Preliminary Report on the Confirmation and Quantitative Determination of Salmeterol in Equine Urine and Serum

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Abstract

Salmeterol is a β_2-adrenergic agonist and an Association of Racing Commissioners International (ARCI) class 3 drug. Trade names of its xinafoate salt are Arial (Dompé). Salmetedur (Menarini), and Serevent (Glaxo). Salmeterol is routinely used to increase ease of breathing in race horses during their training. Due to its stimulant and bronchodilator properties its administration to a horse just prior to race time has the potential to affect the horse's performance, therefore a reliable method of analysis for this compound is necessary.

This paper investigates and describes a method for the identification and quantitation of salmeterol in equine urine and serum using liquid-liquid extraction followed by liquid chromatography and tandem mass spectrometry (LC-MS/MS). Serum concentrations at 30 minutes were generally below the minimum level of quantitation. Urine salmeterol concentrations peaked at about 2 hours post-dose following administration of 500 ug both intravenously and intratracheally at concentrations of 14 ng/ml and 4 ng/ml, respectively. Urinary salmeterol was detectable for 6-8 hours following dosing by either method.

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Introduction

Salmeterol, (±)-4-Hydroxy-α1-[[[6-(4-phenylbutoxy)hexyl]amino]methyl]-1,3-benzenedimethanol; (Fig. 1) is a β_2-adrenergic agonist and an Association of Racing Commissioners International (ARCI) class 3 drug. In human medicine Salmeterol is used for conditions such as asthma and chronic obstructive pulmonary disease (Dal Negro, et al., 2003; Zimmermann et al., 2003; Coraux et al., 2003; Lyseng-Williamson and Posker, 2003; Lindqvist et al., 2003; Reid et al., 2003; Caverley et al., 2003). Due to their anti-bronchoconstrictor properties, β_2-receptor agonists are commonly used to increase ease of breathing in racehorses during their training (Hendrikson and Rush, 2001). Due to the stimulant and bronchodilator properties of salmeterol, its administration to a horse just prior to race time may have the potential to affect the horse's performance. For this reason, a reliable method of analysis for salmeterol is necessary.

This paper investigates and describes a method for the identification and quantitation of salmeterol in equine urine and serum using liquid-liquid extraction followed by liquid chromatography and tandem mass spectromentry (LC-MS/MS).

Briefly, salmeterol-d_{12} xinafoate, was synthesized for use as an internal standard. Salmeterol and its internal standard were extracted from alkaline urine or serum with dichloromethane. The extract was evaporated to dryness, redissolved in HPLC mobile phase, and injected into a liquid chromatograph interfaced with a tandem mass spectrometer operating in the electrospray ionization [positive] (ESI^+) mode. Ion fragmentation mechanisms specific for salmeterol and the internal standard salmeterol-d_{12} were monitored by MS/MS to identify and quantitate salmeterol. In order to insure that the analytical method possessed adequate sensitivity for use in the regulatory control of the use of this medication, samples from horses which had been administered salmeterol were analyzed.

Materials and Methods

Horses and Sample collection –

Two mature Thoroughbred mares weighing 542 and 572 kg were used for this study. The animals were maintained on grass hay and feed (12% protein), which was a 50:50 mixture of oats and an alfalfa-based protein pellet. Horses were fed twice a day. The animals were vaccinated annually for tetanus and dewormed quarterly with ivermectin. A routine clinical examination was performed prior to each experiment to assure that these animals were healthy and sound. During experimentation, horses were provided water and hay *ad libitum*. All animal care was in compliance with the guidelines issued by the Division of Laboratory Animal Resources and was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Kentucky.

