347

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 bronchodilating and central nervous system stimulant 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 describes a method for the identification and quantitation of salmeterol in equine urine using liquid-liquid extraction followed by liquid chromatography and tandem mass spectrometry (LC-MS/MS). Urine salmeterol concentrations peaked at about 2 h post-dose following administration of 500 ug both intravenously and intratracheally at concentrations of 14 ng mL^{-1} and 4 ng mL^{-1}, respectively. Serum concentrations at 30 min were below the minimum level of quantitation.

Keywords: LC-MS/MS, bronchodilator, mass spectroscopy

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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. [1-7] The drug is classified as a long-acting bronchodilator, with its most efficacious use accomplished by its administration prior to any bronchoconstricting activity. Due to their bronchodilator properties, β_2-receptor agonists are commonly used to increase ease of breathing in racehorses during their training. [8] Due to the bronchodilating and central nervous system stimulant properties of these agents, their administration to a horse just prior to race time may have the potential to affect the horse's performance. For this reason, reliable methods of analysis for these compounds in the blood and urine of horses are necessary.

The xinafoate salt of salmeterol can occur in either of two polymorphic states, granular produced by fast-cooling crystallization, and micronized, prepared from the granular by micronization. These polymorphs can be distinguished by differential scanning calorimetry [9]. Trade names of its xinafoate salt are Arial (Dompé), Salmetedur (Menarini), and Serevent (Glaxo). Salmeterol has a relatively low melting point of 76°C, while the melting point of its xinafoate salt is 137-138°C. Salmeterol is sparingly soluble in water, freely soluble in methanol, and slightly soluble in ethanol, chloroform, and isopropanol [10].

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

In brief, 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, we analyzed samples from horses which had been administered salmeterol

Experimental

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.

Salmeterol (500ug) as its xinafoate salt was administered 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 h into Vacutainer serum tubes (Becton Dickinson, Rutherford, NJ), then centrifuged at 900 x g for 15 min 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 h after administration. At 24 h 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 its 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) were obtained in high chemical and isotopic purity as their xinafoate salts [11] .

Sample and Calibrator Preparation

Urine samples (4 mL) to be analyzed and blank urine samples for use in the preparation of standard curves were subjected to β-glucuronidase hydrolysis as first described by Combie et al. [12]. and more recently by Lehner et al. [13]. Serum was processed without hydrolysis.

Standard solutions of salmeterol and salmeterol-d_{12} were prepared in methanol. Using a micropipetor, calibrators in the range of 0 to 25 ng mL^{-1} were prepared by 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^{-1}. Identical quantities of internal standard were added to each unknown sample to be analyzed.

Extraction Procedure

Sample solutions were made alkaline by addition of 200 µL of concentrated NH_4OH (Fisher). Dichloromethane (4 mL) was added and the tubes were capped and rotated on a rotorack (Fisher Model 346) for 15 min. 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

Hewlett-Packard 1050 High Pressure Liquid Chromatograph (HPLC) is interfaced with a Micromass (Beverly, MA) Quattro II tandem quadrupole mass spectrometer (MS/MS) with electrospray ionization source.

Chromatography

Chromatography was accomplished on a Phenomenex Luna phenyl/hexyl column (30 mm x 1.0 mm x 3 micron) with a flow of 0.150 mL min^{-1}. An acetonitrile-water-formic acid mobile phase gradient was used as described in Table 1. The extract was redissolved in 60 µL of mobile phase and 10 µL 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 (Fig. 2).

Tuning parameters for maximum sensitivity for salmeterol were determined by direct infusion of 10 ug mL^{-1} salmeterol in 0.05% formic acid (aq):acetonitrile (1:1, v:v). The peak shape and intensity of the monoprotonated salmeterol *m/z* 416 ion were optimized by adjustment of capillary, HV lens, cone voltage, skimmer lens, and RF lens settings. Skimmer lens offset was left at 5 V. Collision gas (argon) and collision energy were adjusted for collisionally-induced dissociation (CID) in the central hexapole by optimizing settings as needed for the second quadrupole. Generally, the collision gas was set to 1-3 x 10^{-3} mbar. Increasing the photomultiplier setting above the regular 650 V increased sensitivity. In general, for positive mode, the source cone voltage was set at +27 V, the collision energy was set at -(11 to 13) V, the capillary of the ESI probe was set at +3.0 k V, the skimmer at 1.4 V, and the HV lens was set at 0.69 kV. Source temperature was set at 120°C.

