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A tandem solid phase extraction, reversed-phase HPLC method for determining SDZ WAG 994 in dog, monkey and rat blood

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Abstract

The development and validation of a sensitive and specific HPLC method for SDZ WAG 994 (I) in dog, monkey and rat blood is described. Sample preparation entailed double solid phase extraction (SPE) of I and the internal standard from 0.5 ml of animal blood using a phenyl and propyl sulfonic acid cation exchange column, sequentially. Chromatographic separation was achieved on a YMC Basic C-8 narrowbore HPLC column and the eluates were detected by UV absorption at 266 nm. The method has a linear response up to at least 1800 ng/ml with a limit of quantification of 1 ng/ml across all species. Analysis of 'blinded' quality control dog and monkey blood samples over 3 or 4 days produced median precisions of 2.89 and 4.77%, and median reproducibilities of 4.86 and 10.9%, respectively. Curve fitting of variability estimates indicated that concentration independent error contributed 3–9% of the total method error for the tandem SPE procedure. Extracted endogenous material from blood matrices, several potential metabolites and cyclohexyladenosine were well resolved from the peaks of interest. The stability of I in dog blood stored at −20°C is at least 6 months. The overall absolute and relative recovery of I using the tandem SPE procedure was 85.5 ± 5.1% and 96.5 ± 5.0%, respectively. The ruggedness of the method has been demonstrated by multiple analyses, from several toxicokinetic studies, performed by different analysts using comparable instrumentation. © 1997 Elsevier Science B.V.

Keywords: Solid phase extraction; Reversed-phase chromatography; Validation; Curve fitting; Adenosine A_1 receptor agonist

1. Introduction

SDZ WAG 994 (6-cyclohexyl-2'-*O*-methyladenosine, I, Fig. 1) is a potent, orally active adenosine A_1 receptor agonist with therapeutic potential in certain cardiovascular and/or metabolic diseases [1,2]. Absorption and distribution studies in rats (0.037 and 1.4 mg kg^{-1} PO, 0.019 mg kg^{-1} IV) and dogs (0.093 and 5.6 mg kg^{-1} PO, 0.093 mg kg^{-1} IV) found that [^{14}C]I is moderately absorbed in the rat (61–76%) and well absorbed in the dog (88–92%) [3]. The parent compound is extensively metabolized and rapidly cleared in both species with less than 2% of any dose being detected unchanged in the excreta. In

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vitro plasma protein binding experiments in rats and dogs, spanning a concentration range of 1–5000 ng ml^{-1}, indicate that [^{3}H]I is 47–60 and 52–59% bound, respectively.

To support the toxicokinetic program for I, a sensitive and specific bioanalytical method suitable for quantitating parent drug levels in animal blood samples of limited volume had to be developed and validated. This contribution describes the evolution and validation of an assay for I, employing tandem solid phase extraction (SPE) and reversed-phase narrowbore HPLC with ultraviolet detection, in dog, rat and cynomolgus monkey blood matrices with a limit of quantification (LOQ) of 1 ng ml^{-1}. Preliminary results from this work have appeared in abstract form [4].

2. Experimental

2.1. Materials

Sodium phosphate monobasic, sodium phosphate dibasic (anhydrous) and ammonium hydroxide were all AR grade and obtained from Mallinckrodt (Chesterfield, MO). Citric acid monohydrate (certified ACS) and 50% sodium hydroxide were purchased from Fisher (Springfield, NJ). High-purity methanol and acetonitrile (HPLC grade) were obtained from Burdick and Jackson (Muskegon, MI). Ultrapure water (18 MΩ cm) was prepared via a Milli-Q system (Millipore, Bedford, MA). Dimethyl sulfoxide (DMSO), 99+, was purchased from Aldrich (Milwaukee, WI). Dog blood with heparin was obtained from Pel-Freez Biologicals (Rogers, AR). Rat and cynomolgus monkey blood, also containing heparin, were purchased from Harlan (Indianapolis, IN). An adenosine based internal standard (II) and I, both in the stable hydrate form, were obtained from the Preclinical Research Department of Sandoz Pharma (Basel, Switzerland). The derivatizing reagents 9-fluorenylmethyl chloroformate (FMOC Cl) and Accu-Fluor® were purchased from Pierce (Rockford, IL) and Waters (Milford, MA), respectively.

