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Liquid chromatographic analysis, stability and protein binding studies of the anti-HIV agent benzoic acid, 2-chloro-5[[(1-methylethoxy)thioxomethyl]amino]-,1-methylethyl ester

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Abstract: A method was developed for the assay of benzoic acid, 2-chloro-5-[[(1-methylethoxyl)thioxomethyl]amino]–, 1-methylethyl ester (NSC 629243) in hamster, mouse, human and, to a limited extent, dog plasma. Protein in 0.5 ml of plasma was precipitated with four volumes of methanol and the supernatant was analysed for NSC 629243 by LC. Liquid chromatography was carried out on a reversed-phase Nova-Pak C_{18} column, with a mobile phase of 60% acetonitrile in water at 1 ml min^{-1}, and the compound was quantified with a UV detector set at 283 nm. Two standard curves of NSC 629243 were needed to cover a concentration range of 0.05–100 μg ml^{-1}. All standard curves had correlation coefficients >0.999. In practice, the minimum quantifiable concentration was approximately 0.05 μg ml^{-1} in 0.5 ml of plasma. NSC 629243 appeared to have good stability at 37°C in hamster, human and dog plasma at concentrations of 1 and 50 μg ml^{-1} (at least 80% remained in plasma after a 4 h incubation). Breakdown occurred in mouse plasma after 1 h at 37°C, with extensive breakdown occurring after 24 h. NSC 629243 was extensively bound to plasma proteins of Syrian hamsters and humans. The extent of binding ranged from a minimum of 88.6% to a maximum of 99.9% over a concentration range of *ca* 1–100 μg ml^{-1}.

Keywords: *Reversed-phase LC; protein binding; stability; anti-HIV agent, NSC 629243.*

Introduction

Benzoic acid, 2-chloro-5-[[(1-methylethoxy)-thioxomethyl]amino]–, 1-methylethyl ester (NSC 629243) (Fig. 1), is a derivative of 2-chloro-5-aminobenzoic acid, in which both the carboxyl and amino groups are derivatized. It contains the thiocarbamate structure, which is similar to the carbamate and dithiocarbamate structures present in many insecticides, fungicides and herbicides [1–3]. This chemical has demonstrated anti-HIV activity in the NCI AIDS antiviral screen, and is undergoing preclinical pharmacology evaluation under the sponsorship of the NCI. Preclinical pharmacokinetic studies in laboratory animals are needed in order to evaluate this drug candidate in humans. However, in order to obtain these data, a specific, sensitive and reproducible analytical method must be first developed.

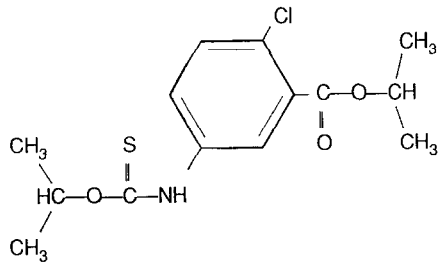

Figure 1
Chemical structure of NSC 629243.

This study reports on the analytical method developed for the analysis of NSC 629243 in mouse, Syrian hamster, dog and human plasma. The method was used to investigate the stability of this drug candidate in plasma of the above-mentioned species, and to study its binding to human and Syrian hamster plasma protein.

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Materials and Methods

Materials

Unlabelled NSC 629243, [7-^{14}C]benzoate-labelled NSC 629243 (49.8 mCi mmol^{-1}), and ring-labelled [^{14}C]NSC 629243 (22 mCi mmol^{-1}) were provided by the Drug Synthesis and Chemistry Branch, OTP, NCI. The chemical and radiochemical purities were reconfirmed prior to use by HPLC using the system described below. 1-Methylethyl-2-chloro-4-fluoro-5[[(1-methylethoxy)thiomethyl]amino]-benzoate (NSC 624577), used as internal standard for the analysis was also obtained from the NCI. All solvents used for chromatography were HPLC grade, purchased from Caledon Laboratories Ltd (Georgetown, Ontario, Canada), and water was deionized and glass-distilled. All HPLC solvents were filtered through 0.45 μm filters and deaerated by sonication and vacuum before use. All chemicals were of analytical grade or better.

