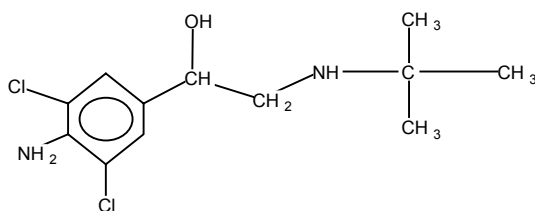


Developed for Testing Integrity Program by Equine Pharmacology Laboratory, Gluck Equine Research Center, University of Kentucky

Confirmation and Quantitative Determination of Clenbuterol in Equine Serum

I. INTRODUCTION

Clenbuterol is a bronchodilator approved by the American Association of Equine Practitioners for use in horses. Since it has the potential to alter athletic performance of racing horses, particularly if the horse has bronchospasm, clenbuterol is classified by the Association of Racing Commissioners International (ARCI) as a class 3 medication, and its detection in post-performance samples may lead to significant sanctions against trainers.



Clenbuterol, m.w. 276

II. SCOPE

This standard operating procedure describes a method for the identification and quantitation of clenbuterol in equine serum. Solid phase extraction (SPE) of clenbuterol from serum is followed by high performance liquid chromatography and tandem mass spectrometry (LC-MS/MS). The method has adequate sensitivity (~10 pg/ml limit of quantitation) for the detection of clenbuterol in serum after the administration of the drug in the low doses used in the intra-tracheal route or by nebulization. Analysis of serum will allow regulators to distinguish improper administrations shortly before race time from therapeutic administrations days before. This is not possible with urine analysis where long-term washout of the drug is known to occur for several days after a legitimate course of oral treatment.

III. METHOD SUMMARY

Clenbuterol is extracted from serum by automated solid phase extraction (SPE). The extract is evaporated to dryness, redissolved in mobile phase and injected into a liquid chromatograph interfaced with a tandem quadrupole mass spectrometer (LC-MS/MS) operating in the electrospray ionization positive (ESI⁺) mode. Multiple reaction mechanisms (MRMs or dynamically monitored ion transitions) for clenbuterol and clenbuterol-d₉ internal standard are measured to identify and quantitate clenbuterol in serum.

IV. REAGENTS

- A. Water, deionized distilled, 18 mΩ/cm
- B. LC Mobile Phases
Prepare from HPLC grade solvents and deionized distilled water and filter through Millipore HVHP04700 0.45 μm filter or equivalent. These solutions must be degassed if the LC system does not do this automatically.
 - 1. Mobile Phase 1
Acetonitrile + 0.05% (v/v) Formic Acid (88% w/v)
To prepare add 570 μl conc. formic acid (88% (w/v)) per liter acetonitrile
 - 2. Mobile Phase 2
H₂O + 5% (v/v) acetonitrile + 0.05% (v/v) Formic Acid (88% (w/v)),
To prepare dissolve 50 ml acetonitrile + 570 μl formic acid (88% w/v) in deionized distilled water and bring to one liter total volume.
- C. Solid Phase Extraction Solvents and Reagents
--organic solvents are HPLC grade; water is deionized distilled.
 - 1. Methanol
 - 2. 1M acetic acid
To prepare add 23 ml glacial acetic acid to 200 ml water, mix, then dilute to a total volume of 400 ml. Store at room temperature.
 - 3. 0.1 M Potassium phosphate buffer, pH 6
To prepare add 1.36 gm potassium phosphate monobasic per liter of water. Adjust to pH 6.0 with 1M KOH (5.6 gm/100 ml water), store at room temperature.
 - 4. Dichloromethane / isopropanol / NH₄OH (~30% (w/v)), 78:20:2 (v:v:v).
Prepare daily. Store at room temperature.
- D. Nitrogen Gas, pre-purified
- E. Standards
 - 1. Clenbuterol hydrochloride
 - 2. Clenbuterol-d₉, (available from Neogen Corp)

V. STANDARD SOLUTIONS

- A. Clenbuterol stock standard
Weigh clenbuterol hydrochloride on an analytical balance and dissolve in methanol to yield a clenbuterol concentration of 1.00 mg/ml (1.13 mg/ml of

clenbuterol-HCl). Prepare this solution monthly and store in a freezer. Allow to come to room temperature before opening container and measuring aliquots.

