Journal of Chromatography, 419 (1987) 225–232
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3666

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR THE EXPERIMENTAL ANTICANCER AGENT OXANTRAZOLE

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(First received November 12th, 1986; revised manuscript received February 20th, 1987)

SUMMARY

Oxantrazole is an anthrapyrazole analogue developed as an anthracycline-like agent with potentially reduced cardiotoxicity. A reversed-phase high-performance liquid chromatographic assay was developed using a C_2 column and mobile solvent system of dimethylformamide–acetonitrile–0.2 *M* ammonium acetate, pH 4.5 (20:5:75, v/v/v) at a flow-rate of 1 ml/min. Drug and internal standard were detected by ultraviolet absorbance at 514 nm. Isolation of drug and internal standard was afforded by elution from C_{18} disposable isolation columns with a mixture of methanol–glacial acetic acid–0.02 *M* sodium acetate, pH 4.0 (12:1:3, v/v/v). The assay was linear ($r^2 > 0.99$) over concentrations of 0.025–2.5 μg/ml and the limit of detection was 10 ng/ml plasma. Oxantrazole was unstable in neutral and particularly alkaline aqueous solutions. Utilizing this assay, plasma pharmacokinetics were determined following intravenous infusion of oxantrazole to beagle dogs. Plasma elimination was rapid with elimination phase half-life values < 45 min.

INTRODUCTION

Oxantrazole (NSC 349174, Warner-Lambert/Parke-Davis CI 942) (Fig. 1) is an anthrapyrazole DNA-binding agent synthesized in an effort to develop an anthracycline analogue with reduced cardiotoxicity [1,2]. The rationale for this particular series of compounds was to prepare molecules with reduced potential for semiquinone free radical production. Oxantrazole stimulated superoxide dis-

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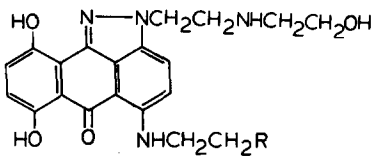

R= CH_2NH_2, Oxantrazole

R= $CH_2CH_2NH_2$, Int. Std.

Fig. 1. Structure of oxantrazole and internal standard.

mutase-sensitive oxygen consumption in rat liver microsomal preparations to a lesser extent than did adriamycin [3]. In addition, release of lactate dehydrogenase in fetal mouse organ cultures was reduced following exposure to oxantrazole as compared to adriamycin [4]. Oxantrazole also bound avidly to DNA [3], introduced DNA single-strand breaks following exposure to L1210 murine [3,5] and human tumors [6] and inhibited DNA synthesis in L1210 cells [3].

Following intraperitoneal administration of oxantrazole to mice, survival was increased for animals implanted with B16 melanoma, M5076 sarcoma, L1210 leukemia and P-388 leukemia [7,8]. In general, oxantrazole was cross-resistant with DNA-binding drug-resistant tumor lines [7,8], and no marked schedule dependency was observed in mice with intraperitoneally implanted L1210 leukemia [8]. The major toxicity of oxantrazole following administration to mice and dogs was reversible myelosuppression. Sites of toxicity included marrow, lymph, testes, liver, hematologic and gastrointestinal cells [9]. Oxantrazole is currently under National Cancer Institute-sponsored preclinical pharmacologic evaluation. In preparation for pharmacologic studies, we have developed a reversed-phase high-performance liquid chromatographic (HPLC) assay for oxantrazole in plasma and have applied the assay to preliminary pharmacokinetic studies in beagle dogs.

EXPERIMENTAL

Reagents

Oxantrazole was supplied as the dihydrochloride salt by the Pharmaceutical Resources Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD, U.S.A.). The internal standard (PD 111816, see Fig. 1) was generously provided by Warner-Lambert/Parke Davis. Disposable extraction columns (C_{18}, 1 ml) were purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). Hibar HPLC columns were purchased from Merck (Darmstadt, F.R.G.). All solvents were of HPLC grade.

Instrumentation

HPLC analyses were performed on an IBM 9533 ternary pump system equipped with either a Hewlett-Packard Model 1040A diode-array ultraviolet–visible

detector or an IBM Model 9523 variable-wavelength ultraviolet detector. The HPLC system was also equipped with an IBM Model 9505 autosampler and IBM Model 9000 minicomputer with appropriate software for HPLC operation and data analysis.