Mouse plasma was obtained from male CDF_1 mice, supplied by the NCI. Beagle dog plasma was obtained from an in-house colony. Human plasma used for analytical method development was purchased frozen from the American Red Cross (Northeast Region, Dedham, MA). Human plasma used for stability studies was obtained from a volunteer. Plasma used for stability studies was freshly collected and prepared using heparin as the anticoagulant.

Liquid chromatography

The LC instrument consisted of two model 6000A solvent delivery systems, a model 680 automated gradient controller, a model 730 data module, a WISP model 710B sample processor (all Waters Associates, Milford, MA), a Kratos Spectroflow model 757 UV/VIS absorbance detector (Kratos Analytical, Ramsey, NJ) set at 283 nm, and a Radiomatic Instruments Flo-One/beta model IC radioactive flow detector (Radiomatic Instruments & Chemical Co., Tampa, FL). A Nova-Pak C_{18} 4 μm stainless steel column (15 cm × 4 mm i.d.) preceded by a Guard-Pak C_{18} precolumn (both Waters Associates), was used. The solvent system was an isocratic elution with 60% acetonitrile in water at a flow rate of 1.0 ml min^{-1}. The retention time of NSC 629243 was *ca* 7.5 min. The retention time of the internal standard NSC 624577 was *ca* 5 min. NSC 629243 was quantified from the peak area ratio relative to the internal standard.

Analytical method development

Extraction of NSC 629243 from plasma. The following methods were evaluated using mouse plasma spiked with a 5 μg ml^{-1} concentration of NSC 629243: Precipitation of plasma protein with methanol, zinc sulphate or trichloroacetic acid; and solid-phase extraction of NSC 629243 from plasma on to Sep-Pak C_{18} cartridges (Waters Associates), followed by elution with either methylene chloride or methanol.

Triplicate samples and triplicate blank plasma were prepared with each method. Following protein precipitation and/or extraction, the samples were subjected to analysis. Following evaluation of the recovery and reproducibility, the final procedure involved the precipitation of protein from plasma with methanol at a 4:1 methanol–plasma ratio, followed by centrifugation. For a concentration range of 10–100 μg ml^{-1} of NSC 629243 in plasma, a volume of the supernatant was taken, and the internal standard NSC 624577 was added in a minimum volume of methanol (33 μl to 2 ml supernatant) at a final concentration of 50 μg ml^{-1}; samples were then analyzed by LC. For a concentration range of 0.05–10 μg ml^{-1}, the same protein precipitation procedure was employed, then 2 ml of the supernatant were evaporated to dryness. The residue was reconstituted in 250 μl of methanol containing the internal standard at a concentration of 50 μg ml^{-1}, and analysed by LC. The working plasma volume was 0.5 ml.

Solubility of NSC 629243 in plasma. NSC 629243 is practically insoluble in aqueous solutions (<0.005 μg ml^{-1} in 0.1 M phosphate buffer, pH 7.4 at 4 or 37°C), and moderately soluble in methanol. In order to establish that the compound was soluble in plasma at the highest concentration to be used, it was added to hamster plasma at 100 μg ml^{-1}, vortexed, and kept on ice for 10 min. Half the volume was filtered through a Whatman No. 50 filter paper. Duplicates of the filtered and unfiltered plasma were analysed for NSC 629243 by LC.