- B. Clenbuterol-d₉ stock internal standard
Weigh clenbuterol-d₉ hydrochloride on an analytical balance and dissolve in methanol to yield a clenbuterol-d₉ concentration of 1.00 mg/ml. (1.13 mg/ml of clenbuterol-HCl). Prepare this solution monthly and store in a freezer. Allow to come to room temperature before opening container and measuring aliquots.
- C. Clenbuterol working standard
Prepare dilutions of clenbuterol stock standard to yield concentrations of 100, 10, 1.0, and 0.0 ng/ml in mobile phase 2. Prepare fresh daily.
- D. Clenbuterol-d₉ working internal standard
Dilute clenbuterol-d₉ stock internal standard solution 1/1000 with mobile phase 2 (10 µl to 10 ml) to a concentration of 1 µg/ml. Prepare fresh daily.

VI. SUPPLIES

- A. Solid phase extraction cartridges, 3 ml, Type CSDAU (World-Wide Monitoring, Clean Screen®)
- B. 15 x 100 mm silanized glass culture tubes
- C. Screwcap autosampler vials with silanized 100 µl polyspring inserts (National Scientific Company)

VII. APPARATUS

- A. Adjustable volume pipettors, 1-10 µl, 10-100 µl, 200-1000 µl (Eppendorf Reference)
- B. Vortex mixer (American Scientific Products)
- C. pH meter with calibration standards (eg. stds of pH 4 and 7)
- D. Nitrogen evaporator (Organomation Multivap or equivalent)

VIII. CALIBRATOR AND SAMPLE PREPARATION

- A. Calibrators are prepared in duplicate in 16 x 100 mm tubes by the addition of a known amount of a clenbuterol working standard solution to 2 ml blank serum to produce a concentration range of 0.01ng/ml to 1 ng/ml as follows:

Clenbuterol spike Concentration, ng/ml	Clenbuterol spike volume, μ l	Clenbuterol calibrator concentration, ng/ml
0.00	10	0.00
1.00	20	0.01
10.0	10	0.05
10.0	20	0.10
100	10	0.50
100	20	1.00

- B. Pipet 2 ml unknown serum + 2 ml pH 6 phosphate buffer into a 16 x 100 mm tube (this is the tube size required for the Zymark extraction apparatus).
- C. For confirmation of presence (qualitative analysis) of clenbuterol in serum do not add internal standard. Proceed to Section IX Solid Phase Extraction.

Note: Clenbuterol-d₉ internal standard must be used only for the quantitation phase of clenbuterol analysis, and must not be added during the confirmation of clenbuterol in the unknown sample. The deuterated analog may contain trace quantities of the non-deuterated compound which could produce a false positive. Any such trace is inconsequential in the quantitation phase.

- D. For quantitative analysis, spike each unknown, blank, and calibrator with 50 μ l of 1 ug/ml clenbuterol-d₉ working internal standard. Mix with gentle vortex.

IX. SOLID PHASE EXTRACTION

- A. Solid phase extraction is run on a Zymark RapidTrace® automated system, but may also be performed using any manual vacuum or positive pressure manifold. (See Appendix I for instrumental set-up for Zymark RapidTrace® extraction)
- B. Condition SPE columns by adding sequentially 3 ml Methanol, 3 ml water, 1 ml 0.1M sodium phosphate buffer, pH 6.0.
- C. Load samples from the 16 x 100 mm tubes and wash the column sequentially with 2ml water, 2 ml 1M acetic acid, 4ml methanol.
- D. Dry column with N₂ for 5 minutes.
- E. Elute column with 3 ml dichloromethane/isopropanol/NH₄OH (78:20:2). Collect eluent in 16 x 100 silanized glass tubes.
- F. Evaporate eluent to dryness under a stream of N₂ in a 35-40°C water bath.