Mass spectrometry involved MRM monitoring of four 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 2. 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 yield for m/z 398 derived from the m/z 416-417 pseudomolecular ion range occurred at m/z 416.2, providing at least 5% improvement over nominal mass 416 (data not shown). Best daughter ion 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, owing to its low mass, was considered less specific than the dehydration product at *m/z* 398; the second dehydration product *m/z* 380 generally paralleled that of *m/z* 398. Low collision energy settings were therefore preferred -to emphasize *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, as well as fragmentation routes to the dehydration products *m/z* 398 and 380.

Liquid/liquid extraction was compared for three organic solvents [ethyl acetate (EA), dichloromethane (DCM) and petroleum ether (pet ether)]. DCM and EA yielded partition coefficients greater 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 processes.

Isocratic HPLC based on techniques similar to those developed for clenbuterol [14] yielded a low retention time peak for salmeterol when optimized to a mobile phase flow rate of 0.135 mL min^{-1} on the 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^{-1} 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^{-1}, which retained the sensitivities described. Salmeterol was eluted in a sharp non-tailing peak at about 9 min under these conditions (Fig. 6). The right portion of Figure 6 displays nested fragment ion peaks (diagnostic ion peaks) at the low collision energy used for quantitative purposes.

Salmeterol calibration curves prepared from extracted standards were linear between 0.025 and 25 ng mL^{-1} with a coefficient of determination $r^2 > 0.99$. Figure 7 illustrates the excellent linearity of the method in the low range, i.e. 0.025-2.5 ng mL^{-1}. The lower limit of detection (LOD) in urine for this method was 0.025 ng mL^{-1} while the lower limit of quantitation (LOQ) was estimated at 0.125 ng mL^{-1}.

Salmeterol (500 ug), as its xinafoate salt, was administered to two horses, one via the intravenous (IV) route and one via the intratracheal (IT) route. One week later each horse was again dosed using the alternative route. While salmeterol is usually administered as an aerosol, these two routes of administration were selected in order to accurately quantify the dose, the most important parameter in any subsequent pharmacokinetic calculations we might make.

Urinary salmeterol concentrations were found to reach peak values at about two h following either route of administration. As shown in Figure 8, intravenous administration gave a peak urinary concentration of about of 14 ng mL^{-1}, while intratracheal administration yielded a maximum concentration of about 4 ng mL^{-1}. Urinary salmeterol concentrations remained above the limit of quantitation for at least 6 h post-dose with either route of administration.

Serum salmeterol concentrations following IV administration of 500 ug salmeterol reached a value of about 0.1-0.2 ng mL^{-1} at 15 min after dosing and declined to about 0.08 ng mL^{-1} by 30 min. 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. Serum samples from longer times had salmeterol concentrations well below the LOQ.

Storage of urine samples for three months at -20°C or subjecting the samples to 4 freeze-thaw cycles had no discernable effect on their salmeterol concentrations.

Discussion

Salmeterol is a potent drug with typical human doses of 50 ug b.i.d. [3,5,7]. Due to correspondingly low doses in the horse, the low concentrations of target analyte in serum and urine required maximum extraction efficiency and adjustment of instrumental parameters to achieve maximum analytical method sensitivity resulting in the lowest possible detection limit.

Dynamic mass calibration of salmeterol parent ion values optimized signal intensity by determination of *m/z* 416.2 parent and 398.2 daughter ion as optimal settings.

Daughter ion abundances were measured at increasing collision energies to determine maximum output intensity; this measurement determined that low collision energies provided the best combination of optimized quantifier ion *m/z* 398 as well as optimized qualifier ions *m/z* 380 and 232.

