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Determination of the anticonvulsant felbamate in beagle dog plasma by high-performance liquid chromatography

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ABSTRACT

An automated internal standard method for the determination of felbamate in 0.1 ml of plasma from pediatric and adult beagle dogs was developed. Plasma proteins are precipitated with acetonitrile and after centrifugation the supernatant is directly injected on a Spherisorb ODS2, 3 μm, 150 mm × 4.6 mm I.D. column with 25% acetonitrile in aqueous phosphate buffer, pH 6.50, as mobile phase with ultraviolet detection at 210 nm. The run time is 10 min, the linear range is 0.150–150 μg/ml felbamate, and the lower limit of quantitation is 0.150 μg/ml.

INTRODUCTION

Felbamate, 2-phenyl-1,3-propanediol dicarbamate (Fig. 1), is a novel orally active and relatively non-toxic anticonvulsant compound with a unique profile of activity in laboratory animals [1,2]. This profile of good activity and minimal toxicity has also been observed in extensive clinical trials [3]. Felbamate is also expected to be used in children. In order to support clinical investigations in very young children pharmacokinetic studies in pediatric animal models were planned. The beagle dog was chosen as the primary model.

Although many pharmacokinetic studies of felbamate in the dog have been carried out, no method for analysis of dog plasma samples has been published yet. The assay for felbamate and other comedications in human plasma described by Remmel *et al.* [4] was not applicable to low-volume dog plasma samples.

This paper describes a high-performance liquid chromatographic (HPLC) method for the determination of felbamate in low-volume 0.1-ml dog plasma samples over the concentration range 0.150–150 μg/ml and its application to a pharmacokinetic study of felbamate in pediatric and adult beagle dogs [5]. It involves protein precipitation with acetonitrile and direct injection of the sample but no extraction. It is not intended and cannot be used for determination of felbamate in human plasma.

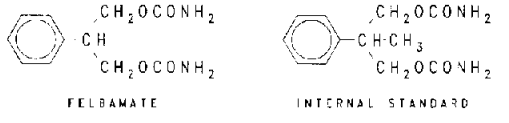

Fig. 1. Structures of felbamate and the internal standard.

EXPERIMENTAL

Chemicals

Felbamate, 2-phenyl-1,3-propanediol dicarbamate, and the internal standard (I.S.), 2-methyl-2-phenyl-1,3-propanediol dicarbamate (Fig. 1), were from Wallace Labs. (Cranbury, NJ, USA). Acetonitrile and methanol, glass distilled, were from Burdick & Jackson (Muskegon, MI, USA), potassium phosphate, trihydrate, dibasic (Cat. No. P-5504) from Sigma (St. Louis, MO, USA), and 85% orthophosphoric acid from Mallinckrodt (Paris, KY, USA); ultrapure water was ob-

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tained from a Milli-Q water system (Millipore, Bedford, MA, USA); heparinized dog plasma was from Pel-Freeze (Rogers, AR, USA).

Instrumentation

The HPLC equipment consisted of Model 600E pump with Model TCM column oven and controller and a Model 712 WISP autoinjector (Waters Assoc., Division of Millipore, Milford, MA, USA). The ultraviolet absorbance detector was a Model 783 from ABI-Kratos (Ramsey, NJ, USA). Data acquisition and processing were performed on a Model 3000 chromatography data system (P-E Nelson, Cupertino, CA, USA).

Preparation of standard solutions

Dog plasma standards were prepared by diluting a 6000 μg/ml felbamate stock solution to 600.0 μg/ml with control dog plasma. Serial dilutions of the 600.0 μg/ml standard with control dog plasma gave the series of thirteen standard concentrations of 150.000, 75.000, 37.500, 18.750, 9.375, 4.688, 2.345, 1.172, 0.585, 0.293, 0.146, and 0.073 μg/ml felbamate. Control dog plasma was used as the blank. A 40.000 μg/ml I.S. solution was prepared by dissolving the I.S. in acetonitrile.

Preparation of dog plasma samples for analysis

The assay used a 0.100-ml aliquot of spiked dog plasma, pipetted into a 2-ml polypropylene centrifuge tube, to which 0.200 ml of the I.S. solution in acetonitrile was added. The tubes were thoroughly vortex-mixed and then centrifuged at 2500 *g* for 15 min at 0°C. A 200-μl aliquot of the supernatant was transferred to an autosampler microvial. The microvials were capped prior to sample analysis.

