Comparison of Clenbuterol and Salbutamol Accumulation in the Liver of Two Different Mouse Strains

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In the European Union, β2-adrenergic agonists like clenbuterol and salbutamol are banned from use as growth promoters. Although clenbuterol and salbutamol both accumulate in the liver, differences in the accumulation rate can be seen among animal species due to different β2-adrenoreceptor distributions. The aim of this study was to compare the accumulation of the two in the liver tissue of two different mouse strains. The study included 200 8-week-old BALB/c and C57/BL/6 mice. One group of BALB/c (40) and one group of C57/BL/6 (40) mice were treated with 2.5 mg/kg body mass clenbuterol per os for 28 days. The remaining two animal groups were treated with salbutamol in the same manner. The animals were then randomly sacrificed on day 1, 15 and 30 post treatments. Despite of the same treatment dose, the results revealed clenbuterol to persist in the liver tissue longer than salbutamol. On post treatment day 30, the concentration of clenbuterol residue in C57/BL/6 and BALB/c mice liver tissue were 0.23 ± 0.02 and 0.21 ± 0.03 ng/g, respectively, while residues of salbutamol were not detected. When comparing the accumulation of both compounds between the two mouse strains, it becomes apparent that no significant difference (P > 0.05) in the accumulation rate can be found.

Introduction

Clenbuterol and salbutamol belong to β2-adrenergic agonists used as bronchodilators and tocolytics in both veterinary and human medicine (1). However, when used in doses 5–10 folds higher than therapeutic, these compounds can provoke an anabolic effect manifested in an increased muscle and decreased adipose tissue mass of the treated animals. In the past, clenbuterol was misused as a growth promoter during the fattening of food-producing animals. Due to its toxic effects, this clenbuterol abuse led to a number of acute intoxications of humans who had consumed meat and especially liver coming from the treated animals (2–5). Therefore, the use of clenbuterol and other β-agonists for growth-promoting purposes was banned in Europe under the Council Directive 96/22/EC (6). The annual monitoring program for these substances is defined under the Council Directive 96/23/EC. One of the possible and commonly implemented matrices for monitoring β-agonists’ abuse is the liver, with research showing a high accumulation of β-agonists in this tissue (7–10). Although β-agonists unanimously accumulate in the liver tissue, differences in this accumulation can be seen among animal species due to different distribution of β2 receptors (11). β2-Agonists can roughly be divided into two chemical groups: clenbuterol-like substances with anilinic moieties and salbutamol-like substances with phenolic, catecholic or resorcinolic moieties (12).

Bearing in mind the difference in chemical properties of clenbuterol and salbutamol and the difference in distribution of β2 receptors among animal species, the aim of this study was to compare the rate of accumulation of clenbuterol and salbutamol in the liver tissue of two different mouse strains after a sub-chronic treatment with the dose of 2.5 mg/kg BM that is equal to clenbuterol’s NOAEL (No Observed Adverse Effect Level).

Materials and methods

Chemicals and apparatus

Clenbuterol hydrochloride and salbutamol (Sigma-Aldrich, Steinheim, Germany) were used for the preparation of solution the animals had been treated with. Clenbuterol D6 and salbutamol D6, used as internal standards, came from RIVM (Bilthoven, the Netherlands). Solid-phase extraction columns (CSDAU 506, 500 mg, 6 mL) used for the cleanup came from I/CT Interchim (Montluçon, France). All other chemicals used with the analysis were of an analytical grade. Separation was carried out using Perkin Elmer 200 HPLC system (Waltham, USA) on which a C18-column having the dimensions of 50 × 2.1 mm and the particle size of 3 μm was mounted. Mass spectrometry was performed using an API 3000 triple quadrupole mass analyzer (Applied Biosystems, Carlsbad, CA, USA).