Synthesis of a deuterated salmeterol internal standard provided uniformity in assay responsiveness. Though clenbuterol was used by Van Eenoo, et al. [15] for an internal standard, we chose not to use it as such 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 varability in quantitative results; no real advantage is provided by clenbuterol in terms of blocking nonspecific sites to which highly lipophilic portions of salmeterol itself might bind during extraction, even with use of silanized glassware. Finally, in instances where a horse has been dosed with multiple beta-agonists in the form of drug cocktails, clenbuterol, if present in serum due to administration of the drug, would interfere with the assay for salmeterol, artificially reducing the salmeterol measured concentration. Synthesis and use of a deuterated salmeterol (Fig. 1) internal standard eliminated these potential problems.

Extraction of salmeterol by direct application of the clenbuterol solid phase extraction (SPE) method [14] yielded poor salmeterol recovery. A single step solvent extraction with dichloromethane resulted in extraction efficiencies greater than 90% with both serum and urine. Another consideration is that we were unable to discover evidence for a salmeterol glucuronide, but nevertheless would hydrolyze urine as a matter of course, a practice in use in other laboratories studying salmeterol.

A gradient HPLC method was developed for the 1 mm diameter phenyl-hexyl column, the geometry of which appears to be optimized for enabling low mobile phase flow rates, adequate sample loading and appropriate amounts of material for electrospray nebulization.

The lower limit of detection in urine of 0.025 ng mL^{-1} and the lower limit of quantitation of about 0.125 ng mL^{-1} were similar to those obtained by Van Eenoo, et al. [15] 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. Other factors that might further improve results could be considered, such as increase of total deuterated salmeterol-d12 concentration to values at the top end of the range of standards in order to block irreversible binding sites in matrix components such as polymers, as well as sonication of urine to enable better interfacing of organic and aqueous phases during extraction, and finally careful silanization of glassware to block irreversible binding sites.

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 h following doses of 500 ug of salmeterol administered both intravenously and intratracheally.

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Figure legends.

Figure 1. Salmeterol, 415 m.w. (top) and salmeterol-d_{12} internal standard (bottom), where d=deuterium atom.

Figure 2. Daughter ion spectrum of salmeterol *m/z* 416 [M+H] ion. 10ug mL^{-1} salmeterol in 0.05% formic acid:ACN, 1:1, infused at 1.2 mL h^{-1} into the Quattro II ESI-MS/MS.

Figure 3. Relationship between ion intensity and collision energy. *M/z* 380 follows a pattern similar to *m/z* 398.

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^{-1} in 0.05% formic acid: acetonitrile, 1:1] was examined by direct infusion into the ESI(+)-MS/MS at 1.2 mL h^{-1}. 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] arising from the monoprotonated *m/z* 416 salmeterol $[M+H]^+$ ion. Dehydration results in *m/z* 398 and 380 ions.

Figure 6. Salmeterol total ion chromatograms. Gradient HPLC of 2.5 ng mL^{-1} 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. Salmeterol standard curve (lower range of concentrations). Non-weighted least squares fit.

Figure 8. Urinary salmeterol concentrations following intravenous and intratracheal administrations.

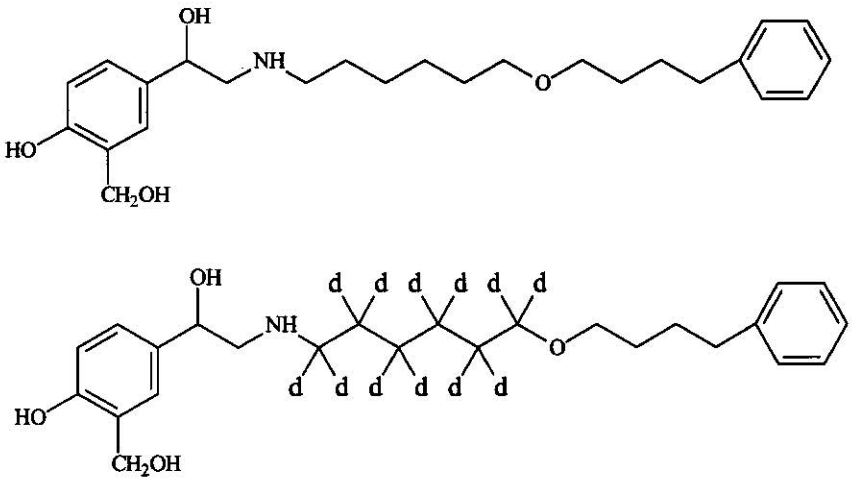

Figure 1.

