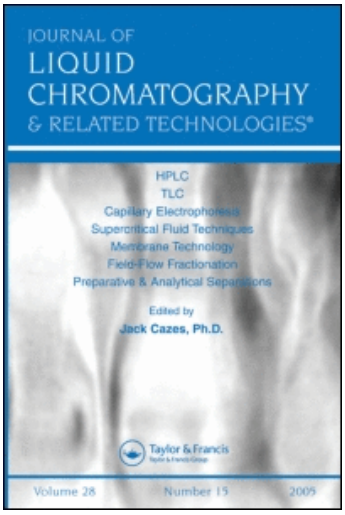

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REVERSE PHASE HPLC DETERMINATION OF AZQ IN BIOLOGICAL FLUIDS

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ABSTRACT

A sensitive and specific reverse phase HPLC method employing a simple sample preparation procedure and utilizing an internal standard was developed to measure the new antitumor agent AZQ in biological fluids. A single chloroform extraction gave drug recoveries of greater than 88% from plasma, urine and CSF in the range of expected physiological concentrations (20-800 ng/ml). Isocratic reverse phase HPLC with UV detection at 340 nm resulted in a limit of quantitation of 5 ng/ml although smaller amounts of the drug could be detected. This assay was successfully applied to determine the single dose plasma pharmacokinetics of AZQ in rats. The potential of this method for determining AZQ disposition and pharmacokinetics in human subjects was demonstrated by analysis of patient CSF.

INTRODUCTION

AZQ (Figure 1) is a potential new antitumor drug that is currently undergoing Phase I and Phase II clinical trial. This aziridinylbenzoquinone is a rationally synthesized compound designed to allow central nervous system (CNS) penetration while retaining antitumor activity (1,2). AZQ shows a broad spectrum of activity against murine model tumor systems, including significant increases in life span in intracerebral L1210 and P388 and cures in intracerebral ependymoblastoma (1,3).

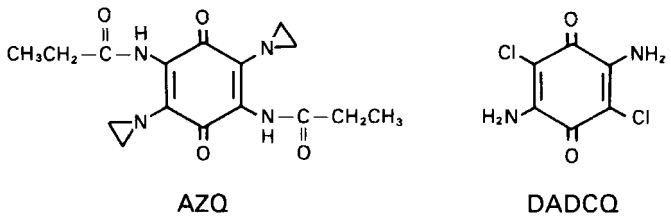

FIGURE 1. Structure of AZQ and the Internal Standard DADCQ.

A low optimum dose combined with appreciable animal toxicity indicated that human clinical trials of AZQ would have to start at doses of 0.5-1.0 mg/m^2. Thus a sensitive and specific analytical method to measure AZQ in plasma was required in order to allow determination of pharmacokinetics. Also of interest was the disposition of intact AZQ in other biological fluids, especially cerebrospinal fluid (CSF), since the drug was designed to enter the CNS. The high polarity and thermal instability of AZQ even after derivatization prohibited sensitive gas phase techniques such as gas chromatography and combined gas chromatography-mass spectrometry from being used to quantitate this compound (4). However, AZQ possessed a strong UV chromophore (λ_{max} = 340, log ε = 4.17) and a nonpolar nature, so high-performance liquid chromatography (HPLC) on a reverse phase column was a feasible alternative. Indeed, reverse phase HPLC provided a convenient method of analyzing formulation mixtures of AZQ (5). However, the concentrations of the drug encountered here were three orders of magnitude greater than those expected in vivo and AZQ did not have to be isolated from a complex biological matrix.

MATERIALS

AZQ (2,5-Diaziridinyl-3,6-bis(carboethoxyamino)-1,4-benzoquinone, NSC 182986) was obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, NCI and