Chromatography conditions

The analytical HPLC column was a Spherisorb ODS2, 3μm, 150 mm × 4.6 mm I.D. (YMC, Morris Plains, NJ, USA). The mobile phase was dibasic potassium phosphate (2.2 g) added to 740 ml of water with the pH adjusted to 6.50 with phosphoric acid. The aqueous volume was adjusted to 750 ml, and 250 ml of acetonitrile were added. The mobile phase was purged with helium. The flow-rate was 0.70 ml/min. The temperature was 40°C, the pressure 76–100 bar, and the injection volume 10 μl. Typical retention times were: felbamate 5.4 min; I.S. 7.3 min. Ultraviolet absorbance detector settings were: wavelength, 210 nm; range, 0.005 a.u.f.s.; rise time, 1.0 s; output, 10 mV; programmed for autozero, range and wavelength reset at each injection. Nelson method parameters were: sampling rate, 0.4 s; peak detection threshold, 0.10 μV/s; area threshold, 10.0; minimum peak width, 10.0 s; delay, 3 min; run time, 10.0 min.

Calculations

Thirteen concentrations of analyte (x), starting with zero, have been used to cover the expected range for method development and statistical evaluation. Each of the concentrations was analyzed daily in triplicate for three days giving for each x a total of nine data points of $y[i,j]$, where $i = 3$ and $j = 3$. Peak heights for felbamate and the I.S. were determined by the Nelson chromatography system and were used directly to calculate peak-height ratios. The data obtained were divided into four sets, 1–4, where sets 1–3 (each with $n = 39$) represent the daily runs and set 4 represents all data combined ($n = 117$). A thirteen-point standard calibration line was obtained from each of the four sets by unweighted linear regression. In addition, a four-point standard curve of stated standard concentration *versus* measured peak-height ratio was chosen to determine the calibration curve parameters that would be used for analyses of actual unknown samples in the pharmacokinetic study. The concentrations of 150.00, 9.374, 0.585, and 0.000 μg/ml standard points were selected (set 5). Paramaters for this set were obtained by concentration–weighted linear regression with weights equal to $1/x$. The linear regression on sets 1–5 was done using the program TableCurve [6], which yielded the ANOVA table as described by Draper and Smith [7]. The ANOVA yielded estimates of the coefficients A and B and their standard errors, r^2, the residuals, the F-ratio, and the variance about the regression line.

The within-day precision, accuracy and total error were calculated for all daily points, $n = i$, for each of the three sets 1–3 (different days). The precision, as represented by the coefficient of vari-

ation (C.V.), and the accuracy, represented by the relative mean error (R.M.E.), were calculated from eqns. 1 and 2. The total error (T.E.), as defined by McFarren *et al.* [8], was calculated using eqn. 3.

$$\text{C.V.} = \frac{100 \times \text{S.D.}}{\bar{x}} \quad (1)$$

$$\text{R.M.E.} = \frac{100 \times [\text{ABS}(\bar{x} - x)]}{x} \quad (2)$$

$$\text{T.E.} = \frac{100 \times [2 \times \text{S.D.} + \text{ABS}(\bar{x} - x)]}{x} \quad (3)$$

S.D. = standard deviation, $\bar{x}$ = mean value of determined concentrations, x = stated standard concentrations.

The between-day precision, accuracy and total error were calculated using all available points ($n = i \times j$) in set 4 from eqns. 1, 2 and 3.

The lower limit of quantitation (LOQ) was arbitrarily set at that lowest standard concentration that had a T.E. $<50\%$. The absolute recoveries for the plasma standards were calculated by direct comparison of the peak heights of plasma standards with peak heights of standards in acetonitrile–water (2:1) and correcting for volume loss during precipitation. Individual daily run recoveries were averaged for each concentration. Average recovery was calculated using only the concentration points yielding T.E. values less than 50% from the plasma data analysis.

RESULTS

Using the developed HPLC conditions for the determination of felbamate in dog plasma no interfering plasma constituent peaks were observed in the area of the felbamate or I.S. peaks (Fig. 2A). The mean (± S.D.) absolute extraction recoveries were 102.1 ± 2.2% for felbamate and 96.6 ± 0.9% for the I.S. Baseline resolution of all peaks was observed at all concentrations. Chromatograms of blank dog plasma containing 0.239 or 150 μg/ml felbamate and 26.667 μg/ml I.S. are shown in Fig. 2B and C.