Standard curves and recovery of NSC 629243 in plasma. Two standard curves in plasma were needed to cover a concentration range of 0.05–

100 μg ml^{-1}; their ranges were 10–100 and 0.05–10 μg ml^{-1}. These curves were prepared using hamster plasma. A methanolic standard curve was used to determine the recovery of NSC 629243 from plasma. To validate the analytical method developed with the hamster plasma, and to evaluate whether the hamster plasma standard curve could be used to analyse the drug candidate in mouse and human plasma samples, the following study was performed. Mouse, hamster and human plasma were spiked at three concentrations of NSC 629243, 0.5, 10 and 100 μg ml^{-1}. Triplicate samples of each concentration were processed and analysed, and triplicate blank plasma samples from each species were also prepared. The concentration and recovery of NSC 629243 in mouse, hamster and human plasma samples were determined using the corresponding hamster plasma standard curves. In addition, the recovery was calculated using the methanolic standard curve.

Stability of NSC 629243 in plasma

The stability of NSC 629243 was investigated at 37°C in Syrian hamster, mouse, dog and human plasma. NSC 629243 was added to plasma samples at concentrations of 1 and 50 μg ml^{-1}. Plasma samples were incubated in a water-bath at 37°C for 0 and 10 min and 1, 2, 4, 6 and 24 h, and then analysed for parent compound by LC. The peak area ratio determined at zero time was used to calculate the percentage of NSC 629243 remaining after each incubation period.

Protein binding studies of NSC 629243

Equilibrium dialysis techniques are unsuitable for plasma protein binding studies with NSC 629243, due to its insufficient solubility in aqueous solutions such as phosphate buffer. Therefore, studies were carried out to determine plasma protein binding by ultrafiltration techniques [4, 5]. In order to perform protein binding studies, non-specific binding to ultrafiltration membranes was first evaluated using human plasma ultrafiltrate.

The results of the non-specific binding studies were satisfactory. Consequently, studies were carried out to determine the extent of binding of NSC 629243 to Syrian hamster and human plasma proteins. Frozen human plasma, and frozen and fresh Syrian hamster plasma, were used in these studies. Frozen plasma was thawed by ultrasonication at room temperature, and was vortexed prior to use. Three concentrations of NSC 629243 in plasma, 1.1, 9.3 and 95.7 μg ml^{-1}, were investigated. ^{14}C-Radiolabelled and unlabelled NSC 629243 were dissolved in methanol to prepare the spiking solutions. Three samples of each of the thawed Syrian hamster and human plasma samples (6–6.5 ml) were spiked with [^{14}C]NSC 629243 at the above-mentioned concentrations. An additional fresh hamster plasma sample (6 ml) was spiked at 95.7 μg ml^{-1} to determine if freezing had any effect on the extent of protein binding. Spiked plasma samples were vortexed, allowed to equilibrate for 10 min at room temperature, revortexed, and then filtered through Whatman No. 1 paper. Samples of the filtrate were counted to determine the initial radioactivity. Three aliquots of each plasma sample (1.8–2 ml each) were transferred to Ultrafree CL ultrafiltration tubes (Millipore Corp., Bedford, MA) with a molecular weight cut-off of 5000 Da. The aliquots were centrifuged in a fixed-angle rotor at 2000 *g* for 4 h, during which *ca* 0.5–0.8 ml ultrafiltrate was collected. Samples of the ultrafiltrate and the plasma retained on the filter were counted to determine the radioactivity. The ultrafiltration tubes were disassembled, rinsed with water, the o-rings were rinsed with methanol, and the filter was combusted in order to determine the total recovery of [^{14}C]NSC 629243.

Two values for protein binding were calculated; the maximum and the minimum. The maximum binding of NSC 629243 to protein was calculated using the following formula:

$$\text{Maximum protein bound (\%)} = \frac{\text{DPM/100 μl original plasma} - \text{DPM/100 μl ultrafiltrate}}{\text{DPM/100 μl original plasma}} \times 100. \quad (1)$$

Original plasma depicts plasma that was transferred to the ultrafiltrate tube prior to centrifugation. The minimum level of protein binding was calculated based on the assumption that the radioactivity remaining in the membrane, the radioactivity extracted from the o-ring, and that which was unaccounted for, were non-protein bound.