2.2. High-performance liquid chromatography

The HPLC systems consisted of a model 10 AD pump, a model CTO-10 A column oven, and a model SPD-10 A ultraviolet detector (all from Shimadzu Instruments, Columbia, MD). A Hitachi AS-4000 (Danbury, CT) or a Perkin Elmer ISS 100 (Norwalk, CT) autosampler was used to inject a 100 μl aliquot onto a Supelco LC 1 (20 mm, 5 μm particle size) guard column (Bellefonte, PA) connected in series with a Basic C-8 (2.0 × 250 mm, 5 μm particle size) analytical column (YMC, Wilmington, NC) thermostated at 50°C. The mobile phase for dog and rat blood analyses consisted of acetonitrile-sodium phosphate buffer (pH 6.0; 20 mM) (35:65, v/v). For monkey analyses, the mobile phase was acetonitrile-sodium phosphate buffer (pH 6.0; 15 mM) (25:75, v/v). All mobile phases were filtered/degassed through a 0.45 μm nylon filter under vacuum. The sodium phosphate buffer was prepared by mixing approximately 880 ml of sodium phosphate monobasic and 120 ml of sodium phosphate dibasic, both at equimolar concentrations, followed by dropwise addition of one or the other solution to achieve the desired pH. The mobile phase flowrate was 0.20 ml min^{-1} (dog and rat) or 0.25 ml min^{-1} (monkey). Column eluates were monitored at 266 nm. Peak heights for I and II were integrated using the CALS laboratory data acquisition system (Beckman, Fullerton, CA). Statistical processing and reporting of the data was accomplished with an in-house LIMS package or PC NONLIN (ver. 3.0).

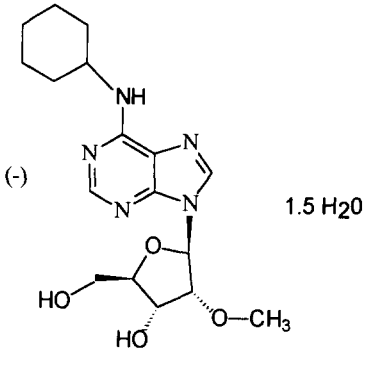

Fig. 1. Structure of SDZ WAG 994 (I).

2.3. Standards and quality control samples

Stock solutions of I and II were prepared at a concentration of 1 mg ml^{-1} in DMSO and serially diluted with water to yield concentrations between 50 and 0.04 μg ml^{-1}. These solutions remained stable for more than 1 month at room temperature. To correct for the stable hydrate form of the solid materials, the balance weights for I and II were multipled by 0.931 and 0.986, respectively.

Nine dog calibration curve standards for I, from 0.449 to 698 ng ml^{-1}, were prepared by spiking 25 ml portions of blood with 0.20–1.7 ml of the appropriate stock solutions. Aliquots (2 ml) of each blood pool were pipetted into polypropylene screw cap tubes and stored at −20°C until analysis. Dog blood quality control (QC) samples, prepared from a different weighing of I, were made at 10 different concentrations ranging from 0.239 to 708 ng ml^{-1}. Monkey and rat blood calibration standards and QC samples for I were made in a similar fashion spanning a concentration range of 1.12–1810 ng ml^{-1} and 1.08–378 ng ml^{-1}, respectively. The analysts were 'blinded' to the actual concentration of the dog and monkey QC samples. Stability, freeze/thaw and recovery experiment samples were prepared and stored in the same manner as calibration standards and QC samples.

