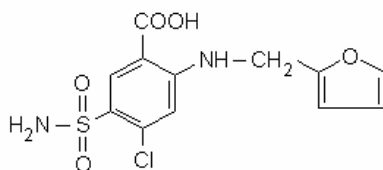


Developed for Testing Integrity Program by Equine Pharmacology Laboratory, Gluck Equine Research Center, University of Kentucky

Confirmation and Quantitation of Furosemide in Equine Serum

I. INTRODUCTION

Furosemide (lasix, salix) is a diuretic commonly used in horses to control exercise-induced pulmonary hemorrhage (EIPH) during racing. The control of its use is of interest to regulatory agencies due to its ability to dilute urine and potentially make the detection of proscribed drugs in urine more difficult.



Furosemide

II. SCOPE

The following method describes the LC/MS/MS procedure for detection, confirmation, and quantitation of furosemide in equine serum samples.

III. SUMMARY

Furosemide is extracted from serum using solid phase extraction (SPE) followed by identification and quantitation by high performance liquid chromatography and tandem mass spectrometry (LC/MS/MS) with negative mode electrospray ionization (ESI(-))

The procedure is a modification of existing SPE methodologies using Supelco DSC-18 Discovery columns (500 mg bed volume) with Liquid Chromatography using a 50 x 1.0mm x 3 micron phenyl-hexyl column (Phenomenex Luna) followed by Mass spectrometry/mass spectrometry detection. Briefly, the method consists of conditioning the SPE columns with methanol, water, and 1.5% (v/v) phosphoric acid in water, application of phosphoric acid-acidified serum samples, washing the columns with 1M acetic acid and water and finally, elution with ethyl acetate:dichloromethane :isopropanol:HCl (conc.), 65:29:5:1. Dried eluents were resuspended in a mobile phase combination consisting of 35%A:65%B, where A was acetonitrile, B was deionized water containing 1% triethylamine and 5% acetonitrile. Chromatography involved 0.15 ml/min isocratic elution with the 35%A:65%B mobile phase. Mass spectrometry used negative mode electrospray with measurement of negative ions. Those fragments arising from m/z 329.3 (M-H) and 330.3 were specific to furosemide, whereas those arising from 334.3 (M-H) and 336.3 were specific to the furosemide-d5 internal standard.

IV. REAGENTS

- A. Methanol
- B. Deionized water, 18 mΩ/cm²
- C. 1.5% Phosphoric acid in water
- D. 3% Phosphoric acid in water
- E. 1M Acetic acid
Prepare by adding 23 ml glacial acetic acid to 200 ml water, mix, then dilute to a total volume of 400 ml. Store at room temperature.
- F. Ammonium hydroxide (conc.) is used to adjust the pH of acetic acid solution
- G. Elution solvent
Dichloromethane / isopropanol / NH₄OH (~30% (w/v)), 78:20:2 (v:v:v).
Prepare daily. Store at room temperature.
- H. 35% acetonitrile + 65% water containing 1% triethylamine and 5% acetonitrile (Mobile phase)
- I. Furosemide standard was purchased from Sigma Chemical Co. (Lot 117H4647, purity >99% by HPLC, Sigma Chemical Co. P.O. Box 14508 St. Louis, MO 63178 USA)
- J. Furosemide-d5 (internal standard) was synthesized in house, as outlined in the appendix.

V. SUPPLIES

- A. Adjustable volume pipettors, 1-10 μl, 10-100 μl, 200-1000 μl (Eppendorf Reference) maintained and calibrated to < 1% error.
- B. Disposable Pasteur pipettes
- C. DCS-18 Discovery SPE columns (500 mg, Supelco)
- D. Disposable 12 × 100 culture tubes
- E. Silanized disposable 16 × 100 culture tubes
- F. Screw top disposable 16 × 125 culture tubes

VI. APPARATUS

- A. Vacuum Manifold (for manual SPE)
- B. Micromass Quattro II LC/MS/MS with ESI capability
- C. Turbo Nitrogen evaporator (Organomation Multivap, Zymark Turbovap, or other evaporator capable of introducing controlled N₂ flow)
- D. Vortex Mixer (American Scientific Products)

VII. STANDARDS

- A. Furosemide stock standard solution (1 mg/ml, 10^6 ng/ml) dissolved in methanol was prepared fresh daily in amber colored vials.
- B. Furosemide-d5 stock internal standard solution (1 mg/ml) dissolved in methanol was prepared fresh daily in amber colored vials.
- C. Furosemide working standard

Prepare dilutions of furosemide stock standard with methanol to yield concentrations of 10, 1.0, 0.1 and 0.0 ng/ul in methanol. Prepare fresh daily.

The stock concentration is 1 mg/ml = 10^6 ng/ml = 1000 ng/ul. In 13 x 100 mm tubes make 1/10 serial dilutions of 100 ul of standard into 900 ul methanol as follows:

Dilution	ng/ml	ng/ul
none (stock)	10^6	1000
1/10	10^5	100
1/10	10^4	10
1/10	10^3	1.0
1/10	10^2	0.1
no standard	0	0.0

These solutions will be used to prepare calibrators.

- D. Furosemide d-5 working internal standard

Dilute stock internal standard with methanol to yield a working solution of 10 ng/ul in methanol.

The stock concentration is 1 mg/ml = 10^6 ng/ml = 1000 ng/ul. In 13 x 100 mm tubes make 1/10 serial dilutions of 100 ul of standard into 900 ul methanol as follows:

Dilution	ng/ml	ng/ul
none (stock)	10^6	1000
1/10	10^5	100
1/10	10^4	10

Prepare fresh daily from stock.

