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Blood and tissue concentrations of Bisantrene measured by a simple fluorometric assay

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Summary. *We have developed a simple sensitive fluorometric assay for the measurement of total Bisantrene in plasma, red blood cells, and tissues to facilitate preclinical and clinical pharmacologic assessment of this active anticancer agent. The assay was used to measure the plasma disappearance and tissue concentrations of Bisantrene in the rabbit. Results are comparable to those reported with HPLC assays and with measurement of radioactivity in combusted tissue following IV administration of radiolabeled Bisantrene. We demonstrated that when a plasma concentration of approximately 50 μg/ml is not exceeded, Bisantrene remains in solution at that concentration. If Bisantrene is introduced into plasma at a concentration exceeding 50 μg/ml, precipitation of the drug is initiated and continues until the plasma concentration is no greater than 15 μg/ml. This finding supports our previous recommendation that in clinical trials Bisantrene should be administered at low concentrations over prolonged periods of time to maximize the bioavailability of the drug by minimizing precipitation of the drug in plasma.*

Introduction

Bisantrene (9,10-anthracenedicarboxyaldehyde bis [(4,5-dihydro-1-H-imidazole-2-yl) hydrazone] dihydrochloride) is an investigational antitumor agent undergoing clinical evaluation in many phase II studies. Current interest in Bisantrene is focused on use in patients with refractory breast carcinoma, metastatic renal carcinoma, metastatic malignant melanoma, myeloma, refractory leukemia, and lymphoma. Administration of Bisantrene by a peripheral vein is frequently complicated by thrombophlebitis and anaphylactoid reactions characterized by fever, wheezing, and chest pain [4]. Infusion of the drug via central venous catheters eliminates peripheral thrombophlebitis but not the systemic "allergic" toxicities.

We showed earlier that Bisantrene has low solubility at physiological pH and precipitates in the veins of animals given the drug at the concentrations and rates of infusion used in clinical studies [8]. We also demonstrated that infusion of the drug over 72 h eliminates all systemic toxicity and produces dose-dependent myelosuppression in man [3]. However, prolonged IV infusion peripherally even at low concentrations (~ 50 ng Bisantrene/ml) produces local venous irritation which, although mild, is troublesome enough to make central line administration of the drug preferable. We are presently attempting to formulate the drug in a manner that will avoid intravascular precipitation and permit rapid administration via peripheral veins without local toxicity. The evaluation of each formulation requires a study of the pharmacokinetics and tissue distribution of Bisantrene given in the new formulation in model systems.

We [8] and other workers [6, 7] have used high-performance liquid chromatography (HPLC) to study the pharmacokinetics of Bisantrene in man and animals. Although the HPLC assays are sensitive, the analysis of the many samples needed for comparative tissue distribution studies by this method is tedious. Because Bisantrene is highly fluorescent and is believed not to be metabolized in vitro [8] or in vivo [10], we developed a fluorometric assay for the measurement of total plasma and tissue concentrations of Bisantrene. The assay is based on a method used by Bachur et al. [1] to measure doxorubicin and fluorescent metabolites.

Materials and methods

Drug. Bisantrene dihydrochloride was obtained from Lederle Laboratories Division, American Cyanamide Company, Pearl River, NJ. It was stored at room temperature as a dry powder. Solutions of Bisantrene for in vivo studies were prepared the same day.

Animals. Male New Zealand white rabbits were given Bisantrene at a dose of 260 mg/m^2 (~ 30 mg) via an ear vein through a winged adaptor (Butterfly, Abbot Hospitals, Inc.). The drug was dissolved in 5% dextrose in water (Travenol, Deerfield, Ill) at 1 mg/ml and infused at approximately 0.5 ml/min. Samples of blood were obtained from a vein in the opposite ear for 4 h. The animals were then sacrificed using a CO_2 chamber and tissue samples were taken.

Sampling and analysis. Blood samples were chilled immediately in ice water. At the end of plasma sampling, plasma, and red blood cells were separated and treated with acid alcohol as described below. Tissue samples were frozen on dry ice and extracted 24 h later. Fresh human whole blood was obtained from single donors. The heparinized plasma and red cells were separated and kept frozen until use.