2.4. Solid phase extraction

Whole blood samples (0.5 ml) from dogs, monkeys, and rats were pipetted into 20 × 125 mm glass disposable screw-cap culture tubes and diluted with 6 ml of 20 mM phosphate buffer, pH 6.0, containing II (120 ng). After a brief vortexing step and room temperature centrifugation at 2000 rpm for 10 min, the supernatant was applied to a Bond Elute (Varian, Harbor City, CA) LRC Phenyl (PH) SPE cartridge (100 mg) previously activated with 1 ml of MeOH and conditioned with 2 ml of 20 mM phosphate buffer, pH 6.0. The PH cartridge was washed with water (4 ml) and 20% MeOH in water (8 ml) under vacuum using a Vac Elut 24 place manifold (Jones Chromatography, Lakewood, CO). The analytes were eluted from the PH cartridge with 80% acetonitrile in water (1 ml) and subsequently loaded onto a Propyl Sulfonic Acid (PRS) SPE cartridge (100 mg), also from Varian, that had been activated with 1 ml of MeOH and conditioned with 2 ml of 20 mM citrate buffer, pH 3.0. The PRS cartridge was washed under vacuum with 1 ml each of water, 10% MeOH in water, and 100% acetonitrile. The analytes were eluted from the PRS cartridge with 2 ml of 0.1% NH_4OH in MeOH and the eluate dried using a Model 3-2200 vortex evaporator set at 45°C (Buchler Instruments, Fort Lee, NJ). The residue was reconstituted in a 200 μl solution of HPLC mobile phase-18 MΩ cm water (1:1, v/v).

3. Results

A systematic characterization of several parameters was made to evaluate method performance. In addition to defining the linearity of response over the working range of the method, evaluations of method bias, precision (within day variability), reproducibility (across day variability), method error, freeze/thaw and long term stability, recovery, specificity and ruggedness were performed. These experiments were patterned after and employ the terminology used in bioanalytical method validation guidelines appearing in the literature [5,6].

Linearity of response was tested by assaying dog blood calibration standards in duplicate on three separate days. As a first test of linearity, the peak height ratio (I/II) for each standard was divided by it's respective concentration to produce a relative response factor (RRF). A t-test (H_o: slope = 0), performed on the RRF's generated on each day vs. analyte concentration, indicated that the slope was not significantly different from zero ($P > 0.05$) thus supporting a linear concentration vs. response relationship. The mean RRF on each of the three analysis days was less than 11% over the entire calibration range. A second statistical test for linearity was performed by applying the equation $y = mx + b$ to the I/II vs. concentration data. Table 1 shows the results from regression analysis. The r^2 was 0.996 or greater on each

Table 1
Regression analysis of peak height ratio vs. concentration for dog blood calibration standards containing I

Weighting function	Analysis day (analyst)		
	One (A)	Two (B)	Three (A)
Weight = 1			
Slope	6.06×10^{-3}	5.35×10^{-3}	5.01×10^{-3}
y-intercept	-5.84×10^{-3}	-4.45×10^{-4}	-6.29×10^{-3}
95% CI on y-intercept	-6.08×10^{-2} to 4.92×10^{-2}	-8.41×10^{-3} to 7.52×10^{-3}	-3.07×10^{-2} to 1.81×10^{-2}
Coefficient of determination(r^2)	0.996	>0.999	>0.999
Weight = $1/y^2$			
Slope	6.02×10^{-3}	5.35×10^{-3}	4.82×10^{-3}
y-intercept	-4.43×10^{-4}	-4.96×10^{-4}	-1.22×10^{-4}
95% CI on y-intercept	-4.54×10^{-2} to 4.46×10^{-2}	-2.58×10^{-2} to 2.48×10^{-2}	-3.97×10^{-2} to 3.9×10^{-2}
Coefficient of Determination (r^2)	0.996	>0.999	0.997

analysis day using weighting functions of 1 or $1/y^2$. The y-intercept was found not to be significantly different from zero based on 95% confidence limits calculated using the equation, 95% CI = y-intercept $\pm$ S.E. x (t, 0.05; n-2 df). The no intercept calibration model ($y = mx$) without weighting, therefore, was judged to be appropriate for dog blood standards. This calibration model was also found to be valid for monkey and rat blood standards. It is likely that the calibration model is linear well beyond 1800 ng ml^{-1}, however, preliminary work and subsequent toxicokinetic studies indicated that the calibration range selected was sufficient.