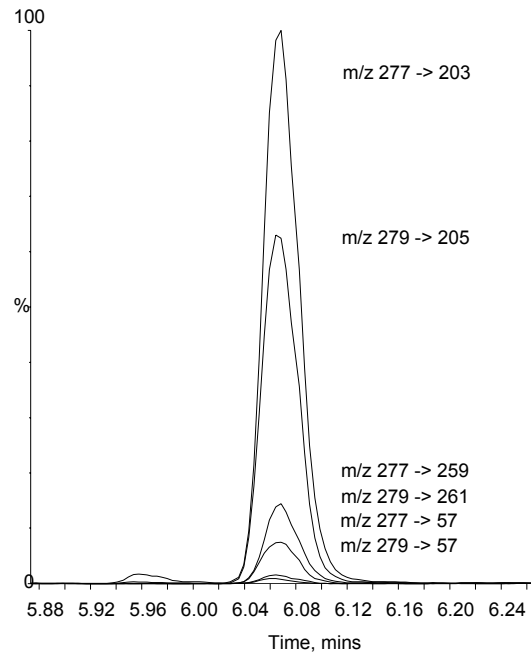
X. LIQUID CHROMATOGRAPHY

- A. Redissolve the extract residue in 50 μ l Mobile Phase 1 and transfer to a micro injection vial. Inject 25 μ l into the HPLC for analysis.
- B. HPLC Instrumentation:
Hewlett-Packard Model 1050 HPLC utilizing a Phenomenex LUNA phenyl-hexyl column (5 μ x 250mm x 2mm id) equipped with a 4mm x 2mm id phenylpropyl guard column and maintained at 30°C.
- C. Mobile Phase Program

Minutes	Mobile Phase Mixture
0 to 1	100% Mobile Phase 1
1 to 5 – linear gradient to:	10.5% Mobile Phase 1 89.5% Mobile Phase 2
5 to 10	10.5% Mobile Phase 1 89.5% Mobile Phase 2
10 to 10.5 – linear gradient to:	100% Mobile Phase 1
10.5 to 15	100% Mobile Phase 1
	Flow rate 0.5 ml/min Throughout

D. Clenbuterol product ion chromatogram

1.0 ng/ml clenbuterol serum standard extracted and chromatographed as described in SOP. Labels indicate the respective nested clenbuterol ion transitions being monitored and acquired across the peak eluted at 6.06 min retention time.



E. HPLC Injection Sequence

The sequence of injection is designed to eliminate or minimize carryover of analyte or standards which might contaminate and invalidate subsequent runs. The following list describes such a sequence. Injection of blanks allows the analyst to rule out the possibility of carryover and to evaluate possible responses from the matrix. Quantitation during the wash runs may also be used to monitor any carryover. A "wash" is an injection of mobile phase used to clear the system of residue from previous injections, and during which collection of analytical data is optional.

Typical Injection Sequence

1 wash	16 0.05 std
2 wash	17 wash
3 0.00 ng/ml std	18 0.00 std
4 blank	19 wash
5 50.0 ng/ml std	20 reagent blank
6 wash	21 biological blank
7 5.00 std	21 sample 1
8 wash	22 wash
9 0.50 std
10 wash	40 blank
11 0.25 std	41 positive control
13 wash	42 wash
14 0.10 std	43 end
15 wash	