HPLC analysis for oxantrazole

Oxantrazole was analyzed by reversed-phase HPLC on a Hibar RP-2 column (25 cm × 0.4 cm I.D., 10 μm) with a mobile solvent system of dimethylformamide (DMF)–acetonitrile–0.2 *M* ammonium acetate, pH 4.5 (20:5:75, v/v/v) at a flow-rate of 1 ml/min. A 5-cm guard column packed with C_3 pellicular resin was routinely used for plasma analyses. Detection of oxantrazole and internal standard was by ultraviolet absorption at 514 nm.

Plasma isolation procedure

Plasma (0.1–1.0 ml) was diluted by the addition of 0.05 *M* sodium phosphate buffer (pH 6.0) to a final volume of 1.0–1.5 ml. A 50-μl volume of a solution of 1.2 *M* citric acid and 25% sodium ascorbate (pH 4.0) was added per ml of plasma. A 100–500 ng amount of the internal standard (a closely related structural analogue of oxantrazole, shown in Fig. 1) was added to each plasma sample. Plasma samples were then applied to C_{18} disposable 1-ml extraction columns which were prepared by washing with 2 ml of methanol, 2 ml of water and 2 ml of 0.05 *M* sodium phosphate buffer (pH 6.0). Following application of the plasma sample, the columns were washed with 2 ml of 0.05 *M* sodium phosphate buffer, pH 6.0. Oxantrazole and internal standard were then eluted with 1 ml of a mixture of methanol–glacial acetic acid–0.02 *M* sodium acetate buffer, pH 4.0. (12:1:3 v/v/v). The eluates were then evaporated to dryness under a gentle stream of nitrogen and the residue dissolved in 100–200 μl of mobile solvent prior to HPLC analysis. Standard curves were prepared by adding known amounts of oxantrazole (0.01–10 μg/ml) and internal standard (0.10–0.50 μg/ml) to control plasma. Standard curves were fit by linear regression and unknown concentrations of oxantrazole determined by use of equations derived from standard curve data.

Pharmacokinetic studies

Dog plasma samples were provided by Charles K. Grieshaber, National Cancer Institute. As part of preclinical toxicology studies, two beagle dogs were administered oxantrazole (3.81 mg/kg) by rapid intravenous infusion. Blood samples were obtained at times from 0 to 24 h after administration of drug. Plasma obtained following centrifugation of whole blood was immediately frozen and remained frozen until analysis.

Analyses of pharmacokinetic data were conducted using the NONLIN least-squares regression analysis program [10] on a CDC Cyber 170-720 computer with interactive graphic analysis. The biexponential decline in plasma concentrations of oxantrazole was fitted to the equation $C = Ae^{-\alpha t} + Be^{-\beta t}$ with a weighting factor of $1/C$. C is the plasma concentration of drug at time t after administration of oxantrazole, A and B are the intercepts at $t = 0$, and α and β are the fast and slow disposition rate constants.

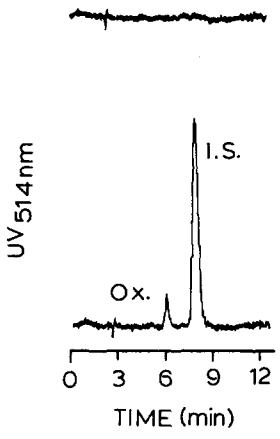

Fig. 2. Chromatograms of control plasma sample (upper panel) and plasma sample containing 50 ng/ml oxantrazole (Ox.) and 500 ng/ml internal standard (I.S.) (lower panel) following column isolation procedure and HPLC analysis.

RESULTS

Initial efforts to develop satisfactory chromatographic conditions for HPLC analysis of oxantrazole were not successful. Routine reversed-phase mobile solvent systems (e.g., methanol or acetonitrile with water or buffers) in combination with C_8 or C_{18} columns resulted in relatively broad, tailing peak shapes. A normal-phase system previously employed for anthracycline analogues which tailed under reversed-phase conditions [11] also resulted in badly tailing peaks. We found, however, that addition of the relatively strong solvent DMF to an acetonitrile–acetate buffer mobile solvent system in conjunction with a C_2 reversed-phase column provided acceptable chromatography. Chromatograms from a control plasma sample and from a plasma sample containing oxantrazole (50 ng/ml) and internal standard (500 ng/ml) obtained following plasma isolation (see below) are illustrated in Fig. 2.