used as received. The AZQ (Lot AJ58.1) was assayed at 96.5 ± 0.8% purity (4). 2,5-Diamino-3,6-dichloro-1,4-benzoquinone (DADCQ, Figure 1) was obtained from Dr. J. S. Driscoll of this laboratory and was recrystallized from hot ethyl acetate before use as an internal standard. Both AZQ and DADCQ were weighed on a Cahn 25 Automatic Electrobalance (Ventron Instruments Corp., Paramount, CA) before being dissolved in 25% CH_3CN/H_2O (v/v) to give standard solutions 1.175×10^{-5} M and 4.94×10^{-5} M, respectively. The standard solutions were stable for at least a month if they were refrigerated. Mono- and dibasic sodium phosphate (J.T. Baker Chemical Co., Phillipsburg, NJ) were used to make a pH 6.5 0.1 M buffer. Acetonitrile and chloroform were "distilled in glass" (Burdick and Jackson, Muskegon, MI) or HPLC grade (Fisher Scientific Co., Fairlawn, NJ) reagents. Chloroform was further purified to remove the 0.5-1% ethanol stabilizer by passage through a 15 mm ID column containing 25 g of basic alumina, activity grade I (AG-10; Bio-Rad, Richmond, CA). The chloroform was used immediately or stored under nitrogen and refrigerated. Acetonitrile and distilled water were filtered through the appropriate 0.45 µm solvent-resistant filters (Millipore Corp., Bedford, MA), before mixing to make the mobile phase.

METHODS

Separations were accomplished at ambient temperature (20°C) on either a 10-µm µBondapak C_{18} column (3.9 mm ID x 300 mm; Waters Associates, Milford, MA) or a 5-µm Ultrasphere-ODS column (4.6 x 250 mm; Altex Scientific Co., Berkeley, CA) using a mobile phase of 25% CH_3CN/H_2O (v/v). The analytical column was preceded by a Waters guard column that was repacked daily with 37-50 µm Bondapak C_{18}/Corasil. The remainder of the high pressure liquid chromatography system was comprised of a Waters Associates Model 6000A solvent delivery system, U6K injector and Model 440 UV absorbance monitor with a 340 nm filter. The mobile phase flow

rate was 1.0 ml/min and injections of standards and biological extracts (100 µl aliquot) were made using a 100 µl Waters gas tight syringe. Peak areas and heights were simultaneously determined on a SP4100 computing and recording integrator (Spectra-Physics, Santa Clara, CA).

Standards were made by adding 5 µl of DADCQ internal standard solution (51 ng) to 1.0 ml aliquots of the appropriate biological fluid in a 15 ml glass conical centrifuge tube and then by spiking with the required volume of AZQ standard solution. Pooled rat and human plasma, pooled monkey CSF (kindly provided by Dr. R. Riccardi, Pediatric Oncology Branch, NCI), and human urine were used. The spiked standards were gently vortexed and allowed to stand for 10 min in an ice bath before extractive workup. After addition of 0.5 ml 0.1 M pH 6.5 phosphate buffer, the standards were extracted with 1 x 5.0 ml $CHCl_3$ by vigorous vortexing for 2 min. The resulting emulsions were centrifuged at 2400 rpm for 5 min on a Dynac table top centrifuge (Clay Adams, Becton Dickinson and Co., Parsippany, NJ). If the emulsions were not completely broken, the samples were frozen in a dry ice-acetone bath and then slowly thawed. Centrifugation again for 5 min at 2400 rpm was sufficient to break the emulsions. The organic layer was transferred to a 10 ml capacity evaporative concentrator tube (Kontes, Vineland, NJ) and evaporated to dryness at room temperature under a stream of prepurified nitrogen. The residue was suspended in 0.5 ml 25% CH_3CN/H_2O by rapid vortexing, and the sample was filtered through a 0.5 µm Fluoropore filter using a stainless steel Swinny holder (Millipore Corp.) before chromatography. One milliliter biological samples were extracted in the same manner after addition of 5 µl DADCQ internal standard solution.

For study of AZQ pharmacokinetics in rat plasma, the drug was dissolved in N,N-dimethylacetamide (Fisher Scientific Co., Fairlawn, NJ) at a concentration of 20 mg/ml before being diluted

to a final concentration of 0.25 mg/ml with pH 6.5 0.01 M phosphate buffer. Male Sprague-Dawley rats, weighing 200-240 g (Taconic Farms, Germantown, NY), were anesthetized with sodium pentobarbital (35 mg/kg ip). A 1 mg/kg dose of AZQ was then given as a single bolus injection via an exposed femoral vein using a 25 gauge needle attached to a 1 ml syringe. Animals were decapitated at varying timed intervals after AZQ injection and blood from each animal was collected in separate heparinized glass beakers kept on ice. Blood was transferred to 15 ml glass centrifuge tubes and plasma obtained by a 15 min centrifugation. One milliliter aliquots of plasma were pipetted into separate 15 ml centrifuge tubes and the samples were either worked up immediately as described above or frozen in dry ice and stored at -20°C for future analysis.