500 ug salmeterol was administered, as its xinafoate salt, both as a single intravenous injection in the right jugular vein and also a single intratracheal injection in a second horse. Blood samples were collected from the left jugular vein for analyses at 0, 0.25, 0.5, 0.75, 1, 2, 3, 4, 5, 6, and 24 hours into Vacutainer serum tubes (Becton Dickinson, Rutherford, NJ), allowed to clot, then centrifuged at 900 x g for 15 minutes at 20°C. After separation, the serum samples were stored at -20°C until assayed. During the first day, complete urine collection was accomplished with a Foley catheter at 0, 1, 2, 3, 4, 5 and 6 hours after administration. At 24 hours after administration, a Harris flush tube (24 Fr x152.4 cm; Seamless, Ocala, FL, USA)

was used to collect the urine samples. Urine was divided into appropriate aliquots and stored at -20°C until assayed.

After one week, the drug was again administered to each of the two horses by the alternative routes of administration. Serum and urine were again obtained in the same manner.

Synthesis of Salmeterol Standard and Salmeterol-d_{12} Internal Standard

A certified salmeterol standard was not commercially available. We therefore elected to synthesize in-house both salmeterol and it deuterated analog for use as standard and internal standard, respectively. Salmeterol xinafoate and salmeterol-d_{12} xinafoate were synthesized from methyl salicylate, 1,6-dibromohexane and its d_{12} analog, and 4-phenyl-1-butanol. The final products (Fig. 1 and Fig. 2) were obtained in high chemical and isotopic purity as their xinafoate salt (Karpiesiuk et al., 2003) .

Sample and Calibrator Preparation

All urine samples were subjected to β-glucuronidase hydrolysis prior to extraction as first described by Combie et al. (1982) and more recently by Lehner et al. (2003). Serum was processed without hydrolysis.

Methanol solutions of salmeterol and salmeterol-d_{12} were prepared. Using a micropipetor, calibrators in the range of 0 to 25 ng/ml were prepared by serial dilution and addition of a known quantity of salmeterol solution to 4 ml aliquots of blank urine or 2 ml of blank serum in a 15 ml test tube with a screw cap. Salmeterol-d_{12} was added to each tube to produce a concentration of 25 ng/ml. Identical quantities of internal standard were added to each 4 ml aliquot of unknown sample to be analyzed.

Extraction Procedure

Samples were made alkaline by addition of 200 µl of concentrated NH_4OH (Fisher). 5 ml of dichloromethane was added and the tubes were capped and rotated on a rotorack (Fisher Model 346) for 15 minutes. The tubes were then centrifuged at 900 x g at room temperature to separate the layers. The top aqueous layer was aspirated to waste, and the remaining dichloromethane layer was carefully decanted into a test tube which was placed in a water bath (Zymark TurboVap LV) at 40°C while the solvent was evaporated to dryness under a stream of nitrogen.

Instrumentation

A Hewlett-Packard 1050 Liquid Chromatograph interfaced with a Micromass (Beverly, MA) Quattro II tandem Mass Spectrometer with electrospray inlet was operated in the positive ion mode.

Chromatography

Chromatography was performed on a Phenomenex Luna phenyl/hexyl 30 mm x 1.0 mm x 3 micron phenyl-hexyl column with a flow of 0.150 ml/min. An acetonitrile-water-formic acid mobile phase gradient was used as described in Table 1. 10 µl of redissolved extract was injected.

Mass Spectrometry

Mass spectrometric parameters for data acquisition were determined by obtaining a daughter ion spectrum for infused salmeterol, then choosing specific ion transitions and optimizing parameters for most sensitive monitoring. Fragments of interest arising from the molecular ion m/z 416 include the m/z 398, 380, 248, 232 and 91 ions (Table 2 and Table 3).

Optimal tuning parameters for detection of salmeterol were determined during direct infusion of 10 ug/ml salmeterol in 0.05% formic acid (aq):acetonitrile, 1:1.

Spectrometry involved MRM monitoring of 4 diagnostic ion transitions arising by fragmentation of the salmeterol m/z 416 $[M+H]^+$ ion, and 2 ion transitions from the internal standard as shown in Table 3. The ion transitions used for quantitation were m/z 416>398 for salmeterol and m/z 428>410 for the internal standard.

Results

Dynamic mass calibration of salmeterol parent ion values in 0.1 amu increments determined that the optimal signal for m/z 398 in the m/z 416-417 range occurred at m/z 416.2, providing at least 5% improvement over nominal mass 416 (data not shown). Best yield also occurred at m/z 398.2.

