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High-performance liquid chromatographic analysis of the anticancer drug oxantrazole in rat whole blood and tissues

Emad Eldin Hassan and James M. Gallo

Department of Pharmaceutics, College of Pharmacy, University of Georgia, Athens, GA 30602 (USA)

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ABSTRACT

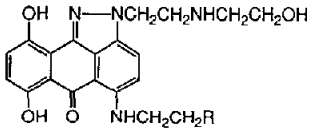
A reversed-phase high-performance liquid chromatographic (HPLC) assay was developed for the antitumor anthrapyrazole analogue, oxantrazole (OX), in rat whole blood and tissues. Blood samples were mixed with equal volumes of a 25% (w/v) aqueous solution of L-ascorbic acid, whereas tissue samples were homogenized with 1.5–3 volumes of an L-ascorbic acid–methanol–water (1:10:1, w/v/v) mixture to prevent oxidative degradation of OX. Samples were then treated with 60% (v/v) perchloric acid (25–30 μl/ml of stabilized sample) to precipitate proteins, and centrifuged, with the resultant supernatants analyzed on HPLC utilizing a C_8 column. The mobile phase for blood and urine samples consisted of 8% (v/v) glacial acetic acid, 13% (v/v) acetonitrile, 79% (v/v) water, 0.16% (w/v) sodium acetate, and 0.05% (w/v) L-ascorbic acid (final pH 2.7), and was pumped at 1.8 ml/min. Tissue samples were eluted at 2 ml/min with a mobile phase consisting of 8% (v/v) glacial acetic acid, 12% (v/v) acetonitrile, 80% (v/v) water, 0.16% (w/v) sodium acetate, and 0.0;5% (w/v) L-ascorbic acid. OX and internal standard were detected at 514 nm and had retention times of 2.3 and 3.1 min, respectively. The limit of quantitation of OX was 25–50 ng/g. Recovery of OX from biological samples ranged from 50 + 0.9% in spleen to 102.8 ± 1.8% in RG-2 glioma. The analytical method was applied to a pharmacokinetic study in rats.

INTRODUCTION

The dose-dependent cardiomyopathy induced by anthracyclines, such as adriamycin, frequently limits their use in cancer therapy [1,2]. Oxantrazole (OX), an anthrapyrazole anticancer agent (see Fig. 1), has been investigated clinically based on its broad spectrum of antitumor activity [3–6] and lower myocardial cell toxicity than adriamycin [7].

Few analytical methods and pharmacokinetic studies of OX are available, and there are no reports of its tissue distribution. Analysis of OX will require consideration of its adsorption onto glass containers at neutral and alkaline pH and its rapid decomposition in plasma [8,9]. OX undergoes a pH-dependent oxidative degradation with an *in vitro* half-life as short as 5 min in human plasma [10]. The degradation process can be inhibited by acids and antioxidants. OX is stable at pH values less than 6 [8].

High-performance liquid chromatographic



R = CH_2 - NH_2, Oxantrazole
R = CH_2 - CH_2 - NH_2, Internal Standard

Fig. 1. Structures of oxantrazole and internal standard.

Correspondence to: Dr. J. M. Gallo, Department of Pharmaceutics, College of Pharmacy, University of Georgia, Athens, GA 30602, USA.

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(HPLC) methods for the determination of OX in plasma and urine have been reported [5,9]. In the plasma assay, OX was isolated by solid-phase extraction from sodium ascorbate–citric acid-stabilized plasma prior to separation on a C_2 reversed-phase analytical column [9]. There have been no reports on the quantitation of OX in whole blood or biological tissues. In this report, a simple HPLC assay of OX in whole blood and tissues in the rat is presented. The procedure was used to characterize the pharmacokinetics of OX in the rat and was applied to the analysis of OX in tissue obtained from rats bearing brain tumors.

EXPERIMENTAL

Chemicals and reagents

OX and a structurally related internal standard, 5-[(4-aminobutyl)amino]-7,10-dihydroxy-2-[2-[(2-hydroxyethyl)amino]ethyl]anthra[1,9-*cd*]-pyrazol-6(2*H*)one, Fig. 1, were generously provided by Warner-Lambert (Ann Arbor, MI, USA). Analytical-grade sodium acetate, glacial acetic acid, and perchloric acid (60%, w/v) and HPLC-grade methanol and acetonitrile were purchased from J. T. Baker (Phillipsburg, NJ, USA). L-Ascorbic acid was obtained from Sigma (St. Louis, MO, USA). Water was double-distilled, deionized water. All other chemicals were of analytical grade.