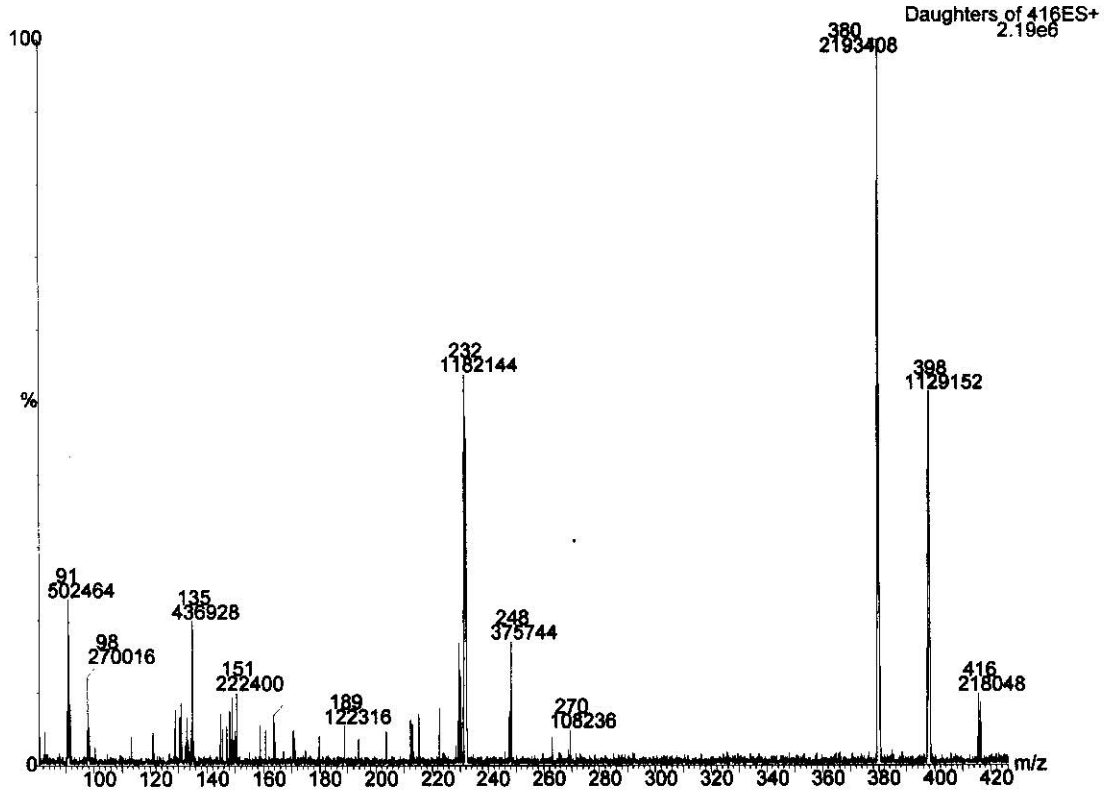
Daughters of 416ES+
2.19e6
100
%
0
380
2193408
232
1182144
398
1129152
91
502464
135
438928
98
270016
248
375744
151
222400
189
122316
270
108236
416
218048
100 120 140 160 180 200 220 240 260 280 300 320 340 360 380 400 420
m/z

Figure 2.