Fluorometric assay. Volumes of 1 ml plasma were each mixed with 2 ml ice-cold acid ethanol (0.45 *N* HCl in 75% ethanol). Samples were vortexed for 30 s and refrigerated in the dark for 12–18 h. They were then centrifuged at 20,000 rpm for 20 min and the clear supernate was used for the assay. Packed red

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blood cells (0.5 ml) were mixed with an equal volume of iced ultrapure water before the acid-ethanol extraction was done as described.

Frozen tissue samples were crushed in a tissue pulverizer and chilled in a dry ice-acetone bath. The pulverized tissue was weighed and 10 ml acid ethanol was added for each gram of tissue. The samples were homogenized in a Polytron sonicator (Brinkman Instruments, Westbury, NY) and centrifuged at 20,000 rpm for 20 min. The supernatant was then decanted into borosilicate tubes and refrigerated until measurement of fluorescence.

To assess the efficiency of extraction of Bisantrene from tissue, different concentrations of Bisantrene in water were added to known quantities of pulverized and sonicated rabbit abdominal muscle. The homogenate was mixed and allowed to stand for 1 h and assayed as described. Results are expressed as the amount of Bisantrene per gram of wet weight of tissue. Known concentrations of Bisantrene, ranging from 10^{-4} to 10^{-9} *M*, in human plasma and in packed red blood cells were extracted with acid ethanol. Reference standards were prepared by dissolving Bisantrene dihydrochloride powder in ultrapure water at 50 mg/ml and then diluting to desired concentrations in acid-ethanol.

Measurement of Bisantrene by fluorescence. Bisantrene dihydrochloride was analyzed with an SLM series 8000 spectrofluorometer for UV-visible absorption and fluorescence emission by Dr Franklyn Prendergast, Department of Pharmacology, Mayo Foundation. Peaks of absorbance were seen at 260 nm and 410 nm. Maximal fluorescence was at 517 nm.

Acid ethanol extracts of plasma and tissue samples were shielded from light, and fluorescence was measured in an Aminco-Bowman spectrofluorometer. Maximum sensitivity was achieved with excitation at 410 nm and emission at 517 nm. Acid-ethanol extracts of appropriate tissues not containing Bisantrene were used as blanks for corresponding experimental samples. Fluorescence of greater than 10 arbitrary units on the Aminco-Bowman instrument were beyond the linear range of fluorescence for Bisantrene in acid alcohol. These samples were diluted in acid ethanol so that the absolute fluorescence reading was less than 10 arbitrary units. We documented that this procedure was valid for concentrations of Bisantrene up to 5 mg/ml.

hplc assay. An HPLC assay for Bisantrene described by Peng et al. [7] was used, except that we added PicA (Waters Associates, Milford, Mass) to the mobile phase. Plasma and tissue samples were injected in acid ethanol onto a Waters C 18 Bondapak column and eluted at 2 ml/min with ammonium acetate (0.4 *N*) and methanol (60 : 40) containing 14 ml PicA; pH 4.3. Detection was at 260 nm. The linearity and reproducibility of the HPLC assay were comparable to those reported by Peng et al. [6]. For these studies samples were analyzed by the fluorometric assay and the HPLC assay on the same day.

Pharmacokinetic analysis. Pharmacokinetic parameters were determined by using the DRUGMODEL pharmacokinetic modeling procedure, a public procedures facility of the PROPHET on-line computer system [2]. The data were fit into a two- and three-compartment model using a zero-order infusion input and first-order elimination with a weighting of 1/y. Visual inspection and quantitative comparison both showed the two-compartment model to best represent the plasma concentration time profile in the rabbit.

Results

Fluorescence of Bisantrene in acid alcohol was proportional to concentration of the drug from 0.5 ng/ml to 5,000 ng/ml. With excitation at 410 nm and emission at 517 nm, tissue and plasma blanks had little fluorescence compared with pure acid ethanol. At concentrations greater than 5 μg/ml, fluorescence was less than expected and at concentrations above 25,000 μg/ml there was a dramatic fall in the fluorescence readings. Dilutions of samples restored linearity to the standard curve. Standard solutions of Bisantrene in acid ethanol showed no change in fluorescence after 7 days at 4° C.