Animals and sampling procedure

The study included 8-week-old white BALB/c (n = 100) and black C57/BL/6 (n = 100) male mice having an average body mass of ~25 g, obtained from a breeding colony of the Ruder Bošković Institute (Zagreb, Croatia). These two strains were selected due to their different hair color, since within this research frame hair accumulation rates of both clenbuterol and salbutamol were studied as well (13). During the experiment, animals were housed four per cage. The bottom of the cage was covered in sawdust (Allspan®, Germany). The mice were fed on a standard laboratory food (4 RF 21 GLP Mucedol, Italy). All animals had free access to food and water. Animals were kept under standard conditions featured by an alternating 12-h light/dark cycles, the temperature of 22°C, and 55% humidity. All experiments were performed according to the ILAR Guide for the Care and Use of Laboratory Animals, the Council Directive (86/609/EEC) and the Croatian Animal Protection Act (Official Gazette of the Republic of Croatia, No 135/06). The animals (n = 160) were randomly divided into four groups.
One group of black mice \((n = 40)\) and one group of white mice \((n = 40)\) were treated with 2.5 mg/kg body mass clenbuterol *per os* daily (through a probe) during 28 days. The remaining two animal groups were treated in the same manner, but with salbutamol. Forty animals, i.e., 20 black and 20 white, were left untreated and served as controls. On post treatment day 1, 15 and 30 the animals were first introduced into anesthesia and then euthanized (by virtue of cervical dislocation) in groups of 40, while their liver tissues were stored at \(-20^\circ C\) pending analysis.

**Sample preparation and clean-up**

Whole liver samples, weighing \(\sim 1.5\) g, were weighed in plastic containers and spiked with 50 \(\mu\)L of the internal standard in the concentration of 100 ng/mL, as well as with 7.5 mL of methanol and 10 mL of 0.2 M acetic acid buffer having \(pH\) of 5.2. After homogenization, the samples were centrifuged (Heraeus, Sepatech, UK) for 15 min at \(4^\circ C\) using a 2,000 \(\times\) \(g\) force. The supernatants were evaporated to one-third of their starting volume under a stream of nitrogen at \(45^\circ C\). After evaporation, 60 \(\mu\)L of glucoronidase/sulphatase were added and the samples were incubated at \(42^\circ C\) overnight. After the incubation, \(pH\) was adjusted to 6.0 and the samples were centrifuged at 2,000 \(\times\) \(g\) for 10 min. The obtained supernatants were loaded onto SPE-columns previously activated using methanol, water and 0.1 M phosphate buffer having \(pH\) 6.0. The columns were subsequently washed with 1 M acetic acid and methanol, while the elution was carried out using a mixture of ethyl-acetate and ammonia in the \(97/3\) ratio. The eluents were evaporated to dryness, and the residues were dissolved in 200 \(\mu\)L of 0.1\% acetic acid.

**Liquid chromatography–tandem mass spectrometry conditions**

HPLC separation was performed on Uptisphere C18-columns (50 \(\times\) 2.1 mm, 3.0 \(\mu\)m Interchim, Montluçon, France) at a flow rate of 0.2 mL/min and at the room temperature. The mobile phase consisted of the constituent A (methanol) and the constituent B (0.1\% acetic acid in water). A gradient elution protocol was employed, as follows: 0–3 min 1-A, 13 min 50-A, 15 min 100-A, 20 min 100-A, 26 min 1-A and 35 min 1%-A. The injection volume was 10 \(\mu\)L. MS–MS analysis was performed using electrospray ionization in the positive ion mode (ESI\(^+\)). With quantitative analysis, the multiple reaction monitoring (MRM) approach was exercised using the most abundant protonated molecular ion \(\rightarrow\) product ion transition for clenbuterol, salbutamol and their respective internal standards. For qualitative purposes, the second transition was monitored to confirm clenbuterol’s and salbutamol’s identity (Table I). The source temperature and capillary voltage were set at 400 °C and at 5,500 \(V\), respectively. The MS conditions were optimized by tuning the analyte-specific parameters, including declustering potential (DP), focusing potential (FP), entrance potential (EP), collision energy (CE) and collision cell exit potential (CXP). The nebulization gas (air) and curtain gas (nitrogen) pressure was set at 9 and 12 psi, respectively, while that of the collision gas (nitrogen) was set at 7 psi. Working parameters were optimized by infusing 1 \(\mu\)g/mL of clenbuterol, salbutamol and internal standard solutions into methanol–acetic acid 0.1\%, 50:50 (v/v), monitoring thereby two fragment ions for clenbuterol and salbutamol, and a single fragment ion for both internal standards. Optimized MS parameters and fragment ions are reported in Table I.

