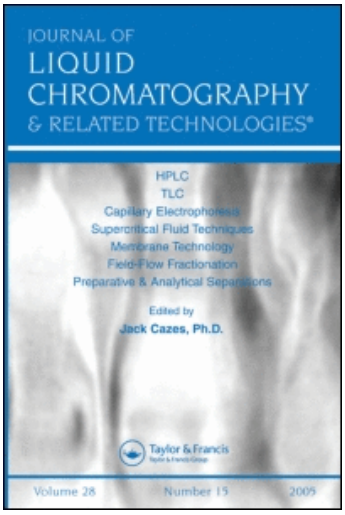

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Moacir Kaiser,[1] Francine Johansson Azeredo,[1] Heloísa de Oliveira Beraldo,[2] and Teresa Dalla Costa[1]

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□ *A simple, rapid, and sensitive high performance liquid chromatographic method using UV detection (HPLC-UV) for the determination of benzaldehyde semicarbazone (BS) in rat plasma was developed and validated using sodium diclofenac as an internal standard (IS). Analyses were performed on an HPLC system using a C_{18} column and isocratic elution with mobile phase composed of water-methanol-acetonitrile (65:30:5, v/v/v) (pH 9.0), at a flow rate of 1.1 mL/min. The eluate was monitored using a UV detector at 282 nm. Plasma samples were deproteinized with methanol and 25 μL of the supernatant were injected into the system. The analysis was performed in 6 min and the retention times of BS and IS were approximately 4.9 and 2.6 min, respectively. The method was linear in the concentration range of 0.1–16.0 μg/mL ($r^2 > 0.98$). The lower limit of quantification (LLOQ) was 0.1 μg/mL. The accuracy of the method was within 15%. Intra and inter-day relative standard deviations were equal or less than 10.19% and 2.10%, respectively. The applicability of the HPLC-UV method for pharmacokinetic studies was tested using plasma samples obtained after oral administration of BS to male Wistar rats. No interference from endogenous substances was observed, showing the specificity of the method developed. The reported method can provide the necessary sensitivity, linearity, precision, accuracy, and specificity to allow the determination of BS in preclinical pharmacokinetic studies.*

Keywords benzaldehyde semicarbazone, HPLC, pharmacokinetic, plasma, validation

INTRODUCTION

Epilepsy is a central nervous system (CNS) malfunction characterized by periodic and unpredictable occurrence of seizures that leads either to generalized hyperactivity involving essentially all parts of the brain or to

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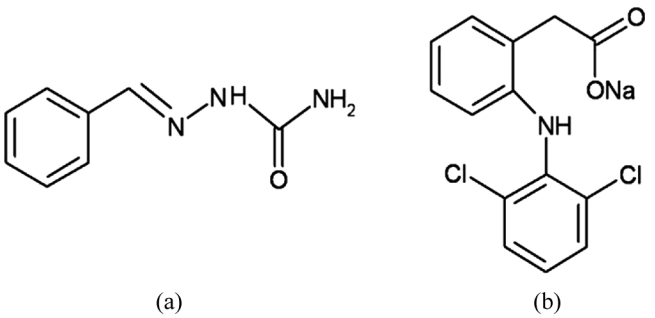
hyperactivity of only a portion of the brain.[1] Around 1%–2% of the population worldwide suffers from this disorder.[2] The current drug therapy of epilepsy suffers from a number of disadvantages, including the fact that the convulsions of approximately 25% of epileptics are inadequately controlled by medication. In addition, many currently used antiepileptic drugs cause significant side effects that may limit their maximal usefulness.[1,3,4] Thus, the evaluation of a new antiepileptic drug for the treatment of epilepsy is of great interest.

A series of aryl semicarbazones have been shown to possess excellent anticonvulsant activity in different experimental models of epilepsy in rodents.[5] A number of these compounds exhibit generally low or absent neurotoxicity and provide greater protection than phenytoin, carbamazepine, and sodium valproate, three known anticonvulsant drugs.[6] Furthermore, the compounds are inexpensive to synthesize and can be easily administered by the oral route.[7,8] One of these prototypes is benzaldehyde semicarbazone (BS) (Fig. 1).

