Stereochemical Composition of Clenbuterol Residues in Edible Tissues of Swine

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A gas chromatography−mass spectrometry method was developed to measure the stereochemical residues of clenbuterol derivatives in edible tissues of swine. Clenbuterol present in tissue extracts was derivatized with phosgene to form clenbuterol oxazolidin-3-one, which was then separated into component enantiomers using a dimethyl β-cyclodextrin capillary gas chromatographic column. Purified clenbuterol stereoisomers, isolated using published liquid chromatographic techniques, were used to determine stereoisomer elution order, stereoisomer racemization potential, and accuracy of the method. The stereochemical composition of clenbuterol could be measured at tissue concentrations of <2 ppb using the method. The dextrorotatory stereoisomer was the predominant clenbuterol stereoisomer present in edible tissues of hogs slaughtered after withdrawal periods of 0, 3, and 7 days, with a (+)/(−) isomer ratio of about 3:1. The prevalence of the dextrorotatory stereoisomer in edible tissues of hogs at all withdrawal periods suggests that stereoselective processes are occurring during the absorption, distribution, metabolism, and (or) excretion of clenbuterol. The effect of clenbuterol dose on its stereochemical composition in edible tissues is unknown but will be an area of further investigation.

Keywords: *Clenbuterol; residues; hogs; stereoisomer; chiral*

INTRODUCTION

Phenethanolamine β-adrenergic agonists were developed for the therapeutic treatment of asthma and preterm labor in humans and have been used in domestic livestock for the same purposes (Boenisch and Quirke, 1992). In the early 1980s it was noted that inclusion of some β-agonists in the diets of livestock improved growth performance, carcass characteristics, and, in some cases, efficiency of feed utilization (Anderson et al., 1990). The positive effects of β-agonists on these economically important production traits have stimulated the development of β-agonists for commercial application (Muirhead, 2000).

In general, phenethanolamines used experimentally for leanness enhancement of livestock are chiral compounds (Smith, 1998a) that exist as mixtures of stereoisomers. This is true even though β-adrenergic receptor activation has a strict requirement for levorotatory stereoisomers (Ruffalo, 1991). Most available β-agonists exist as racemates because the cost associated with the manufacture of stereochemically pure compounds is prohibitive. For phenethanolamine β-agonists, levorotatory stereoisomers are generally hundreds of times more potent than dextrorotatory stereoisomers (Ruffolo, 1991) at activating β-adrenergic receptors.

Few data have been published investigating the stereospecificity of β-agonist-induced leanness enhancement, but the available data suggest that levorotatory isomers are responsible for the bulk of the leanness-enhancing activity demonstrated with racemic mixtures. For example, Ricke et al. (1999) demonstrated that the *RR* stereoisomer of ractopamine HCl enhanced leanness in rats, whereas carcass compositions of rats dosed with the *SR* and *SS* isomers did not differ from controls. The *RS* isomer of ractopamine reduced carcass adipose tissue content but did not influence other variables. Consistent with these observations were those of Shappell et al. (2000), who noted that the *RR* stereoisomer of ractopamine caused a cAMP response of cultured muscle cells similar to that of racemic ractopamine but greater than that evoked by the *RS*, *SR*, or *SS* stereoisomers. Another β-agonist developed for use as a leanness-enhancing agent for the livestock industry, L644,969, was developed and tested as the *RR* stereoisomer (Zhang and Grieve, 1995).

Clenbuterol is a β-adrenergic bronchodilating agent (Nazzal, 1985) that has been used illicitly in cattle and hogs as a leanness-enhancing agent. Intoxications of humans have occurred worldwide after the consumption of meat products from clenbuterol-treated cattle (Martínez-Navarro, 1990; Pulce et al., 1991; Sporano et al., 1998) and swine (Anonymous, 1998; Chan, 1998). In some Asian countries illegal β-agonist use is so extensive that it has been estimated that annual β-agonist imports provide sufficient material for inclusion in the diets of 90% of the hogs in production (Melville, 2000). Clenbuterol exists as a racemic mixture (U.S. Pharmacopeia, 1998), and the bulk of its biological activity resides with the levorotatory stereoisomer (Martin et al., 1985; Waldeck and Widmark, 1985). Clenbuterol toxicity is manifested by tachycardia, nervousness, shortness of breath, and tremor (Kuiper et al., 1998), which are side effects common to most other phenethanolamine β-agonists (Spangler, 1989). These symptoms are caused by the direct stimulation of β-receptors (Boenish and Quirke, 1992), presumably with levorotatory stereoisomers (Ruffalo et al., 1991).

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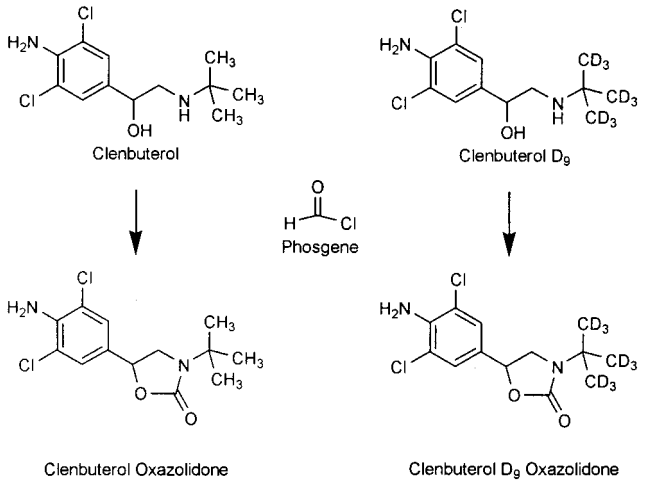

Figure 1. Formation of the clenbuterol and clenbuterol D_9 oxazolidone derivatives by reaction with phosgene as described by Wilson et al. (1994). Racemization of clenbuterol was not observed.