**Validation process**

Validation was carried out according to the Commission Decision 2002/657/EC (14). Within the frame of the validation process, decision limit (CC\(_a\)), detection capability (CC\(_b\)), precision, recovery, repeatability, in-house reproducibility and linearity (matrix effect) were studied. The decision limit (CC\(_a\)) was determined by analyzing 20 representative blank liver samples, while the detection capability (CC\(_b\)) was determined by analyzing 20 liver samples fortified at the level of 0.3 ng/g. Linearity and the matrix effect were evaluated by means of correlation coefficients obtained from the calibration curves for both the solvent and the matrix. The linearity of the LC–MS–MS response was checked by repeated analyses (three replicates) of clenbuterol and salbutamol standard solutions at the concentration levels of 3.125, 6.25, 12.5 and 25.0 ng/mL containing a fixed amount of clenbuterol D6 and salbutamol D6 (25 ng/mL). Linear regression analysis was performed by virtue of plotting the ratio of the analyte standard area into the internal standard area against the analyte concentration. The linearity was also evaluated by virtue of analyzing blank liver samples (10 g) fortified at levels 0.125, 0.250, 0.375, 0.500 and 0.625 ng/g, in which 12.5, 25.0, 37.5, 50.0 and 62.5 \(\mu\)L of standard solution containing 100 ng/mL of clenbuterol and salbutamol, and 50.0 \(\mu\)L of internal standard solution of clenbuterol D6 and salbutamol D6 at 100 ng/mL was added. Recovery and precision were determined by analyzing 20 blank liver samples fortified in 20 replicates at 0.30 ng/g for both analytes. The ion suppression arising on the grounds of matrix components was compensated by isotope dilution method.

**Statistical analysis**

Differences in concentrations of analytes seen on different withdrawal days, as well as differences between the two mouse strains, were tested for their statistical significance using ANOVA. Statistical analysis was performed using the StatisticaVer. 6.1. Software (StatSoft, serial number AGA304B21198B61, 1984–2003, USA).

**Results and Discussion**

**Validation results**

Within the frame of the validation process, no interferences with either clenbuterol or salbutamol identification were found owing...
to a highly specific MRM acquisition method and the use of appropriate deuterated internal standards. Figures 1 and 2 show a typical HPLC–MS/MS–MRM chromatogram of a blank BALB/c and C57BL/6 mouse liver sample.

Linearity was tested in the range of 3.125 to 25.0 ng/mL using standard solutions (correlation coefficient 0.996 for clenbuterol and 0.991 for salbutamol) and in the range of 0.125 and 0.625 ng/g using spiked samples (correlation coefficient 0.998 for clenbuterol and 0.984 for salbutamol). For samples containing analytes in the range tested for linearity as stated above, the addition of internal standard was adequately increased, while the final solution was diluted so as to fall within the linearity range. The recovery values calculated from 20 spiked samples at 0.30 ng/g were 85.6% for clenbuterol and 84.2% for salbutamol. For samples containing analytes in the range tested for linearity as stated above, the addition of internal standard was adequately increased, while the final solution was diluted so as to fall within the linearity range. The recovery values calculated from 20 spiked samples at 0.30 ng/g were 85.6% for clenbuterol and 84.2% for salbutamol. The precision of the method was evaluated in terms of relative standard deviation from the recovery experiment (6.8 for clenbuterol and 7.8% for salbutamol). Validation procedure revealed the reduction of the extract volume of about 2/3 when compared with the initial one, to be sufficient to annihilate any effect of residual methanol on subsequent enzymatic hydrolysis. The summary of the validation results, including CCa, CCb, linearity, recovery, precision and repeatability of both compounds, is presented in Table II.

Pursuant to the Commission Decision 2002/657/EC, certain requirements imposed on quantitative methods' performance and mass spectrometric detection have to be met (14). These requirements include, for example, repeatability CVs, recovery range, linearity, etc. On the whole, validation process carried out within the frame of this study resulted in good recovery, linearity, precision and repeatability values, as well as in satisfying decision limit (CCa) and detection capability (CCb) values, so that the method can be considered suitable for clenbuterol and salbutamol determination in liver samples. Detection and quantification limit values obtained within this study frame are comparable with those obtained by other researchers (10, 15).

**Accumulation of clenbuterol and salbutamol**

Our previous research was focused on clenbuterol accumulation and depletion in the pig liver after a prolonged administration of a growth-promoting dose (9, 10, 16). Literature sources bring some data on different distribution of β2-receptors in different animal species, resulting in different accumulation of β-agonists (11). The aim of this study was to compare the accumulation of clenbuterol and salbutamol in the liver tissue of two different mouse strains after a sub-chronic treatment. As of now, data on differences in accumulation of different β-agonists in different animal breeds are lacking. This study also attempted to establish possible differences in accumulation patterns of the compounds under study across various mouse strains, so as to allow for further deduction on possible differences in accumulation patterns across various farm animal breeds. The determined concentrations are shown in Table III.