Results of calibration runs for the validation range of 0–150 μg/ml felbamate, seen in Fig. 3A, showed excellent linearity ($r^2 > 0.9999$). Calibration plots and the corresponding 95% prediction

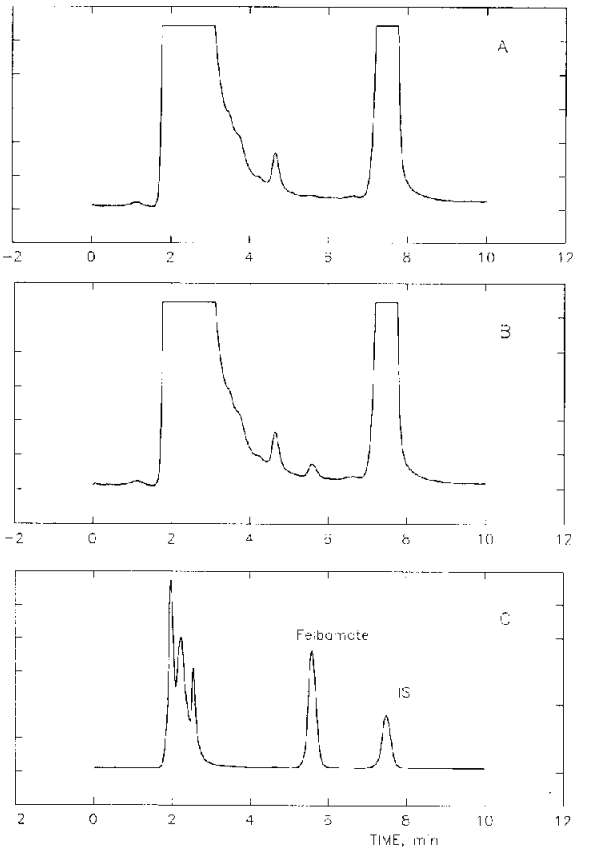

Fig. 2. Chromatograms of beagle dog plasma extracts. (A) Blank sample containing only I.S.; (B) sample containing 0.293 μg/ml felbamate and I.S.; (C) sample containing 150 μg/ml felbamate and I.S. The I.S. concentration in all samples was 26.667 μg/ml.

interval lines for the 0–20 μg/ml subrange are seen in Fig. 3B. The linear model $y = A + Bx + \varepsilon$ was selected for calibration. Estimates of the line parameters A (intercept) and B (slope), the error term ε (variance about the regression), the intercept and slope standard errors $\text{S.E.}_{(A)}$ and $\text{S.E.}_{(B)}$, the correlation coefficient r^2 and the F-ratio (significance of regression), obtained for all five sets by least squares and ANOVA, are listed in Table I. The calculated F values were much larger than the value $F_{1,37,0.95} = 4.1$ from statistical tables indicating an excellent fit of the linear model to the data. The residuals for the regression, shown in Fig. 4A and B, are evenly and randomly distributed around the zero line with no evidence of bias. The overall error ε for set 4 has a within-day and between-day component. The size of the within-day component for sets 1–3 varied from 54 to 90% of the overall error.