Although studies of clenbuterol residue depletion (Meyer and Rinke, 1991; Malucelli et al., 1994; Smith and Paulson, 1997; Smith, 1998b) and clenbluterol metabolism (Schmid et al., 1990; Zalko et al., 1997, 1998) have been conducted in a number of species, the stereochemical elimination of the drug from edible tissues of livestock has only been described for broilers slaughtered with no withdrawal period (Smith, 1998b). In broilers, (+)-clenbuterol represented almost 75% of the parent clenbuterol present in liver; (−)-clenbuterol represented <25% of the total clenbuterol residue. These results indicate that the inactive stereoisomer of clenbuterol was preferentially absorbed or retained in edible tissues.

The purpose of this study was (1) to develop a method of sufficient sensitivity and accuracy to measure clenbuterol stereoisomers in edible tissues of animals treated with clenbuterol and (2) to measure clenbuterol stereoisomers in edible tissue of swine provided 1 ppm of dietary clenbuterol HCl for 7 consecutive days and slaughtered after 0, 3, or 7 day withdrawal periods.

MATERIALS AND METHODS

Animals and Study Design. The experimental design, dose preparation, test animals, collection of tissue, radiochemical analysis, and quantitative analysis of tissue clenbuterol levels were described in another paper (Smith, 2000). Briefly, nine barrows and nine gilts were fed 1 ppm of dietary [^{14}C]-clenbuterol for 7 consecutive days. Three barrows and three gilts were each slaughtered after withdrawal periods of 0, 3, and 7 days. Tissues were collected at slaughter and subsequently analyzed for total radiocarbon content and for parent clenbuterol (Smith, 2000).

Isolation and Characterization of Clenbuterol Stereoisomers. [^{14}C]Clenbuterol (radiochemical purity = 98.8%; 9.14 μCi/mg) stereoisomers were purified by HPLC using the method of Abou-Basha and Aboul-Enein (1996). Individual stereoisomers were collected into and quantified from volumetric flasks using liquid scintillation counting (LSC). Each isomer was dissolved in methanol, and its polarity was determined by measuring the relative optical rotation with a polarimeter (Jasco, Tokyo, Japan). The first eluting peak was dextrorotatory [(+)-clenbuterol], and the second eluting peak was levorotatory [(−)-clenbuterol]. Solvent was evaporated from each stereoisomer, and the residue was derivatized with phosgene (Figure 1) according to the method of Wilson et al. (1994). The elution order of clenbuterol oxazolidin-3-one derivatives was determined on capillary GC (described below) by injecting the purified clenbuterol isomer derivatives onto the GC column and noting their relative retention times.

Gas Chromatography—Mass Spectrometry (GC-MS). Samples dissolved in toluene were introduced into the mass spectrometer (VG Autospec, Micromass, Beverly, MA) using a Hewlett-Packard model HP5890 gas chromatograph equipped with a Hewlett-Packard 7673A autosampler. Samples (2 μL) were cool-on-column injected onto a 30 m × 0.25 mm Chirdaldex B-DM cyclodextrin capillary GC column (Advanced Separations Technologies, Inc., Whippany, NJ) protected with a 1-m retention gap constructed of deactivated fused silica (0.53 mm i.d.; J&W Scientific, Folsom, CA). Clenbuterol oxizolidin-3-one stereoisomers were eluted from the column by holding the column temperature constant at 200 °C for the duration of each chromatographic run. The injector temperature was 186 °C, and the helium flow rate was set at 65 psi. Four ions were monitored each for clenbuterol (*m*/*z* 243, 245, 302, and 304) and the clenbuterol D_9 (Toronto Research Chemicals, Toronto, ON, Canada) internal standard phosgene derivatives (*m*/*z* 249, 251, 311, and 313). Protons of clebuterol's *tert*-butyl group were replaced with deuterium on the internal standard (Figure 1). Peak areas of the individual ions were integrated for the respective stereoisomers of clenbuterol and clenbuterol D_9 and were summed to obtain a total peak area. The dextrorotatory stereoisomer eluted prior to the levorotatory stereoisomer.

Effect of Clenbuterol Concentration on Stereoisomer Assay. A standard curve consisting of 0.5, 2.5, 7.5, 10, 25, and 50 ppb of racemic clenbuterol fortified with 10 ppb of clenbuterol D_9 was constructed. Samples were derivatized with phosgene and injected onto the GC. Peak area ratios for clenbuterol and clenbuterol D_9 stereoisomers were evaluated for consistency over the concentration range.