VIII. CALIBRATORS PREPARATION

- A. Calibrators consist of 1 ml blank serum samples spiked to produce furosemide concentrations of 0, 5, 25, 50, 100, 250, 500 ng/ml, and each containing 100 ng/ml furosemide-d5 as an internal standard.
- B. Prepare duplicate calibrators in the range of 0–500 ng/ml by spiking furosemide working standards into 1 ml of blank serum in 12 x 100 culture tubes as specified in the following table:

Furosemide std spike concentration, ng/ml	Furosemide spike volume, ul	Furosemide calibrator concentration, ng/ml
0	50	0.00
10 ²	50	5.0
10 ³	25	25
10 ³	50	50
10 ³	100	100
10 ³	250	250
10 ⁴	50	500

- C. Pipet 1 ml of each unknown serum sample into a 12 x 100 culture tubes.
- D. Pipet into each calibrator, blank, and unknown tube 100 ul of 10³ ng/ml furosemide-d5 internal standard.

IX. PROCEDURE

- A. Add 1 ml serum to a 12 × 100 culture tube.
- B. Add 1 ml 3% phosphoric acid solution to serum-containing tube, vortex well.
- C. Condition SPE columns sequentially with 1 ml methanol, 1 ml distilled water and 1 ml 1.5% phosphoric acid.
- D. Do not allow column to dry before applying serum samples.
- E. Apply serum samples to the preconditioned columns.
- F. Rinse columns with 1 ml 1 M Acetic acid and dry columns under pressure (15 inches of mercury) for 6 minutes.
- G. Rinse columns with 1 ml distilled water and dry columns under pressure (15 inches of mercury) for 4 minutes.
- H. Elute column with 2 ml of 65:29:5:1 Ethyl acetate:dichloromethane: isopropanol:HCl (conc.) for several minutes.
- I. Evaporate samples to dryness under a stream of nitrogen in Turbo Vap LV evaporator at 38-40 °C.
- J. Resuspend dried eluents in 75 µl mobile phase.
- K. Inject 7 µl aliquot into the LC/MS/MS.

X. LIQUID CHROMATOGRAPHY—MASS SPECTROMETRY

The following table shows the organization of a typical sample table to run the samples on the Quattro LC/MS/MS system.

File name	File text	MS file	Inlet file	Vial #	Inject vol ul	Tuning file	Sample type	Samp conc
Furos-LC-301	mobile phase wash	FUROSEMIDE-MRM	Furosemide-1mm col-isocrat-2 min	60	7	Furosemide-Apr23-2002-ESI-	Analyte	0
Furos-LC-302	DSC-18 SPE isocrat LC fuso&d5 0ng/ml-0	FUROSEMIDE-MRM	Furosemide-1mm col-isocrat-2 min	1	7	Furosemide-Apr23-2002-ESI-	Standard	0
Furos-LC-303	DSC-18 SPE isocrat LC fuso&d5 0ng/ml-0'	FUROSEMIDE-MRM	Furosemide-1mm col-isocrat-2 min	2	7	Furosemide-Apr23-2002-ESI-	Standard	0
Furos-LC-304	mobile phase wash	FUROSEMIDE-MRM	Furosemide-1mm col-isocrat-2 min	60	7	Furosemide-Apr23-2002-ESI-	Analyte	0
Furos-LC-305	DSC-18 SPE isocrat LC fuso&d5 5ng/ml-A	FUROSEMIDE-MRM	Furosemide-1mm col-isocrat-2 min	3	7	Furosemide-Apr23-2002-ESI-	Standard	5
Furos-LC-306	DSC-18 SPE isocrat LC fuso&d5 5ng/ml-A'	FUROSEMIDE-MRM	Furosemide-1mm col-isocrat-2 min	4	7	Furosemide-Apr23-2002-ESI-	Standard	5
Furos-LC-307	mobile phase wash	FUROSEMIDE-MRM	Furosemide-1mm col-isocrat-2 min	60	7	Furosemide-Apr23-2002-ESI-	Analyte	0
Furos-LC-308	DSC-18 SPE isocrat LC fuso&d5 25ng/ml-B	FUROSEMIDE-MRM	Furosemide-1mm col-isocrat-2 min	5	7	Furosemide-Apr23-2002-ESI-	Standard	25
Furos-LC-309	DSC-18 SPE isocrat LC fuso&d5 25ng/ml-B'	FUROSEMIDE-MRM	Furosemide-1mm col-isocrat-2 min	6	7	Furosemide-Apr23-2002-ESI-	Standard	25
Furos-LC-310	mobile phase wash	FUROSEMIDE-MRM	Furosemide-1mm col-isocrat-2 min	60	7	Furosemide-Apr23-2002-ESI-	Analyte	0
Furos-LC-311	DSC-18 SPE isocrat LC fuso&d5 50ng/ml-C	FUROSEMIDE-MRM	Furosemide-1mm col-isocrat-2 min	7	7	Furosemide-Apr23-2002-ESI-	Standard	50
Furos-LC-312	DSC-18 SPE isocrat LC fuso&d5 50ng/ml-C'	FUROSEMIDE-MRM	Furosemide-1mm col-isocrat-2 min	8	7	Furosemide-Apr23-2002-ESI-	Standard	50
Furos-LC-313	mobile phase wash	FUROSEMIDE-MRM	Furosemide-1mm col-isocrat-2 min	60	7	Furosemide-Apr23-2002-ESI-	Analyte	0
Furos-LC-314	DSC-18 SPE isocrat LC fuso&d5 100ng/ml-D	FUROSEMIDE-MRM	Furosemide-1mm col-isocrat-2 min	9	7	Furosemide-Apr23-2002-ESI-	Standard	100
Furos-LC-315	DSC-18 SPE isocrat LC fuso&d5 100ng/ml-D'	FUROSEMIDE-MRM	Furosemide-1mm col-isocrat-2 min	10	7	Furosemide-Apr23-2002-ESI-	Standard	100
Furos-LC-316	mobile phase wash	FUROSEMIDE-MRM	Furosemide-1mm col-isocrat-2 min	60	7	Furosemide-Apr23-2002-ESI-	Analyte	0
Furos-LC-317	DSC-18 SPE	FUROSEMIDE-	Furosemide-1mm	11	7	Furosemide-	Standard	250