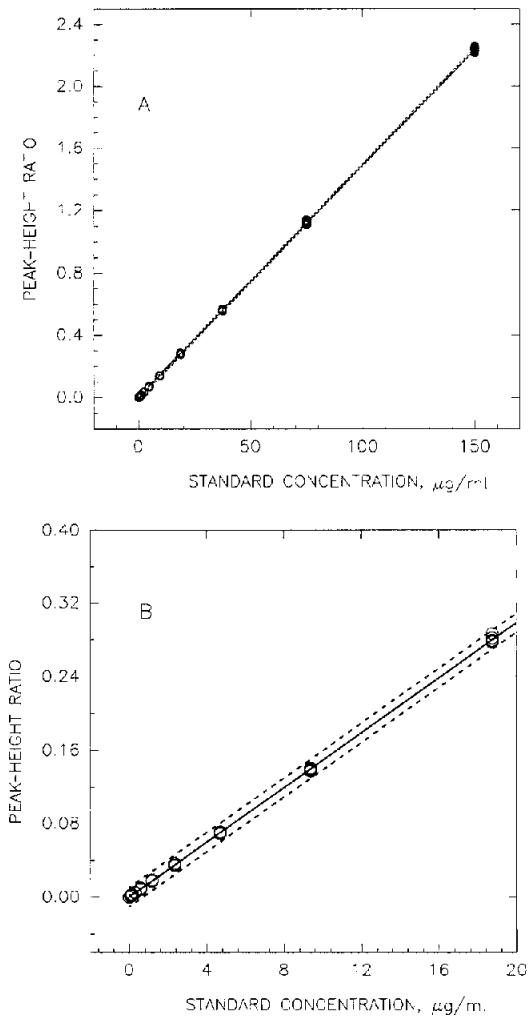

Fig. 3. Calibration line for set 4, $n = 117$ (full line), and its prediction intervals (dotted line) for the range 0–150 μg/ml (A) and the subrange 0–20 μg/ml felbamate (B); $y = 0.00316 + 0.0149x$ using unweighted regression.

Table II lists the mean of determined concentrations $\bar{x}$ and the C.V. for five selected standard concentrations out of the thirteen concentrations used in the calibration in sets 1–4 using the linear model parameters from Table I. For completeness, the R.M.E. and T.E. are listed in Table III. The mean determined concentrations $\bar{x}$, the C.V., R.M.E. and T.E. for the "short" set 5, based on concentration weighted regression, are listed in Table IV. The C.V.s for sets 1–3 (Table II) do account for a large part of the C.V. of set 4 which again included the within-day and between-day component of precision for the listed concentrations. Based on the complete calibration sets 1–4 the LOQ, arbitrarily set as that lowest standard concentration with a T.E. less than 50%, was a concentration of 0.146 μg/ml.

The assay was used on over 2700 dog plasma samples originating from a pharmacolinetic study [5]. Each daily set of unknown study samples contained twelve standard calibration samples, *i.e.* three subsets of four concentrations each with concentrations identical to those of set 5. These were used to obtain calibration line parameters A and B by weighted linear regression. The calibration during analysis of study samples resulted in 41 individual four-concentration calibration sets. The mean (± S.D.) values for parameters A and B were 0.0004308 ± 0.0002140 and 0.0145536 ± 0.001461, respectively. These parameter values are very close to the values in Table I. Typical chromatograms from two dogs in the pharmacokinetic study are shown in Fig. 5A and B and the full plasma concentration *ver-*

TABLE I

CALIBRATION LINE PARAMETERS AND COEFFICIENTS FOR DATA SETS 1–5 OBTAINED BY LEAST SQUARES AND ANOVA

Set No.[a]	n	A	S.E.$_{(A)}$	B	S.E.$_{(B)}$	r^2	F	ε
1	39	−0.0005116	0.0006220	0.0150177	0.00000129	0.999973	1345340	0.001077
2	39	0.0014243	0.0008641	0.0148389	0.00000180	0.999946	680570	0.001497
3	39	0.0000034	0.0005136	0.0148489	0.00000107	0.999981	1928790	0.000889
4	117	0.0003157	0.0005537	0.0149018	0.00000115	0.999931	1671630	0.001661
5	36	0.0003160	0.0001767	0.0148901	0.00000188	0.999946	88893560	0.000221

[a] Set 1 = triplicates run, day 1; set 2 = triplicates run, day 2; set 3 = triplicates run, day 3; set 4 = day 1–3 runs combined; set 5 = day 1–3, four concentrations, weighted.