Daughter ion abundances were measured at increasing collision energies to determine maximum output intensity, as shown in Figure 3. Ion m/z 91 generally provided the best response, but was considered less specific than the dehydration product at m/z 398; the second dehydration product m/z 380 generally parallels that of m/z 398. Low collision energy settings were therefore preferred for m/z 398 and 380, retaining fairly strong signal at the additional m/z 232 qualifier. Figure 4 contrasts the full daughter ion spectra at low and high collision energies; note the general lack of peaks at the higher setting, despite the intense m/z 91. Figure 5 shows the structural origins assigned to fragments m/z 91 and 232.

Liquid/liquid extraction was investigated for three organic solvents, [ethyl acetate (EA), dichloromethane (DCM), and petroleum ether (pet ether)]. DCM and EA yielded partition coefficients greated than 95% with ammonia-basified water. DCM was deemed the best choice of solvent since it formed less emulsion during the serum and urine extraction process.

Isocratic HPLC based on conditions similar to those developed for clenbuterol (Lehner et al., 2001) yielded a low retention time peak for salmeterol when optimized to a mobile phase flow rate of 0.150 ml/min on a 1 mm i.d. Luna phenyl-hexyl column. Injection of 10-300 ng on column (equivalent to injection of 100% of the extract of 1 ml in the10-300 ng/ml sample range)

resulted in a linear response with some curvilinearity above 200 ng, probably owing to the relatively low loading capacity of the column. Injection of 20 pg, 200 pg, 2 ng and 20 ng on column revealed linearity of the method over a four log-unit span. A gradient HPLC method described in the methods section was then developed for the same column at 0.15 ml/min which retained the sensitivities described. Salmeterol was eluted in a sharp non-tailing peak at about 9 minutes under these conditions (Fig. 6). The right portion of Figure 6 displays nested fragment ion peaks at the low collision energy used for quantitative purposes.

Salmeterol calibration curves prepared from extracted standards were curvilinear between 0.025 and 50 ng/ml with a coefficient of determination $r^2 > 0.99$. Figure 7 is a typical standard curve. Over the lower concentration range of 0.025 to 2.5 ng ml the standard curve was linear with a first-order regression plot having a coefficient of determination $r^2 > 0.99$. The lower limit of detection in urine for this method was 0.025 ng/ml while the lower limit of quantitation was estimated at about 0.125 ng/ml.

500 ug salmeterol was administered to two horses, one via the intravenous (IV) route and one the intratracheal (IT) route. One week later each horse was again dosed using the opposite route. Urinary salmeterol concentrations were found to reach peak values at about two hours following either route of administration. As shown in Table 4, intravenous administration gave an average maximum urinary concentration of about 14 ng/ml, while intratracheal administration gave a maximum concentration of about 4 ng/ml. Urinary salmeterol concentrations remained above the limit of quantitation for at least 6-8 hours post-dose for both routes of administration.

Serum salmeterol concentrations following IV administration of 500 ug salmeterol reached an average value of approximately 0.17 ng/ml at 15 minutes post-dose and declined to about 0.08 ng/ml by 30 minutes (Fig. 10). This 15-minute value is approximately equivalent to our measured lower limit of quantitation in serum, suggesting that this method, using our instrumentation, has insufficient sensitivity for routine analysis of serum for salmeterol.

Discussion

Salmeterol is a highly potent long-acting drug, with typical human doses of 50 ug b.i.d. (Lindqvist et al., 2003; Reid et al., 2003; Zimmerman et al., 2003). Due to correspondingly low doses used in the horse, low concentrations of target analyte are found in serum and urine of the horse. Maximum extraction efficiency and adjustment of instrumental parameters to achieve maximum sensitivity and thus, the lowest possible detection limit, were attempted in an effort to measure these low concentrations.