Assay Response to Variations in Stereochemical Composition. [^{14}C]Clenbuterol stereoisomers were purified by chiral HPLC and derivatized as described above. Each clenbuterol oxazolidinone stereoisomer was accurately diluted into volumetric flasks, and the mass per unit volume was determined using LSC. A series of these clenbuterol stereoisomer derivatives was created so that (+)-clenbuterol represented 0, 5, 10, 25, 40, 50, 60, 75, 90, 95, and 100% of the total clenbuterol, respectively; standards were mixed at a total clenbuterol concentration of 25 ppb. Solutions were analyzed by GC-MS, the respective peak areas were integrated, and the proportion of each isomer was determined. The percent composition of each peak area was calculated by dividing the peak area for each stereoisomer by the summed peak area of both stereoisomers. The stereochemical purity of each purified stereoisomer was not 100%, so reported values were adjusted for stereochemical purity.

Tissue Samples. Tissues were extracted, and clenbuterol was derivatized with phosgene using the method of Wilson et al. (1994) as described by Smith (2000). The samples were extracted and stored at −40 °C until analysis. Standard samples were stored for >6 months under these conditions with no appreciable change in stereochemical composition. Because of low clenbuterol concentrations, sample extracts from hogs slaughtered with 3- or 7-day withdrawal periods (100 μL total volume) were evaporated to dryness at room temperature. The dried residue was then reconstituted in 10−25 μL of toluene prior to GC analysis. Peak areas of clenbuterol and clenbuterol D_9 were monitored. Between-day variation was assessed by evaluating the stereochemical composition of clenbuterol D_9 stereoisomers fortified into tissue samples.

Quality Assurance. The stereochemical composition of the internal standard (clenbuterol D_9) was measured in each sample. On a theoretical basis, the stereochemical composition of clenbuterol D_9 should remain constant regardless of tissue, animal, or withdrawal period. Deviation of 5% from the expected stereoisomer ratio of 50:50 (acceptable isomer range was 47.5:52.5 to 52.5:47.5) was used as a criterion for reanalysis. In addition, chlorine isotope ratios for ions at *m*/*z* 245/243, 304/302, and 313/311 were monitored (Wilson et al., 1994) for clenbuterol and clenbuterol D_9 in each sample set

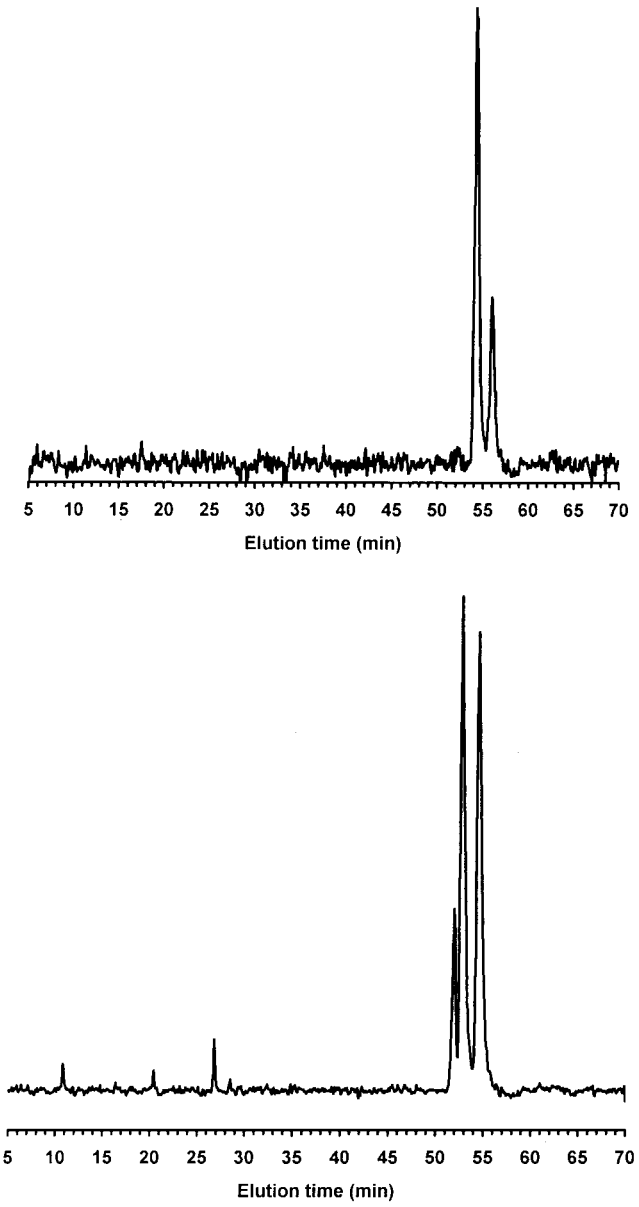

Figure 2. Example chromatograms: (A, top) total ion chromatogram (sum of selected ion signals for *m*/*z* 243, 245, 302, and 304) of native clenbuterol present in hog liver after extraction and derivatization with phosgene; (B, bottom) total ion chromatogram (sum of selected ion signals for *m*/*z* 249, 251, 311, and 313) of the internal standard (clenbuterol D_9) in the same liver sample as shown for the top panel. The small peak eluting at ~51.5 min was present only in the selected ion chromatograph for *m*/*z* 251 of the internal standard; when present in sample chromatograms, the signal for *m*/*z* 251 was not used for the calculation of stereochemical composition. The (+) isomer of clenbuterol eluted prior to the (−) stereoisomer for native clenbuterol and the deuterated internal standard.