BS was assayed (dose range of 30–300 mg/kg p.o.) in the maximal electroshock screen (MES) in rats. The pharmacodynamic results demonstrated an inhibitory effect on induced seizures with low neurotoxicity, except at the highest dose (300 mg/kg).[5] In addition to the anticonvulsant activity, it has been reported that BS has an analgesic effect, as demonstrated by different experimental models in doses ranging 10–50 mg/kg i.p.[9] This analgesic effect was already reported for several anticonvulsant drugs through the blockade of the sodium channel mechanism, as described to carbamazepine.[10] These results instigated the need to evaluate the preclinical pharmacokinetics of BS, which could become a prototype drug to treat epilepsy.

A preclinical pharmacokinetic investigation requires reliable bioanalytical methodology for the measurement of the drug involved. Thus, a sensitive and specific high performance liquid chromatography (HPLC)



(a) (b)

FIGURE 1 Chemical structure of BS (a) and Sodium diclofenac (b).

method with UV detection was developed for the quantitation of BS in rat plasma using sodium diclofenac (Fig. 1) as the internal standard (IS). The bioanalytical methodology was validated considering the specificity, linearity, precision (intra-day and inter-day), accuracy, and limit of quantification. The applicability of this method for pharmacokinetic studies was tested using plasma samples obtained after oral administration of BS to Wistar rats.

EXPERIMENTAL

Chemical and Reagents

BS was synthesized at the Chemistry Department of the Federal University of Minas Gerais, Brazil, as described in the literature.[5] Sodium diclofenac was obtained from Delaware (Porto Alegre, Brazil). HPLC grade methanol and acetonitrile were purchased from Tedia® (Fairfield, USA). Ammonium hydroxide 30% was purchased from Merck® (Darmstat, Germany). HPLC water, Millipore's® Milli-Q System, was used throughout the analysis.

Chromatographic Equipment and Conditions

A Waters HPLC system equipped with Waters® 600 pump controller, automatic injector 717 Plus-Waters®, detector Waters® 2487 dual λ absorbance, and Software Millennium 32® was used. The chromatography was performed on a Waters NovaPak® C_{18} HPLC column (3.9×150 mm) preceded by a guard column (3.9×20 mm, 4 μm particle size) packed with the same material. All samples and standard solutions were chromatographed at room temperature. The mobile phase contained a mixture of LC-grade water, LC-grade methanol, and LC-grade acetonitrile (65:30:5, v/v/v). The pH was corrected to 9.0 with ammonium hydroxide 30%. The detection was at 282 nm, where the maximum absorbance of the BS was observed. A flow rate of 1.1 mL/min was used and 25 μL samples were injected. The autosampler was set up to inject sample aliquots every 6.0 min.

Preparation of Standard Stock Solutions and Quality Control Samples

A standard stock solution of BS was prepared in a mixture of acetonitrile and water (50:50, v/v) with a final concentration of 400 μg/mL. Appropriate dilutions of the stock solution were made with the same solution mixture. The calibration curve samples were prepared by spiking 90 μL

of Wistar rat plasma with 10 μL of the appropriate standard working solution to result in the final BS concentrations of 0.1, 0.2, 0.5, 1.0, 4.0, 8.0, 16.0 μg/mL. The standard stock solutions were prepared in duplicate from separate weightings: one set was used to prepare the calibration curve samples and one set was used to prepare the quality control (QC) samples.

A standard stock solution of sodium diclofenac (IS) was prepared in a mixture of acetonitrile and water (50:50, v/v) to produce a concentration of 500 μg/mL diluted to give 250 μg/mL. The standard stock solutions, calibration curve, and QC samples were freshly prepared on each analysis day.

Sample Preparation

Prior to the chromatographic analysis, 100 μL samples were deproteinized by addition of 300 μL of ice cold methanol and 10 μL of IS (250 μg/mL), vortexed for 20 s and centrifuged at $6800 \times g$ at 4°C for 10 min. The final concentration of sodium diclofenac (IS) in the samples was 25 μg/mL. All plasma samples, including calibration curve, QC, and samples from pharmacokinetic experiments, were processed in the same manner.