Results

Analytical method development

The data for an initial methanolic standard curve, with a concentration range of 0.033–33 μg ml^{-1}, were fitted to a linear regression model. The correlation coefficient was 0.9998. Protein precipitation with trichloroacetic acid and zinc sulphate resulted in recovery values for the compound from plasma of 20 and 43%, respectively. Solid-phase extraction, followed by elution with methanol or methylene chloride, resulted in recoveries from plasma of 25 or 84%, respectively. Protein precipitation using four volumes of methanol for each volume of plasma resulted in an average recovery of 85%. This method was selected because the solid-phase extraction procedure failed to give reproducible results with a different lot of Sep-Pak cartridges. The concentrations of NSC 629243 in filtered and unfiltered hamster plasma samples which had been spiked with the drug candidate at 100 μg ml^{-1} were very similar, indicating that the compound was soluble in plasma at 100 μg ml^{-1}.

Table 1 shows the parameters of standard curves of NSC 629243 in methanol and in hamster plasma. In all cases, the correlation coefficients were >0.999. The recovery of NSC 629243 from the Syrian hamster plasma samples (which were used for the standard curve) based on methanol standard curves is presented in Table 2. Recovery values ranged from *ca* 83–110% of the nominal concentrations.

In order to evaluate whether the analytical method could be used with other species, the method was validated in human and mouse plasma, while Syrian hamster plasma was used for comparison. Three concentrations (0.5, 10 and 100 μg ml^{-1}) were prepared in each plasma, in addition to three blank samples. Table 3 shows the concentrations and recovery of NSC 629243 in plasma of mice, hamsters and humans, calculated based on the Syrian hamster plasma standard curve. These results indicate that satisfactory recovery values were obtained. In addition, there were no interfering peaks in blank plasma of the above species. These findings demonstrate that a standard curve prepared in plasma of one species may be used to determine an unknown concentration in plasma samples from the other two species within the range studied.

Stability of NSC 629243 in mouse, hamster, human and dog plasma

In order to determine the appropriate con-

Table 1
Parameters of standard curves

Type curve	Concentration range* (μg/ml)	Correlation coefficient	Slope	Intercept
Hamster plasma	0.05–10	0.999883	0.58	0.026
	10–100	0.999973	0.076	0.007
Methanol	0.05–25	0.999900	0.386	−0.012

*Each standard curve included at least five concentrations. Duplicate LC analyses of each of two replicates at each concentration were performed.

Table 2
Recovery of NSC 629243 from hamster plasma

	Conc. of NSC 629243 in plasma (μg ml^{-1})	
Added	Found* (mean ± SD)	% Recovery (mean ± SD)
0.05†	0.055 ± 0.003	110.26 ± 6.53
0.25†	0.23 ± 0.01	93.27 ± 5.72
1†	0.96 ± 0.01	96.03 ± 1.42
5†	4.16 ± 0.22	83.11 ± 4.40
10†	8.28 ± 0.67	82.84 ± 6.75
10‡	9.13 ± 0.31	91.33 ± 3.09
12.5‡	11.97 ± 0.25	96.87 ± 1.98
25‡	22.80 ± 0.07	91.20 ± 0.27
50‡	45.85 ± 1.34	91.70 ± 2.68
100‡	91.08 ± 3.00	91.08 ± 3.00

*Mean of three replicates, with duplicate injections of each.
†These samples were concentrated by evaporation to dryness, then reconstituted.
‡These samples were not concentrated before analysis.