Clenbuterol concentration determined in the liver samples of BALB/c mice on day 1, 15 and 30 post treatment were...
On the same post-treatment days, clenbuterol concentrations determined in C57BL/6 liver samples were 16.92 ± 3.85, 0.68 ± 0.18 and 0.23 ± 0.02 ng/g, respectively. Although clenbuterol concentration established in C57BL/6 liver samples on day 1 after the treatment withdrawal was higher when compared with that found in BALB/c mice livers, no significant difference (P > 0.05) in the accumulation rate was detected, so that clenbuterol residues could be found in both strains even 30 days after the treatment withdrawal. Figures 3 and 4 display typical HPLC–MS/MS–MRM chromatograms of clenbuterol found in BALB/c and C57BL/6 mouse liver samples on day 1 after the treatment withdrawal.

Salbutamol concentration determined in the liver samples of C57BL/6 mice on day 1 after the treatment withdrawal (17.90 ± 2.34 ng/g) was significantly (P < 0.05) higher than that determined in BALB/c mice liver samples (3.28 ± 0.33 ng/g). Despite a significant difference (P < 0.05) in salbutamol concentration determined on day 1 post treatment, the accumulation of salbutamol in the liver samples of the two mouse strains was equal; on post-treatment day 30, salbutamol residues were not

<table>
<thead>
<tr>
<th>Day after withdrawal</th>
<th>Mouse strain</th>
<th>C57BL/6</th>
<th>BALB/c</th>
<th>C57BL/6</th>
<th>BALB/c</th>
<th>No. of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BALB/c</td>
<td>0.24 ± 0.03</td>
<td>0.21 ± 0.03</td>
<td>1.71 ± 0.28</td>
<td>&lt;0.1</td>
<td>13</td>
</tr>
<tr>
<td>15</td>
<td>BALB/c</td>
<td>0.21 ± 0.03</td>
<td>0.23 ± 0.02</td>
<td>1.71 ± 0.28</td>
<td>0.1</td>
<td>13</td>
</tr>
<tr>
<td>15</td>
<td>C57BL/6</td>
<td>0.86 ± 0.18</td>
<td>1.71 ± 0.28</td>
<td>1.71 ± 0.28</td>
<td>0.1</td>
<td>13</td>
</tr>
<tr>
<td>30</td>
<td>C57BL/6</td>
<td>0.86 ± 0.18</td>
<td>1.71 ± 0.28</td>
<td>1.71 ± 0.28</td>
<td>0.1</td>
<td>13</td>
</tr>
</tbody>
</table>

Table II
Summary of the validation results for both clenbuterol and salbutamol

<table>
<thead>
<tr>
<th>Compound</th>
<th>CCx (ng/g)</th>
<th>CCp (ng/g)</th>
<th>Correlation coefficient in solvent</th>
<th>Correlation coefficient in matrix</th>
<th>Recovery ± SD (ng/g)</th>
<th>Precision RSD (%)</th>
<th>Repeatability RSD (%)</th>
</tr>
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<tbody>
<tr>
<td>C1lenbuterol</td>
<td>0.082</td>
<td>0.100</td>
<td>0.996</td>
<td>0.998</td>
<td>85.6 ± 5.8</td>
<td>6.6</td>
<td>7.5</td>
</tr>
<tr>
<td>Salbutamol</td>
<td>0.094</td>
<td>0.107</td>
<td>0.991</td>
<td>0.984</td>
<td>84.2 ± 6.5</td>
<td>7.8</td>
<td>8.7</td>
</tr>
</tbody>
</table>

Table III
Clenbuterol and salbutamol concentrations (mean ± SD) determined in the liver samples of two different mouse strains after the treatment withdrawal

13.07 ± 0.27, 0.24 ± 0.03 and 0.21 ± 0.03 ng/g, respectively. On the same post-treatment days, clenbuterol concentrations determined in C57BL/6 liver samples were 16.92 ± 3.85, 0.68 ± 0.18 and 0.23 ± 0.02 ng/g, respectively. Although clenbuterol concentration established in C57BL/6 liver samples on day 1 after the treatment withdrawal was higher when compared with that found in BALB/c mice livers, no significant difference (P > 0.05) in the accumulation rate was detected, so that clenbuterol residues could be found in both strains even 30 days after the treatment withdrawal. Figures 3 and 4 display typical HPLC–MS/MS–MRM chromatograms of clenbuterol found in BALB/c and C57BL/6 mouse liver samples on day 1 after the treatment withdrawal.