Validation Procedures

Standard calibration curves and QC samples were analyzed in triplicate on two consecutive days. The linearity of the calibration curves based on peak area ratio (area of analyte/area of IS) as a function of the nominal concentration was assessed by weighted (1/concentration) least square regression. Slopes, intercepts, and determination coefficients were calculated. A statistical analysis using ANOVA ($\alpha = 0.05$) was conducted to certify the linearity of the calibration curves. The selectivity of the method was investigated for potential interferences of endogenous substances using six independent batches of rat plasma samples, including hemolyzed plasma. Recovery experiments (extraction efficiency) was performed by comparing the analytical results for extracted samples ($n = 6$) with unextracted (spiked blank plasma extract) standards that represent 100% recovery. Comparison between the unextracted samples, spiked, and plasma residues was done in order to eliminate a matrix effect, giving a true recovery. The intra and inter-day precision and accuracy of the analytical methods were evaluated by triplicate processing and following analysis of QC samples (0.3, 6.5, 12.8 μg/mL). Precision was calculated as the relative standard deviation (R.S.D.) of the experimental concentrations and accuracy by the comparison between the experimental and nominal samples concentration. The criteria for acceptability of the data included accuracy within ±15% deviation from the nominal values and precision within

±15% R.S.D., except for the lower limit of quantification (LLOQ), where it should not exceed 20% of R.S.D.[11]

Stability Studies

The stability of BS in rat plasma was tested using low (0.3 μg/mL) and high (12.8 μg/ml) quality control samples for three freeze thaws, short term, long term, and processed sample stability. For the short term stability, the frozen plasma samples (−20°C) were kept at room temperature for 4 h before sample preparation. The freeze thaw stability of the BS in plasma was determined over three freeze thaw cycles within 3 days. In each freeze thaw cycle, the spiked plasma samples were frozen at −20°C for 24 h and thawed at room temperature. The long term stability was evaluated after keeping the plasma samples frozen at −20°C for 70 days. The stability of the prepared plasma samples was tested after keeping the samples in an autosampler at 4°C for 12 h. The samples were analyzed and the results were compared with those obtained for the freshly prepared samples.

Pharmacokinetic Studies

The animal experiments were approved by the Federal University of Rio Grande do Sul Ethics in Research Committee (#2007794).

The pharmacokinetic study was carried out in five male Wistar rats. The animals were fasted overnight (12 h) and had access to water throughout the experimental period. The animals were fed 4 h after drug administration. BS was administered at a 100 mg/kg oral dose (p.o.), suspended in 0.9% NaCl solution containing 10% of polissorbate 80 (Tween 80®) and 25% of dimethyl sulfoxide (DMSO) through gavage. Blood samples were collected into heparinized tubes at different time points (0, 0.25, 0.5, 1, 2, 4, 6, 8, and 10 h) from the tail vein and centrifuged at 15000 rpm, 4°C for 10 min to separate the plasma. Plasma (100 μL) samples were prepared as described under Sample Separation Section for BS quantification.

RESULTS AND DISCUSSION

Selectivity and Recovery

The selectivity of the analytical methodology can be observed in Fig. 2 by comparing representative chromatograms of independent blank plasma (a), blank plasma spiked with IS (25 μg/mL) (b), and a rat plasma sample containing BS and IS (c). BS and IS were well separated from each other. No additional peaks due to endogenous substances were observed that