	isocrat LC fuso&d5 250ng/ml-E	MRM	col-isocrat-2 min			Apr23-2002-ESI-		
Furos-LC-318	DSC-18 SPE isocrat LC fuso&d5 250ng/ml-E'	FUROSEMIDE- MRM	Furosemide-1mm col-isocrat-2 min	12	7	Furosemide- Apr23-2002-ESI-	Standard	250
Furos-LC-319	mobile phase wash	FUROSEMIDE- MRM	Furosemide-1mm col-isocrat-2 min	60	7	Furosemide- Apr23-2002-ESI-	Analyte	0
Furos-LC-320	DSC-18 SPE isocrat LC fuso&d5 500ng/ml-F	FUROSEMIDE- MRM	Furosemide-1mm col-isocrat-2 min	13	7	Furosemide- Apr23-2002-ESI-	Standard	500
Furos-LC-321	DSC-18 SPE isocrat LC fuso&d5 500ng/ml-F'	FUROSEMIDE- MRM	Furosemide-1mm col-isocrat-2 min	14	7	Furosemide- Apr23-2002-ESI-	Standard	500
Furos-LC-322	mobile phase wash	FUROSEMIDE- MRM	Furosemide-1mm col-isocrat-2 min	60	7	Furosemide- Apr23-2002-ESI-	Analyte	0
Furos-LC-323	DSC-18 SPE isocrat LC precision 10ng/ml-1	FUROSEMIDE- MRM	Furosemide-1mm col-isocrat-2 min	15	7	Furosemide- Apr23-2002-ESI-	Analyte	0
Furos-LC-324	DSC-18 SPE isocrat LC precision 10ng/ml-2	FUROSEMIDE- MRM	Furosemide-1mm col-isocrat-2 min	16	7	Furosemide- Apr23-2002-ESI-	Analyte	0
Furos-LC-325	mobile phase wash	FUROSEMIDE- MRM	Furosemide-1mm col-isocrat-2 min	60	7	Furosemide- Apr23-2002-ESI-	Analyte	0
Furos-LC-326	DSC-18 SPE isocrat LC precision 100ng/ml-3	FUROSEMIDE- MRM	Furosemide-1mm col-isocrat-2 min	17	7	Furosemide- Apr23-2002-ESI-	Analyte	0
Furos-LC-327	DSC-18 SPE isocrat LC precision 100ng/ml-4	FUROSEMIDE- MRM	Furosemide-1mm col-isocrat-2 min	18	7	Furosemide- Apr23-2002-ESI-	Analyte	0
Furos-LC-328	mobile phase wash	FUROSEMIDE- MRM	Furosemide-1mm col-isocrat-2 min	60	7	Furosemide- Apr23-2002-ESI-	Analyte	0
Furos-LC-329	DSC-18 SPE isocrat LC precision 400ng/ml-5	FUROSEMIDE- MRM	Furosemide-1mm col-isocrat-2 min	19	7	Furosemide- Apr23-2002-ESI-	Analyte	0
Furos-LC-330	DSC-18 SPE isocrat LC precision 400ng/ml-6	FUROSEMIDE- MRM	Furosemide-1mm col-isocrat-2 min	20	7	Furosemide- Apr23-2002-ESI-	Analyte	0
Furos-LC-331	mobile phase wash	LONG SHUTDOWN	Shutdown	60	7	Furosemide- Apr23-2002-ESI-	Analyte	0
Furos-LC-332	mobile phase wash	LONG SHUTDOWN	Final shutdown	60	7	Furosemide- Apr23-2002-ESI-	Analyte	0

LC-MS TUNING AND SET-UP PARAMETERS

Quattro II LC/MS/MS Tuning Parameters: ESI-

The Quattro II has a coaxial electrospray probe supplied with nitrogen gas for HPLC effluent nebulization. The gas cell is supplied with argon gas for collision-induced dissociation (CID). The instrument is tuned by infusing 10 µg/ml furosemide in acetonitrile: 0.5% v/v NH₄OH and optimizing response and peak shape. Typical settings are as follows:

Source Page (ESI)

Capillary:	3.04	kVolts
HV Lens:	0.64	kVolts
Cone:	28	Volts
Skimmer Offset:	5	Volts
Skimmer:	1.6	Volts
RF Lens:	0.0	Volts
Source Temp:	120	°C

MS1

Ion Energy:	0.6	Volts
Ion Energy Ramp:	9.6	Volts
LM Resolution:	14.0	
HM Resolution:	14.0	
Lens 5:	100	Volts
Lens 6:	4	Volts
Multiplier 1:	712	Volts

MS2

Ion Energy:	4.0	Volts
Ion Energy Ramp:	1.9	Volts
LM Resolution:	14.5	
HM Resolution:	14.5	
Lens 7:	250	Volts
Lens 8:	10	Volts
Lens 9:	0	Volts
Multiplier 2:	702	Volts

Pressures

Analyser Vacuum:	3.3e-5	mBar
Gas Cell (Argon):	3.2e-3	mBar

Ions are acquired in a single function:

Function 1

Scans in function: 410

Cycle time (secs): 0.030

Inter Channel delay (secs): 0.00

Retention window (mins): 0.000 to 2.000

Ionization mode: ESI-

Data type: MRM (Multiple reaction monitoring) data

Function type: MRM of 9 channels

Chan	Reaction	Dwell(secs)	Cone Volt.*	Col.Energy*
1	: 329.30 > 285.00	0.02	28.0	22.0
2	: 329.30 > 204.70	0.05	28.0	22.0
3	: 329.30 > 77.60	0.02	28.0	22.0
4	: 330.30 > 205.80	0.02	28.0	22.0
5	: 330.30 > 204.90	0.02	28.0	22.0
6	: 334.30 > 290.00	0.02	28.0	22.0
7	: 334.30 > 205.80	0.05	28.0	22.0
8	: 336.30 > 291.80	0.02	28.0	22.0
9	: 336.30 > 207.80	0.02	28.0	22.0

*These values are set by Tune Page.

HPLC Parameters, Isocratic Elution:

HP1050 LC Initial Conditions

Solvents

A% 35.0

B% 65.0

C% 0.0

D% 0.0

Flow (ml/min) 0.150

Stop Time (mins) 2.0

Min Pressure (bar) 0

Max Pressure (bar) 400

Solvent: A=acetonitrile; B=filtered deionized distilled water containing 1% triethylamine, 5% acetonitrile.

Column: Phenomenex Luna 3u phenyl-hexyl column, dimensions 30 mm x 1.00 mm, p/n 407352.

Guard column: Phenomenex Security Guard containing a 4 mm x 2.0 mm ID phenylpropyl cartridge, p/n AJO-4350.

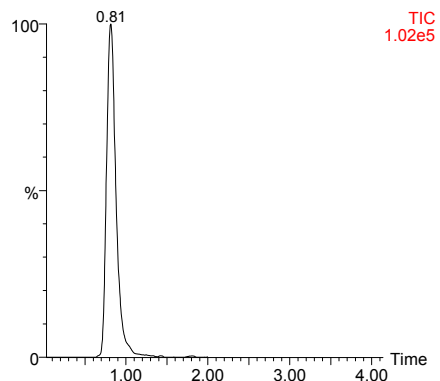


Figure 1. Example of furosemide chromatography. DSC-18 SPE extracted 400 ng/ml furosemide calibrator. TIC of furosemide and d5-furosemide is shown.

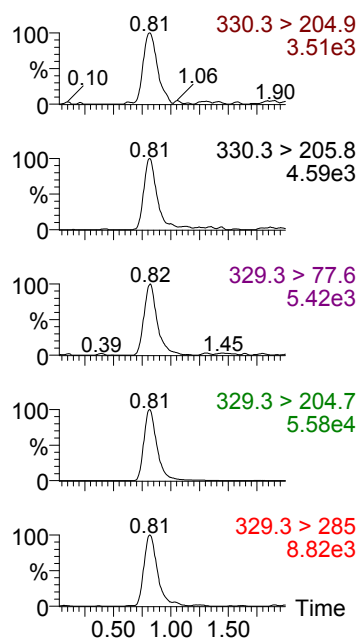


Figure 2. Ion chromatograms of transitions specific to furosemide (from Fig. 1 TIC): m/z 330.3->204.9, m/z 330.3->205.8, m/z 329.3->77.6, m/z 329.3->204.7, m/z 329.3-> 285 [top to bottom].

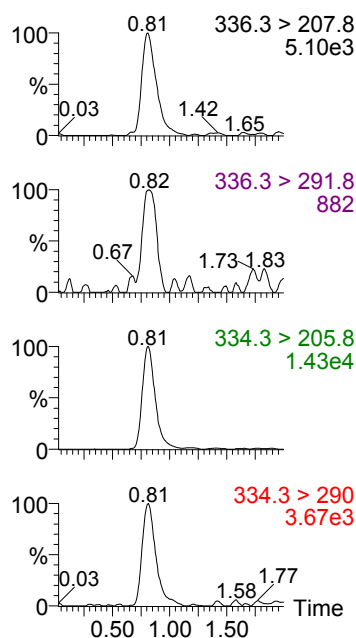


Figure 3. Ion chromatograms of transitions specific to furosemide-d5 (from Fig. 1 TIC): m/z 336.3->207.8, m/z 336.3->291.8, m/z 334.3->205.8, m/z 334.3->290.0 [top to bottom].