(tissue within a withdrawal period). A deviation of 15% from the theoretical ^{37}Cl:^{35}Cl ratio of 0.65 was cause for reanalysis.

RESULTS

Assay Validation. Analysis of the clenbuterol oxazolidone derivative (Figure 1) by chiral GC resulted in essentially baseline resolution of each enantiomer at 200 °C (Figure 2), but complete resolution was achieved only with run times of >50 min. Resolution was decreased with increasing temperature and with increased sample mass. The mass spectra of clenbuterol oxazolidone stereoisomers were consistent with the mass spectrum of clenbuterol oxazolidone reported by Wilson et al. (1994); namely, major ions were formed at *m*/*z* 243, 245, 302, and 304. Ions at *m*/*z* 243 and 245 represent the simultaneous loss of CO_2 and a *tert*-butyl methyl group; ions at *m*/*z* 302 and 304 are molecular ions (Wilson et al., 1994). Mass spectra of clenbuterol D_9 oxazolidone stereoisomers were also consistent with replacement of clenbuterol's *tert*-butyl protons with deuterium (*m*/*z* 249, 251, 311, and 313). Ions at *m*/*z* 203 and 201 (loss of CO_2 and the *tert*-butyl group) were not monitored because of their low intensities. For some tissue samples, selected ion monitoring of *m*/*z* 251 showed an interference with the dextrorotatory stereoisomer of clenbuterol D_9. The interference, visible in Figure 2, originated from the tissue matrix and was never present in blank samples. For tissue sets in which the interference was present, peak areas derived from the *m*/*z* 251 ion chromatogram were not added to the total peak areas of either clenbuterol D_9 stereoisomer.

Derivatization of either clenbuterol stereoisomer resulted in only one clenbuterol oxazolidone peak in the GC chromatogram, indicating that racemization was not occurring during the derivatization procedure. Nor did stereoselective derivatization occur as evidenced by the consistent measurement of an equal ratio of clenbuterol stereoisomers into clenbuterol-fortified blank tissues and as evidenced by the equal ratio of clenbuterol D_9 stereoisomers fortified into all samples.

The (+)-[^{14}C]clenbuterol stereoisomer isolated by HPLC contained 21.4% of the (−)-clenbuterol stereoisomer, and (−)-clenbuterol contained 6.3% of the (+) stereoisomer. The stereochemical impurities were obtained during the stereochemical purification of the [^{14}C]-clenbuterol stereoisomers; the HPLC column was overloaded, resulting in incomplete resolution of each stereoisomer. Figure 3 shows the correlation of formulated versus actual stereochemical composition after adjustment for the stereochemical purity of the ^{14}C-labeled standards. Correlation coefficients (r^2) for the resulting curves were 0.9968 for the (+) and (−) isomers.

Table 1 shows the performance of the assay over a range of clenbuterol concentrations (0.5−50 ppb) fortified with a constant amount (10 ppb) of clenbuterol D_9 internal standard. Over the range of the clenbuterol standard curve, the stereochemical composition was 51.9 ± 1.2% (+)-clenbuterol and 48.1 ± 1.2% (−)-clenbuterol. There was a significant ($P < 0.01$) linear decrease of the (+)-clenbuterol stereoisomer with increasing clenbuterol concentration, but the correlation coefficient was only 0.62, indicating some association between clenbuterol concentration and the percentage of the (+) isomer, albeit weak. The most likely explanation for the observed phenomenon was that with greater concentrations of clenbuterol, resolution between the enantiomers decreased, resulting in slight differences in peak area integrations. Figure 4 shows this association graphically. The internal standard averaged 49.0 ± 0.3% (+)-clenbuterol and 51.1 ± 0.3% (−)-clenbuterol. Between-day variability of the assay, assessed by measuring the stereochemical composition of the internal standard fortified into tissue samples, is shown in Table 2. The average stereochemical composition of the internal standard was consistent with that measured for the linearity experiment [49.1% (+)-clenbuterol for tissues versus 49.0% for the standard curve experiment] with a standard deviation of 1.1%.

