.9	0.25	4.7	+3.2	1.0	2.1	+0.5	+5.0	104	96	98
Mapenterol	0.03	17	+13	0.25	2.8	+2.1	1.0	2.8	+0.3	+8.7	80	94	105
Indacaterol	0.15	13	+4.5	0.50	5.3	+0.8	2.5	1.6	+0.4	+1.2	100	110	106
Salmeterol	0.03	18	-0.4	0.25	2.9	+3.7	1.0	0.5	-0.4	-3.8	110	88	99

**Table 4. Patients under chronic therapy. General features, treatment information, and  $\beta$ -agonists concentration determined in urine.**

CHRONIC THERAPY							
$\beta$ -agonist	Patient	Sex	Age	Time in therapy (months)	Time from last administration (h)	Dose ( $\mu$ g)	Urine concentration (ng/mL)
Salmeterol	010	F	50	36	1	25+25	0.37
	012	F	72	2	3	50+50	0.02
	039	F	64	6	3	50+50	0.08
	043	F	65	36	3	50+50	0.38
	044	F	39	3	3	25+25	0.07
	050	M	56	60	3	25+25	0.55
	017	F	64	10	5	50+50	0.22
	035	M	43	4	5	50+50	0.11
	058	F	28	2	6	50+50	ND
	022	M	81	3	7,5	50+50	0.16
	041	F	59	72	8	50+50	0.16
	040	M	38	24	48	50+50	ND
Indacaterol	006	F	66	0.5	24	150	3.93
Formoterol	054	F	30	2	0.5	12+12	4.05
	004	F	60	1	1	6+6	1.20
	016	F	83	0.25	1.5	6	0.50
	046	F	27	120	2	12+12	41.2
	029	M	53	15	3	12+12	1.78
	031	F		3	3	6+6	1.99
	037	M	23	60	3	4,5+4,5	0.81
	057	F	47	3	3	12+12	1.16
	020	F	46	48	4	12+12	0.14
	030	F	48	0.25	4	12+6	15.9
	056	M	20	1	4	4,5+4,5	0.63
	014	F	34	3	5	6+6	0.77
	019	F	49	1	5	4,5 + 4,5	0.30
	028	M	54	2.5	5	9	2.00
	005	M	75	0.25	6	12+12+12	0.62
	032	F	45	2	6	6+6	0.46
	038	F	49	60	6	4,5 + 4,5	2.69
	055	F	54	36	7	6+6	2.20
	011	M	56	72	8	4,5	0.23
	042	F	65	132	8	4,5+4,5	0.30
	021	F	70	5	14	6+6	1.20
	023	F	26	1.5	14	6+6	0.05
	013	F	30	2	14	12	0.14
	033	M	16	10	15	12+12	0.88
	045	M	29	8	15	6+6	ND
	018	F	54	36	17	6+6	ND
	034	F	41	12	17	4,5 + 4,5	0.10
	025	F	43	0.75	20	12+12	0.12
	048	F	34	12	30	6	1.88
	047	F	62	1.5	48	6	ND
009	F	13	1	52	6+6	< LOQ	
Salbutamol	059	M	38	12	3	200	57
	006	F	66	16 years	18 days	50+50	42.1

\*ND = not detected

**Table 5. Patients under acute therapy. General features, treatment information, and  $\beta$ -agonists concentration determined in urine.**

ACUTE THERAPY						
$\beta$ -agonist	Patient	Sex	Age	Time in therapy (h)	Dose ( $\mu$ g)	Urine concentration (ng/mL)
Formoterol	015	M	30	0.25	4.5	<LOQ
	007	F	38	0.5	5+5	2.0
	002	M	61	4	200	0.65
	053	F	52	6	6	0.41
	027	M	33	8	18	1.3
	049	M	29	16	9	ND
	001	F	33	17.5	4.5	<LOQ
	003	M	33	18.5	4.5	<LOQ
	036	F	24	48	4.5+4.5	0.09
Salbutamol	051	F	48	0.5	400	171
	052	F	53	1	400	926
	060	M	34	1	400	103
	003	M	33	1.5	300	171
	008	F	70	2	200	1.20
	013	F	30	2	400	138
	024	M	21	2	400	406
	040	M	38	3	400	295
	027	M	33	12	1312	41.4
026	F	29	15	200	12.8	

\* ND = not detected