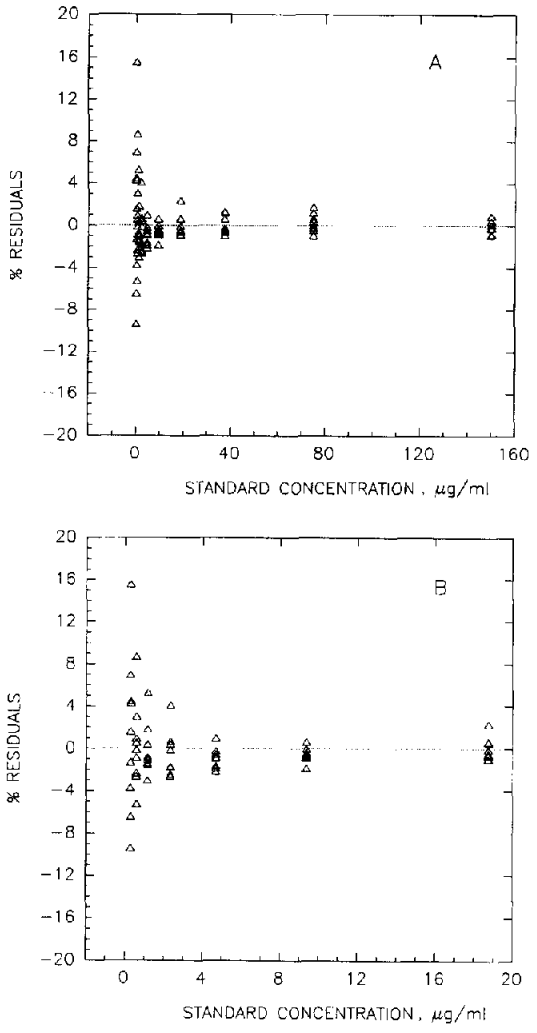

Fig. 4. Residuals plots (as percentage of stated concentration) for calibration points of set 4 for the full range (A) and subrange 0–20 μg/ml (B).

sus time curves for these two dogs are plotted in Fig. 6.

DISCUSSION

A large number of plasma samples was expected to be generated in the pharmacokinetic study [5]. From previous work in our laboratories we expected the concentrations for the majority of the study plasma samples within the 24 h post-dose to be in the range 0.5–75 μg/ml. A sufficiently sensitive, reproducible and efficient assay for felbamate in a large number of low-volume dog plasma samples was needed to support the wide range of drug concentrations. The precipitation of plasma proteins with acetonitrile and injection of the supernatant, used in our assay to improve its ruggedness, is an established technique applied in antiepileptic drug analysis [9,10].

As is evident from Table I and Fig. 3A and B, the calibration lines were linear with very little variation within the concentration range 0.15–150 μg/ml. The actual study samples were scheduled to be analyzed over a period of weeks. The assessment of the variability in between-day precision was very important. The overall precision for assays carried out on the same equipment over a period of days has two components, the between-day and the within-day precisions. We have used the calibration sets 1–3 to estimate the within-day components of precision (Table II) and accuracy and total error (Table III). In set 4, where the variability included both the between-day and within-day components, the precision

TABLE II

MEAN DETERMINED CONCENTRATIONS AND COEFFICIENTS OF VARIATION FOR FIVE CONCENTRATIONS OF UNWEIGHTED CALIBRATIONS SETS 1–4

Standard concentration (μg/ml)	Mean observed concentration (μg/ml)				C.V. (%)			
	Set 1	Set 2	Set 3	Set 4	Set 1	Set 2	Set 3	Set 4
0.146	0.198	0.122	0.177	0.165	0.26	8.52	8.03	15.21
0.585	0.623	0.540	0.602	0.588	2.88	5.20	1.09	4.28
2.343	2.346	2.295	2.346	2.328	1.21	3.50	1.59	2.24
18.750	18.664	18.902	18.719	18.760	0.58	1.51	0.26	1.02
150.000	150.055	149.745	149.974	149.924	0.27	0.55	0.34	0.71

TABLE III

RELATIVE MEAN ERRORS AND TOTAL ERRORS FOR FIVE CONCENTRATIONS OF UNWEIGHTED CALIBRATION SETS 1 TO 4

Standard concentration (μg/ml)	Relative mean error (%)				Total error (%)			
	Set 1	Set 2	Set 3	Set 4	Set 1	Set 2	Set 3	Set 4
0.146	35.43	16.49	21.02	12.93	36.16	30.72	40.46	47.29
0.585	6.36	7.74	2.81	0.38	12.50	17.34	5.06	8.98
2.343	0.11	2.04	0.11	0.63	2.55	8.92	3.31	5.09
18.750	0.45	0.81	0.16	0.05	1.62	3.86	0.68	2.11
150.000	0.03	0.16	0.01	0.05	0.59	1.27	0.70	1.48

TABLE IV

MEAN DETERMINED CONCENTRATIONS, COEFFICIENTS OF VARIATION, RELATIVE MEAN ERRORS AND TOTAL ERRORS FOR CONCENTRATIONS-WEIGHTED SET 5