XI. MASS SPECTROSCOPY

A. Mass Spectrometer -- Mass spectral data is acquired on a Quattro-II tandem quadrupole MS operated in the ESI⁺ mode.

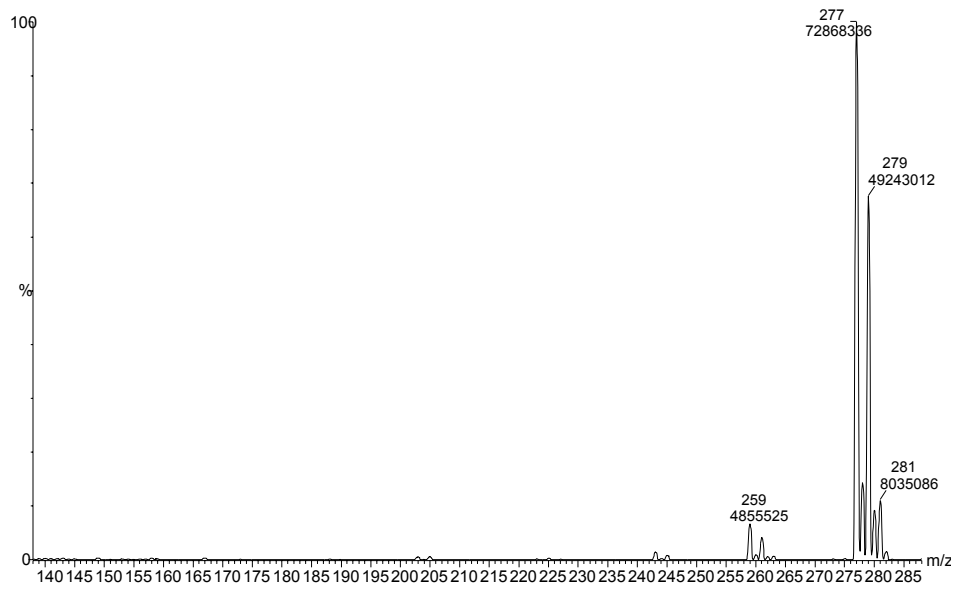
1. Tuning Parameters
2. Acquisition Parameters

Scans in function:		1397		
Cycle time (secs):		0.020		
Inter Channel delay (secs):		0.000		
Retention window (mins):		0.000 to 10.000		
Ionization mode:		ES+		
Data type:		SIR or MRM data		
Function type:		MRM of 14 channels		
Chan	Reaction	Dwell(secs)	Cone Volt.	Col.Energy
1	136.00 >65.00	0.01	19.0	20.0
2	136.00 >119.00	0.01	19.0	20.0
3	136.00 > 91.00	0.03	19.0	20.0
4	144.00 > 97.00	0.03	19.0	20.0
5	150.00 > 91.00	0.03	19.0	20.0

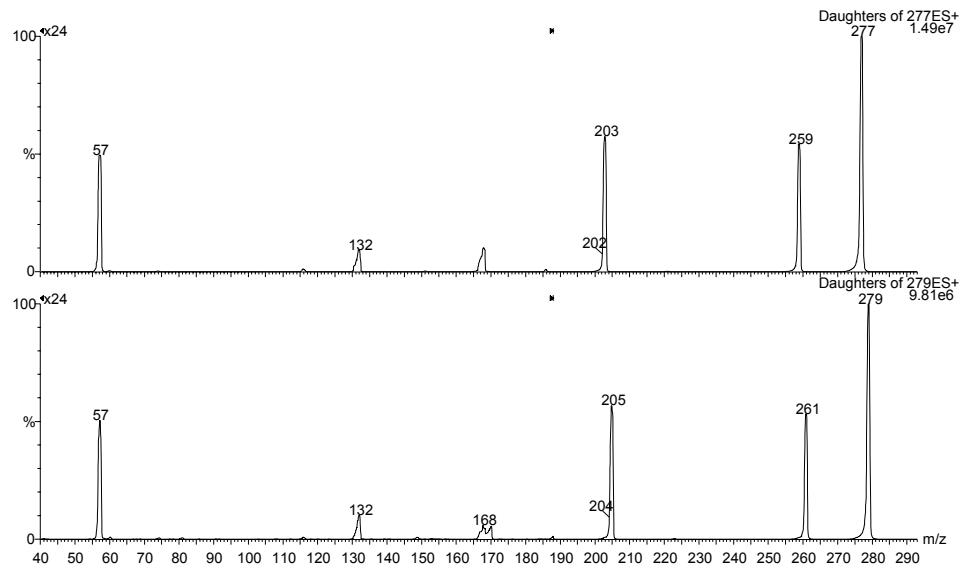
6	150.00 > 65.00	0.01	19.0	20.0
7	150.00 > 119.00	0.01	19.0	20.0
8	161.00 > 97.00	0.03	19.0	20.0
9	174.00 > 91.00	0.01	19.0	20.0
10	174.00 > 119.00	0.01	19.0	20.0
11	188.00 > 91.00	0.03	19.0	20.0
12	188.00 > 70.00	0.01	19.0	20.0
13	188.00 > 119.00	0.01	19.0	20.0
14	196.00 > 93.00	0.03	19.0	20.0