Recoveries of AZQ and DADCQ from plasma, urine and CSF were determined by comparison of the absolute HPLC peak area or peak height of extracted spiked standards to calibration curves generated from comparable standards directly made up in 25% CH_3CN/H_2O. A blank of each different biological fluid was also run. The peak area or peak height ratio of AZQ to DADCQ internal standard was computed for each spiked standard and plotted against AZQ concentration for the range 20-880 ng/ml to generate a calibration curve (Figure 2). A calculator least-squares program (TI-55, Texas Instruments, Dallas, TX) was used to define the best straight line through each set of standard points in the body fluids examined. Initial pharmacokinetic parameters for AZQ were calculated from the rat plasma concentration curve by the method of residuals (6). The experimental data points were then fit to the biexponential function representing a two-compartment open model ($C = Ae^{-\alpha t} + Be^{-\beta t}$) by using MLAB, an on-line computer modeling laboratory utilizing an iterative, non-linear least squares program.

TABLE 1

Recovery of Spiked AZQ and DADCQ from Biological Fluids

Biological Fluid	AZQ Recovery	AZQ Range	DADCQ[1] Recovery
rat plasma	97 ± 9%	43-855 ng/ml	80 ± 6%
human plasma	88 ± 3%	22-880 ng/ml	88 ± 3%
human urine	92 ± 4%	21-214 ng/ml	88 ± 5%
monkey CSF	89 ± 7%	21-428 ng/ml	88 ± 3%

1. The concentration of DADCQ was 51 ng/ml in all cases.

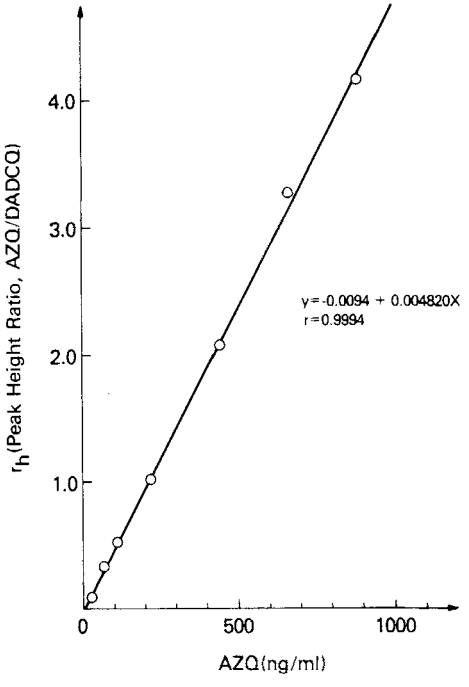

FIGURE 2. Typical Calibration Curve for Human Plasma.

RESULTS

Both AZQ and DADCQ could be easily and efficiently extracted from plasma, urine and CSF (Table 1). Recoveries were examined for the expected range of AZQ concentrations in rat plasma as determined from optimal therapeutic doses of ^{14}C-AZQ (7). Linear and reproducible calibration curves could be constructed from extracted spiked standards for all biological fluids examined (Figure 2):

rat plasma: $y = 0.0996 + 0.004934x$ ($r = 0.9998$)
human plasma: $y = -0.0094 + 0.004820x$ ($r = 0.9994$)
human urine: $y = -0.0073 + 0.004565x$ ($r = 0.9995$)
monkey CSF: $y = -0.0005 + 0.004344x$ ($r = 0.9976$)

HPLC analysis of extracted 1.0 ml aliquots of rat plasma from animals given a bolus injection of 1 mg/kg AZQ resulted in chromatograms similar to Figure 3. Because of detection at 340 nm

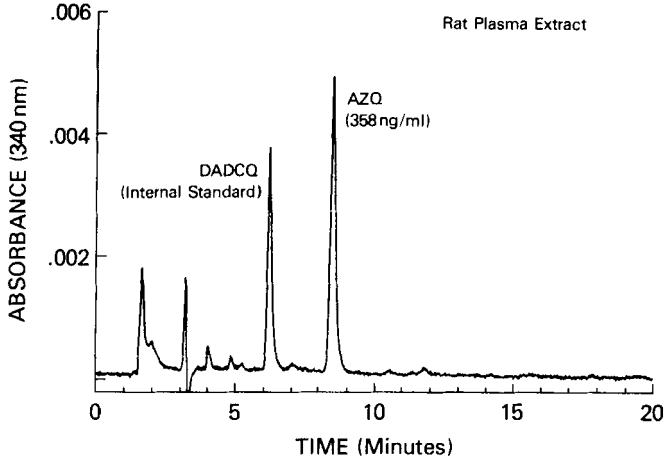

FIGURE 3. Representative HPLC Analysis of AZQ in Rat Plasma. Conditions are as described in Methods. A male Sprague-Dawley rat was intravenously administered a single 1 mg/kg dose of AZQ. The animal was sacrificed 60 min after drug injection and the plasma analyzed.

