SUPPLEMENTARY MATERIAL

Analysis of β_2 -agonists in cattle hair samples using a rapid UHPLC-ESI-MS/MS method

Vita Di Stefano^{1*}, Rosa Pitonzo², Vita Giaccone³, Angelina Alongi³, Andrea Macaluso³, Nicola Cicero⁴, Gabriella Cancemi¹, Vincenzo Ferrantelli³

Affiliation

¹*Department of Biological, Chemical and Pharmaceutical Science and Technology (STEBICEF), University of Palermo, Palermo, Italy*

²*CGA, UNINETLAB, University of Palermo, Palermo, Italy*

³*Istituto Zooprofilattico Sperimentale della Sicilia "A. Mirri", Palermo, Italy*

⁴*Dipartimento di Scienze dell'Ambiente, della Sicurezza, del Territorio, degli Alimenti e della Salute (S.A.S.T.A.S.), Messina, Italy*

*Corresponding author

Dr. Vita Di Stefano, Department of Biological, Chemical and Pharmaceutical Science and Technology (STEBICEF), University of Palermo, Palermo, Italy

E-mail: vita.distefano@unipa.it Tel +39 091 23891948

Abstract

A simple and efficient method was developed for simultaneous analysis of 5 illegal residual β_2 -agonists in cattle hair. β_2 -agonists were quantified by ultra high performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (UHPLC-ESI-MS/MS) operating in positive multiple-reaction monitoring mode (MRM). The method was validated as quantitative confirmatory method according to the EU Decision 2002/657/EC: instrumental linearity, specificity, precision, recovery, decision limit (CC α) and detection capability (CC β) were evaluated. The recovery were greater than 90% and the method appeared suitable for the control of these β_2 -agonists in cattle hair samples with LOQ values between 4.9 and 5.5 $\mu\text{g/Kg}$.

This method could represent a simple and cheap approach to confirm β_2 -agonists contamination of cattle for feeding in a not invasive way and before slaughter operations.

Keywords: UHPLC-ESI-MS/MS; β_2 -agonists; cattle hair; Commission Decision 2002/657/EC

Experimental

Method validation

The developed method was fully validated as quantitative confirmatory method according to the Commission Decision 2002/657/EC. Parameters taken into account were instrumental linearity, specificity, precision, recovery, decision limit ($CC\alpha$) and detection capability ($CC\beta$). The instrumental linearity was evaluated by drawing five points calibration curves in methanol, containing a fixed amount of ISTD (mapenterol- d_{11} , 4 $\mu\text{g/L}$), and analytes concentrations ranging from 0.5 to 10 $\mu\text{g/L}$. Specificity was tested by analyzing 20 blank bovine hair samples.

Matrix calibration curves were obtained by spiking bovine hair samples with the five β_2 -agonists, resulting in three weekly analytical series, each series with three concentration levels (4–8–12 $\mu\text{g/kg}$) and six samples per concentration level (6 samples $\times$ 3 concentration levels $\times$ 3 series = 54 analysis).

Method recovery and precision were evaluated using these matrix curves results; recovery was expressed in terms of percentage of measured concentration to fortified concentration ratio and precision as relative standard deviation. The same results were used to calculate decision limit ($CC\alpha$) and detection capability ($CC\beta$) according to the matrix calibration curve procedure described in the Commission Decision 2002/657/EC.

Linearity, Specificity, Recovery and Precision, $CC\alpha$ and $CC\beta$

The instrumental response was evaluated for all analytes in a concentration range from 0.5 to 10 $\mu\text{g/L}$ using standard solutions in methanol that contained a fixed amount of labeled ISTD (4 $\mu\text{g/L}$). Five concentration in three replicates for concentration level were used to calculate the linear regression by the least square method. The calibration curves were obtained by plotting the peak area ratio of each standard to ISTD versus drug concentration.

The correlation coefficients of the calibration curves indicated a good fit for all the analytes ($R^2 > 0.999$). Chromatograms of standard solutions are shown in Figure S1.

The specificity was evaluated by analyzing 20 blank hair samples. No interfering peaks of endogenous compounds at the retention times of all β_2 -agonists are present.

The blank hair samples were spiked with five β_2 -agonists at three different concentrations (4, 8 and 12 $\mu\text{g/kg}$). Results are shown in Table S1. The average recoveries for all analytes were greater than 90%. Precision was expressed in terms of repeatability and reproducibility as % relative standard deviation (% RSD).

According to the concept of the European Commission Decision 2002/657/EC, the $\text{CC}\alpha$ and $\text{CC}\beta$ have been estimated. The decision limit $\text{CC}\alpha$, defined as “the limit at and above which it can be concluded with an error probability of α that a sample is non-compliant”, was determined by the matrix calibration curve. Three different series of blank bovine hair samples fortified at three levels were analyzed. Average calibration curves were calculated and the signal was plotted against the added concentration. The corresponding concentration at the y-intercept plus 2.33 times the standard deviation of the within-laboratory reproducibility of the intercept equals the decision limit. The values of $\text{CC}\alpha$ are reported in Table S1.

The detection capability ($\text{CC}\beta$), defined as “the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of β ”, was determined by the matrix calibration curve. Three different series of blank bovine hair samples fortified at three levels were analyzed. Average calibration curves were calculated and the signal was plotted against the added concentration. The corresponding concentration at the decision limit plus 1.64 times the standard deviation of the within-laboratory reproducibility of mean measured content at the decision limit equals the detection capability. The values of $\text{CC}\beta$ are reported in Table S1.

