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Research Article

Development and Validation of LC-MS/MS Method for Determination of Ten Beta Agonists in Bovine Urine

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Abstract

The use of β-agonists in livestock production is prohibited in many countries because the residues of β-agonists in food pose a potential risk to human health. The present work describes the development and validation of the LC-MS/MS method for detection of ten β-agonists in bovine urine according to Commission Decision 2002/657/EC requirements. Linearity of the method resulted with coefficient of correlation >0.990. The decision limits (CCα) ranged from 0.127 ng/mL to 0.646 ng/mL, and the detection capability (CCβ) resulted in the range 0.140 ng/mL to 0.739 ng/mL. The observed recoveries in the fortified bovine urine samples were satisfactory at every fortification level with values from 73.67% to 118.80%. The coefficient of variation (CV, %) at three fortification levels for each β-agonist was in complete agreement with the requirements from Commission Decision 2002/657/EC. The CV for intraday precision varied from 1.619% to 15.472% and the CV for interday precision varied from 2.695% to 10.441%. From the obtained validation results the proposed method is an appropriate method for determination of β-agonists in bovine urine.

Keywords: *β-agonists, Bovine, Urine, Validation, LC-MS/MS*

Sığır İdrarında 10 Beta Agonist Varlığının Belirlenmesi İçin LC-MS/MS Metodu Geliştirilmesi ve Validasyonu

Öz

Bir çok ülkede β-agonistlerin hayvancılıkta kullanılması yasaklanmıştır, çünkü gıda ürünlerinde bulunan β-agonist kalıntıları insan sağlığı için risk oluşturmaktadır. Bu çalışma, komisyon kararı 2002/657/EC ye uygun olarak, sığır idrarındaki 10 adet β-agonistin tespitini sağlayan metod gelişimini ve validasyonunu içermektedir. Doğrusallık korelasyon katsayısı >0.990 ile sonuçlanmıştır. Karar limitleri (CCα) 0.127 ng/mL ile 0.646 ng/ml arasında ve tespit kapasitesi (CCβ) 0.140 ng/mL ile 0.739 ng/ml arasında bir değer almıştır. Geri kazanım %73.67 ile %118.80 arasında bulunmuştur. Korelasyon varyasyonu (CV, %) her üç konsantrasyon seviyesi için Komisyon Kararı 2002/657/EC 'ye tamamen uygun olarak yapılmıştır. Gün içi kesinlik CV değeri %1.619 ile %15.472 arasında ve günler arası CV kesinlik değeri %2.695 ile %10.441 arasında değişmektedir. Bu çalışmada elde edilen validasyon değerlerine göre geliştirilen metod sığır idrarında β-agonist tespiti için uygundur.

Anahtar sözcükler: *β-agonistler, Sığır, İdrar, Validasyon, LC-MS/MSS*

INTRODUCTION

β-agonists are a group of synthetic compounds derived from cathecholamines, such as adrenaline and noradrenaline. They are therapeutically used in human medicine for the treatment of bronchoconstrictions and as bronchospasmolytic. In the veterinary medicine β-agonist are used for treatment of bronchoconstrictions, such us chronic obstructive pulmonary disease in horses, and as bronchodilators, tocolytics and heart tonics [1-4]. Moreover, β-agonists are illegally used in livestock production as growth promoters. After the application of 5-10 times

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higher doses than the therapeutic doses they improve carcass composition with increased muscle development (live weight-gain, improvement of feed conversion and increase of the muscle to fat tissue ratio) and reduced fat deposition [4-7]. The residues from illegally used β-agonist accumulate and persist in liver, muscle, retina, pigmented and other tissues, and for these reasons they incur a risk to human health [5,7-9]. In 1990, 22 people in France were affected after consumption of veal liver with residues of clenbuterol. In Spain, the same year, 135 people were affected, and in 1992 a total of 232 cases of poisoning were registered after consumption of liver or meat with clenbuterol residues. The main symptoms found were: tremors, tachycardia, nervousness and general malaise [10,11]. The Commission of European Communities has banned the use of β-agonists as growth promoter in livestock production with Council Directive 96/22/EC, while Council Directive 96/23/EC prescribes the measures to monitor certain substances and residues thereof in live animals and animal products [12,13]. The most commonly used confirmation and identification technique for β-agonists in biological matrices is mass spectrometry performed with gas chromatography (GC-MS/MS) or liquid chromatography. The LC-MS/MS method is used more often than GC-MS/MS method, because derivatisation step is the critical step in GC-MS/MS technique, while in the LC-MS/MS method the derivatisation is not required [7,11,13]. The aim of this study was to develop and validation the LC-MS/MS method for detection of ten β-agonist (clenbuterol, brombuterol, mabuterol, cimbuterol, isoxsuprine, clenpenterol, ractopamine, salbutamol, zilpaterol and terbutaline) in bovine urine, applying thereby the analytical criteria stipulated under the Commission Decision 2002/657/EC [14].