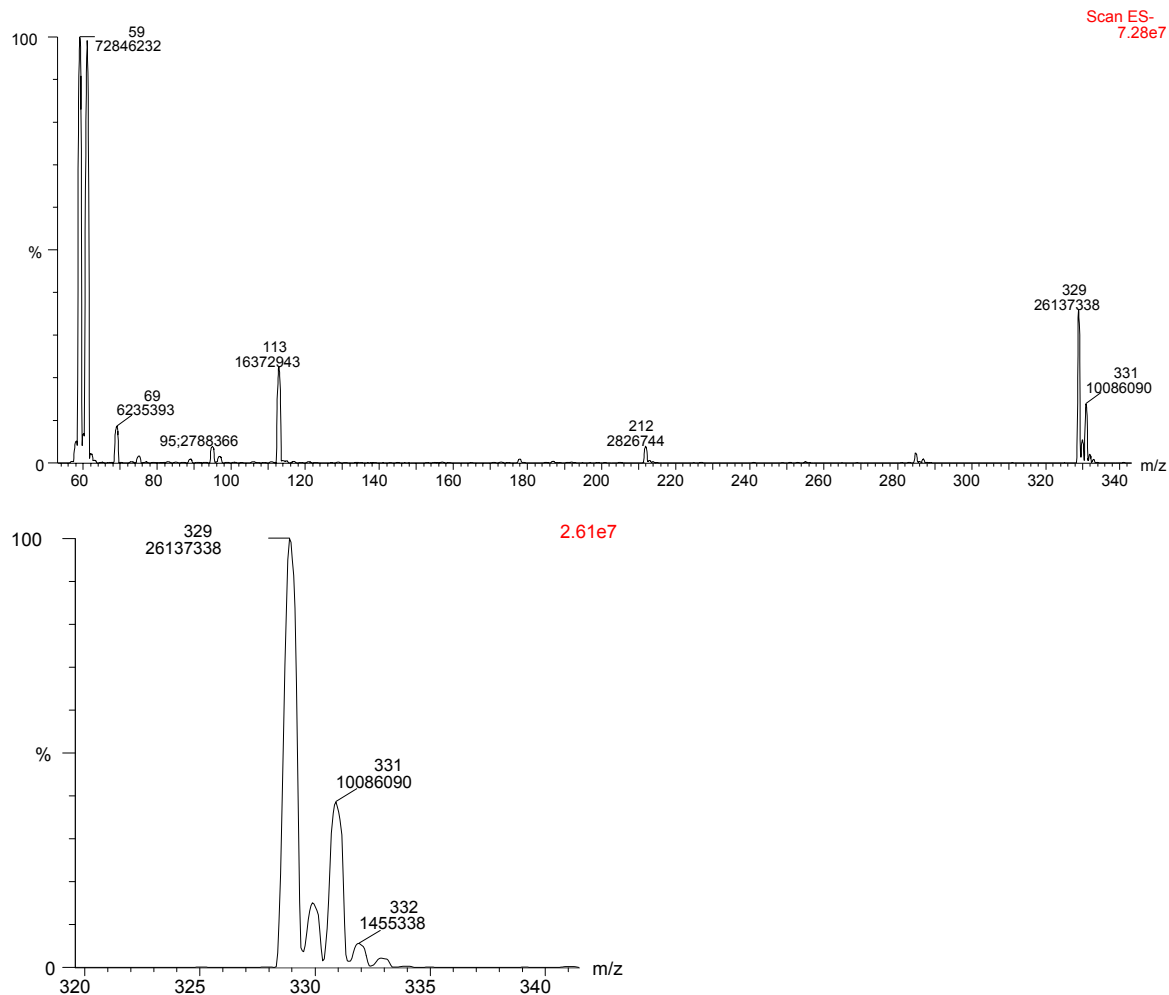


Figure 4. Furosemide mass spectrum, obtained by ESI(-) scan of 10 ug/ml furosemide in acetonitrile:0.5%NH₄OH, infused by means of Harvard syringe pump at 1.2 ml/hr. Left, full scan, m/z 50-350. Right, blow up of m/z 320-340 region, displaying [M - H] pseudomolecular ions. Note that the ion ratios, 329:330:331:332:333:334:335, measured at 1 : 0.151 : 0.386 : 0.056 : 0.022 : 0.0004, respectively, compare well with calculated values of 1 : 0.152 : 0.384 : 0.055 : 0.021 : 0.002, respectively.