Table 2 summarizes the results from the analysis of dog blood QC samples. These analyses were performed in quintuplicate on 3 separate days by two different analysts who were 'blinded' to the actual concentration of each sample. This data was used to characterize method bias, precision and reproduciblity (%CV), and method error so as to reasonably assign an LOQ. An evaluation of method bias is presented graphically in Fig. 2. The individual QC sample results are shown as a log-log plot to help distinguish concentration values and to directly evaluate relative error accross the concentration range. No consistent deviation from the line of identity was seen suggesting that there is no systematic deviation, or bias, between actual and theoretical results. Excluding the 0.239 and 0.492 ng/ml QC sample results in Table 2 (below the LOQ), precision ranged from 0.971 to 8.96% (median 2.89%) and reproducibility ranged from 2.41 to 8.89% (median 4.86%).

Quantitative estimates of method error were calculated using PC NONLIN to fit the %CV (Table 2) and 95% confidence limit data (Table 3), obtained from the dog blood 'blinded' QC sample analyses, to an adaptation of the Snyder and van der Wal equation [7]:

$$y = [e_a^2 + 1.5(100\sigma_n)^2/x^2]^{0.5}$$

where, y is the method error (%), e_a is the concentration independant error, σ_n is the standard deviation of the concentration measurement as the analyte concentration approaches zero (dependent error), and x is the theoretical analyte concentration. Results from this analysis are presented graphically in Fig. 3. The calculated LOQ was 1.16 and 1.53 ng ml^{-1} with 15% (%CV) and 20% (95% confidence limit) method error while the concentration independent error was 3.58% (%CV) and 8.90% (95% confidence limit), respectively. Based on these results, precision and reproducibility acceptance criteria of $\leqslant$20% (% CV) and method bias information, a conservative LOQ of 1 ng ml^{-1} for a 0.5 ml dog blood sample was set.

Table 2
Results from the analysis of 'blinded' QC samples in quintuplicate on three separate days

Day (analyst)	Theoretical concentration of I in dog blood (ng m^{-1})									
	0.239	0.492	0.957	1.81	8.81	19.1	47.6	191	372	708
One (A)										
Mean	0.241	0.518	0.932	1.69[a]	8.57	17.6	44.8	188	351	704
S.D.	0.138	0.0557	0.0769	0.0614	0.438	0.390	1.58	8.17	9.76	17.5
% CV	57.0	10.8	8.24	3.64	5.12	2.21	3.54	4.34	2.78	2.49
Two (B)										
Mean	0.0146	0.323	0.838	1.60	8.98	19.7	47.1	214	389	726
S.D.	0.0201	0.0330	0.0471	0.0532	0.230	0.192	0.698	2.07	6.91	15.6
% CV	137	10.2	5.62	3.33	2.56	0.977	1.48	0.971	1.78	2.15
Three (A)										
Mean	0.212	0.436	0.932	1.70	9.14	19.0	44.8	205	375	714
S.D.	0.128	0.0570	0.0835	0.0493	0.466	0.554	1.28	7.05	5.05	13.9
% CV	60.2	13.1	8.96	2.91	5.10	2.92	2.86	3.44	1.35	1.94
Overall										
Mean	0.156	0.425	0.091	1.66	8.90	18.8	45.6	202	372	715
S.D.	0.145	0.0947	0.0801	0.0689	0.441	0.962	1.61	12.4	17.7	17.2
% CV	93.0	22.3	8.89	4.16	4.96	5.13	3.54	6.12	4.76	2.41
Overall accuracy[b]	65.3	86.4	94.1	91.7	99.0	98.4	95.8	94.2	100	99.8

[a] $n = 4$.
[b] Accuracy = $(1 - |\text{observed} - \text{expected}| / \text{expected}) \times 100$.