B. Confirmation of Presence and Identity of Clenbuterol

1. Full scan ESI+ mass spectrum of clenbuterol standard at 10ug/ml, direct infusion in acetonitrile:0.05% w/v formic acid, 1:1 at 1.2 ml/hr.



- ESI⁺ product ion spectra of clenbuterol [M+1] molecular ions a) m/z 277 and b) 279 m/z. Spectra were obtained by direct infusion of a standard. The m/z region from 40 to 185 is expanded for clarity of detail.



- Ion transitions monitored to confirm the identity of clenbuterol 277>259, 277>203, 279>261, 279>205, and 277>57, 279>57. The relative abundances of these ion transitions will vary from instrument to instrument and with tuning parameters, therefore they must agree with the relative abundances of standards run on the same instrument after tuning for the specific compound.

D. Criteria for reporting a positive result by LC/MS/MS analysis

- Biological and system blanks must demonstrate an absence of the compound in question.
- The unknown must have an HPLC elution time within $\pm 0.2\%$ (or 12 seconds, whichever is greater) of that of an authentic standard run under identical conditions.
- A minimum of 3 specified diagnostic ion transitions must be obtained. A diagnostic ion transition is a molecular ion or fragment transition characteristic of the analyte. MS/MS techniques may employ precursor ions which are greater than 5% of the abundance of the most abundant diagnostic ion. The molecular ion must be included as a precursor ion if it has a relative abundance greater than 5% of the base peak. Diagnostic ion transition chromatograms must show a peak heights greater than 3x baseline noise.
- Relative abundances (measured as an integrated chromatographic peak) of diagnostic ion transitions must be within $\pm 35\%$ of those of the standard.

Background subtraction, if employed, must be consistent across a batch of samples and must be appropriate to the individual sample.

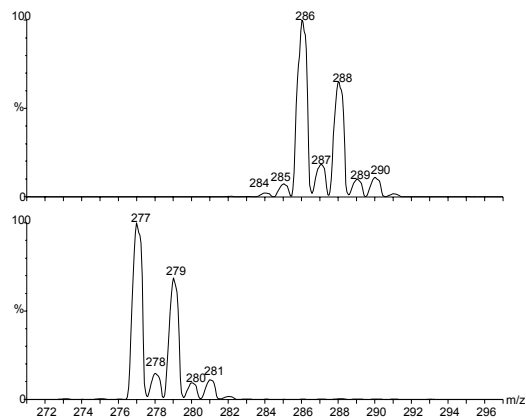
Due to potential interferences, such as those from the biological matrix for example, there may be occasions when experience of the mass spectroscopist should take precedence over one or more of the above criteria. In these circumstances details of the decision and rationale must be documented.

E. Quantitation of Clenbuterol

1. Clenbuterol-d₉ is used as the internal standard for quantitative determination of clenbuterol.
2. ESI⁺ spectra of clenbuterol and clenbuterol-d₉ consist predominantly of the molecular ions.

Molecular ion clusters of clenbuterol (277 m/z) and clenbuterol-d₉ (286 m/z) standards show the characteristic Cl₂ pattern.

High isotopic purity of the internal standard is desirable. Note the lack of nondeuterated clenbuterol (277 m/z) in this deuterated internal standard (top). The isotopic purity of our internal standard was calculated to be % clenbuterol-d₉.



Traces of the nondeuterated drug in the internal standard do not effect calculation of concentration in the unknown since they do not effect the slope of the standard curve.