Standard concentration (μg/ml)	Concentration found (mean ± S.D.) (μg/ml)		C.V. (%)	Mean error (%)	Relative mean error (%)	Total error (%)
0.000	−0.021	0.000	0.00	0.021	0.00	0.00
0.585	0.588	0.025	4.28	−0.002	0.46	9.06
9.375	9.329	0.061	0.66	0.045	0.48	1.80
150.000	150.043	1.076	0.71	−0.043	0.02	1.46

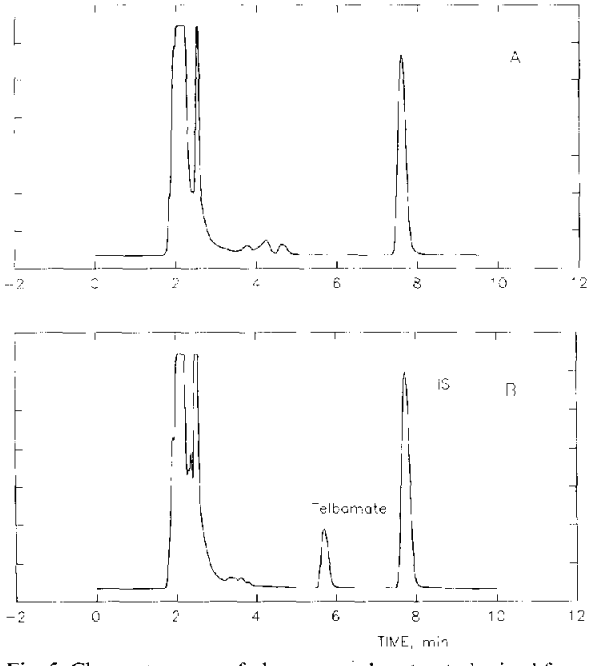

Fig. 5. Chromatograms of plasma sample extract obtained from a pediatric female beagle dog before dosing (A) and from an adult male dog at 8 h after the tenth dose with a felbamate concentration of 18.71 μg/ml (B). The I.S. concentration in both samples was 26.667 μg/ml.

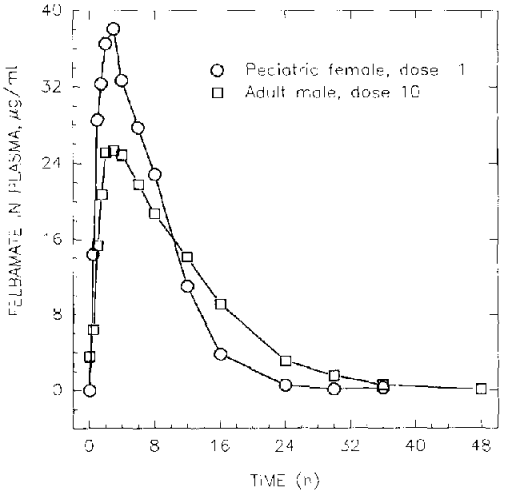

Fig. 6. Plasma felbamate concentration *versus* time curves for the two dogs in Fig. 5.

was in most cases slightly higher than for sets 1–3. This indicated that the within-day variability was the major contributor, however, estimation of the size of this contribution over the whole range from the C.V. values was difficult.

A better estimate of the contribution of the within-day variability to the overall variability was obtained from the regression ANOVA (see Table I) that separated out the error term ε of the model (variance about the regression). We have selected the square root of the variance about the regression as the measure of the error term. The ε sets 1, 2, and 3 was 65, 90, and 54% of that for set 4. This method confirmed that most of the variability in the precision was caused by within-day factors.

The parameters for the four-concentrations calibration set 5 were obtained by weighted least squares (Table IV) in order to get the best possible results from calibration during analysis of actual study samples with a minimum number of calibration standards. It gave essentially the same total error as the unweighted full thirteen-concentrations calibration set 4. Therefore, the parameters from set 5 were used as criteria to accept or reject the daily calibrations during study sample analysis.

A precision better than 5% was desirable and for our calibration sets 1–5 it applied to all concentrations starting with 0.585 μg/ml. Any study sample values below 0.6 μg/ml were used with caution, all values below the LOQ (<0.150 μg/ml) were discarded.

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