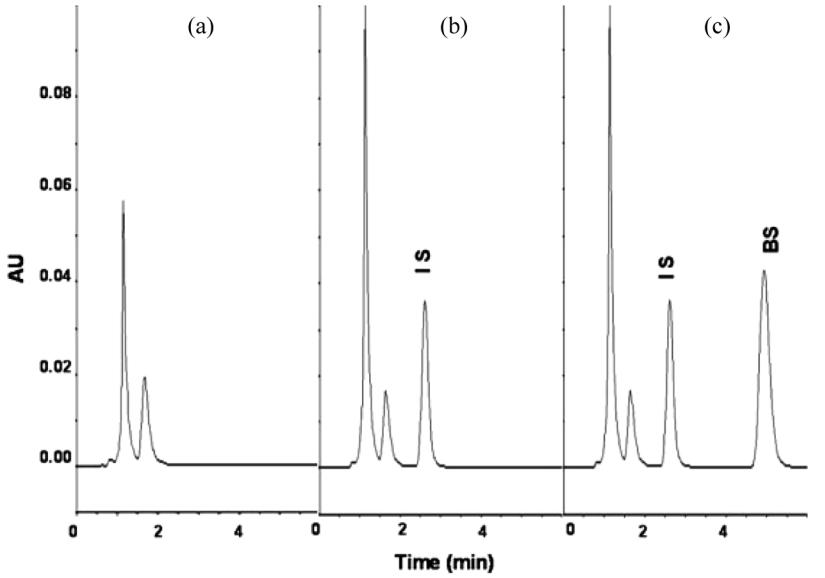


FIGURE 2 Representative HPLC chromatograms of the method: (a) blank rat plasma, (b) rat plasma spiked with IS (25 μg/mL), and (c) 2 h plasma sample (11.9 μg/mL) from a rat dosed with BS at 100 mg/kg p.o and IS (25 μg/mL).

would interfere with the detection of the target compounds. The retention times of BS and IS were approximately 4.9 and 2.6 min, respectively (Fig. 2c).

Cold acetonitrile and cold methanol were tested as a solvent for extracting BS from rat plasma. When acetonitrile was used as the extracting solvent, the recovery showed a lower yield compared with extraction with methanol (data not shown). Thus, methanol was selected as a solvent for extraction. The extraction recovery of BS and IS was $85.5 \pm 7.4\%$ and $79.4 \pm 2.8\%$, respectively.

Quantitation Limit

The LLOQ was 0.1 μg/mL, which was the lowest concentration of analyte in a sample that could be determined with acceptable precision and accuracy[11] under the stated experimental conditions for this method.

Linearity

The standard calibration curves were linear over the BS concentration ranges of 0.1–16.0 μg/mL in rat plasma. Regression results and statistics from calibration standard curves on two successive days are shown in Table 1. Calibration curves were linear using weighted (1/concentration)

TABLE 1 Calibration Curve Parameters and Statistics for BS in Rat Plasma

Curve	Slope	y-Intercept	Determination Coefficient
Day 1			
1	0.1467	−0.0070	0.9994
2	0.1370	−0.0038	0.9996
3	0.1345	−0.0019	0.9999
Day 2			
1	0.1413	−0.0082	0.9995
2	0.1439	−0.0059	0.9983
3	0.1459	0.0009	0.9998
Mean ($n=6$)	0.1416		
S.D.	0.0049		
R.S.D. (%)	3.49		

S.D., standard deviation; R.S.D., relative standard deviation.

linear regression ($r^2=0.9997$, $y=0.1416x+(-0.0037)$, where x is concentration and y is the peak area ratio of the BS to the IS). All back calculated values of the individual calibration standards were within 15% of the spiked value, except the LLOQ, which was within 20%.[11]

Precision and Accuracy

The intra- and inter-day relative standard deviation values for BS are shown in Table 2. The intra-assay and inter-assay precision for QC samples were less than or equal to 10.19% and 2.10%, respectively. The method had an accuracy within 15% (Table 3). Considering analyte measurement in the biological matrix, the criteria of acceptance for accuracy and precision was met.[11]

TABLE 2 Intra and Inter-Day Variation of BS in Rat Plasma

		Measured Concentrations		
Spiked Concentration	Day	Mean (μg/mL)	S.D.	R.S.D. (%)
Intra-day variation				
0.3 μg/mL	1	0.31	0.0316	10.19
	2	0.31	0.0284	9.13
6.5 μg/mL	1	6.12	0.4771	7.80
	2	6.30	0.2592	4.11
12.8 μg/mL	1	12.42	0.9684	7.80
	2	12.56	0.9225	7.35
Inter-day variation				
0.3 μg/mL		0.31	0.0003	0.09
6.5 μg/mL		6.21	0.1304	2.10
12.8 μg/mL		12.49	0.0967	0.77

Values are for n = 3 observations; S.D., standard deviation and R.S.D., relative standard deviation.

TABLE 3 Accuracy for the Analysis of BS in Rat Plasma

Concentration (μg/mL)	Range (μg/mL)	Accuracy (%)
0.3	0.27–0.34	91.48–114.50
6.5	5.69–6.64	87.61–107.72
12.8	11.37–13.47	88.81–104.95

n = 6 observations.