Development of a method to isolate oxantrazole from plasma and other aqueous solutions required preliminary characterization of oxantrazole stability in aqueous solution as a function of pH. Oxantrazole is stable only in acidic aqueous solutions. Decomposition of parent drug was observed following incubation in neutral and particularly in alkaline aqueous solutions, but was retarded in the presence of the antioxidant ascorbic acid (Fig. 3). Based on these results, ascorbic acid was routinely added to plasma or other aqueous solutions prior to plasma isolation. Significant adsorption of oxantrazole to glass and plastic containers was observed with neutral and alkaline aqueous solutions. Adsorption appeared to be retarded in acidic solutions. For experiments in which it was inappropriate to add ascorbic acid (e.g. plasma stability studies), stock solutions of oxantrazole were prepared in sodium acetate buffer (pH 4–5) and these solutions kept on ice prior to their addition to plasma.

Efforts to isolate and concentrate oxantrazole from aqueous solutions by extraction with organic solvents were not successful. Recoveries of oxantrazole

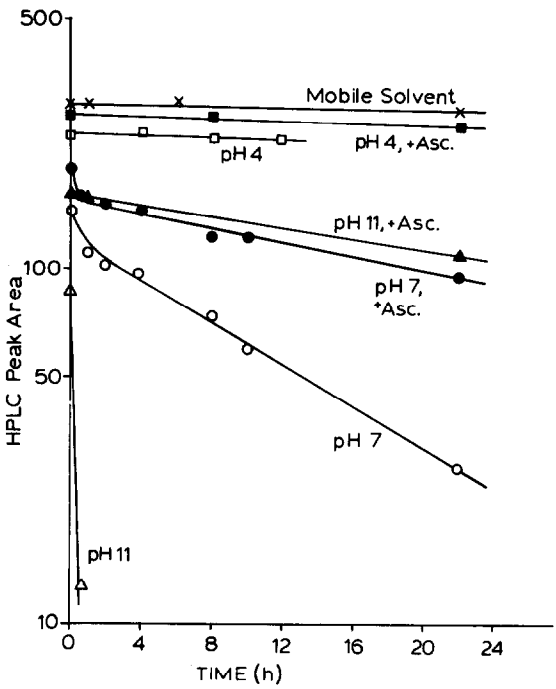

Fig. 3. Stability of oxantrazole as a function of time in mobile solvent (×) (see Experimental for buffer composition), sodium acetate buffer pH 4 (□), sodium acetate buffer pH 4 and ascorbic acid (■), sodium phosphate buffer pH 7 (○), sodium phosphate buffer pH 7 and ascorbic acid (●), glycine buffer pH 11 (△) and glycine buffer pH 11 and ascorbic acid (▲).

from aqueous solutions were less than 20% following extraction at pH values of 4–11 with ethyl acetate, chloroform and hexane. We attempted to isolate oxantrazole following application of aqueous solutions to a variety of disposable extraction columns (C_{18}, C_8, C_2, cyano, silica). Methanol and methanol-buffer mixtures did not provide efficient recovery of oxantrazole from these columns. However, recoveries of oxantrazole and internal standard were 80–90% following elution from C_{18} disposable extraction columns with a mixture of methanol, glacial acetic acid and sodium acetate buffer. In preliminary studies using plasma isolated from a variety of whole blood sources, an interfering peak with retention time very similar to that of oxantrazole was noted in some, but not all chromatograms following isolation and HPLC analysis. Further study revealed that the peak was detected only following analysis of samples in which heparin was used as the anticoagulant. Addition of heparin to buffer samples followed by column isolation and HPLC analysis afforded chromatograms with the same impurity. The impurity was detected at all wavelengths monitored, including 514 nm, with the single-beam detector. However, this impurity was not observed with the diode-array detector at 514 nm when a reference wavelength of 590 nm was monitored simultaneously. This problem was avoided by using the diode-array detector and also by using citrate as the anticoagulant.

When the C_{18} column isolation procedure was employed with plasma contain-

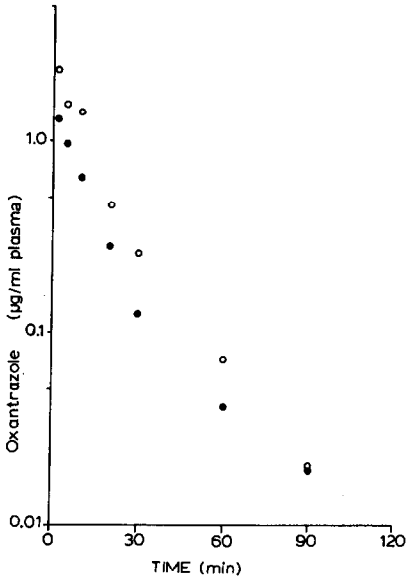

Fig. 4. Plasma time–concentration data for two beagle dogs following bolus intravenous administration of oxantrazole (3.81 mg/kg, 80 mg/m^2).

