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INTRODUCTION

Clenbuterol is a β -2-agonist and an effective bronchial dilator used for the treatment of human asthma, but clenbuterol also has significant anabolic effects and is used regularly as a drug of abuse by athletes and for livestock due to its muscle growth stimulant and fat catabolism properties. However, it is clenbuterol's anabolic properties that make it a drug which is regularly monitored by veterinary and human doping control laboratories. A specific chirally discriminating analytical method for the determination of both enantiomers of clenbuterol in rat plasma has been developed. The dynamic range was 0.500 - 500ng/ml for both the (R) and (S) clenbuterol enantiomers using a urea type pirkle stationary phase.

Figure 1: Structural formulae of (S) and (R) - Clenbuterol



METHODOLOGY

Sample Preparation and Extraction - Plasma Samples

Control rat plasma samples were spiked with solutions containing racemic clenbuterol. The samples were prepared for extraction by accurately pipetting 100 μ l of plasma (Li hep) into an eppendorf tube and 100 μ l of 1N NaOH added to basify the plasma. The samples were then extracted by liquid-liquid extraction by pipetting 400 μ l of 7:3 v/v methyl tert butyl ether (MTBE): Hexane and gently shaking for ten minutes. The samples were left at room temperature to allow the layers to clarify, and then placed in a -80 $^{\circ}$ C freezer for 15 minutes to allow the aqueous layer to freeze. The organic layer was decanted to a culture tube and centrifuged to dryness under vacuum. The samples were prepared for analysis by re-constituting the dry extract in 100 μ l of mobile phase.

Sample Analysis

The LC-MS/MS system comprised of a CTC HTC PAL Auto sampler, an Agilent HP1100 series quaternary pump, degasser and column oven coupled to a Heated Nebuliser APCI interface of an Applied Biosystems MDS SCIEX API 3000 triple quadrupole mass spectrometer operating in the positive mode. The chiral chromatography was performed on a urea type pirkle phase Phenomenex Chirex 3022 (250 x 4.6mm 5 μ). The separation was performed isocratically using a mobile phase of 0.2% formic acid in 65/25/10 v/v/v Hexane/ 1, 2- Dichloroethane/Ethanol, at a flow rate of 1ml/min with a run time of 25 minutes. An injection volume of 30 μ l was used and the flow from the column was diverted to waste for the first 10 minutes.

(S) - Clenbuterol and (R) - Clenbuterol were determined by monitoring the following mass transitions:

(S) - Clenbuterol: 277.10 - 202.94
(R) - Clenbuterol: 277.10 - 202.94

RESULTS

Back-calculated results for the plasma calibration samples are shown in Table 1 and Table 3. The Calibration curves showed good linearity (Correlation coefficient >0.99) over the range 0.500 – 500ng/ml for both (S) and (R) enantiomers. The quality control accuracy and precision data presented in Table 2 and Table 4 show that for all levels (0.500, 1.50, 50.0, 400 and 500 ng/ml) that they are all satisfactory. Typical calibration curves are displayed in Figure 2 and Figure 3 respectively for (S) and (R) – clenbuterol. Typical chromatograms are shown in Figure 4 and Figure 5. Sharp, asymmetrical peaks with good sensitivity were obtained for both enantiomers. The chromatograms show that the clenbuterol enantiomers are chromatographically resolved as determined by the R_s value = 1.64 for the separation.

CONCLUSION

A sensitive and versatile Chirally discriminating bio-analytical LC-MS/MS method was developed to determine concentrations of (S) – Clenbuterol and (R) – Clenbuterol in rat plasma.

TABLES

Table 1: Back Calculated Concentrations from the Calibration Curve for (S) - Clenbuterol

Batch ID	(S) - Clenbuterol Nominal Concentrations (ng/ml)							
	0.500	1.00	5.00	20.0	50.0	100	200	500
TSS V9009 Batch 003	0.519	0.909	5.14	18.3	49.6	104	231*	425
	0.500	2.08*	5.29	19.6	50.2	107	221	414*
Mean	0.510	0.909	5.22	19.0	49.9	106	221	425
Bias	2.0	-9.1	4.4	-5.0	-0.2	6.0	10.5	15.0
SD	0.0134	NC	0.106	0.919	0.424	2.12	NC	NC
CV (%)	2.6	NC	2.0	4.9	0.9	2.0	NC	NC

* results not used in calculation

Table 2: (S) - Clenbuterol Quality Control Accuracy and Precision Statistics

Batch ID	(S) - Clenbuterol Nominal Concentrations (ng/ml)				
	0.500	1.50	50.0	400	500
TSS V9009 Batch 003	0.451	2.11*	51.1	398	377*
	0.443	1.40	53.5	370	460
	0.390*	1.44	51.4	391	446
	0.413	1.55	52.8	362	453
	0.440	1.26*	52.3	371	447
	0.437	1.58	50.6	348	452
Mean	0.437	1.49	52.0	373	452
Bias	-12.6	-0.7	4.0	-6.8	-9.6
SD	0.0143	0.0862	1.10	18.5	5.59
CV (%)	3.3	5.8	2.1	4.9	1.2