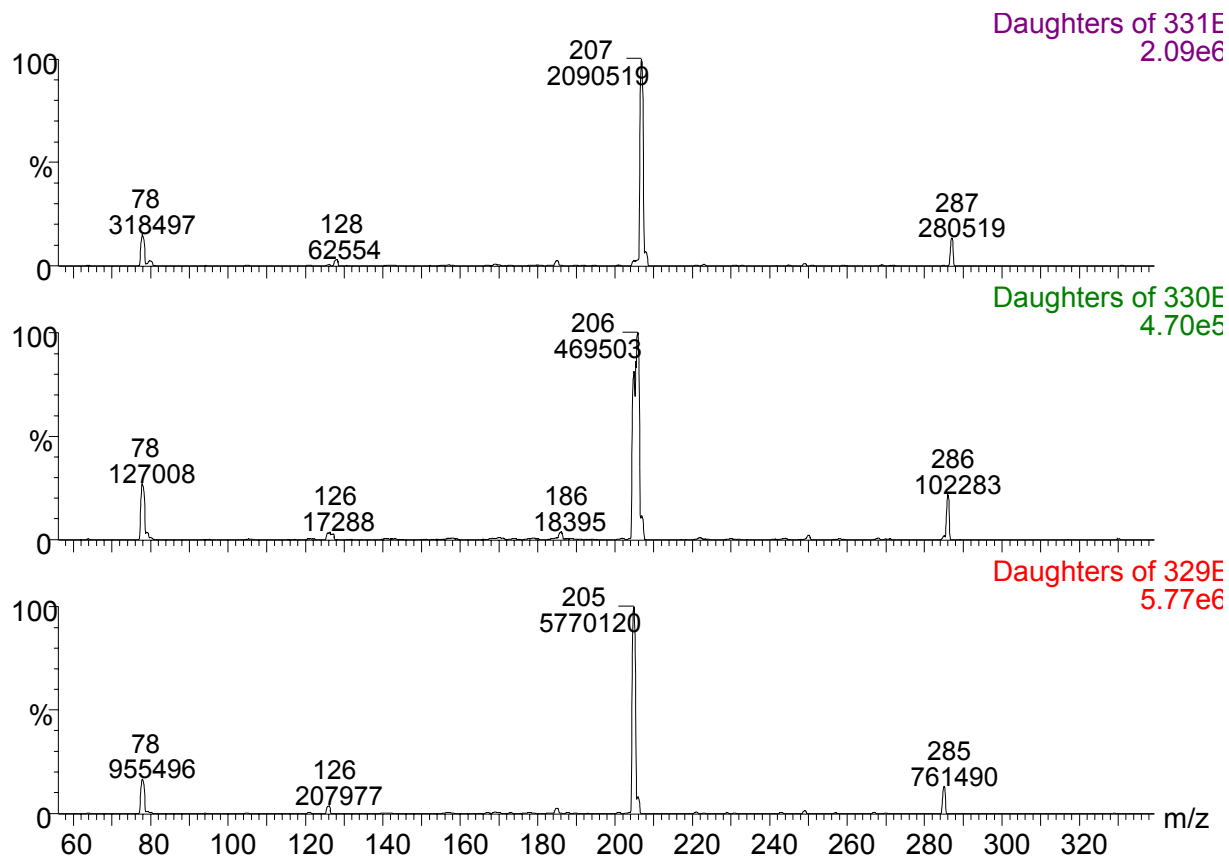


Figure 5. Furosemide daughter ion spectra, obtained as in Fig. 4. Daughters of m/z 331, 330 and 329 [top to bottom, respectively] are shown.

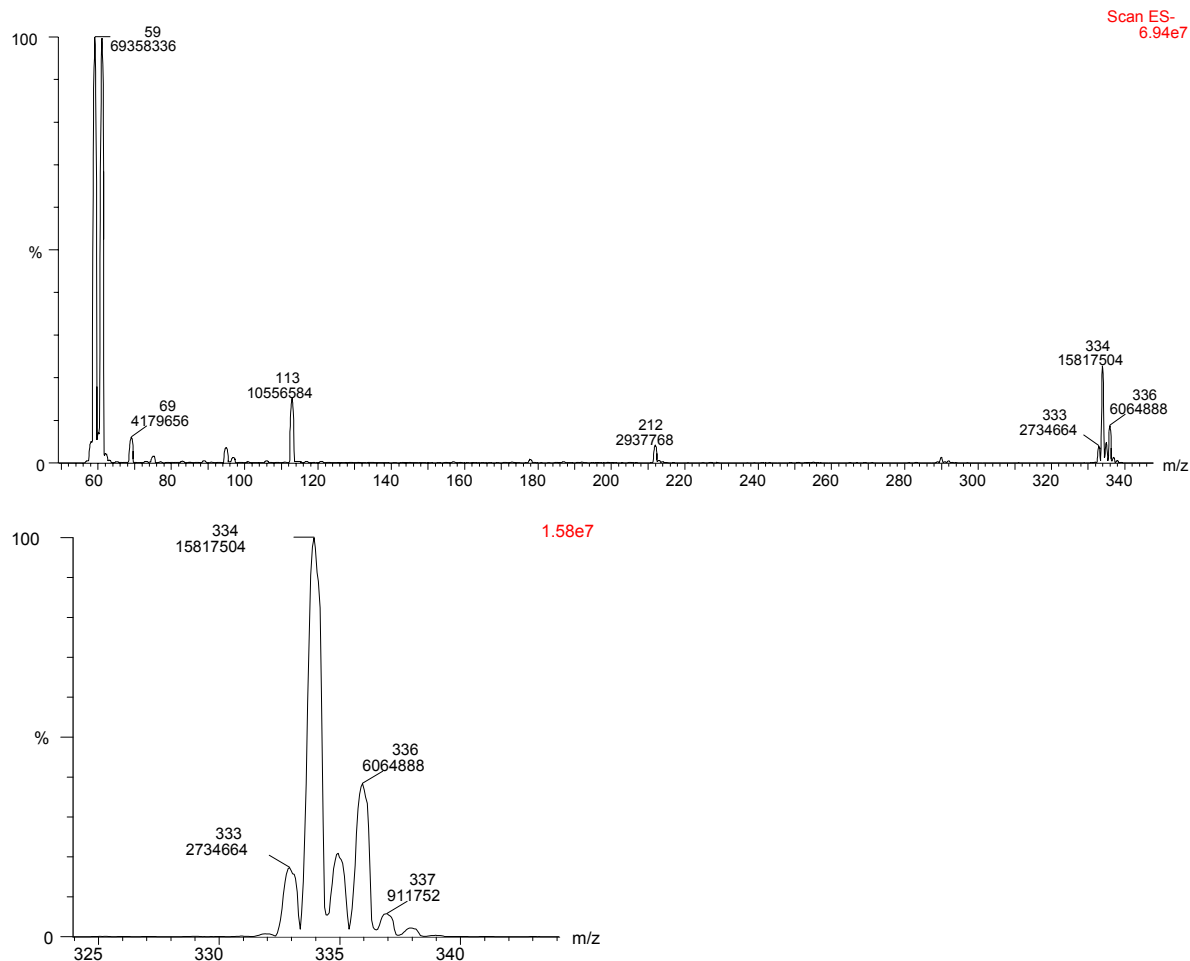


Figure 6. Furosemide-d5 mass spectrum, obtained by ESI(-) scan of 10 ug/ml furosemide-d5 in acetonitrile:0.5%NH₄OH, infused by means of Harvard syringe pump at 1.2 ml/hr. Left, full scan, m/z 50-350. Right, blow up of m/z 325-345 region, displaying [M-H] pseudomolecular ions. Note that the ion ratios, 334:335:336:337:338:339:340, measured at 1 : 0.207 : 0.380 : 0.057 : 0.022 : 0.0029, respectively, compare well with calculated values of 1 : 0.152 : 0.384 : 0.055 : 0.021 : 0.002, respectively, particularly in light of minor deuterated contaminants d4 at 17%, d3 at 0.68%, and d2 at 0.08% based on the relative intensities at m/z 333, 332 and 331, respectively. Nondeuterated furosemide-d0 was similarly measured at 0.05% which did not interfere with quantitation.