Table 3
Quantification and recovery of NSC 629243 from mouse, hamster and human plasma samples based upon hamster plasma standard curve

Species	Plasma conc. ($\mu g\ ml^{-1}$) Added	Found*	% Recovery
Mouse	0.5	0.49 ± 0.01	98.88 ± 1.99
	10	9.46 ± 1.25	94.62 ± 12.49
	100	80.97 ± 6.22	80.97 ± 6.22
Hamster	0.5	0.50 ± 0.01	101.18 ± 9.12
	10	9.99 ± 0.39	99.86 ± 3.93
	100	95.59 ± 5.00	95.59 ± 5.00
Human	0.5	0.51 ± 0.05	100.03 ± 1.99
	10	9.90 ± 0.53	98.99 ± 5.29
	100	93.01 ± 2.39	93.01 ± 2.39

*Values are means ± SD of six determinations from three replicates.

ditions of handling plasma samples containing NSC 629243, the stability of the agent in plasma was evaluated. The results of the stability studies are shown in Table 4. At 37°C, NSC 629243 appeared to be stable in human plasma for at least 4 h at 1 $\mu g\ ml^{-1}$ and for at least 24 h at 50 $\mu g\ ml^{-1}$. In dog plasma, the compound was stable for at least 24 h at the two concentrations studied at the same temperature. The apparent decrease in stability observed in dog plasma at 4 h (77.5% ± 11.6% remaining) followed by increase in stability at later time points is most likely due to experimental error. In hamster plasma at 37°C, NSC 629243 was stable for at least 2 h at 1 μg and for at least 6 h at 50 $\mu g\ ml^{-1}$. In mouse plasma, the compound was least stable as indicated by the fact that only 55 and 65% of the original concentrations remained after 1 h of incubation at 37°C for the 1 and 50 $\mu g\ ml^{-1}$ concentrations, respectively. No attempt was made to identify the breakdown products of NSC 629243.

Plasma protein binding of NSC 629243

The data in Table 5 indicate that NSC 629243 was extensively bound to plasma proteins. The extent of binding ranged from 88.6% (minimum) to 99.9% (maximum); it was similar for both Syrian hamster and human plasma, and was unaffected by the concentration over the range studied (*ca* 1–100 $\mu g\ ml^{-1}$). Based on the fact that the extent of binding was unaffected by the concentration over the range studied, the actual values for protein binding were probably closer to the maximum, at least for the 1.1 and 9.3 $\mu g\ ml^{-1}$ concentrations. The extent of protein binding was similar for both the fresh and frozen hamster plasma. The total recovery of [^{14}C]NSC 629243 was *ca* 92–98%.

Table 4
Stability of NSC 629243 in plasma at 37°C

Species	Conc. ($\mu g\ ml^{-1}$)	NSC 629243 remaining (%)* 10 min	1 h	2 h	4 h	6 h	24 h
Human	1	95.8 ± 1.2	92.3 ± 1.6	93.3 ± 0.6	90.3 ± 0.9	83.9 ± 5.2	73.8 ± 8.3
	50	96.4 ± 1.4	93.7 ± 2.3	91.7 ± 1.2	87.4 ± 0.9	89.5 ± 2.1	88.5†
Mouse	1	80.3 ± 2.6	54.5†	31.8 ± 6.3	24.8 ± 2.6	21.4 ± 3.7	16.7 ± 4.2
	50	90.8 ± 2.1	65.4 ± 4.2	41.5 ± 0.9	6.7 ± 0.8	13.0 ± 1.3	5.2 ± 0.9
Dog	1	84.9 ± 3.0	90.3 ± 8.8	102.8 ± 4.8	77.5 ± 11.6	98.0 ± 5.1	92.9 ± 10.3
	50	100.8 ± 1.2	103.2 ± 0.3	102.4 ± 1.4	100.4 ± 1.3	100.2 ± 1.7	101.2 ± 5.2
Hamster	1	93.9 ± 12.4	95.5 ± 5.2	89.2 ± 5.1	80.7 ± 2.7	71.7 ± 2.4	63.9 ± 2.8
	50	96.3 ± 2.7	89.7 ± 1.1	91.5 ± 1.0	85.5 ± 0.6	84.8 ± 2.0	76.3 ± 1.3

*Data represent the mean ± SD of triplicate determinations.
†Only two samples.