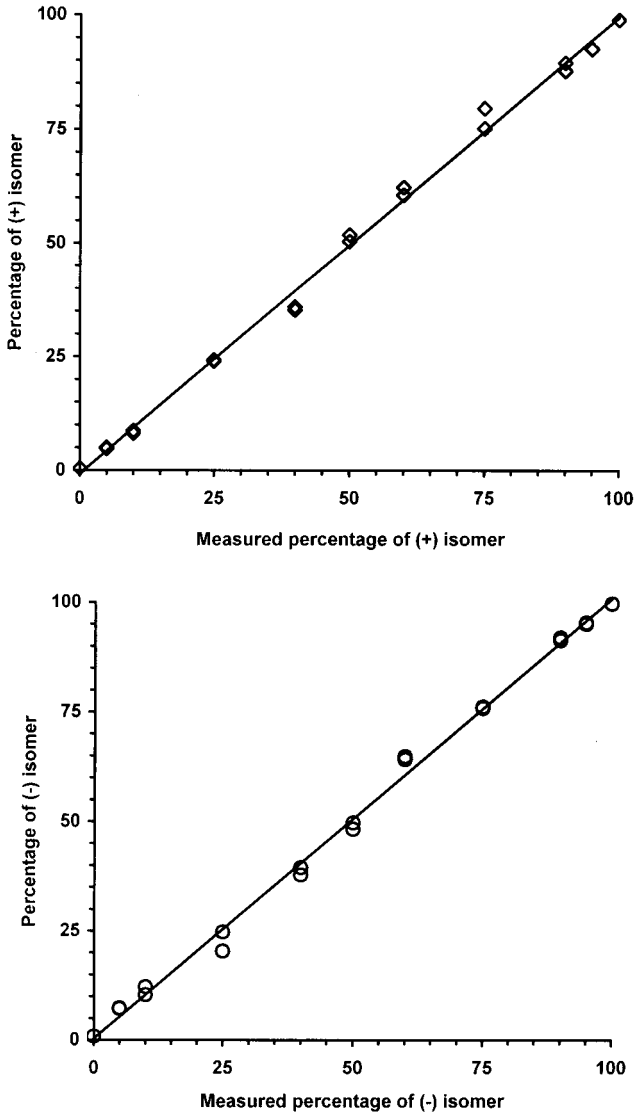

Figure 3. Correlations between formulated and measured stereoisomer compositions of dextrorotatory (top) and levorotatory (bottom) clenbuterol stereoisomers. [^{14}C]Clenbuterol stereoisomers were trapped from a chiral HPLC column, and solutions were formulated in duplicate to contain 25 ppb of clenbuterol containing 0, 5, 10, 25, 40, 50, 60, 75, 90, 95, and 100% of the (+)-clenbuterol stereoisomer. Clenbuterol was derivatized with phosgene, and the stereoisomeric composition was measured using GC as described under Materials and Methods. Data shown are corrected for stereochemical purity of each stereoisomer.

Tissue Analysis. Table 3 shows the stereochemical composition of clenbuterol in tissues of hogs fed 1 ppm of clenbuterol HCl for 7 consecutive days and slaughtered with withdrawal periods of 0, 3, or 7 days (Smith, 2000). The stereochemical composition of clenbuterol was not measured in adipose tissue because of an unknown component in the matrix that eliminated stereochemical resolution, decreased instrument sensitivity, and reduced column performance extensively. Clenbuterol residues in kidneys and skeletal muscle were not present in sufficient concentrations in animals slaughtered with a 7-day withdrawal period for the stereochemical analyses.

(+)-Clenbuterol was the predominant stereoisomer remaining in liver, lung, kidney, and skeletal muscle at each withdrawal period. The degree to which the (+) isomer exceeded the (−) stereoisomer varied with withdrawal period, however. With the exception of withdrawal day 0, the composition of clenbuterol stereoisomers for all tissues at days 3 and 7 was fairly constant.

Table 1. Chiral Composition of a Clenbuterol Standard Curve Fortified with 10 ppb of the Internal Standard, Clenbuterol D_9

sample (ppb[a])	% composition			
	clenbuterol		clenbuterol D_9	
	(+) isomer	(−) isomer	(+) isomer	(−) isomer
0.5-a	NI[b]	NI	48.8	51.2
0.5-b	NI	NI	48.9	51.1
2.5-a	53.3	46.7	48.7	51.3
2.5-b	52.9	47.1	48.9	51.1
7.5-a	52.9	47.1	48.8	51.2
7.5-b	52.8	47.2	48.8	51.2
10-a	51.3	48.7	48.7	51.3
10-b	52.1	47.9	49.4	50.6
25-a	52.1	47.9	48.5	51.5
25-b	51.1	48.9	48.9	51.1
50-a	51.5	48.5	49.1	50.9
50-b	48.7	51.3	49.7	50.3
mean	51.9	48.1	49.0	51.1
SD	1.2	1.2	0.3	0.3
% COV	2.3	2.5	0.6	0.6

[a] Sample concentrations are those for racemic clenbuterol. [b] NI, not integrated due to low signal-to-noise ratio.

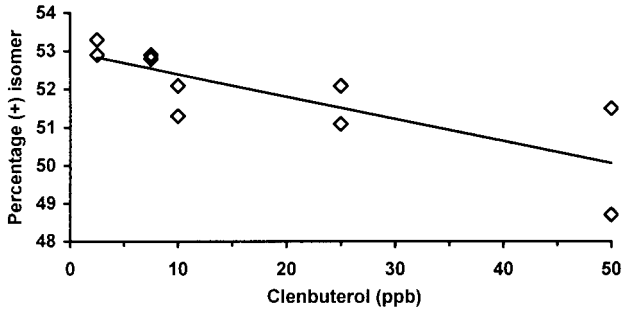

Figure 4. Relationship between the clenbuterol concentration and the integrated percentage of the (+)-clenbuterol stereoisomer. There was a statistically significant linear decrease in ($P < 0.01$) (+)-clenbuterol percentage with increasing clenbuterol concentration, but a relatively weak association ($r^2 = 0.62$) between the two variables, suggesting that the practical importance of the association was not significant.