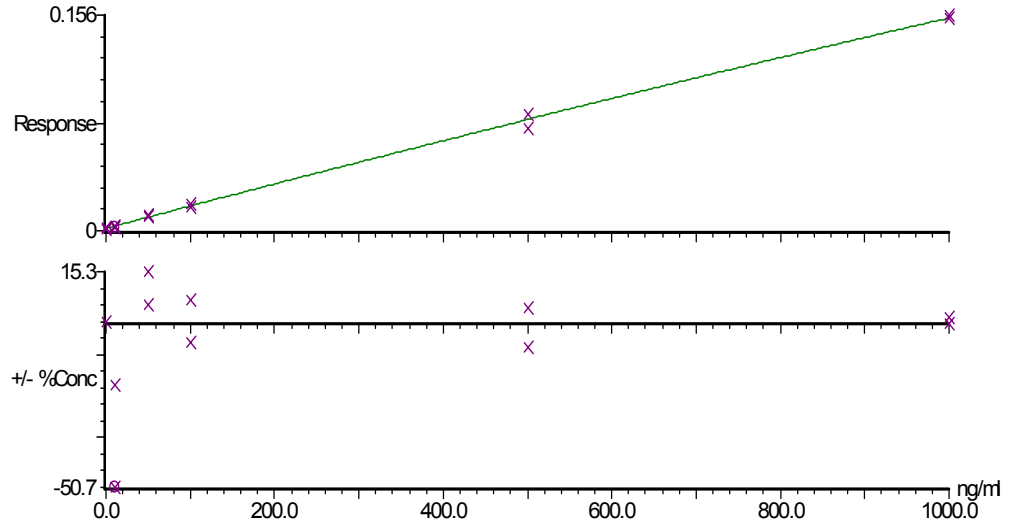
3. Quantitation of clenbuterol is based on the 277>203 m/z transition corrected by the clenbuterol-d₉ internal standard 286>204 m/z transition.

F. Calculation of Clenbuterol Concentration

1. Unknown concentrations are calculated by comparison to a plot of peak area ratios (clenbuterol / clenbuterol-d₉) vs. clenbuterol concentration.

2. Typical Clenbuterol Standard Curve produced by Quattro II MassLynx

Compound 1 name: Clenbuterol
Coefficient of Determination: 0.998414
Calibration curve: $-1.05755e-8 * x^2 + 0.000162887 * x + 0.00176147$
Response type: Internal Std (Ref 2), Area * (IS Conc. / IS Area)
Curve type: 2nd Order, Origin: Exclude, Weighting: 1/x, Axis trans: None



3. Over a narrow range of concentrations a linear response is observed. A second order regression curve reasonably accommodates the nonlinear response which is commonly observed when working over a several-fold difference in concentrations. The standard curve may be plotted and unknowns calculated using most available spreadsheet software packages. The standard curve presented above was constructed using the Quattro II MassLynx quantitation software which performs a second-order regression calculation with the inclusion of a “weighting” factor which generally gives enhanced accuracy at the lower concentrations.

G. Criteria for reporting quantitative clenbuterol results

1. Criteria for identification (Section XI.D.) must be satisfied.
2. Correlation coefficient of the standard curve must be greater than 0.98

APPENDIX I – Zymark Solid Phase Extraction Sequence

	Step	Source	Output	Vol	ml/min	Liquid Sense
1	Condition	MeOH	Org/Aq	3	20	No
2	Condition	dH2O	Aq	3	20	No
3	Condition	NaPO4	Aq	1	20	No
4	Load	Sample	Aq	4	1.5	No
5	Rinse	dH2O	Aq	2	20	No
6	Purge Cannula	dH2O	Cannula	5	20	No
7	Rinse	Acetic	Aq	2	20	No
8	Rinse	MeOH	Org/Aq	4	20	No
9	Purge Cannula	dH2O	Cannula	1	20	No
10	Dry	- - -	Time =	5	min	No
11	Purge Cannula	MeOH	Cannula	5	20	No
12	Collect	DCM+	Fract 1	3	1.5	No
13	Purge Cannula	dH2O	Cannula	3	20	No