Results from the duplicate analysis of spiked dog blood samples over three freeze/thaw cycles showed that the observed concentration values of 8.85 ± 0.17, 377 ± 5 and 705 ± 6 ng/ml ($n = 6$) compared very favorably with the actual values of 8.81, 372 and 708 ng/ml. Recovery results for I, also performed at three different concentrations, are shown in Table 4. Overall absolute recovery for the tandem SPE procedure, determined by comparison of extracted blood standards against directly injected aqueous standards, was $85.5 \pm 5.1\%$. Overall relative recovery, based on the comparison of extracted blood standards to extracted aqueous standards, was $96.5 \pm 5.0\%$. The stability of I in dog blood stored at $-20°C$ is at least 6 months as triplicate analyses performed on 1.87, 372, and 708 ng ml^{-1} standards at bi-monthly intervals had a recovery of 94.4% or greater.

Cross-validation of the method to cynomolgus monkey blood was performed by analysing several sources of blank monkey blood matrix as well as 'blinded' QC samples. Due to the presence of small chromatographic interferences seen in the blank matrix at the retention time of I, the mobile phase and flow rate were changed slightly to obtain the desired resolution. Results from the QC sample analyses are presented in Table 5. The median precision was 4.77% and the median reproducibility was 11.9%, while the overall accuracy ranged from 86.9–98.4%. Based on the acceptance criteria outlined above for dog blood, an LOQ of 1 ng ml^{-1} for monkey blood was justified. Similarly, analysis of rat blood QC samples in quadruplicate at 1.96, 126 and 306 ng ml^{-1}, in connection with an IV toxicokinetic study, yielded statistical results of 2.06 ± 0.09, 121 ± 10 and 278 ± 17 ng/ml. These findings, coupled with the absence of endogenous interferences, also support a 1 ng ml^{-1} LOQ.

Potential metabolites and cyclohexyladenosine (CHA) were well resolved from I and II. In addition, analysis of several sources of blank dog and rat blood matrix did not indicate the presence of interferences at the retention times of interest.

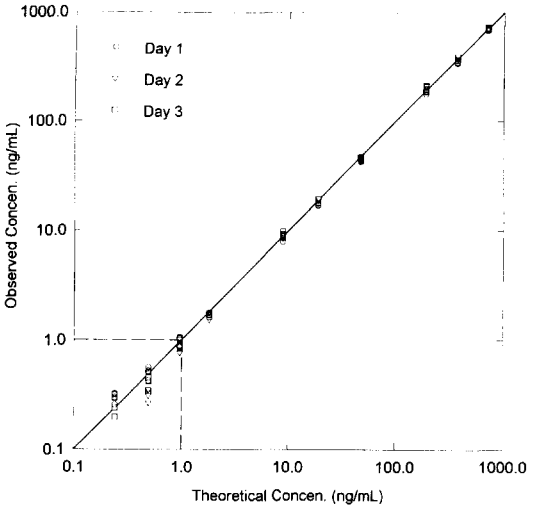

Fig. 2. Log-log plot of individual daily dog blood concentration results from the analysis of 'blinded' QC samples containing I.

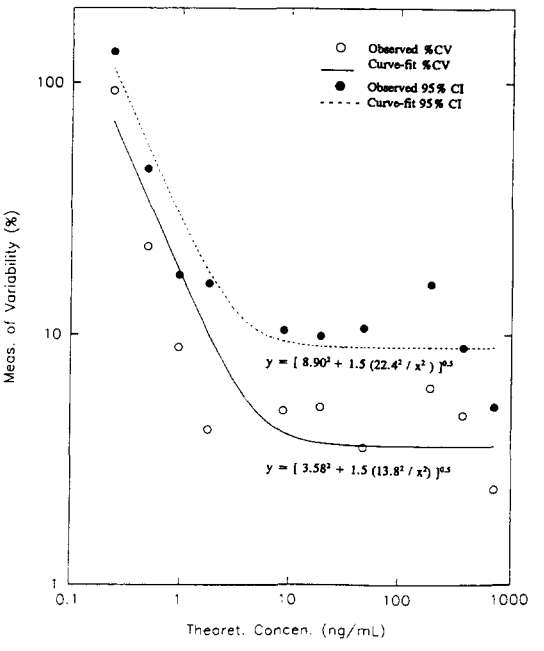

Fig. 3. Plot of observed and curve fit dog blood %CV and 95% confidence limit data for 'blinded' QC samples analysed over 3 days.