Table 2. Between-Day Variation of the Stereochemical Composition of the Clenbuterol D_9 Internal Standard Fortified into Tissue Samples

tissue	withdrawal period (days)	% stereoisomer[a]	
		(+)	(−)
liver	0	48.9	51.1
kidney	0	48.7	51.2
lung	0	49.1	51.0
liver	3	51.4	48.6
kidney	3	48.2	51.8
lung	3	49.5	50.5
liver	7	49.2	50.8
kidney	7	not measured	not measured
lung	7	47.9	52.1
mean		49.1	50.9
SD		1.1	1.1
SE		0.1	0.1
% CV		2.2	2.2
range		47.9−51.4	48.6−52.1

[a] Means for each tissue and withdrawal period are based on 14 fortified samples consisting of duplicate samples of fortified control tissue and duplicate samples from 6 hogs.

Table 3. Stereochemical Composition (Means ± SD) of Clenbuterol Stereoisomers in Tissues of Swine Fed [^{14}C]Clenbuterol HCl for 7 Consecutive Days and Slaughtered with Withdrawal Periods of 0, 3, or 7 Days[a]

withdrawal, days	tissue	sex	parent clenbuterol[b], ppb	% stereoisomer (+)	% stereoisomer (−)	stereochemical residue (+), ppb	stereochemical residue (−), ppb
0	liver	barrow	122.4 ± 25.9	68.2 ± 2.3	31.8 ± 2.3	83.7 ± 19.3	38.8 ± 8.6
		gilt	115.1 ± 38.4	68.3 ± 1.9	31.8 + 1.9	78.9 ± 34.5	36.2 ± 14.0
	kidney	barrow	77.4 ± 14.5	56.6 ± 1.2	43.4 ± 1.2	43.9 ± 8.3	33.5 ± 5.0
		gilt	64.8 ± 3.0	58.1 ± 0.2	41.9 ± 0.2	37.7 ± 2.5	27.2 ± 1.7
	lung	barrow	186.8 ± 39.1	56.2 ± 1.5	43.8 ± 1.4	105.3 ± 31.9	81.5 ± 22.0
		gilt	186.1 ± 30.6	56.4 ± 0.3	43.7 ± 0.3	104.8 ± 19.2	81.3 ± 16.0
	skeletal muscle	barrow	20.5 ± 3.6	55.2 ± 2.0	44.8 ± 2.0	11.4 ± 2.2	9.2 ± 1.2
		gilt	19.4 ± 1.1	54.6 ± 0.5	45.4 ± 0.5	10.6 ± 0.7	8.8 ± 0.6
3	liver	barrow	12.9 ± 3.9	83.5 ± 3.0	16.5 ± 3.0	10.8 ± 3.3	2.1 ± 0.3
		gilt	14.4 ± 1.3	83.2 ± 2.7	16.9 ± 2.8	12.0 ± 1.4	2.4 ± 0.2
	kidney	barrow	3.3 ± 1.1	83.7 ± 1.3	16.3 ± 1.3	2.8 ± 0.8	0.5 ± 0.2
		gilt	3.2 + 0.9	81.7 ± 4.4	18.3 ± 4.3	2.7 ± 0.6	0.6 ± 0.1
	lung	barrow	8.9 ± 5.6	84.2 ± 1.5	15.8 ± 1.5	7.5 ± 4.3	1.4 ± 0.7
		gilt	8.9 ± 1.4	82.7 ± 6.3	17.4 ± 6.2	7.2 ± 1.5	1.5 ± 0.3
7	liver	barrow	2.5 ± 0.8	74.6 ± 2.1	25.5 ± 2.1	1.8 ± 0.2	0.6 ± 0.2
		gilt	2.1 ± 0.4	71.7 ± 2.7	28.3 ± 2.7	1.5 ± 0.3	0.6 ± 0.1
	kidney	barrow	0.4 ± 0.1	nt[c]	nt	nt	nt
		gilt	0.3 ± 0.02	nt	nt	nt	nt
	lung	barrow	1.5 ± 0.5	77.6 ± 2.3	22.5 ± 2.3	1.0 ± 0.1	0.3 ± 0.1
		gilt	1.0 ± 0.4	74.7 ± 0.5	25.3 ± 0.5	0.7 ± 0.2	0.3 ± 0.1

[a] Values are means from three animals within each sex and withdrawal period. [b] Data from Smith (2000). [c] Not tested.

After a 0-day withdrawal period, kidney, lung, and skeletal muscle (+)-clenbuterol represented ~56% of the total clenbuterol residue; however, in liver (+)-clenbuterol represented ~68% of the total clenbuterol residue. After a 3-day withdrawal period, the total amount of clenbuterol had dropped to <10% of that present in animals slaughtered with no withdrawal period, but (+)-clenbuterol residue had increased to >80% of the total residue for all tissues. After 7 days, the (+) isomer had decreased to ~75% of the total clenbuterol residue.