Equipment

The chromatographic system consisted of a Waters (Milford, MA, USA) Model 510 pump, a Model 480 variable-wavelength detector, an Allcott (Norcross, GA, USA) Model 738 autosampler, and a Hewlett-Packard (Avondale, PA, USA) Model 3396A integrator. Chromatographic separations were performed with a Zorbax RX-C_8 analytical column (150 mm × 4.6 mm I.D., 5 μm), with a 12.5 mm × 4 mm I.D. guard cartridge purchased from Mac-Mod Analytical (Chadds Ford, PA, USA).

Chromatography

The composition of the mobile phase was empirically optimized to determine the optimal acetonitrile and acetic acid concentrations. Chromatographic parameters such as resolution and peak asymmetry factor were utilized for the empirical optimization procedure [11]. Mobile phase for blood and urine analyses consisted of 8% (v/v) glacial acetic acid, 13% (v/v) acetonitrile, 79% (v/v) water, 0.16% (w/v) sodium acetate trihydrate, and 0.05% (w/v) L-ascorbic acid. The mobile phase had a final pH of 2.70 and was pumped at 1.8 ml/min. For all tissues, the mobile phase acetonitrile concentration was 12% (v/v), water was 80% (v/v), while all other components were at the same concentration as for blood analysis. The flow-rate of the mobile phase for tissue analysis was 2 ml/min. All chromatographic separations were performed at ambient temperature.

Sample preparation

A blood sample (0.2 ml) was incubated with an equal volume of 25% (w/v) ascorbic acid, 10 μl of internal standard (25 μg/ml), and 10 μl of 7.8% (w/v) sodium citrate at room temperature for 15 min to hemolyze the blood. A 20-μl volume of 60% (v/v) perchloric acid was added to precipitate proteins, followed by centrifugation at 6875 *g* for 15 min. A 50-μl aliquot of the clear supernatant was injected onto the HPLC system. Urine samples were analysed directly after adding the internal standard and diluting with mobile phase.

Tissue samples were homogenized in a 1:1.5 ratio (g/ml) with an L-ascorbic acid–methanol–water (1:10:1, w/v/v) mixture over dry ice. Tissue homogenate, 0.4 or 0.7 ml, was mixed with 10 μl of the internal standard (12.5 or 25 μg/ml). Each sample was then treated with 10 or 20 μl of 60% (v/v) perchloric acid and centrifuged at 6875 *g* for 10 min, and 50 or 100 μl of the resultant supernatants were injected onto the HPLC system.

Assay validation

Absolute recovery of OX and the internal standard was determined from their peak-height ratios obtained from the analysis of citrated whole blood, urine, and tissue homogenates containing known amounts of OX and internal standard, and the analysis of similarly prepared samples in

water. The precision of the blood analysis method was evaluated in terms of within-day and between-day variability, while the precision of tissue analyses was estimated by within-day variability only. Accuracy of the analytical methods was determined by comparing actual concentrations to predicted concentrations calculated from equations obtained by linear regression of sample peak-height ratios and OX concentrations.

Pharmacokinetic studies

Five adult male Sprague–Dawley rats (body weight 280–295 g) were administered OX as an intravenous bolus dose of 3 mg/kg through a silastic cannula, surgically implanted in the right jugular vein. The OX dose was administered as a 1.5 mg/ml solution in normal saline. Following dosing, animals were individually housed in plastic metabolism cages to facilitate the collection of urine into polypropylene beakers containing 5 ml of 25% (w/v) ascorbic acid solution. Serial blood samples (0.2 ml) were withdrawn from the cannula over a time period of 10 h. Fluid volume was maintained by injecting donor rat plasma (0.2 ml) after each blood collection. After the terminal blood sample at 10 h, the volume of urine was measured, and urine and blood samples were stored at −20°C until analysis.

A preliminary OX tissue distribution study was performed using Fisher 344 rats bearing RG-2 gliomas. A 2-μl volume of a 10^5 cells/ml suspension of RG-2 cells (kindly provided by Dr. Darnell Bigner, Duke University Medical School, Durham, NC, USA) was implanted in the right hemisphere of the brain. Once animals became symptomatic, approximately three weeks after implantation, a 3 mg/kg dose of OX was administered intraarterially into the right external carotid artery in a retrograde manner. Animals were killed by decapitation at various times after OX administration and blood and tissue samples collected, rapidly forzen in freon, and then stored at −80°C until analysis by HPLC.