The accuracy of the fluorometric method was compared with that of the HPLC method by assaying a number of plasma samples obtained from animals after infusion of Bisantrene by both methods. Table 1 shows that there is good agreement between the two techniques, the maximum difference being 15% among seven determinations. The precision of the fluorometric assay was studied by assaying in triplicate two different concentrations of drug in plasma from one of the infusion experiments. As shown in Table 2, the fluorometric assay was highly reproducible, the coefficient of variation being 0.2% at both concentrations. The sensitivity of currently used HPLC assays for Bisantrene in plasma ranges from 10 ng/ml to 50 ng/ml, depending on the extraction procedure [6–8]. Our fluorometric assay compares favorably with these methods. The lower limit of detection for the fluorometric assay is 5 ng/ml; accurate measurement is achieved at 20 ng/ml.

The efficiency of extraction of Bisantrene from serum ranged from 89% at 50 μg/ml to 97% at 0.5 μg/ml. These results are similar to an 80% efficiency of extraction of Bisantrene from plasma with ethyl acetate followed by drying under nitrogen and reconstitution in methanolic acid, as described by Peng et al. [6]. More recently, Peng et al. [7]

Table 1. Comparison of fluorometric and HPLC bisantrene assay[a]

Fluorometric concentration (μg/ml)	HPLC concentration (μg/ml)
1.99	1.80
1.97	2.12
1.61	1.81
1.13	1.25
1.02	1.20
0.90	0.91
0.86	0.81

[a] Plasma samples were assayed on the same day by each method. For both methods the concentration was calculated by comparison with a known concentration of bisantrene in plasma

Table 2. Precision of fluorometric assay

Sample	Concentration (μg/ml)[a] (mean + SD)	Coefficient of variation (%)
A	8.07 + 0.02	0.2
B	4.37 + 0.01	0.2

[a] Each concentration represents the mean of three separate analyses performed on a sample of plasma

reported an extraction efficiency of greater than 90% using methanolic acid. We also achieved efficient extraction of Bisantrene from homogenized tissue to which Bisantrene was added in known amounts using acid ethanol. The results of recovery of Bisantrene from homogenized rabbit muscle are shown in Table 3.

We used the fluorometric assay to study the distribution of Bisantrene between plasma and red blood cells. Following incubation of whole blood with Bisantrene at several different concentrations greater amounts of drug were associated with red blood cells than with plasma. The ratio of the concentration of Bisantrene in red cells to the concentration of bisantrene in plasma was 3 : 1 at low concentrations (1 μg/ml) of drug, as initially reported from our laboratory by Powis [9]. At higher concentrations of Bisantrene, the amount of drug associated with red cells compared with plasma increased to a maximum of 5 : 1 whole blood at concentration of 10 μg/ml and greater. The nature of the partitioning of Bisantrene between plasma and red blood cells is unclear. Preparations of lysed red cells contained similar amounts of Bisantrene to intact red cells. Wu and Nicolau [10], using radioactive Bisantrene, reported that after Bisantrene was added to whole blood 90% of the radiolabel was present in the red cell fraction. A lysate of the red cells contained up to 60% of radiolabel, the remainder being present in the membrane fraction.

Several investigators [7, 9] have reported that Bisantrene is highly protein-bound and that ultrafiltrates of plasma contain small amounts of the drug. Using the fluorometric assay, we confirmed that ultrafiltration of plasma containing Bisantrene removes virtually all the drug. Because the solubility of Bisantrene in human blood appears to be a major factor determining local toxicity (thrombophlebitis) and the systemic toxicity (anaphylactoid reactions), we studied the solubility of the drug in buffers and blood in further detail. When we added Bisantrene in large excess to phosphate-buffered saline, pH 7.4 (PBS), the drug precipitated slowly over several hours. After 24 h the concentration of Bisantrene in PBS was less than 5 μg/ml. When the drug was added to PBS to give a concentration no greater than 50 μg/ml, less precipitation occurred after prolonged standing at room temperature. After several days the drug concentration was 30 μg/ml. When Bisantrene was added slowly to achieve a concentration of 50 μg/ml in human rabbit or calf plasma, the drug remained in the plasma at 50 μg/ml. When the drug was added in excess to plasma, resulting in immediate precipitation of some drug, a steady state concentration no greater than 15 μg/ml was achievable. Powis and Kovach [9] reported previously that the maximum solubility of Bisantrene was 0.45 μg/ml in PBS and 13 μg/ml in plasma. These values were determined by adding a large excess of drug to the appropriate fluid and shaking vigorously for prolonged periods of time.