XI. QUANTITATION

The quantitation is based on the ratio of furosemide m/z 329.3->204.7 transition to furosemide-d5 m/z 334.3->205.8 transition. The largest peaks in the 0.3 minute time window centered on the correct furosemide retention time are smoothed twice over an 8 scan range by the Savitzky-Golay algorithm, with subsequent curve fitting done according to generation of a linear graph, excluding the point of origin, with no fit weighting or axis transformation.

Compound 1 name: Furosemide (329.0)
Correlation coefficient: $r = 0.999719$, $r^2 = 0.999438$
Calibration curve: $0.0206168 * x + -0.101944$
Response type: Internal Std (Ref 2), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: Null, Axis trans: None

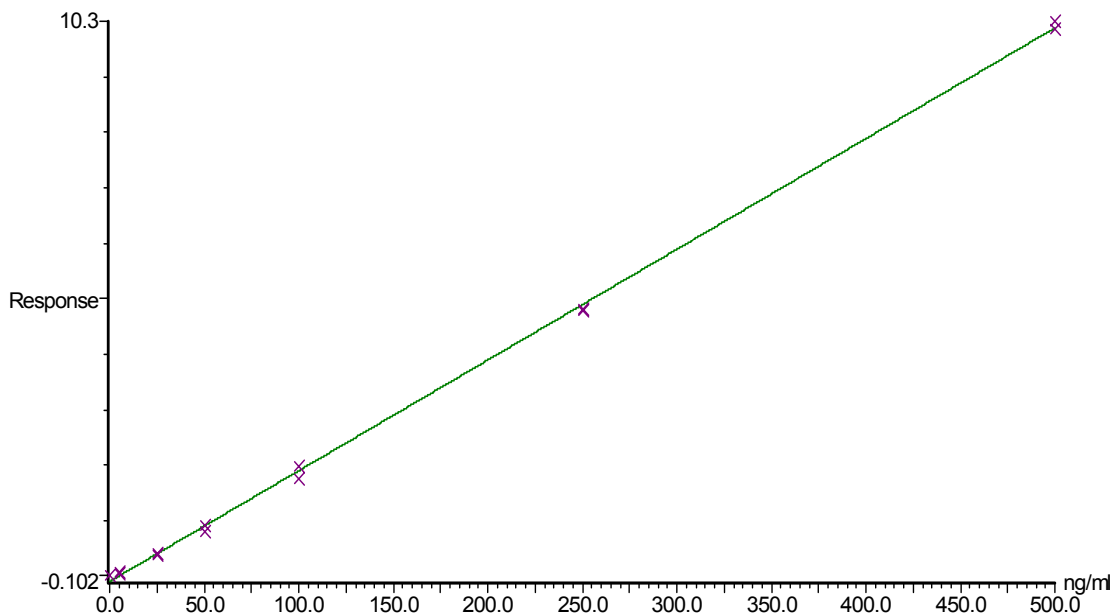


Figure 7. Typical calibration curve generated by MassLynx 3.4 software indicating coefficient of determination of 0.999438.

XII. VALIDATION OF THE ANALYTICAL METHOD

Linearity of the method was verified from the coefficient of determination r^2 of the standard curves from six consecutive runs. The standard curve for the assay was linear from 5 ng/ml to 500 ng/ml, with a mean coefficient of determination r^2 of 0.9986 ± 0.00068 (SEM) ($n=6$). (Figure 7) The standard concentrations' residuals were well within 20% of the nominal

values for the standard concentrations indicating adequate precision. The y-intercepts of all the regression lines were within the 95% confidence intervals for zero indicating lack of interference with the method.

Extraction efficiency was determined from six runs of spiked serum samples at three concentrations, 25, 100, and 250 ng/ml ranged between 96 and 101% (Table 1c).

The lower limit of detection (LOD) was calculated utilizing the analyte's peak height compared to the baseline noise in the 329.3>204.7 m/z fragmentation chromatogram. By this method, the LOD was defined as the lowest concentration of analyte producing a peak greater than or equal to three times the baseline noise was 2.2 ng/ml.

Additionally, the LOD was calculated statistically, as suggested by Miller and Miller (1984), from the standard curves of six consecutive runs. The concentration calculated from the mean of the responses at zero concentration (y-intercepts) was determined. The LOD, defined as the concentration calculated from the mean response at zero concentration plus 2 standard deviations (the upper 95% confidence limit for zero), was 1.8 ng/ml.

The lower limit of quantitation (LOQ) was defined as the concentration calculated from the mean of the zero responses plus five times the standard deviation (Miller and Miller, 1984) was 3.9 ng/ml. More rigorous determination of LOQ was not done since these low concentrations have little if any regulatory significance when compared to the regulatory threshold of 100 ng/ml.

Within-run accuracy and precision was determined by analyzing 6 replicate spiked samples at each of three concentrations, 10, 100, and 400 ng/ml (Table 1a). The within-run accuracy ranged between 93% and 99% for spiked samples at three concentrations. The precision was determined by the coefficient of variation (CV) for the assay which ranged from 1.4% to 15.3% (Table 1a).

Between-run accuracy and precision was determined by analyzing samples at three concentrations in six consecutive runs (Table 1b). The between-run accuracy and precision ranged between 100% and 104% with a CV from 3.5 to 12.8% (Table 1b).