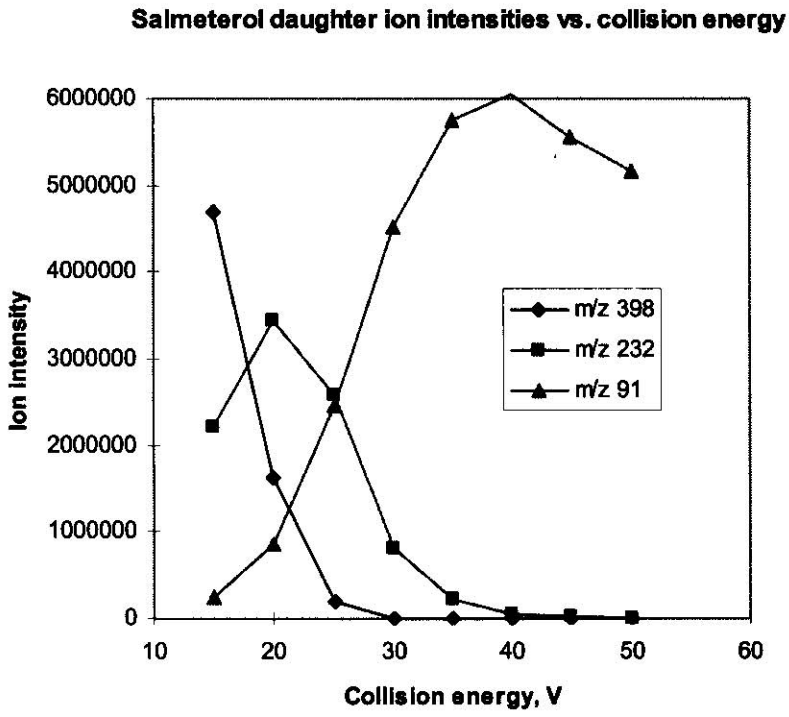
Salmeterol daughter ion intensities vs. collision energy
Ion intensity
6000000
5000000
4000000
3000000
2000000
1000000
0
10
20
30
40
50
60
Collision energy, V
m/z 398
m/z 232
m/z 91

Figure 3.

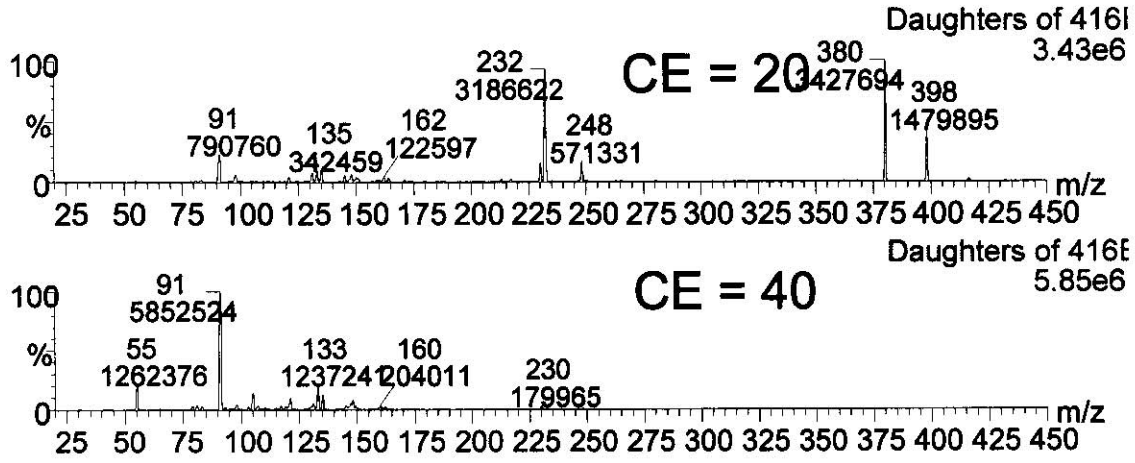
Daughters of 416I
3.43e6
CE = 20
100
%
0
232
3186622
380
3427694
398
1479895
91
790760
135
342459
162
122597
248
571331
m/z
25 50 75 100 125 150 175 200 225 250 275 300 325 350 375 400 425 450
Daughters of 416I
5.85e6
CE = 40
100
%
0
91
5852524
55
1262376
133
123724
160
204011
230
179965
m/z
25 50 75 100 125 150 175 200 225 250 275 300 325 350 375 400 425 450

Figure 4.