interfering artifacts were kept to a minimum, and the AZQ and DADCQ peaks could be readily integrated. The limit of quantitation was found to be about 10 ng/ml for rat plasma and 5 ng/ml for human plasma using a 1.0 ml sample size; the limit of detection was somewhat less. Rat plasma blanks consistently had a very small peak with almost the same retention time as AZQ. This accounted for the positive non-zero intercept in the calibration curve. No such interference was encountered with human plasma and urine or monkey CSF.

DISCUSSION

Extraction with chloroform was chosen as the means of isolating AZQ, a drug designed to have an adequate lipophilic character (1), because it was efficient, simple and applicable to all the body fluids under consideration. Since plasma lipids and other chloroform-soluble components could be expected to partition into the organic phase, assay sensitivity and specificity depended on UV detection at the μ_{max} of AZQ (340 nm) and adjustment of k' on the octadecylsilane column to achieve sharp peaks yet adequate separation. In a situation where low levels of drug were expected, extraction with a volatile organic solvent allowed facile concentration of the extract. In practice, a two-fold concentration was found adequate to attain sensitivity in the low nanogram range. With injection of a 100 µl aliquot, less than a nanogram of AZQ on-column could be detected.

A common problem in the use of an organic solvent to extract a biological medium is the formation of emulsions. This was encountered in almost all cases with plasma and on a more random basis with CSF and urine. However, application of the freeze-thaw technique outlined in Methods resulted in sharp phase separation for every sample. Evaporation of this chloroform plasma extract and attempted solution of the residue in 25% CH_3CN/H_2O resulted in a suspension where the undissolved materials were

probably lipids. This phenomenon was not so noticeable with CSF and urine extracts. Filtration through a 0.5 μm Fluoropore membrane eliminated most of this material. As an added precaution a guard column, which was repacked daily, was used to protect the analytical column from any strongly adhering components such as lipids.

Although AZQ is not ionized in the range of physiologically occurring pH's, it does undergo pH-dependent decomposition (4,5). Accordingly, all biological fluids were buffered to pH 6.5, the pH of maximum AZQ stability, before extraction.

Studies using ^{14}C-AZQ in rats and dogs have suggested that AZQ undergoes extensive metabolism (7). It should be emphasized that chloroform extraction will not isolate the more polar metabolites, nor will those less polar metabolites that are extracted be detected if the quinone chromophore is no longer intact. Thus the assay is quite specific for the parent drug; and only closely related contaminants, decomposition products or metabolites are likely to be detected in addition to AZQ.

The choice of an internal standard in an HPLC assay can be quite critical. The internal standard should have a chromatographic behavior that is similar but not identical to the compound(s) being determined and should be sensitive to the method of detection. The internal standard should also be chemically similar to the analyte, since minimization of sample handling errors requires that it be carried through the isolation procedure. If the chemical similarity is close enough, the internal standard might even be used as a carrier for very small amounts of the unknown during sample workup. Finally, the internal standard should be a compound that is readily available, either commercially or through a simple synthesis. DADCQ was chosen as the internal standard for this analysis because it met all of the above criteria. This tetrasubstituted benzoquinone is retained by an octadecylsilane column yet elutes before AZQ,

has strong absorbance at 340 nm, is chloroform extractable (see Table 1) and hydrolytically stable, and is readily available since it was an intermediate in the initial synthesis of AZQ (1,8).

This assay was applied to determine the single dose plasma pharmacokinetics of AZQ in rats receiving an optimum dose (1 mg/kg) of the drug (7). A typical chromatogram is shown in Figure 3. Both AZQ and the DADCQ internal standard are clearly defined with no obvious interferences. In those cases where the AZQ concentration was expected to exceed 1 µg/ml (i.e. immediately after drug injection) and be outside the range of the calibration curve, the amount of internal standard added to the sample before workup was doubled. Upon subsequent HPLC analysis, a 50 µl aliquot was injected and the calculated concentration doubled to obtain the true level. For maximum sensitivity the method required at least 1.0 ml of biological fluid. In a human such a sample size presented no problem, but for 200-240 g rats where the total blood volume was on the order of a few milliliters, serial sampling of the type necessary for pharmacokinetics would severely perturb the system. Therefore, rats of similar age, sex and weight were sacrificed at prescribed times after injection of AZQ and only a single sample was obtained from each animal. The data from these composite samples was then used to define the plasma pharmacokinetics.