Chemicals

β_2 agonists analytical standards mapenterol hydrochloride (MPT), mabuterol hydrochloride (MBT), clenpenterol hydrochloride (CPT), clenbuterol hydrochloride (CBT) and brombuterol hydrochloride (BBT) were purchased from Sigma-Aldrich (Milan, Italy). The purity of the analytes was $\geq 99.0\%$. Structures of these compounds are shown in Figure 1.

Mapenterol d_{11} hydrochloride (MPT- d_{11}) was used as internal standard (ISTD) and was purchased from Sigma-Aldrich (Milan, Italy).

Hydrochloric acid solution 1N, sodium chloride solution 1N, sodium chloride and sodium carbonate were obtained from Sigma-Aldrich (Milan, Italy).

Methanol, acetonitrile and formic acid 99.9% were LC-MS grade, water was HPLC gradient grade, n-hexane and dichloromethane were analytical grade. All solvents were supplied from VWR (VWR International PBI Srl Milan, Italy).

The stock standard solutions of β_2 agonists (1 mg/mL) were prepared in methanol and stored in dark glass bottles at -20°C for 1 month. The working mixed standard solutions of β_2 -agonists (each at 50 μ g/L) were prepared daily by dilution with methanol of the stock standard solutions.

The stock solution of mapenterol-d₁₁ (ISTD) was prepared in methanol at concentration of 10 μ g/mL. This solution was stored in dark glass bottles at -20°C. Working standard solution of ISTD at 50 μ g/L was prepared daily in methanol.

Sample preparation

Hair samples were collected from living animals in the neck region. They were washed with deionized water to remove external contamination and, after drying overnight at 40°C, they were pulverized with a household mill. 0.25 g of sample were weighed into a 50 ml polypropylene tube and spiked with 40 μ l of a 50 μ g/L solution of ISTD.

After 15 min, 5 ml of NaOH 1N was added and this mixture was left in a waterbath overnight at 45°C and, after being cooled to room temperature, the pH was adjusted to 6.0 with hydrochloric acid 1N. A volume of 4 ml of a mixture of 1.5 g of NaCl and Na₂CO₃ (90:10 w/w) in hexane/methylene chloride (50:50 v/v) were added to the sample, vortexed for 5 min and centrifuged for 10 minutes at 3500 g.

2.0 mL of supernatant were transferred to a test tube and evaporated to dryness under a stream of nitrogen at 40°C. The residue was dissolved in 250 μ L of methanol just before injection, transferred to HPLC vials and analyzed into LC-MS/MS system.

UHPLC-ESI-MS/MS analysis

The ultra-performance liquid chromatography tandem mass spectrometry method with electrospray ionization (UHPLC-ESI-MS/MS) was performed with a Thermo Scientific TSQ Vantage triple quadrupole mass spectrometer, operating in positive ion mode (ESI), coupled with a Thermo Scientific Accela U-HPLC system with thermostated autosampler and column compartment.

LC separations were obtained using a Hypersil Gold C₁₈ 1.9 μ m (50 x 2.1 mm) column, operating at 30°C. LC eluents were: A 0.1% (v/v) aqueous formic acid solution, B 0.1% (v/v) acetonitrile formic acid solution and C 0.1% (v/v) methanol formic acid solution. The gradient elution program was 0–0.5 min 5% B and 3% C; 0.5–3.5 min 94% B and 3% C, 3.5–5.5 min 94% B and 3% C; 5.5–6 min

5% B and 3% C, 6-11 min 5% B and 3% C. The injection volume was 15 μ L (partial loop 5 μ L) and the flow rate was set at 400 μ L/min.

The parameters optimized were: capillary temperature 310°C; vaporizer temperature 340°C; spray voltage 1500 V; sheath and auxiliary gas flow rates 35 and 30 (arbitrary unit), respectively. The gas used for the nebulizer, dissolve, and cone was nitrogen. Ultra high purity argon gas was used as collision gas at 1.5 mTorr and peak resolution of 0.7 FWHM was used on Q1 and Q3. The scan time for each monitored transition was 0.02 s and the scan width was 0.1 m/z. MS/MS parameters for each compound are shown in Table 1. Acquisition data were recorded and elaborated using XcaliburTM version 2.1.0.1139 software from Thermo Scientific.

Product ion scans of each analyte were performed by direct infusion (10 μ L/min) of 1 μ g/mL individual β_2 agonists standard solutions with the built-in syringe pump through a T-junction, mixing with the blank column eluate (200 μ L/min).

The product ion spectra of the investigated β_2 -agonists after positive electrospray ionization showed common and individual fragmentation patterns upon collision-induced dissociation (CID); selected ions are listed in Table 1.

Supplemental file Legend

Figure S1: Chromatogram of β_2 -agonists standard solutions (50 $\mu\text{g/L}$) analyzed by LC–MS/MS

Table S1: Validation parameters according to European Commission 2002/657/EC

Table S1

Analyte	Spike level ($\mu\text{g/kg}$)	Recovery (%) (n=18)	Repeatability (RSD %) (n=6)	Reproducibility (RSD %) (n=18)	CC α ($\mu\text{g/kg}$)	CC β ($\mu\text{g/kg}$)
MPT	4	93	3.7	20.7	6.1	8.0
	8	99	6.0	12.7		
	12	98	4.7	11.1		
MBT	4	91	5.8	8.3	5.1	8.0
	8	95	3.5	6.3		
	12	93	2.2	4.9		
CPT	4	92	2.6	8.4	5.1	6.2
	8	97	3.6	9.5		
	12	96	2.5	11.9		
CBT	4	98	7.7	12.1	6.3	8.7
	8	99	4.5	8.9		
	12	98	3.7	11.2		
BBT	4	95	7.1	11.9	5.7	7.3
	8	95	3.9	8.7		
	12	92	3.7	8.5		

Figure S1