DISCUSSION

Phenethanolamine β-adrenergic agonists have been used as illegal feed additives in animal production in Europe (Kuiper et al., 1998; Elliott et al., 1996), Asia (Anonymous, 1998; Chan 1998), Mexico (Smith and Buchenau, 1999), and the United States (Mitchell and Dunnavan, 1998). In some instances, illegal β-agonist use has resulted in human intoxications after the consumption of tainted meat (Pulce et al., 1991; Martínez-Navarro, 1990; Sporano et al., 1998). To the author's knowledge, human poisonings have resulted only after the consumption of clenbuterol-contaminated meat products and not with other β-adrenergic agents.

For phenethanolamine β-agonists used in human medicine for the treatment of asthma or premature labor, therapeutic and acute side effects are associated with levorotatory stereoisomers (Ruffolo, 1991; Handley, 1999). Dextrorotatory stereoisomers are considered to be inactive at the β-adrenergic receptor (Page and Morley, 1999) but may contribute to unwanted side effects (paradoxical bronchospasm) associated with long-term therapeutic use (Handley, 1999; Yamaguchi and McCullough, 1996). Classical acute side effects of phenethanolamine β-agonist overdose in humans include tachycardia, muscle tremor, and nervousness (Spangler, 1989). These signs are attributable to the direct actions of phenethanolamines on tissue β-receptors and require a threshold dose (Reed, 1985).

In addition, parent clenbuterol, and not metabolites, are presumably responsible for toxic effects. For example, oxidation of mabuterol's (a clenbuterol analogue) *tert*-butyl group decreased its β_2-selective physiological effects from 2- to 10-fold (Horiba et al., 1984). Other major metabolites of clenbuterol include sequential oxidation of the alkyl side chain to ultimately form benzylic acid analogues (Schmid, 1990) and oxidation at the aromatic amine to form hydroxyarylamine-, nitroso-, and nitro-clenbuterol analogues (Zalko et al., 1997). Metabolites that destroy the phenethanolamine backbone of clenbuterol would not interact with the β-adrenergic receptor and would be expected to lose activity (Smith, 1998).

Humans poisoned by clenbuterol after the consumption of clenbuterol-contaminated meat products have exhibited tachycardia, palpitations, nervousness, tremors, gastrointestinal symptoms, vertigo, myalgia-arthralgia, malaise, and cephalagia (Brambilla et al., 1997, 2000; Pulce et al., 1991; Martínez-Navarro, 1990; Salleras et al., 1995). The major side effects observed during clenbuterol intoxication (tachycardia, tremors, and nervousness) correlate well with β-adrenergic receptor mediated tissue responses to β-agonists (Morgan et al., 1990; Boenisch and Quirke, 1992). It is probable that the symptoms exhibited by humans acutely poisoned through the consumption of clenbuterol-contaminated meat are caused by clenbuterol's levorotatory stereoisomer. Such a supposition is consistent with the fact that the levorotatory stereoisomer of clenbuterol was ~1000 times more effective than the dextrorotatory isomer in assays conducted on the rat trachea and soleus muscle (Waldeck and Widmark, 1985). This supposition is also consistent with data from another β-agonist, salbutamol, indicating that its dextrorotatory stereoisomer had no effect on either heart rate or tremor in humans (Lipworth et al., 1997; Boulton and Fawcett, 1997) and had no intrinsic activity in evoking mechanical responses of isolated guinea pig skeletal muscles (Prior et al., 1998).

Numerous tissue residue studies have been published that have described the disposition and tissue residues of clenbuterol and other β-adrenergic agonists in livestock species [reviewed by Smith (1998)]. Residue deple-

Table 4. Documented Ocurrences of Human Clenbuterol Intoxication after Consumption of Contaminated Livestock Tissues

date	location	no. of humans affected	tissue source	clenbuterol residue, ppb	reference
1990	Lyon, France	22	beef liver	375−500	Pulce et al. (1991)
1990	Asturias, León, and Palencia, Spain	135	beef liver	160−290	Martínez-Navarro (1990)
1992	Catalonia, Spain	113	veal liver	19−5395	Salleras et al. (1995)
1996[a]	Caserta, Italy	62	beef heifer muscle	4500	Brambilla et al. (1997)
1996[a]	Caserta, Italy	62	beef heifer muscle	800−7400	Sporano et al. (1998)
1997	Assisi, Italy	15	veal muscle	1140−1480	Brambilla et al. (2000)
1998	Hong Kong, China	8	pork lung	not reported	Anonymous (1998)

[a] Caserta, Italy, incident is documented twice.

tion studies utilizing radiolabeled β-adrenergic agonists to describe total residues and residues of a marker (specific drugs or metabolites that deplete in a known relationship to total residues) compound have been limited to β-agonists that have undergone fairly intense regulatory scrutiny such as ractopamine (Dalidowicz et al., 1992) and clenbuterol (Heitzman, 1997). Residue depletion studies utilizing radiolabeled test articles are valuable because the depletion of total residues (parent drug plus metabolites) can be relatively easily measured and because regulatory agencies use marker compounds to estimate total residues present in edible tissues (Guyer and Miller, 1994; Weber, 1992; Woodward, 1992). With the exception of Smith (1998), no previously published residue depletion study has investigated the stereochemical composition of β-agonist tissue residues in livestock species.