Representative chromatograms for dog blood extracts are shown in Fig. 4. Selected time vs. concentration plots from dog, monkey and rat toxicokinetic studies are overlayed in Fig. 5. The ruggedness of the method was demonstrated via validation experiments and support of the toxicokinetic program for I by four different analysts using comparable HPLC instrumentation.

4. Discussion

Three major areas were concurrently addressed during method development; analyte detection, chromatographic separation and sample extraction. To achieve an LOQ in the pg-ng ml^{-1}

Table 3
Confidence level analysis of 'blinded' QC sample results for I in dog blood

True value (ng ml^{-1})	Observed value (ng ml^{-1})	Number of observations	Mean absolute difference from the true value ± S.D.(ng ml^{-1})	95% Confidence limits	
				(ng ml^{-1})	% True value
0.239	0.156	15	0.136 ± 0.093	0.319	133
0.492	0.425	15	0.092 ± 0.068	0.225	45.8
0.957	0.901	15	0.088 ± 0.039	0.165	17.2
1.81	1.66	14[a]	0.153 ± 0.069	0.228	15.9
8.81	8.90	15	0.319 ± 0.307	0.919	10.4
19.1	18.8	15	0.847 ± 0.530	1.89	9.88
47.6	45.6	15	2.09 ± 1.52	5.06	10.6
191	202	15	14.4 ± 8.1	30.3	15.8
372	372	15	14.4 ± 9.6	33.2	8.92
708	715	15	14.2 ± 11.3	36.4	5.14

[a]1 sample lost in analysis.

Table 4
Recovery of I from dog blood

Condition	Theoretical value (ng ml^{-1})			Overall recovery
	1.87	292	656	
Absolute recovery[a] Mean% (S.D.)	82.0 (5.1)	83.6 (2.4)	90.8 (3.0)	85.5 (5.1)
Relative recovery[a] Mean % (S.D.)	91.5[b] (2.8)	100.2 (0.4)	96.0 (6.1)	96.5 (5.0)

[a] $n = 3$.
[b] $n = 2$.

range, the possibility of fluoresence detection was examined. Native fluoresence for I in 20 mM phosphate buffer, pH 6.0, was observed (266 nm_{ex}, 315 nm_{em}). The quantum yield, however, was not sufficient to permit reproducible quantification in the 1–5 ng ml^{-1} range, a finding consistent with that reported for an A_2 adenosine receptor agonist [8]. Furthermore, chromatograms from tandem SPE extracted blood blanks had numerous endogenous fluorophores. Since the cyclohexyl moiety at position N^6 of I precludes the use of derivatizing agents like chloroacetaldehyde, a molecule long used to tag adenosine type compounds [9,10], reagents for secondary amines, namely FMOC Cl [11] and Accu-Fluor® [12], were investigated. These materials, used under conditions similar to those cited in the preceding references, did not produce a fluorescent derivative of I. A possible explanation for this is N^6 protection by the cyclohexyl group. Spectroscopic analysis of I and II, on the other hand, revealed a very strong UV absorption band at 266 nm. This mode of detection was found to yield much cleaner chromatograms and the potential for sub-ng ml^{-1} quantitation.