Extraction of salmeterol by direct application of the clenbuterol solid phase extraction (SPE) method (Lehner et al., 2001) yielded poor salmeterol recovery. A single step solvent extraction with dichloromethane resulted in salmeterol extraction efficiencies greater than 90% with both serum and urine.

Though clenbuterol was used by Van Eenoo, et al. (2002) for an internal standard, we chose not to use it due to the following potential disadvantages: significant difference in the HPLC retention times of clenbuterol and salmeterol may contribute to assay variability; somewhat dissimilar chemical classes produce moderate differences in extractability which can produce variability in quantitative results; no real advantage is provided by clenbuterol in terms of blocking nonspecific sites to which salmeterol itself might bind during extraction, even with use

of silanized glassware. Synthesis and use of a deuterated salmeterol internal standard (Fig. 2) eliminated these potential problems.

The lower limit of detection in urine of 0.025 ng/ml and the lower limit of quantitation of about 0.125 ng/ml is similar to that obtained by Van Eenoo, et al. (2002) who used different instrumentation. Recent advances in mass spectrometry and chromatography, such as the Quattro Z-spray inlet and micro-bore capillary columns, may have the potential to significantly lower these limits of detection and quantitation.

In summary, we have developed a LC-MS/MS method for the analysis of salmeterol in the horse. While we were unable to achieve adequate sensitivity for the analysis of the low concentrations of salmeterol found in serum, we were able to identify and quantitate urinary salmeterol for at least 6-8 hours following doses of 500 ug of salmeterol administered both intraveneously and intratracheally.

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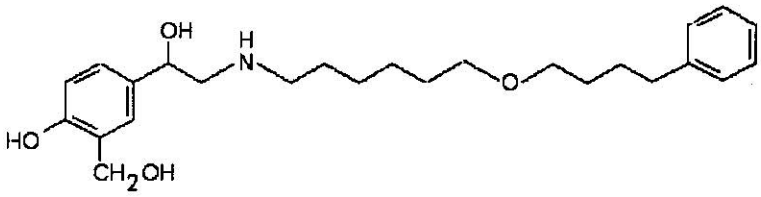

Figure 1. Salmeterol

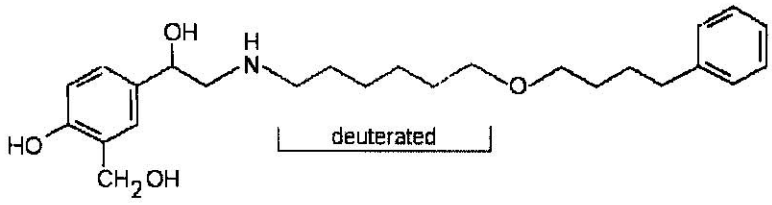

Figure 2. Salmeterol-d_{12} internal standard

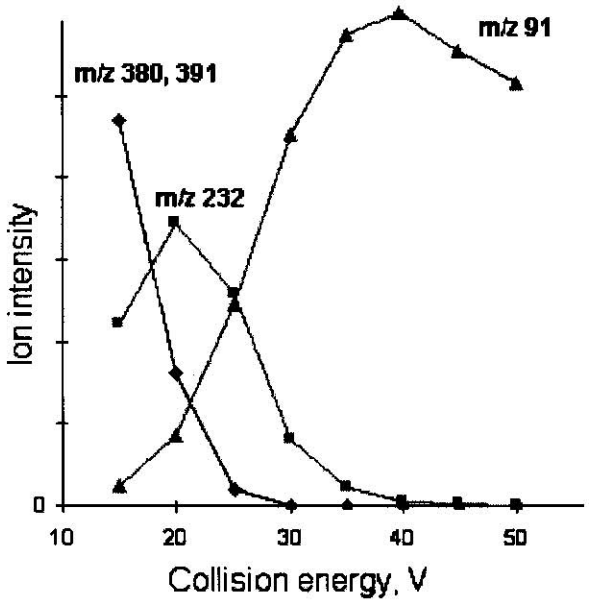

Figure 3. Relationship between ion intensity and collision energy.