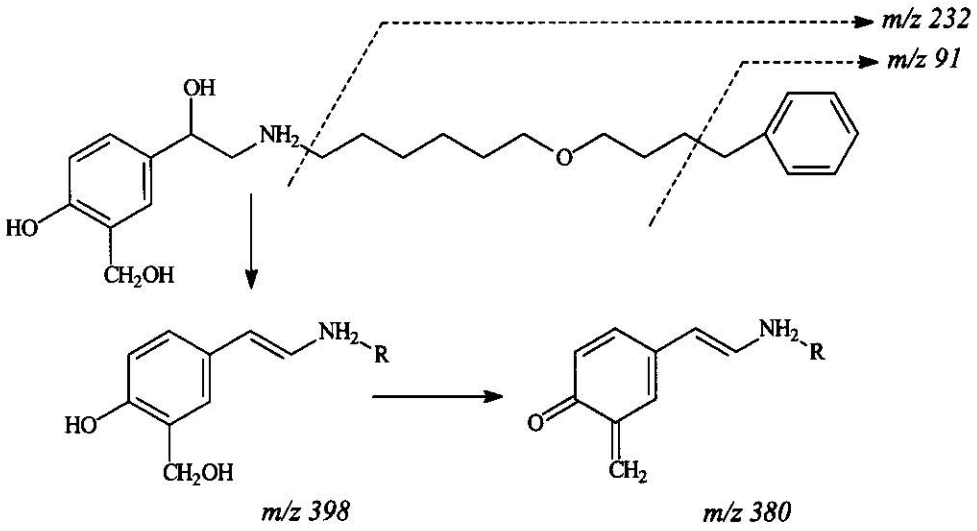
m/z 232
m/z 91
OH
NH2
HO
CH2OH
O
NH2
R
HO
CH2OH
m/z 398
NH2
R
O
CH2
m/z 380

Figure 5.

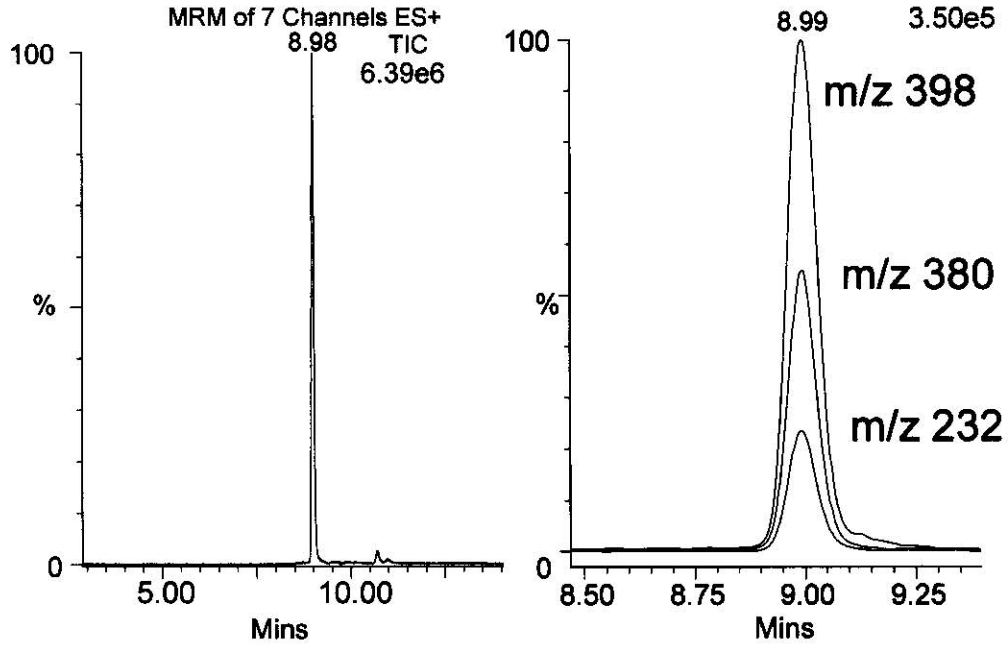
MRM of 7 Channels ES+
8.98
TIC
6.39e6
100
%
0
5.00
10.00
Mins
8.99
3.50e5
m/z 398
m/z 380
m/z 232
100
%
0
8.50
8.75
9.00
9.25
Mins

Figure 6.