Table 5
Binding of NSC 629243 to Syrian hamster and human plasma proteins

Conc. in plasma ($\mu g\ ml^{-1}$)	Human protein bound (%)* Minimum	Maximum	Recovery (%)	Syrian hamster protein bound (%)* Minimum	Maximum	Recovery (%)
1.1	93.9 ± 4.2	99.8 ± 0.1	97.6 ± 3.5	90.2 ± 1.5	99.8 ± 0.1	92.7 ± 1.2
9.3	88.6 ± 2.4	99.7 ± 0.0	91.6 ± 2.4	89.4 ± 0.8	99.9 ± 0.1	91.8 ± 1.2
95.7	87.9 ± 1.9	99.9 ± 0.0	91.9 ± 1.7	89.8†	99.9†	92.6†
95.7 (fresh plasma)	—	—	—	90.4 ± 1.7	99.8 ± 0.1	92.8 ± 1.5

*Values are mean ± SD of three replicates unless otherwise noted.
†Mean of two replicates.

Discussion

The analytical method developed for NSC 629243 covers a concentration range of 0.05–100 μg ml^{-1} in 0.5 ml plasma. The method was used successfully to analyse NSC 629243 in mouse, Syrian hamster and human plasma, and to a limited extent in dog plasma. However, it should be pointed out that the stability of this compound in mouse plasma was very limited at 37°C. The method was subjected to extensive evaluation prior to use [6]. The correlation coefficients for the standard curves were >0.999. A standard curve prepared in mouse, Syrian hamster or human plasma may be used to determine the concentration in plasma sample of the other two species within at least the 0.5–100 μg ml^{-1} concentration range. The recovery of NSC 629243 from hamster plasma, based on a methanol standard curve, was satisfactory. The recovery of NSC 629243 from mouse and human plasma was similar to that of hamster plasma, with the exception of mouse plasma, where lower recovery (*ca* 74%) was observed at the 100 μg ml^{-1} concentration only. The limit of detection was less than 0.05 μg ml^{-1}. The minimum limit of quantification was calculated to be 0.29 μg ml^{-1} (95% confidence limit) and 0.49 μg ml^{-1} (99% confidence limit) [7]. In practice, we were able to quantify 0.05 μg ml^{-1} in 0.5 ml plasma.

The precision and accuracy of the method were calculated as the relative standard deviation and the relative error, respectively, for each of the three replicates from hamster plasma standard curve. The precision for the 0.05–10 μg ml^{-1} standard curve ranged from 1.0 to 8.3% with a mean of 5.4%, and for the 10–100 μg ml^{-1} curve, from 0.3 to 3.1% with a mean of 2.2%. The accuracy ranged from a mean of 4–17% for the lower concentration standard curve and from 4.1 to 8.9% for the higher concentration standard curve. The precision and accuracy were better for the higher concentration standard curve, where no concentration and reconstitution took place.

The stability of NSC 629243 was evaluated in Syrian hamster, mouse, dog and human plasma at 1 and 50 μg ml^{-1} concentrations at 37°C. Overall, the stability of NSC 629243 in plasma of the four species was in the following descending order: dog > human > Syrian hamster ⩾ mouse. These data suggest that the stability profile of NSC 629243 in human plasma was closer to that of the Syrian hamster than that of the mouse.

Protein binding studies with NSC 629243 were carried out with human and Syrian hamster plasma at *ca* 1, 10 and 100 μg ml^{-1} concentrations. NSC 629243 was extensively bound to plasma protein. The extent of protein binding was from 88.6% (minimum) to 99.9% (maximum), with more bias toward the higher value. The extent of protein binding was similar for both Syrian hamster and human plasma, was unaffected over the concentration range studied, and was similar for fresh and previously frozen Syrian hamster plasma.

In conclusion, this study demonstrates a suitable and simple analytical method for the analysis of NSC 629243 in plasma. NSC 629243 is extensively bound to plasma proteins. This drug candidate is more stable in human, dog and Syrian hamster plasma than it is in mouse plasma.

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