We used the fluorometric assay to study the plasma elimination and distribution of Bisantrene given IV over 60 min to rabbits at the dose used in most clinical trials, 260 mg/m^2. Plasma elimination fit a two-compartment model with a terminal half-life of elimination of 205 min, which is similar to that previously reported from our laboratory with an HPLC method. Animals were sacrificed 4 h after initiation of the 1-h infusion, and various tissues were selected for measurement of Bisantrene concentrations. The mean Bisantrene concentrations in the liver, kidney, heart, and muscle of four rabbits receiving identical doses of Bisantrene are shown in Table 4. The standard deviations in the concentrations among the four animals are small. The distribution of Bisantrene in the rabbits is very similar to that reported by Wu and Nicolau [10], who used measurement of radioactivity in tissue after administration of ^{14}C-Bisantrene.

Table 3. Bisantrene extraction from homogenized calf bladder

Actual concentration (μg/gm tissue)	Measured concentration (μg/g tissue)	Percent recovery
1,000	1,057.4	105.7
200	231.4	115.7
50	47.2	94.4
20	16.5	82.4
5	4.8	95.8

Table 4. Rabbit tissue concentrations[a] of bisantrene following IV infusion

Tissue	Bisantrene concentration (μg/g tissue) (Mean + SD)
Liver	72.8 + 8.6
Kidney	288.6 + 53.9
Heart	29.2 + 3.5
Muscle	7.7 + 2.6

[a] All tissue concentrations are from four separate animals

Discussion

We have developed a simple fluorometric assay for the measurement of Bisantrene in plasma, red blood cells, and tissues. The assay has a sensitivity comparable to more time-consuming and expensive HPLC methods. Because there is no evidence that Bisantrene is metabolized in vivo [10], it seems likely to us that the fluorometric assay is no less specific than other assays in which the parent compound is separated from other plasma tissue components and measured. The fact that the distribution and relative concentration of Bisantrene in different organs of the rabbit measured in this study are comparable to values reported by Wu and Nicolau [10] in the dog using measurements of radioactivity after combustion of tissue supports our belief that the fluorometric assay accurately measures total tissue concentrations of Bisantrene.

Bisantrene is currently being evaluated in phase-II and phase-III clinical trials to determine its role in the treatment of a variety of human cancers. In our opinion the optimal method of drug delivery is not clear. In most clinical studies the drug is being administered by central line over 1 h or several hours to avoid peripheral thrombophlebitis. However, these short-term infusions are invariably associated with anaphylactoid reactions, which are troublesome at best and dose-limiting in some patients. We believe the data reported here support our initial recommendation [9] that the drug should be administered slowly at a low concentration to minimize intravascular precipitation in plasma. In vitro, when Bisantrene is added to plasma to a concentration of 50 μg/ml, the drug remains in solution at that concentration. When the concentration of Bisantrene is increased to greater than 50 μg/ml there is some immediate precipitation of the drug, followed by continuing precipitation to a steady state concentration of no greater than 15 μg/ml. We speculate that a similar phenomenon occurs in vivo and is responsible for the allergy-like toxicities of the

drug. We have demonstrated in ongoing clinical trials that when Bisantrene is given at a total dose of approximately 260 mg/m^2 is 9 liters fluid over 72 h only dose-dependent myelosuppression is seen, and no allergy-like toxicities [3]. We and several other groups are exploring a variety of formulations of Bisantrene designed to eliminate intravascular precipitation, upon the assumption that such a formulation could be administered via a peripheral vein without producing thrombophlebitis. The necessity for administration of the drug by central venous line to avoid peripheral vein irritation is a significant limitation to the use of Bisantrene. We expect that the simple, sensitive, and specific fluorometric assay reported will facilitate further preclinical and clinical research of this new clinically active anticancer drug.

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