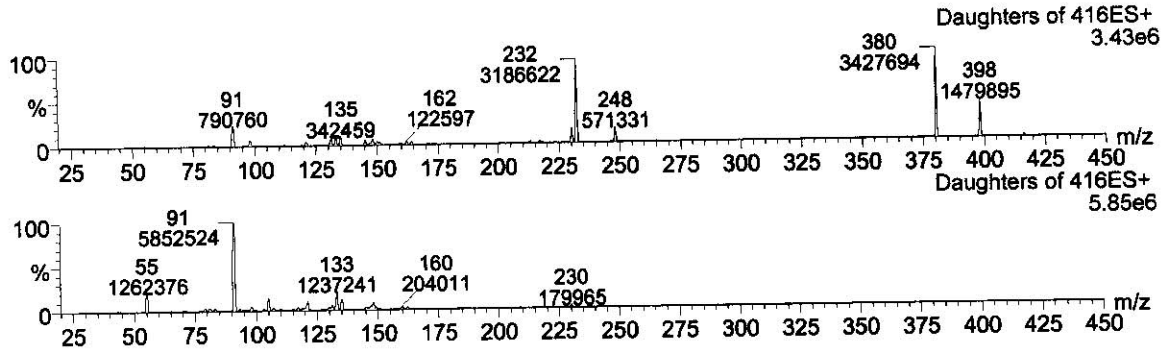

Figure 4. Daughter ion spectra at different collision energies. Salmeterol daughter ion spectra at low (top; CE=20) and high (bottom; CE=40) collision energy settings. Salmeterol [10 ug/ml in 0.05% formic acid: acetonitrile, 1:1] was examined by direct infusion into the ESI(+)-MS/MS at 1.2 ml/hr. Peak labels indicate m/z value [top value] and intensity [bottom value].

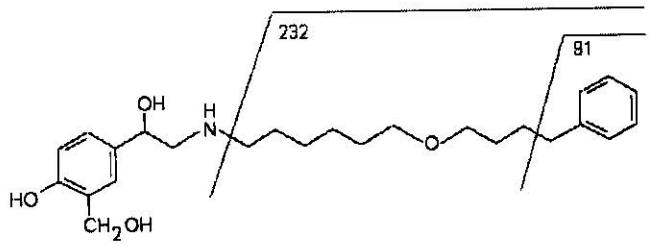

Figure 5. Salmeterol fragmentation. The figure shows the assigned origins of fragments m/z 91 [benzyl group] and 232 [phenylbutyl-O-hexyl ether fragment].

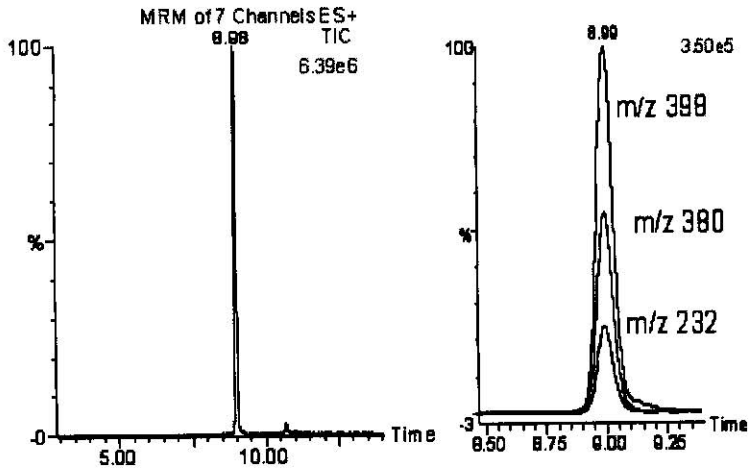

Figure 6. Salmeterol total ion chromatograms. Gradient HPLC of 2.5 ng/ml salmeterol standard extracted from urine; detection is with ESI(+)-MS/MS. The left panel shows total ion chromatogram (TIC) on a 1 mm bore 3u phenyl-hexyl column (Phenomenex Luna), right shows the array of nested fragment ion peaks at a collision energy of 16.