Pharmacokinetic data analysis

Area-moment data analysis was applied to the measured blood OX concentrations to obtain pharmacokinitic parameters. For each animal, area under the blood concentration–time curve (AUC) and area of the first moment–time curve (AUMC) were determined by Lagrange polynomial interpolation and integration from time zero to the last measured sample time, with extrapolation to time infinity using the least-squares terminal slope [12]. The following parameters were estimated:

Cl_T = dose/AUC, V_{ss} = dose × AUMC/$(AUC)^2$, $t_{1/2} = 0.693/k$, $Cl_R = X_u/AUC$, where Cl_T is total systemic clearance, V_{ss} is the volume of distribution at steady state, $t_{1/2}$ is the elimination half-life, k is the terminal disposition rate constant, Cl_R is renal clearance, and X_u is the amount of OX excreted unchanged in urine.

RESULTS

Chromatography

Mobile phases without acetic acid resulted in

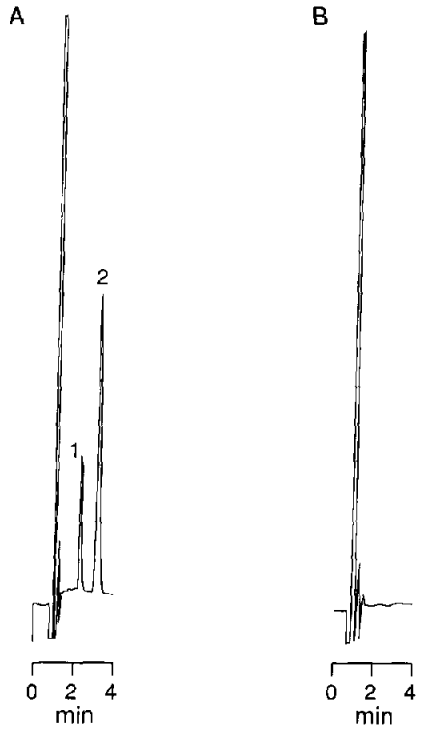

Fig. 2. Chromatograms from rat whole blood. (A) Sample containing 0.540 μg/ml oxantrazole (1) and 25 μg/ml internal standard (2). (B) blank blood. See text for conditions of analysis.

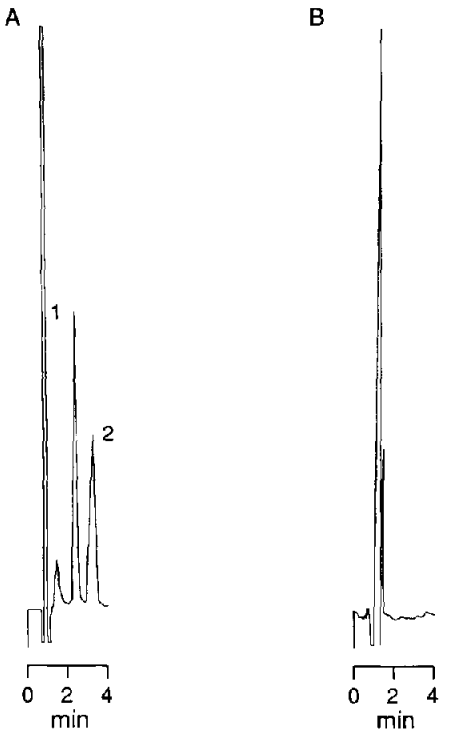

Fig. 3. Chromatograms from rat liver. (A) Sample containing 9.64 μg/g oxantrazole (1) and 12.5 μg/g internal standard (2); (B) blank liver. See text for conditions of analysis.

OX and internal standard peaks being asymmetric, broad, and of long retention times. However, incorporation of acetic acid in the mobile phase improved peak shape and decreased retention times of OX and the internal standard, while maintaining good peak separation.

Figs. 2 and 3 show blank and sample chromatograms for blood and liver samples, respectively. Other blank tissue homogenate chromatograms were similar to the chromatograms obtained from blank liver homogenate.