AZQ exhibits biphasic behavior in rat plasma (Figure 4). A very rapid redistribution phase is followed by a slower yet still fast elimination phase with a half-life of 26.5 min. Because the half-life of the initial phase is on the order of 3-5 minutes, sampling was not rapid enough to accurately define it. Each point in Figure 4 represents multiple animals and the error bar represents the range of concentrations for these different animals. AZQ plasma concentrations could be easily determined over four half-lives. Indeed, in some instances the parent drug could

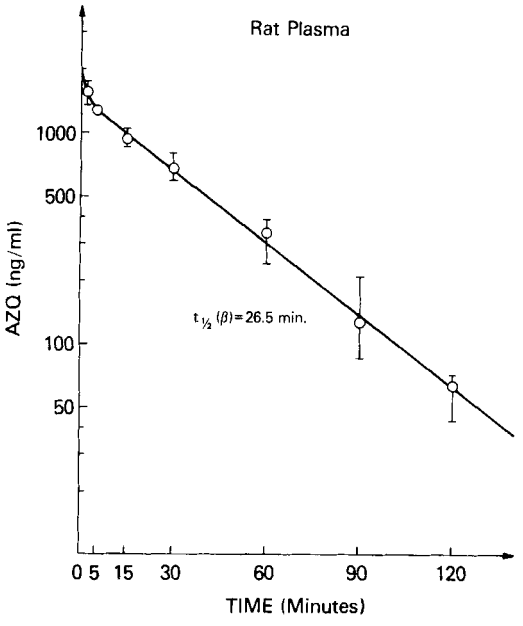

FIGURE 4. Plasma Pharmacokinetics of a Single 1 mg/kg Dose of AZQ in Male Sprague-Dawley Rats. Each point represents the mean AZQ concentration in 3 or more animals. The brackets about each point signify the range of measured AZQ concentrations.

still be detected after 4 hr at concentrations of less than 10 ng/ml.

CONCLUSION

The data presented above show that this HPLC assay is suitable for measuring AZQ concentrations in human patients. The method possesses sufficient sensitivity and specificity to determine single dose AZQ pharmacokinetics, even at the lowest initial dose (e.g. 1 mg/m^2) employed in a Phase I clinical trial. A primary question about the disposition of AZQ is the ability of the drug to enter the CNS, since it was specifically designed to do

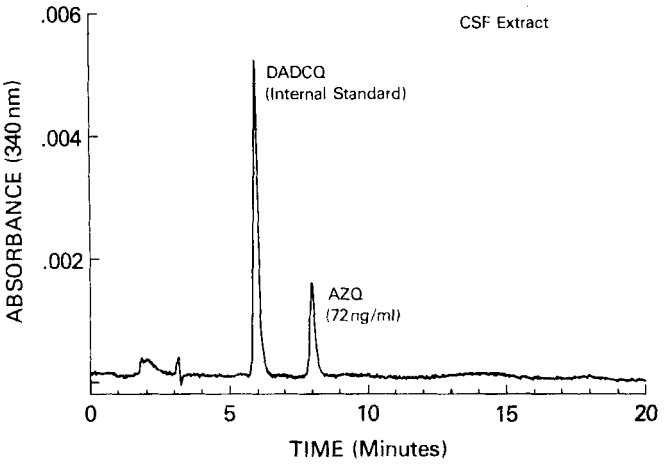

FIGURE 5. HPLC Analysis of AZQ in Human CSF. The sample was obtained by lumbar puncture 90 min after intravenous administration of a 15 mg/m^2 dose of AZQ.

this. AZQ penetration into CSF has been demonstrated in non-human primates (rhesus monkeys) by using ^{14}C-AZQ (9). Figure 5 presents the first evidence that intact AZQ likewise enters the CSF in humans. Here one can measure the unchanged drug directly without relying on radioactivity and the uncertainty generated by metabolism or decomposition. Determination of AZQ disposition and pharmacokinetics in human patients in Phase I clinical trials is currently in progress.

ACKNOWLEDGEMENTS

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