MATERIAL and METHODS

Chemicals and Apparatus

Standards and Internal Standards: Clenbuterol, ractopamine, isoxsuprine, terbutaline and terbutalin-d9 were from Sigma-Aldrich, brombuterol, mabuterol, clenpenterol, cimbuterol, clenbuterol-d6, brombuterol-d9, mabuterol-d9, clenpenterol-d5 and cimbuterol-d9 from Witega, isoxsu-prine-d5 and ractopamine-d6 HCl from EURL, Rikilt, zilpaterol and zilpaterol d-7 from Toronto Research Chemicals Inc., sabutamol from Riedel-de Haen and salbutamol d-9 from Dr. Ehrenstorfer GmbH were supplied. All standards and internal standards that were used in this research are donations from EURL Berlin Germany.

Reagents: Ethyl acetate, acetonitrile (LC-MS grade), methanol (HPLC grade), water (LC-MS grade), sodium acetate (pro analysis (p.a.), ammonium hydroxide 32% and HCl 37% were purchased from Carlo Erba, formic, acetic acid, β glucoronidase/aryl sulphatase from Helix pomatia and potassium hydroxide (p.a) were purchased from Sigma Aldrich, while ascorbic acid (p.a) and potassium dihydrogen phosphate (p.a) were purchased from Alkaloid.

Apparatus: The LC-MS/MS system was purchased from Waters. The LC system equipped with binary pump, vacuum degasser, thermostated autosampler and thermostated column manager. The MS/MS detector is triple quadruple with ESI source. For separation of β-agonists were used C18 column from Phenomenex, with dimensions 50×2.1 mm and particle size from 2.6 µm. MassLynx software version 4.1 was used for data acquisition and calculation of results. For solid phase extraction were used Discovery® DSC-MCAX cartridges, 300 mg, 6 mL from Supelco.

Standards and Sample Preparation

For construction of calibration curve β-agonists were divided in three groups, as follows: group I (clenbuterol, brombuterol, mabuterol), group II (cimbuterol, clenpenterol, isoxsuprine) and group III (ractopamine, terbutaline, zilpaterol, salbutamol). The standards were prepared in the blank urine (matrix-matched calibration). In the first step 10 mL blank urine and 10 mL samples were spiked with internal standards at 0.5 ng/mL. After that, in the blank urine was added mix of standards from group I, II and III for construction of calibration curve. The concentration of standards for group I were: 0.05, 0.125, 0.25, 0.375, 0.5 and 0.75 ng/mL, for group II: 0.125, 0.25, 0.375, 0.5, 0.75 and 1.0 ng/mL and for group III: 0.25, 0.5, 0.75, 1.0, 1.5 and 2.5 ng/mL. 5 mL sodium acetate buffer (pH=5) was added in the urine and then 50 µL β-glucoronidasa/aril sulfatasefrom Helix pomatia were added and incubated at 37°C over night. After cooling to room temperature, 5 mL of phosphate buffer pH 6 was added, and the samples were centrifuged at 4°C, on 4000 rpm, 10 min. After centrifugation in the supernatant, 0.2 mL methanol was added. In the next step, SPE cartridges were conditioned with 2 mL of methanol, 2 mL of water and 2 mL of phosphate buffer (pH=6). The supernatant were loaded to SPE cartridges and then cartridges were washed with 1 mL of 1 M acetic acid and evaporated to dryness followed by washing with 6 mL of methanol and evaporating to dryness. The elution was performed with 6 mL of a mixture consisting of ethyl acetate and 32% ammonia at a 97:3 ratio. The samples were evaporated to dryness under stream of nitrogen at 35°C. Then residues were dissolved in 200 µL of mobile phase consisting of 0.1% formic acid in water (A) and 0.1% formic acid (B) in acetonitrile, at a 95:5 ratio. 10 µL of the final extract was injected into LC-MS/MS system.

LC-MS/MS Conditions

The chromatographic separation on β-agonists was performed on C18 column at 40°C and flow rate of 0.8 mL/min. The gradient elution program is given in *Table 1*.