Retention times for OX and the internal standard were 2.3 and 3.1 min, respectively, in all samples except in urine. In urine samples, OX and internal standard had slightly different retention times of 1.9 and 2.6 min, respectively, due to the difference in polarity of the injected sample.

Sample preparation and assay validation

Assay validation data are summarized in Table I. Recovery of OX from biological samples ranged from 50% in spleen to complete recovery for liver, RG-2 glioma, kidney, and urine samples. Coefficients of variation ranged from 0.0% in urine to 5.3% for blood samples. Biases

TABLE I

RECOVERY OF OXANTRAZOLE (OX) AND PRECISION AND ACCURACY OF OX ASSAY IN VARIOUS BIOLOGICAL MATRICES OF THE RAT

Sample	Added concentration (μg/ml)	Recovery (mean ± S.D.) (%)	Coefficient of variation (%)		Predicted OX concentration (μg/ml)	Bias[a] (%)
			Within-day	Between-day		
Blood[b]	0.2	72.9 ± 0.0	0.0	5.3	0.21	4.5
	1.0	76.2 ± 1.2	1.7	1.0	0.98	1.9
	5.0	68.6 ± 1.1	1.6	1.9	4.93	1.3
Brain[c]	1.0	80.1 ± 4.7	5.9	–	0.99	1.1
Heart	5.0	55.6 ± 1.8	3.2	–	5.43	8.6
Kidney	20.0	97.8 ± 1.5	1.5	–	19.92	0.4
Liver	5.0	101.3 ± 2.6	2.6	–	5.20	4.7
Lung	5.0	61.2 ± 0.8	1.3	–	4.88	2.4
RG-2 glioma	1.6	102.8 ± 1.8	1.8	–	1.61	0.5
Spleen	5.0	50.0 ± 0.9	1.8		5.10	2.0
Urine	0.2	100 ± 0.0	0.0	–	0.20	0.7

[a] $\text{Bias} = \frac{\text{Predicted concentration-added concentration}}{\text{added concentration}} \times 100\%$.

[b] Blood samples obtained from Sprague–Dawley rats, $n = 3$.

[c] Tissue samples obtained form Fisher 344 rats bearing RG-2 gliomas, $n = 3$.

TABLE II

PHARMACOKINETIC PARAMETERS OF OXANTRAZOLE (OX) AFTER INTRAVENOUS BOLUS ADMINISTRATION OF 3 mg/kg TO NORMAL SPRAGUE–DAWLEY RAT (n = 5)

	Cl (l/h/kg)	V_{ss} (l/kg)	$t_{1/2}$ (h)	Cl_R (l/h/kg)	Percentage dose excreted unchanged in urine
Mean	1.13	0.59	1.35	0.04	3.6
S.D.	0.42	0.21	0.51	0.02	0.7

ranged from 0.3 to 5%. Standard curves in blood (n = 3) were linear from 0.05 to 5.0 μg/ml, with r^2 values greater than 0.999. Linear characteristics were also obtained for all tissues, with $r^2 \geq 0.99$.

Pharmacokinetic studies

Fig. 4 is a representative OX blood concentration–time profile in a rat administered 3 mg/kg OX intravenously. OX blood concentrations rapidly declined in a biexponential fashion, with a terminal half-life of 1.35 ± 0.51 h. Pharmacokinetic parameters of OX in rats are summarized in Table II. Total clearance was 1.13 l/h/kg and the volume of distribution at steady state was 0.59 ± 0.21 l/kg. The percentage of the dose eliminated unchanged in urine was 3.64 ± 0.69% in 10 h and the renal clearance was 0.04 ± 0.02 l/h/kg.

Maximum OX tissue concentrations, C_{max}, and the time to reach that concentration, t_{max}, for Fischer 344 rats is shown in Table III. The highest OX concentration was achieved in kidneys, 40.6 μg/g after 0.25 h, whereas the left brain had the lowest C_{max}, being 0.40 μg/g at 0.5 h. Lung, heart, spleen, and RG-2 glioma tissues showed similar maximum OX concentrations (4.8–7.9 μg/g).