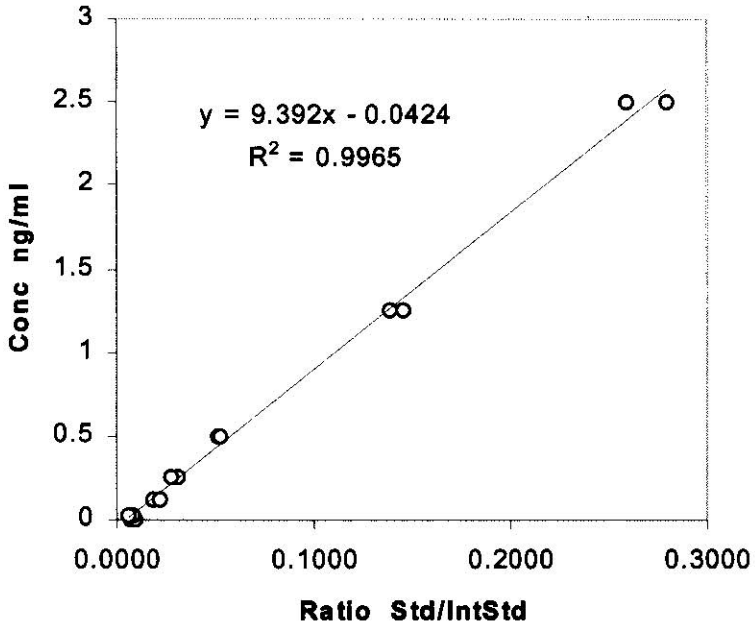
y = 9.392x - 0.0424
R2 = 0.9965
Conc ng/ml
Ratio Std/IntStd
3
2.5
2
1.5
1
0.5
0
0.0000
0.1000
0.2000
0.3000

Figure 7.

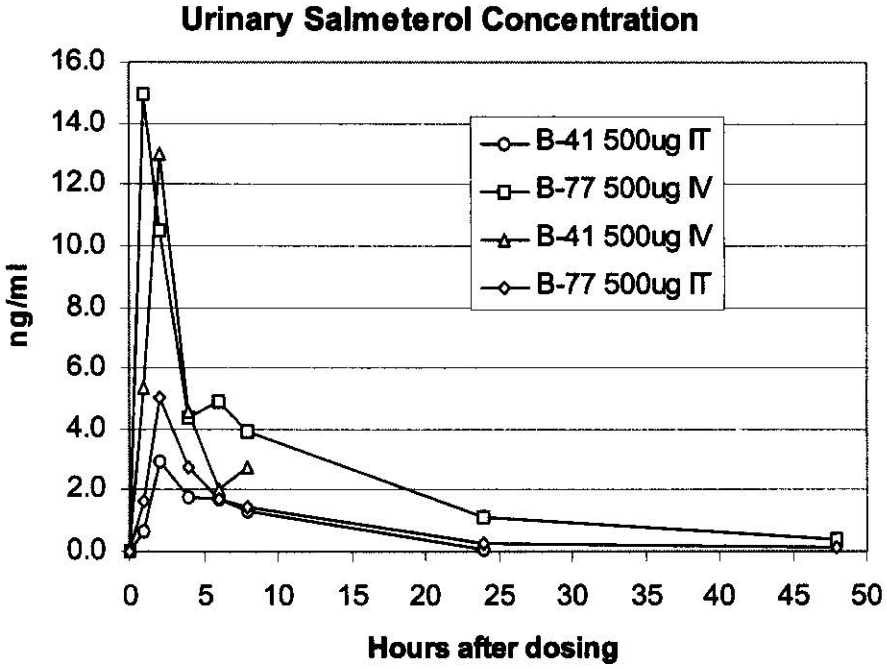
Urinary Salmeterol Concentration
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
ng/ml
0
5
10
15
20
25
30
35
40
45
50
Hours after dosing
B-41 500ug IT
B-77 500ug IV
B-41 500ug IV
B-77 500ug IT

Figure 8.

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. Ion Transitions Monitored – MRM of 6 Mass Pairs (ESP+)

Inter Channel Delay (Secs): 0.01
Repeats : 1

Channel	Parent, *m/z*	Daughter, *m/z*	Dwell, sec	Compound
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	