Data from this study and that of Smith (1998) indicate that the (+) stereoisomer of clenbuterol is retained in edible tissues of swine and poultry to a greater extent than the levorotatory stereoisomer. In broiler liver (animals slaughtered with no withdrawal period) dextrorotatory clenbuterol comprised ~73% of the total clenbuterol residue regardless of the feed level (0.5, 1.0, or 2.0 ppm of dietary clenbuterol). In the current study, the proportion of the dextrorotatory stereoisomer exceeded that of the levorotatory isomer by about the same magnitude. The stereochemical composition of tissue residues of other β-adrenergic agonists has not been measured in livestock species, but it has been reported that the dextrorotatory stereoisomer of salbutamol is retained in human lung to a greater extent than is the levorotatory isomer (Dhand et el., 1999). Salbutamol's levorotatory stereoisomer is cleared more rapidly than the dextrorotatory isomer by bronchial tissue (Eaton et al., 1996), liver, gastrointestinal tissue, and platelets (Walle et al., 1993) to a degree that the oral bioavailability of the levorotatory stereoisomer is <50% (Boulton and Fawcett, 1996).

Consumers poisoned after the consumption of clenbuterol-contaminated swine tissues (Chan, 1998) had to have eaten at least a therapeutic dose of clenbuterol. Because edible tissues of swine contain a greater proportion of the inactive (+)-clenbuterol isomer than the active (−) isomer, the total residue of clenbuterol had to be sufficient to deliver at least a therapeutic dose of the (−) isomer. The recommended oral therapeutic dose of clenbuterol is 10−20 μg (Boenisch and Quirke, 1992). Assuming a racemic stereochemical composition of the human drug, the therapeutic dose of the (−) isomer for humans would be 5−10 μg. Table 4 clearly shows that the levels of clenbuterol residue ingested by humans intoxicated via contaminated meat products has varied widely but are high, especially relative to levels of clenbuterol residues measured in experimentally treated animals [which are generally <200 ppm; reviewed by Smith (1998)]. For this study, the greatest clenbuterol residues were measured in lung from animals slaughtered with no withdrawal period (~190 ppb). As discussed by Smith (2000) the dose received by these animals was ~42 μg/kg of body weight, a dose considerably greater than the "growth promoting" dose of ~16 μg/kg per day cited by other investigators [Meyer and Rinke, 1991; Zalko et al., 1998; see Smith (2000)]. Nevertheless, animals receiving illegal clenbuterol have been administered much larger amounts of clenbuterol than animals in experimental situations. Because the clenbuterol dose could have a significant effect on the stereochemical residue remaining in tissues, it is possible that animals administered doses >50 μg/kg per day could have proportions of the (−)-clenbuterol isomer in excess of that measured in this study.

For clenbuterol, studies have not been conducted to determine why dextrorotatory clenbuterol is retained in edible tissues to a greater extent than the levorotatory isomer. Previously, stereosepecific clenbuterol assays were not of sufficient sensitivity to measure clenbuterol stereoisomers in tissues of livestock administered growth-promoting doses. For example, liquid chromatography based stereochemical separations of clenbuterol developed by Abou-Basha and Aboul-Enein (1996) and Aboul-Enen and Serignese (1999), with quantitation limits of ~0.1 nmol (27 ng) or greater, are of insufficient sensitivity to measure tissue residues. Stereochemical clenbuterol separations based on capillary electrophoresis (CE) may have the necessary sensitivity (Altria et al., 1993; Gausepohl and Blaschke, 1998), but CE is not as widely available as mass spectroscopy as an analytical tool.

For the current study, the extraction method of Wilson et al. (1994) was used for sample preparation and the quantitative determination of clenbuterol (Smith, 2000). Although the method of Wilson et al. (1994) has been fairly assessed as "long and tedious" (Abukhalaf et al., 2000), the method has been validated for multiple tissues (Wilson et al., 1994), can be used for quantitative analysis (Smith, 2000), has been used by regulatory agencies for surveillance purposes (Mitchell and Dunnavan, 1998), and, as demonstrated in this study, is useful for the preparation of samples for stereochemical analysis. The method was of sufficient sensitivity so that 1 ppb of clenbuterol residue could be measured (hog lung, Table 3; 7-day withdrawal; gilts). Because the (−) isomer represented ~25% of the total clenbuterol residue, the stereochemical assay successfully measured ~0.25 ng of residue/g of tissue.

The use of clenbuterol D_9 as an internal standard was valuable in the stereochemical assay because its stereoisomer ratio remained constant regardless of tissue or clenbuterol concentration. It was assessed in fortified

control samples, blank samples, and tissue samples from treated hogs. Column deterioration was readily detected by the evaluation of the stereochemical composition of the internal standard; when the stereochemical composition of the internal standard departed from a 1:1 ratio, an instrumental or column problem was indicated. For the present assay clenbuterol D_9 served both quantitative (Smith, 2000) and qualitative purposes for the stereochemical assay.

In conclusion, a sensitive and specific stereochemical assay was developed that allowed the stereochemical assessment of clenbuterol in edible tissues collected from swine. Results of the stereochemical assay showed that the levorotatory stereoisomer of clenbuterol is preferentially retained in edible tissues of swine collected after 0-, 3-, or 7-day withdrawal periods. The degree of the stereoselective retention of clenbuterol in edible tissues may be a function of the clenbuterol dose administered to the animal, but this hypothesis awaits verification in future work.

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