1a. Within-run Accuracy and Precision

Concentration (ng/ml)	Measured concentration (ng/ml; mean \pm SEM) (n=6)	Accuracy (%) (mean \pm SEM)	C.V. (%)
10	9.31 \pm 0.6	93.1 \pm 5.8	15.3
100	98.63 \pm 1.5	98.6 \pm 1.5	3.6
400	397.83 \pm 2.7	99.1 \pm 0.6	1.4

1b. Between-run Accuracy and Precision

Concentration (ng/ml)	Measured concentration (ng/ml; mean \pm SEM) (n=6)	Accuracy (%) (mean \pm SEM)	C.V. (%)
10	10.3 \pm 0.5	100.3 \pm 5.32	12.8
100	103 \pm 3.0	103 \pm 3.0	7.2
400	415 \pm 5.8	104 \pm 1.5	3.5

1c. Extraction Efficiency

Concentration (ng/ml)	Extraction efficiency (%) (mean \pm SEM) (n=6)	C.V. (%)
25	96 \pm 4.1	10.0
100	100.8 \pm 1.8	4.5
250	100.4 \pm 1.5	3.8

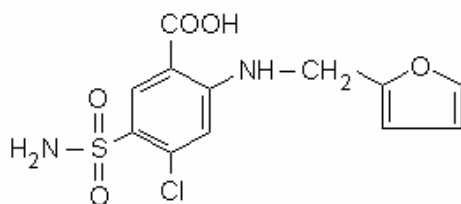
Table 1. Within-run and between-run assay accuracy and precision (a, b), extraction efficiency (c) of the LC-MS/MS assay used to quantify furosemide in horse serum samples.

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APPENDIX I -- SYNTHESIS OF DEUTERATED FUROSEMIDE

Deuterium-labelled furosemide was synthesized for use as an internal standard in the analysis for furosemide,

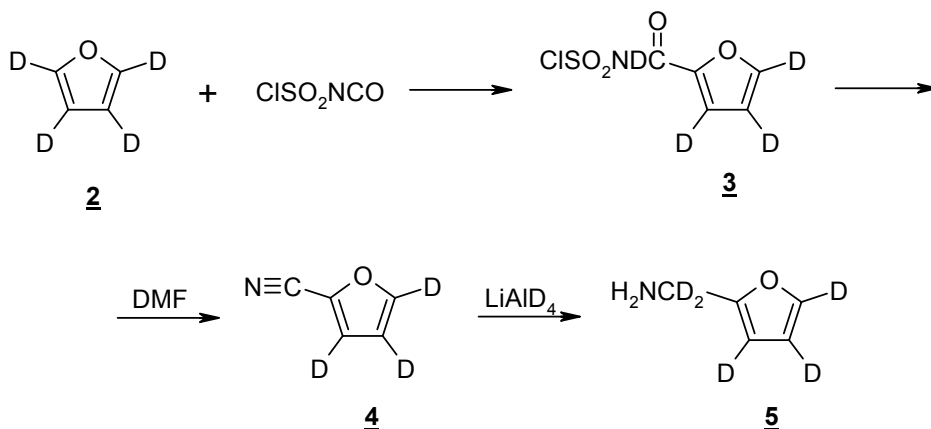


Furosemide **1**

Experimental Approach:

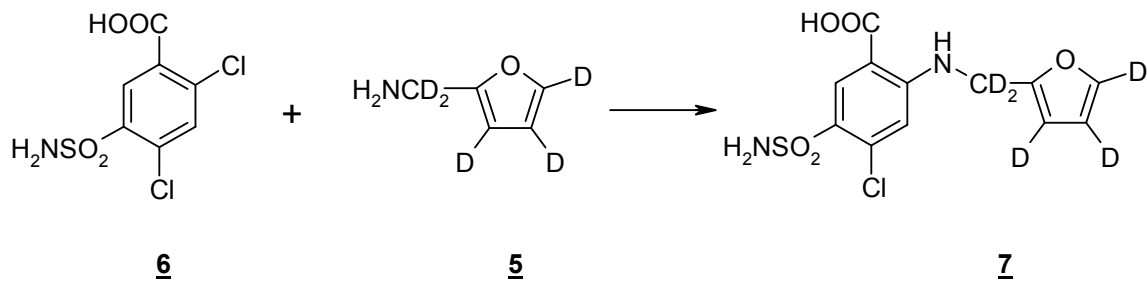
The initial synthesis of furosemide was published in the patent literature [1]. Furosemide was prepared from 4-chloro-2-fluorobenzoic acid by chlorosulfonation and ammonolysis to the corresponding sulfonamide followed by reaction with furfurylamine. Later the same authors showed a similar, but a little bit easier route using 2,4-dichlorobenzoic acid instead of 4-chloro-2-fluorobenzoic acid [2].

In order to synthesize deuterated furosemide, we decided to introduce five deuterium atoms into the furosemide molecule with the furfuryl fragment. Therefore the first and the most important part of the synthesis was the preparation of furfurylamine-d₅ (**5**).



This was achieved in the following way. First, direct cyanation [3] of furan-d₄ (**2**) by chlorosulphonyl isocyanate in ethanone at -78 °C led with moderate yield to the intermediate (2-furylcarbonyl-d₃)sulphamoyl chloride (**3**), which after addition of dimethylformamide (DMF) was converted into 2-furonitrile-d₃ (**4**). The next step was a reduction of the cyano group into an aminomethylene-d₂ group by lithium aluminum deuteride, which resulted in introduction of the next two deuterium atoms in the furfuryl system and gave the desired furfurylamine-d₅ (**5**). Deuterated furfurylamine was next reacted with 2,4-dichloro-5-sulfamoylbenzoic acid (**6**) by

using a modified procedure of Sturm [2]. As a result, furosemide-d₅ (**7**) was obtained in high chemical and isotopic purity. This product has been characterized using GC/MS and ¹H-NMR.



In summary, we have synthesized deuterated furosemide by a simple and convenient route, enabling its use as an internal standard in analytical studies on equine serum containing furosemide and thereby providing a very useful and helpful agent in the quantitation of this diuretic.

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