* outside acceptance criteria, results not used in calculation

Table 3: Back Calculated Concentrations from the Calibration Curve for (R) - Clenbuterol

Batch ID	(R) - Clenbuterol Nominal Concentrations (ng/ml)							
	0.500	1.00	5.00	20.0	50.0	100	200	500
TSS V9009 Batch 003	0.572	0.910	5.14	18.5	51.7	105	236*	435
	0.448	1.93*	5.13	19.9	51.5	109	231	426
Mean	0.510	0.910	5.14	19.2	51.6	107	231	431
Bias	2.0	-9.0	2.8	-4.0	3.2	7.0	15.5	-13.8
SD	0.0877	NC	0.00707	0.990	0.141	2.83	NC	6.364
CV (%)	17.2	NC	0.1	5.2	0.3	2.6	NC	1.5

* results not used in calculation

Table 4: (R) - Clenbuterol Quality Control Accuracy and Precision Statistics

Batch ID	(R) - Clenbuterol Nominal Concentrations (ng/ml)				
	0.500	1.50	50.0	400	500
TSS V9009 Batch 003	0.411	1.99*	50.1	399	383*
	0.488	1.46	54.0	377	471
	0.452	1.53	53.2	395	448
	0.432	1.65	54.0	377	462
	0.418	1.20*	53.4	382	466
	0.585	1.58	51.3	353	477
Mean	0.464	1.56	52.7	381	465
Bias	-7.2	4.0	5.4	-4.8	-7.0
SD	0.0653	0.0802	1.60	16.3	10.9
CV (%)	14.1	5.2	3.0	4.3	2.4

* outside acceptance criteria, results not used in calculation

FIGURES

Figure 2: Representative Calibration Curve for (S) - Clenbuterol

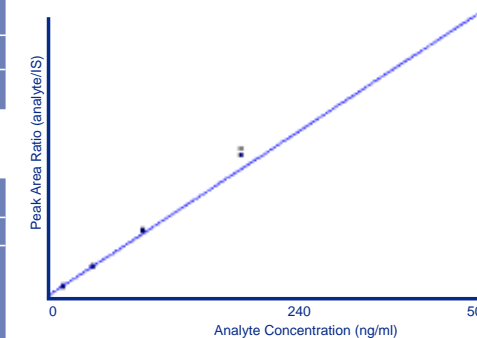


Figure 3: Representative Calibration Curve for (R) - Clenbuterol

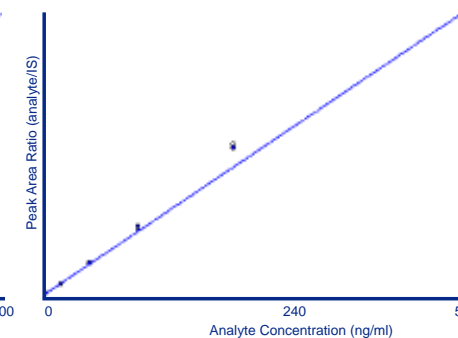


Figure 4: Representative Chromatogram from a Calibration Standard Sample containing 0.50ng/ml of (S) - Clenbuterol and (R) - Clenbuterol

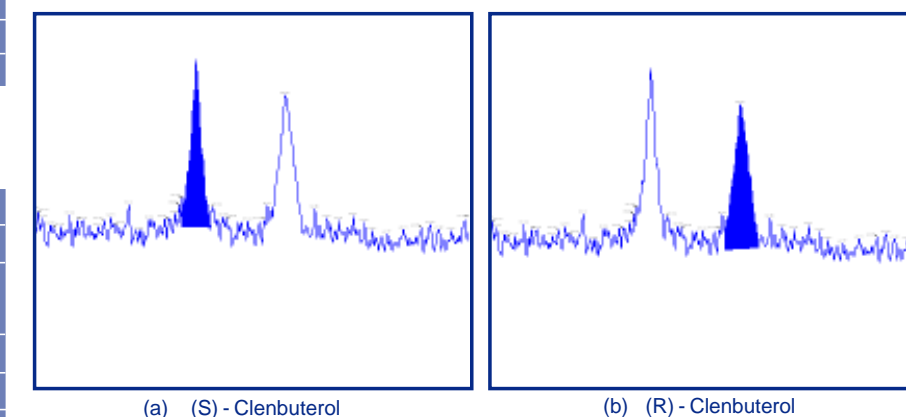


Figure 5: Representative Chromatogram from a Calibration Standard Sample containing 500ng/ml of (S) - Clenbuterol and (R) - Clenbuterol