Salbutamol concentration determined in the liver samples of C57BL/6 mice on day 1 after the treatment withdrawal (17.90 ± 2.34 ng/g) was significantly (P < 0.05) higher than that determined in BALB/c mice liver samples (3.28 ± 0.33 ng/g). Despite a significant difference (P < 0.05) in salbutamol concentration determined on day 1 post treatment, the accumulation of salbutamol in the liver samples of the two mouse strains was equal; on post-treatment day 30, salbutamol residues were not
Figure 3. Typical HPLC–MS–MS–MRM chromatogram of BALB/c mouse liver sample on day 1 after withdrawal; (a) clenbuterol D6 MRM 283.2→203.0 (clenbuterol D6 concentration 0.5 ng/g); (b) clenbuterol MRM 277.2→203.0; (c) clenbuterol MRM 277.2→259.1 (clenbuterol concentration 12.95 ng/g).

Figure 4. Typical HPLC–MS–MS–MRM chromatogram of C57BL/6 mouse liver sample on day 1 after withdrawal; (a) clenbuterol D6 MRM 283.2→203.0 (clenbuterol D6 concentration 0.5 ng/g); (b) clenbuterol MRM 277.2→203.0; (c) clenbuterol MRM 277.2→259.1 (clenbuterol concentration 17.51 ng/g).
Figure 5. Typical HPLC–MS–MS–MRM chromatogram of BALB/c mouse liver sample on day 1 after withdrawal; (a) salbutamol D6 MRM 246.2→228.2 (salbutamol D6 concentration 0.5 ng/g); (b) salbutamol MRM 240.2→148.1; (c) salbutamol MRM 240.2→222.2 (salbutamol concentration 3.45 ng/g).

Figure 6. Typical HPLC–MS–MS–MRM chromatogram of C57BL/6 mouse liver sample on day 1 after withdrawal; (a) salbutamol D6 MRM 246.2→228.2 (salbutamol D6 concentration 0.5 ng/g); (b) salbutamol MRM 240.2→148.1; (c) salbutamol MRM 240.2→222.2 (salbutamol concentration 16.63 ng/g).
detected either in BALB/c or C57BL/6 liver samples. Figures 5 and 6 display typical HPLC–MS/MS–MRM chromatograms of salbutamol found in BALB/c and C57BL/6 mouse liver samples on day 1 after the treatment withdrawal.

The results of this study are comparable to earlier data on clenbuterol and salbutamol accumulation in farm animals (8, 9, 17). As formerly revealed, clenbuterol residues resting in the liver tissue of treated animals are detectable no later than 14 days after withdrawal; this study, on the other hand, showed that such residues are detectable even 30 days after the substance withdrawal. This difference can be attributed to a higher clenbuterol dose applied within our study frame. As for salbutamol accumulation rates, the results of this study showed that its residues can be detected up to 15 days after withdrawal, as also demonstrated by the research conducted by Malucelli et al. (8), who managed to detect salbutamol in the liver tissue of the treated broiler chickens 14 days after withdrawal. When comparing the accumulation of clenbuterol and salbutamol in the liver of the two mouse strains under study, it becomes apparent that despite the same treatment dose clenbuterol persists in the liver tissue longer than salbutamol. Clenbuterol residues were detected in the liver samples of both mouse strains even 30 days after the treatment withdrawal, while on the same post-treatment day salbutamol residues were nonexistent. Previous research was focused either on clenbuterol or salbutamol alone, not on both compounds in parallel, and did not engage different breeds of the same animal species. Our research, on the other hand, brings a comparison of accumulation rates of two different compounds in the liver tissue of two different mouse strains.

Conclusion
Clenbuterol concentration determined in the liver tissues of C57BL/6 mice on day 30 post treatment was 0.23 ng/g, while that in the liver tissues of BALB/C mice equaled to 0.21 ng/g. Unlike clenbuterol, salbutamol residues were detected in the liver of both mouse strains 15 days after the treatment withdrawal. The obtained data showed that clenbuterol cumulated in the liver tissue of both mice strains longer than salbutamol. Comparison of accumulation of both compounds between the two mouse strains made it clear that no significant difference in the accumulation rate can be expected between the two. This presumably applies to farm animals as well, so that no significant difference in accumulation patterns among various animal breeds is to be expected.

Acknowledgments
The authors thank Prof. Bruno Le Bizec, LABERCA, France, for his help during the development and validation of the method in use.

References