The mass spectrometry conditions were as follows:

Table 1. *Gradient elution program for mobile phase A and B.*

Time (min)	Flow (mL/min)	Mobile Phase A (%)	Mobile Phase B (%)
Initial	0.8	95	5
1.0	0.8	80	20
4.0	0.8	60	40
8.0	0.8	95	5
12.0	0.8	95	5

electrospray ionization ESI+, capillary voltage 3.0 kV, source temperature 150°C, desolvation temperature 400°C, cone gas 100 L/h, and desolvation gas 300 L/h. The multiple reaction monitoring (MRM) mode was used for the LC-MS/MS chromatograms acquisition of β-agonists. The conditions are given in *Table 2*.

Method Validation

The method was validated according to the criteria established by the Commission Decision 2002/657/EC for banned substances. The linearity was obtained from the calibration curve in matrix. Selectivity was determined by analyzing 20 blank bovine urine samples. The blank bovine urine were obtained from untreated cattle. Decision limit (CCα) was obtained by fortificationon 18 blank urine with β-agonist standards as follow: for group I at 0.1 ng/mL, for group II at 0.25 ng/mL and for group III at 0.5 ng/mL. CCα was calculated from the corresponding concentration at the y-intercept plus 2,33 times the standard deviation of the within-laboratory reproducibility. The Detection capability (CCβ) was obtained as corresponding concentration at the decision limit plus 1,64 times the standard deviation of the within-laboratory reproducibility (2002/657/EC). The accuracy (recovery), intra- and interday precision were determined at three different levels by fortified 18 replicates (6 replicates per level) of blank urine with β-agonist standards for group I at 0.2, 0.3 and 0.4 ng/mL, for group II 0.5, 0.75 and 1.0 ng/mL and 1.0, 1.5 and 2.0 ng/mL for group III.

RESULTS

The LCMS/MS method for determination of ten β-agonists in bovine urine was developed. The calibration curves obtained for all β-agonists were linear and gave a good coefficient of correlation (R^2) >0.99 *(Table 3)*.

Blank urine samples did not contain any traces of β-agonists and there was also no contamination observed. Moreover, no interference on β-agonist identification was found owing to the highly specific MRM acquisition method and the use of appropriate internal standards. It is concluded that the methods showed good selectivity. CCα and CCβ values for target β-agonists in bovine urine were determined according to Commission Decision 2002/657/EC. The results for CCα and CCβ and recommended concentration (RC) according to CRL guidance paper are summarized in *Table 4* [15].

The CCα ranged from 0.127 ng/mL to 0.646 ng/mL, and the CCβ ranged from 0.140 ng/mL to 0.739 ng/mL. It is concluded that the methods showed relevant CCα and CCβ according to the 2002/657/EC *(Table 4)* [13]. Nielen et al.[16], in the development of the LC-MS/MS method have determined a CCα from 0.01 -0.28 µg/l, and CCβ from 0.09-0.99 µg/L for group of 18 β-agonists in bovine and porcine urine, while in the study from VanHoof et al.[17], the CCβ of zilpaterol, ritodrine, ractopamine and isoxsuprine for calf urine and faeces was lower or equal to 1 µg/kg and for formoterol, the CCβ was lower or equal to 5 µg/kg [16,17]. According to literature data, besides the urine, with LC-MS/MS method can be detected low concentration of β-agonists in other biological matrices. For example, the CCα ranged from 0.1 to 0.3 µg/kg for bovine liver, 1-3 µg/kg for bovine retina, and CCβ from 0.2-0.5 µg/kg for liver and 2-5 µg/kg for retina, while in the bovine hair CCα resulted in the range 0.2-1.0 µg/kg. Application to feed showed CCβ value of less than 5.0 µg/kg [5,17-19]. From the results obtained from this study and from literature data it can be concluded that the LC-MS/MS method shows relevant CCα and CCβ values for β-agonists in biological samples. The validation results for accuracy and precision are shown in *Table 5*. Accuracy was expressed as recovery of the method (%). The observed recoveries were satisfactory for 10 β-agonists at every fortification level with values from 73.67% to 118.80%.