Previous work describing the C-18 reversed-phase separation of adenosine and related analogs in plasma served as a starting point for the HPLC separation of I [8,10,13]. Preliminary work using a C-18 stationary phase with buffered mobile phases containing up to 50% acetonitrile, gave excessively long retention times for I and II. These compounds were eluted from a Supelco C-8 DB column (5 μm, 4.6 × 15 cm) in under 10 min using a buffered mobile phase containing 30% acetonitrile and a flow rate of 1 ml min^{-1}. To improve on the peak tailing observed with this column and to avoid the use of triethylamine or ion-pairing agents such as pentanesulfonic acid [8], a YMC Basic C-8 column of similar dimensions was successfully substituted. For method validation experiments, the YMC column packing material was used in a 2.0 × 250 mm column to exploit the concentration enhancing characteristics of narrowbore HPLC and, thereby, decrease the LOQ by roughly a factor of 5. The strength of the mobile phase was optimized to give the shortest possible runtime while permitting sufficient k′ values so as to accommodate the large injection volume to flowrate ratio.

Several procedures for extracting I were examined including simple liquid/liquid extraction of blood, buffered from pH 3–9, with various organic solvents. This approach was unacceptable because there were materials co-eluting with I as well as several late eluting peaks. Recovery of I from whole blood using these procedures never exceeded 65%. SPE using C-18, C-8, C-2, cyano, and PH bonded stationary phases, therefore, was systematically explored. Several different washing/eluting solvents were studied to define conditions giving the cleanest extract and the best recovery of I. A PH SPE column using the procedure outlined in this report yielded an extract that was chromatographically clean at the retention times of interest with over 90% recovery for I. Small late eluting peaks, however, were still encountered thereby requiring unacceptable runtimes.

A second SPE procedure was developed as a final cleanup step. Two types of sulfonic acid cation exchange SPE columns were tried. The first column, containing a phenyl spacer group be-

Table 5
Results from the analysis of 'blinded' QC samples in quintuplicate on four separate days

Analysis day	Theoretical concentration of I in monkey blood (ng ml^{-1})					
	1.82	3.03	7.58	189	947	1580
One						
Mean	1.38[a]	2.26	6.07	193	1040	1640
S.D.	0.0776	0.0492	0.187	5.52	115	53.1
% CV	5.64	2.18	3.07	2.86	11.1	3.24
Two						
Mean	1.65	2.66	6.13	186	987*	1570
S.D.	0.137	0.353	0.199	3.44	48.3	15.1
% CV	8.31	13.2	3.25	1.85	4.89	0.963
Three						
Mean	2.18		7.73	183	993	1600
S.D.	0.159		0.729	3.62	2.64	36.3
% CV	7.31	LA[b]	9.43	1.98	2.66	2.27
Four						
Mean	1.90	3.48	6.42	162	830	1380
S.D.	0.423	0.814	1.053	4.20	43.6	65.8
% CV	22.2	23.4	16.4	2.59	5.26	4.77
Overall Mean	1.80	2.80	6.59	181	962	1550
S.D.	0.374	0.708	0.915	12.3	105	113
% CV	20.8	25.3	13.9	6.78	10.9	7.32
Overall accuracy[c]	98.9	92.4	86.9	95.8	98.4	98.1

[a] $n = 4$.
[b] Lost in analysis.
[c] Accuracy = $(1 - |\text{observed} - \text{expected}| / \text{expected}) \times 100$.

tween the silica backbone and the sulfonic acid moiety, irreversably retained I. The PRS column, however, sufficiently retained I during a 100% acetonitrile washing step and completely released I with 1% ammonium hydroxide in MeOH. Secondary pi-pi interactions between the aromatic spacer group and I could account for the excessive binding. Efforts to combine the hydrophobic and cation exchange properties offered by the PH and PRS SPE cartridges, via mixed mode SPE packings, did not produce samples as clean as those obtained with the tandem SPE procedure.

To ascertain the contributions of concentration dependant and concentration independent parameters on method error, curve fitting of the variability estimates (%CV and 95% confidence limits) was performed. The equation used did not optimally account for the sharp break seen in the experimental data below 0.957 ng ml^{-1}. It is possible that the e_a term in the equation is a composite of several terms not calculable from the present study results [14]. Attempts at weighting the data to obtain a better estimate did not improve the fit nor appreciably decrease the calculated LOQ. At sub-ng ml^{-1} levels, the detection mode may be the overriding factor in analyte detection. The modeled 3–9% contribution by sources of concentration independent error (ie, tandem SPE, solvent evaporation) when analyte concentration values approach zero is consistent with the findings of other workers using manual liquid/liquid and automated sample extraction techniques [14]. The LOQ estimates obtained by curve fitting are 20–50% higher than those found via precision and accuracy criteria. The model values do serve as a first approximation and would likely be closer to 1 ng ml^{-1} if the model fit the sharp break in the curve.