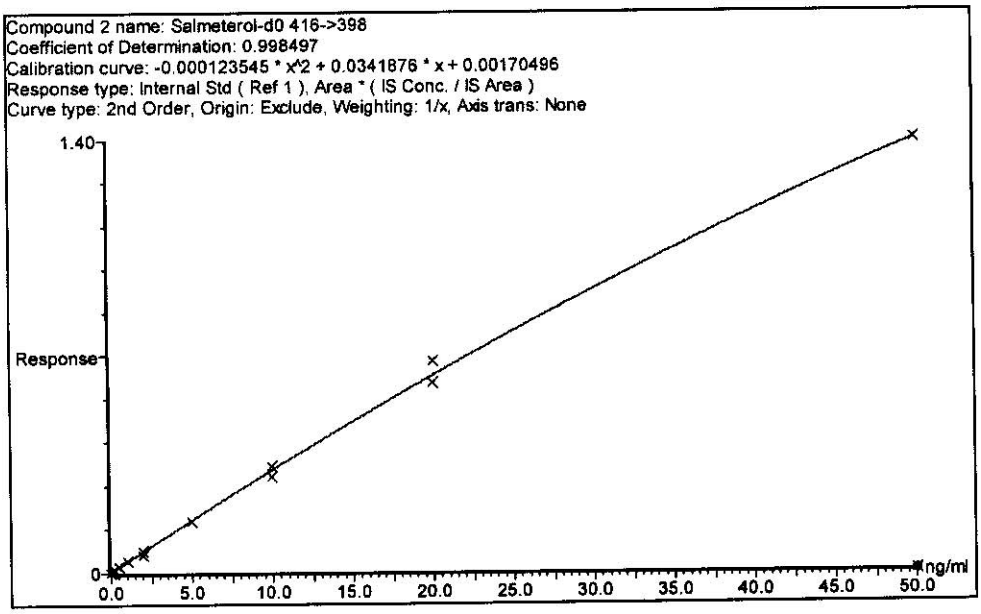

Figure 7. Typical salmeterol standard curve generated by Masslynx 3.4 software. Coefficient of determination $r^2 = 0.998$.

Table 1. HP 1050 HPLC Gradient Timetable

Time	A%	B%
0.00	10.0	90.0
1.20	10.0	90.0
5.00	89.5	10.5
8.00	89.5	10.5
8.50	10.0	90.0
17.00	10.0	90.0

Mobile phase system:
A = acetonitrile + 0.05% formic acid
B = HPLC-grade water + 5% acetontirile + 0.05% formic acid.

Table 2. Principal ions in daughter ion spectrum of salmeterol m/z 416 $[M+H]^+$ ion. 10 ug/ml salmeterol in 0.05% formic acid:ACN, 1:1, infused at 1.2 ml/hr into the Quattro II ESI-MS/MS.

Mass	% Relative Abundance	Mass	% Relative Abundance
416	9	189	7
398	52	151	9
380	100	135	14
270	5	98	10
248	13	91	17
232	56		

Table 3. Ion Transitions Monitored –
MRM of 6 Mass Pairs (ESI+)

Channel	Parent	Daughter	Dwell	
1	416.20	398.20	0.02	salmeterol
2	416.20	380.20	0.02	
3	416.20	248.20	0.02	
4	416.20	232.00	0.02	
5	428.20	410.20	0.02	salmeterol-d_{12}
6	428.20	392.20	0.02	

Table 4. Concentration (ng/ml) of salmeterol in urine following a dose of 500 ug administed both intravenously (IV) and intratracheally (IT).

Hour	Horse B-77 IT	Horse B-41 IT	Horse B-77 IV	Horse B-41 IV
0	**	**	**	**
1	1.6	0.7	15.0	5.37
2	5.0	3.0	10.5	13.0
4	2.7	1.8	4.4	4.6
6	1.7	1.7	4.9	2.0
8	1.5	1.3	3.9	2.8
24	0.26	0.1*	1.1	**
48	0.1*	**	0.4	**

* Below limit of quantitation
** Below limit of detection