The precision of the method was determined by calculating the coefficient of variation (CV). The CV for intraday precision varied from 1.619% to 15.472% and the CV for interday precision varied from 2.695% to 10.441%. The coefficient of variation at three fortification level for each β-agonist was in complete agreement with the requirements from Commission Decision 2002/657/EC, and demonstrating the excellent method precision. Most methods for detection of β-agonist with LC-MS/MS technique reported in the literature showed good accuracy, intraday and interday precision. In the method for testing of ractopamine in bovine and sheep urine the recovery ranged from 108.4 to 117.8%, and the intraday precision was 0.9% at 35 ng/mL and 5.64% at 0.25 ng/mL, while the interday precision was 0.95% at 35 ng/mL and 4.32% at 0.25 ng/mL [18]. Recovery from 50-120%, and precision around 14% were detected in the method for detection of zilpaterol in calf urine [16]. Fesser et al.[5], detected recovery from 98-118%, for samples fortified at levels between 0.5-2.0 µg/kg (liver) and 5-20 µg/kg (retina) with good precision (CV ranging from 6 to 20%). Good recovery (83-90% for salbutamol, clenbuterol and ractopamine) and precision (CV=1.5-11%) were detected in feed by Zhang et al.[20]. Also, the recovery (97-109.4%) and precision (CV=0.1-9.5%) for ractopamine in swine and cattle tissues were satisfactory [21].

Table 2. *Parameters of MRM condition and retention times of the β-agonists.*

Standards	Retention Time (min)	Monitored Reactions Precursor		Collision Energy (v)	Adequate Internal Standards	Retention Time (min)	Monitored Reactions Precursor		Collision Energy (v)
		Parent Ion (m/z)	Daughter Ions (m/z)				Parent Ion (m/z)	Daughter Ions(m/z)	
Clenbuterol	2.22	276.97	202.95 131.87 167.77	16 30 30	Clenbuterol-d6	2.29	283.03	203.56 132.19	16
Brombuterol	2.55	366.90	292.84 211.42 57.00	20 34 38	Brombuterol-d9	2.49	375.93	293.87 212.39	18
Mabuterol	2.66	310.95	236.99 216.96 57.00	18 26 30	Mabuterol-d9	2.65	320.07	237.94 66.04	18
Clenpenterol	2.60	291.00	202.92 131.89 167.79	16 30 28	Clenpenterol-d5	2.59	296.00	203.10 132.01	16
Isoxsuprine	2.50	302.04	106.96 164.01 120.95	30 16 28	Isoxsuprine-d5	2.70	308.15	168.05 107.09	16
Cimbuterol	1.49	234.03	159.98 142.94 57.00	16 28 26	Cimbuterrol-d9	1.51	243.07	160.96 143.72	16
Ractopamine	1.94	302.04	106.96 164.01 120.95	28 16 24	Ractopamine-d6	1.99	308.10	168.05 120.95	16
Salbutamol	1.27	240.03	147.96 165.98 56.94	20 14 24	Salbutamol-d9	1.29	249.08	148.59 166.99	20
Zilpaterol	1.31	262.03	185.01 202.05 156.98	24 22 32	Zilpaterol-d7	1.23	269.08	185.15 203.04	24
Terbutaline	1.26	226.00	152.00 106.97	14 30	Terbutaline-d9	1.25	235.07	152.83 66.05	16

Table 3. *Linearity of the method*

β-agonists	Matrix Match Calibration	
	Range (ng/mL)	R^2
Clenbuterol	0.05-0.75	0.995214
Brombuterol	0.05-0.75	0.993479
Mabuterol	0.05-0.75	0.996597
Clenpenterol	0.125-0.75	0.994935
Isoxsuprine	0.125-0.75	0.990097
Cimbuterol	0.125-0.75	0.995858
Ractopamine	0.25-2.50	0.993357
Salbutamol	0.25-2.50	1.000000
Zilpaterol	0.25-2.50	0.994861
Terbutaline	0.25-2.50	0.992348

Table 4. *CCα and CCβ for β-agonists in bovine urine, recommended concentration for β-agonists in urine*

β-agonist	CCα (ng/mL)	CCβ (ng/mL)	RC (ng/mL)
Clenbuterol	0.158	0.188	0.2
Brombuterol	0.144	0.170	0.2
Mabuterol	0.127	0.140	0.2
Clenpenterol	0.299	0.329	0.5
Isoxsuprine	0.29	0.320	0.5
Cimbuterol	0.259	0.282	0.5
Ractopamine	0.577	0.619	1.0
Salbutamol	0.584	0.657	1.0
Zilpaterol	0.646	0.739	1.0
Terbutaline	0.565	0.702	3.0

DISCUSSION

In the similar, but screening quantitative method for detection of β-agonists in bovine and porcine urine, feed and hair, from Nielen et al.[16], accuracy of the method for urine was from 85-111 %, recovery at 0.5 µg/L for clenbuterol was 74.0%, recovery for another β-agonists at 1.0 µg/L was from 54-85%, the interday precision of the method was from 3-26% and intraday precision was from 5-32% [16]. The use of analogue isotope-labelled internal standards