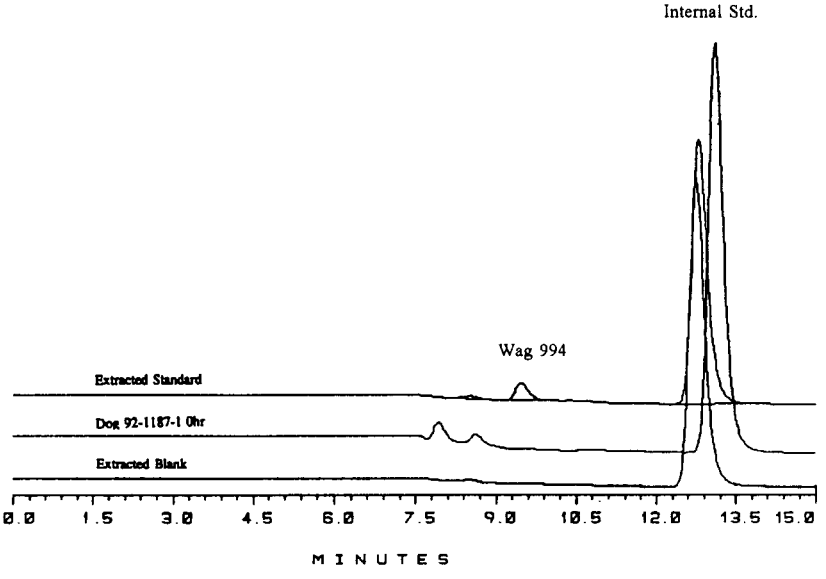

Fig. 4. Representative chromatograms showing an extracted dog blood blank, an 11.2 ng ml^{-1} extracted dog blood standard and an extracted pre-dose dog blood toxicokinetic study sample.

Results from the rigorous validation studies in dog blood presented here support an LOQ of at least 1 ng ml^{-1} for I using 0.5 ml of blood. This value is based on the reliable and reproducible quantification of I, using conservative acceptance critieria, in order to support a Good Laboratory Practices (GLP) regulated toxicokinetic program, and should not be confused with detectable analyte signal (s/n ratio of 3–5). Indeed, results from the analysis of 'blinded' dog blood QC samples showed that samples containing I could be detected at 0.239 ng ml^{-1} and quantified at 0.492 ng ml^{-1} with a precision of 22.3%. The same can also be said for I contained in monkey and rat blood matrices. The method has proven to be very useful in the pre-clinical development of I across several animal species.

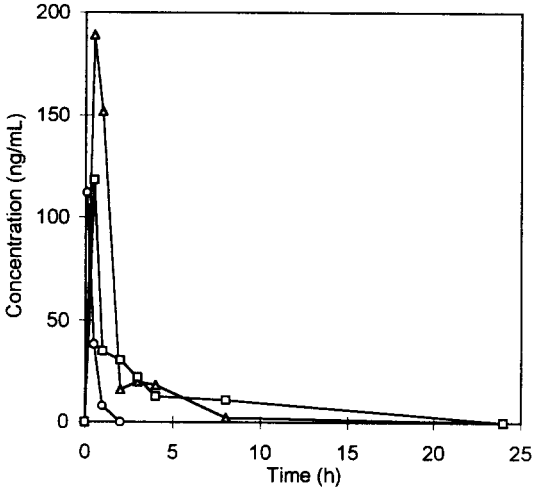

Fig. 5. Selected time vs. concentration plots from dog (triangles, 3 mg kg^{-1} PO), monkey (squares, 2 mg kg^{-1} PO), and rat (circles, 0.3 μg kg^{-1} IV) toxicokinetic studies.

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