Stability

The stability was assessed under a variety of conditions (Table 4). Three freeze thaw cycles with the quality control samples did not affect the quantification of the BS (>97.0%). The analyte was stable in plasma samples at room temperature for 4 h (>98.0%) and in plasma samples after freezing at −20°C for 70 days (>92.0%). The stability of the processed samples was determined at 4°C in the autosampler for 12 h. The BS did not degrade under these conditions (>95.0%).

Applicability of the Analytical Method

To investigate the suitability of this analytical method for pharmacokinetic studies, male Wistar rats received BS as a single dose (100 mg/kg) by the oral route (n = 5). The mean plasma concentration time profile of BS is shown in Figure 3. The results indicated that the analytical method is suitable to measure plasma concentrations of the compound in preclinical studies. The peak plasma concentration (C_{max}) was 14.4 ± 2.6 μg/mL, achieved between 1 and 2 h. The estimated half-life ($t_{1/2}$) was found to be 1.0 ± 0.2 h and the area under the plasma concentration-time ($AUC_{0-\infty}$) was 69.1 ± 18.8 μg · h/mL. The study could be conducted for almost six

TABLE 4 Stability Results of BS in Rat Plasma Expressed as Drug Percentage in Relation to Day One

	Drug Percentage			
	0.3 μg/mL		12.8 μg/mL	
Stability	Mean ± S.D.	R.S.D. (%)	Mean ± S.D.	R.S.D. (%)
Short-term stability[a]	99.29 ± 2.78	2.80	98.57 ± 1.16	1.17
Freeze-thaw stability[b]	99.18 ± 2.94	2.96	97.73 ± 6.07	6.21
Long-term stability[c]	93.53 ± 5.19	5.55	92.20 ± 1.28	1.39
Processed sample stability[d]	98.87 ± 1.04	1.05	95.86 ± 0.20	0.21

Values are for n = 3 observations; S.D., standard deviation, R.S.D., relative standard deviation; [a]At room temperature for 4 h; [b]Three freeze–thaw cycles within 3 days for 24 h and thawed at room temperature; [c]At −20°C for 70 days; [d]In an autosampler at 4°C for 12 h.

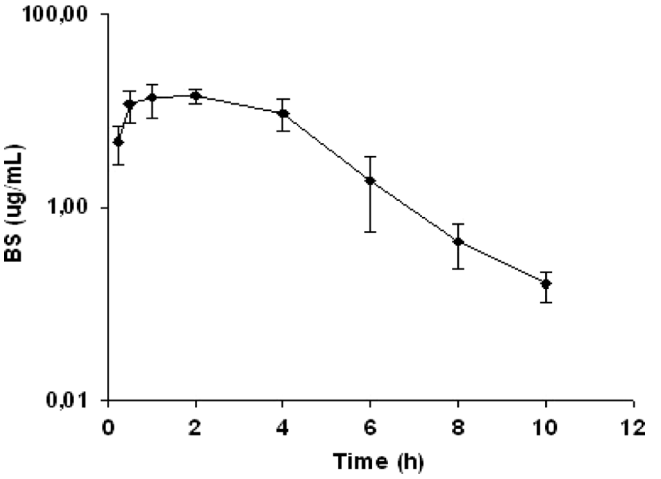


FIGURE 3 Mean plasma concentration-time profile of BS after single oral dose of 100 mg/kg to male Wistar rats (mean ± S.D., n = 5).

half-lives and the extrapolated AUC_{extrap} was approximately 0.6%, proving that the method has adequate sensitivity to properly analyze the BS pharmacokinetics in preclinical studies.

CONCLUSION

A simple and efficient HPLC method with UV detection was developed and validated for the determination of BS in rat plasma using sodium diclofenac as IS. This method showed adequate sensitivity, linearity, precision, and accuracy and it has been successfully applied to determine the BS concentration time profiles in a pharmacokinetic study in rodents.

Detailed pharmacokinetic evaluation of BS after administration of different doses by diverse routes in a complete preclinical investigation will be reported in a separate article.

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