Table 5. *Accuracy and precision of the method*

β-agonists	Added Concentration (ng/mL)	Intraday Precision			Interday Precision		
		Mean Concentration (n=6)(ng/mL)	Mean Recovery (%)	CV (%)	Mean Concentration (n=6)(ng/mL)	Mean Recovery (%)	CV (%)
Clenbuterol	0.2	0.206	103.00	3.225	0.176	88.00	7.545
	0.3	0.292	97.33	12.513	0.231	77.00	5.205
	0.4	0.370	92.50	9.791	0.363	90.75	4.730
Brombuterol	0.2	0.213	106.50	11.764	0.205	102.50	7.966
	0.3	0.305	101.67	11.764	0.307	102.33	4.471
	0.4	0.470	117.5	9.831	0.458	114.50	5.888
Mabuterol	0.2	0.198	99.00	7.591	0.162	81.00	5.592
	0.3	0.277	92.33	3.957	0.249	83.00	7.301
	0.4	0.365	91.25	11.610	0.450	112.50	4.467
Clenpenterol	0.5	0.474	94.80	7.671	0.471	94.20	6.051
	0.75	0.623	83.07	2.184	0.713	95.07	4.248
	1.0	1.188	118.80	4.714	1.184	118.40	3.217
Isoxsuprin	0.5	0.385	77.00	7.365	0.410	82.00	2.695
	0.75	0.558	74.40	7.596	0.606	80.80	3.477
	1.0	0.993	99.30	4.038	0.972	97.20	6.715
Cimbuterol	0.5	0.503	100.60	7.154	0.458	91.60	4.163
	0.75	0.739	98.53	4.924	0.666	88.80	4.345
	1.0	1.122	112.2	8.638	1.052	105.20	3.889
Ractopamine	1.0	0.872	87.20	1.619	0.853	85.30	3.383
	1.5	1.321	88.07	4.148	1.317	87.80	3.235
	2.0	1.929	96.45	3.235	1.810	90.50	5.200
Salbutamol	1.0	0.849	84.90	3.648	0.873	87.30	5.296
	1.5	1.105	73.67	5.828	1.275	85.00	9.789
	2.0	1.815	90.75	11.319	1.998	99.90	4.985
Zilpaterol	1.0	0.812	81.20	8.402	0.976	97.60	8.756
	1.5	1.351	90.07	5.676	1.487	99.13	5.734
	2.0	1.782	89.10	15.472	2.060	103.00	5.467
Terbutaline	1.0	0.953	95.30	9.316	1.091	109.10	9.770
	1.5	1.490	99.33	7.613	1.597	106.47	6.043
	2.0	2.037	101.85	10.949	2.168	108.40	10.441

for all β-agonists in this study has resulted with better interday and intraday precision, as well as better recovery (>73%) compared with the study of the Nielen et al.[16] where authors used only 3 isotope-labelled internal standards which not completely correct recovery loss and ionization suppression. Moreover, in the process of identification and quantification of the substances in the analytical methods the number of transitions play important role. In this study are included 1 precursor and 3 daughter ions for all β-agonists and 1 precursor and 2 daughter ions for internal standards and terbutalin therefore the method fulfills the requirement for a minimum of 4 identification points for a confirmatory method. On the other hand in the study of Nielen et al.[16], authors used 1 precursor ion and 1 daughter ion which is suitable for screening method because in the method yield 2.5 identification points. The confirmatory methods are more accurate, more sensitive, more precise than screening methods and enable the identification and quantification of analytes. According to 2002/657/EC "confirmatory method provide full or complementary information enabling the substance to be unequivocally identified and if necessary quantified at the level of interest", while "screening method means methods that are used to detect the presence of a substance or class of substances at the level of interest" [13]. The results from this study showed good validation parameters for detection of β-agonists in bovine urine; therefore, this method will

be used in routine analysis for detection of β-agonists in bovine urine.

In summary, the LC-MS/MS method for detection of ten β-agonists was validated according to Commission Decision 2002/657/EC and European Union requirements. The method validation study demonstrated acceptable linearity, selectivity, CCα and CCβ, accuracy and precision. On the basis of validation results we can conclude that the method is suitable for determination of low concentration of β-agonist residues in bovine urine samples. The method will be used for monitoring the abuse of β-agonists in bovine urine and evaluating the potential risk to human health.

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