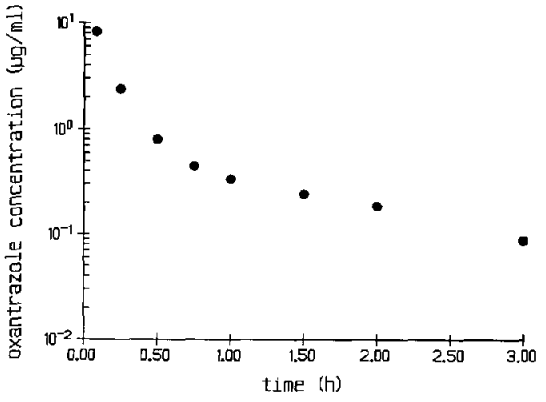

Fig. 4. OX blood concentration–time profile after an intravenous bolus dose of 3 mg/kg OX to the rat.

DISCUSSION

Chromatographic peak tailing of analytes, as was observed for OX in weakly acidic mobile phases, is primarily due to ion exchange and/or hydrogen bonding between the amino groups of the analyte and the free silanol groups of the stationary phase [11]. Various methods are available to reduce peak tailing of basic compounds and include ion pairing of protonated amino groups

TABLE III

OBSERVED C_{max} AND t_{max} OF OXANTRAZOLE (OX) AFTER A 3 mg/kg INTRAARTERIAL DOSE TO RG-2 GLIOMA-BEARING FISHER 344 RATS

Tissue	t_{max} (h)	C_{max} (μg/ml)[a]
Blood	0.08	4.5 ± 0.8
Brain		
Right hemisphere	1.00	1.4 ± 0.4
Left hemisphere	0.50	0.40 ± 0.18
RG-2 glioma	0.08	7.9 ± 6.9
Heart	0.75	4.8 ± 0.5
Kidney	0.25	40.6 ± 17.5
Liver	0.08	21.1 ± 6.1
Lung	0.08	6.4 ± 1.5
Spleen	0.50	5.2 ± 2.5

[a] All means based on n = 3; assuming tissue density = 1.

TABLE IV

PHARMACOKINETIC PARAMETERS OF OXANTRAZOLE IN MAN AND EXPERIMENTAL ANIMALS

Species	Cl_T (l/h/kg)	V_{ss} (l/kg)	$t_{1/2}$ (h)	Cl_R (l/h/kg)	Ref.
Man	2.3	0.8	0.5	0.12	5
Dog	8.7	3.2	0.6	–[a]	9
Mouse	9.1	28.9	5.5	–[a]	10
Rat	1.1	0.6	1.4	0.04	

[a] Not reported.

of the analyte with large anionic molecules, such as heptanesulfonate [13] or lauryl sulfate [14], and inclusion of a low-molecular-mass basic compound in the mobile phase to block the reaction between free silanol groups of the stationary phase and the protonated analyte [15]. Ion-pairing methods produce symmetric chromatographic peaks by inhibiting ion–dipole interactions between the analyte's amino group and free silanol groups of the stationary phase. The strategy pursued to eliminate OX's peak tailing was to select a stationary phase of minimal acidity (*i.e.* least available silanol groups) and to use a mobile phase of high acidity. The high acidity or high proton concentration of the mobile phase would reduce electronic densities surrounding the silanol groups as well as the analyte's amino group, decreasing the possibility of analyte–silanol hydrogen bonding. The abundant proton population may also suppress peak tailing by an ion-exchange reaction in a manner similar to that of the low-molecular-mass amines.

Immediate mixing of whole blood samples with an ascorbic acid solution provided an acidic, antioxidant environment required for maximum OX stability. Ascorbic acid, at a 25% (w/v) concentration, also served to hemolyze red blood cells. The methanol–ascorbic acid–water tissue homogenizing mixture was selected to improve sample recovery and to eliminate interfering peaks in chromatograms obtained from tissue samples prepared in purely aqueous ascorbic acid solutions.

The relatively high volume of distribution (8.2 times blood volume) is consistent with the ability of OX to bind to intracellular tissue components; OX is a DNA-chelating agent. OX mean clearance value is lower than the hepatic blood flow-rate in rats (2.812 l/h/kg body weight) [16] and indicates OX is extracted by the liver to a moderate degree. The rat pharmacokinetic parameters obtained in this study were relatively closer to those reported for man [5] than those obtained in mice and dogs [9,10] (Table IV). These observations indicate that the rat is a suitable animal model for further pharmacokinetic investigations.

In conclusion, a rapid and sensitive HPLC analytical method has been developed for OX analysis in biological samples obtained from pharmacokinetic studies.

ACKNOWLEDGEMENTS

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