ing oxantrazole, the limit of detection of oxantrazole under standard assay conditions was 10 ng/ml in plasma. The assay was linear over a broad range (0.025–2.5 µg/ml, $r^2 \geq 0.997$), and the coefficients of variation were 8 and 6.5% for concentrations of 0.25 and 2.5 µg/ml, respectively ($n=8$). Variability of standard curve points was reduced for curves determined on the same day as compared to those conducted over several days.

The HPLC assay was applied to determination of oxantrazole concentrations in plasma following rapid intravenous administration of 3.81 mg/kg (approximately 80 mg/m^2) to two beagle dogs. The plasma time–concentration data for the two dogs are illustrated in Fig. 4. Oxantrazole was rapidly eliminated from plasma of both dogs, with parent drug detected in plasma samples for only 90 min after drug administration. Plasma pharmacokinetic parameters for the two dogs are summarized in Table I.

TABLE I

BEAGLE DOG PHARMACOKINETIC PARAMETERS

Abbreviations: $t_{\frac{1}{2}\alpha}$ = distribution phase half-life; $t_{\frac{1}{2}\beta}$ = elimination phase half-life; Cl_{TB} = total body clearance; Vd_{ss} = steady-state volume of distribution.

Dog (No.)	Dose		$t_{\frac{1}{2}\alpha}$ (min)	$t_{\frac{1}{2}\beta}$ (min)	Cl_{TB}		Vd_{ss}	
	mg/kg	mg/m^2			l/min/m^2	l/min/kg	l/m^2	l/kg
B1285M	3.81	80	7.2	44.5	3.84	0.18	93	4.4
B1215F	3.81	80	3.5	14.9	2.37	0.11	54	1.9

DISCUSSION

Development of plasma isolation and HPLC methods for the analysis of oxantrazole were cumbersome due to the unique nature of this molecule. Oxantrazole possesses lipophilic character by virtue of the anthrapyrazole ring system leading to adsorption on glass, plastic, membranes and filters under neutral or alkaline conditions. The lipophilic nature of oxantrazole also explains the conditions required to elute the drug from HPLC columns and isolation columns. The *para*-oriented hydroxyl substituents on this ring system lead to facile oxidation of oxantrazole. The side-chains provide the molecule with polar character (including ionized moieties) preventing efficient extraction into organic solvents.

Oxantrazole is relatively unstable in neutral and alkaline aqueous solutions. In addition, this agent decomposes more rapidly in the presence of fresh or thawed frozen human plasma [6,12]. For these reasons, it is important to carefully determine the stability of oxantrazole under conditions used for study in the laboratory, and the use of an internal standard with similar physical chemical properties is a major benefit for such studies. These problems are reflected in the relatively poor coefficients of variation noted for the plasma assay. Citric acid (to reduce pH) and ascorbic acid (to minimize oxidation) are routinely added to all samples prior to storage and/or work-up.

Addition of heparin to whole blood samples as an anticoagulant interfered with detection and quantitation of oxantrazole. An interfering peak was observed at 514 nm with the single-beam variable-wavelength detector, but not with the diode-array detector using a reference wavelength of 590 nm. We interpreted this as evidence that turbidity or related phenomena were responsible for the peak (rather than absorption of energy), and that this effect was quantitatively similar for detection at 514 and 590 nm, thus eliminating the signal with the diode-array detector. No interfering peaks were observed in plasma samples obtained from whole blood to which citric acid was added as anticoagulant.

Oxantrazole was detected in dog plasma for only 90 min following bolus intravenous administration of 80 mg/m^2. Analysis of plasma disappearance data indicates a very rapid clearance for this agent (Table I). This may be due in part to the decomposition of oxantrazole observed in plasma. Results of mouse pharmacokinetic studies currently underway suggest somewhat lower rates of drug elimination [6]. Preclinical pharmacologic and biochemical studies with oxantrazole continue in our laboratory. The assay described in this manuscript is currently being employed for mouse pharmacokinetic studies, plasma stability studies and determination of oxantrazole stability in cell culture media.

ACKNOWLEDGEMENTS

Supported in part by NCI CM37601 and Research Career Development Award CA00755 (M.M.A.).

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