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Note

Determination of diaziridinyl-3,6-bis(2-hydroxyethylamino)-1,4-benzoquinone in plasma by solid-phase extraction and high-performance liquid chromatography

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Among many hundreds of synthetic compounds evaluated by the National Cancer Institute for antitumour activity, the aziridinylbenzoquinones showed good activity against murine leukaemia L1210 cells. 2,5-Diaziridinyl-3,6-bis(carboethoxyamino)-1,4-benzoquinone (AZQ, NSC 182986, Fig. 1) was eventually selected [1] as the first of these compounds for phase I and II clinical trials [2–4] and has shown good results against brain and other tumours [5,6]. During work on the synthesis of aziridinylbenzoquinone analogues, 2,5-diaziridinyl-3,6-bis(2-hydroxyethylamino)-1,4-benzoquinone (BZQ, NSC 224070, Fig. 1) also showed good antitumour activity [1].

BZQ is currently in a phase I clinical trial here in Bath, U.K. In this report we present a relatively simple extraction and high-performance liquid chromatographic (HPLC) method for the detection of BZQ in plasma together with some preliminary pharmacokinetic data and results of other experiments performed with the drug.

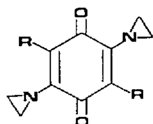

Fig. 1. Structures of AZQ ($R = -NHCO_2CH_2CH_3$) and BZQ ($R = -NHCH_2CH_2OH$).

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EXPERIMENTAL

Drug preparation

BZQ was synthesised by Dr. D.E.V. Wilman of the Institute of Cancer Research (Sutton, U.K.). The drug was dissolved in sterile 150 m*M* sodium bicarbonate at 1 mg/ml (with ultrasonication) for maximum stability [7].

Drug administration and blood sampling

The drug solution was given intravenously over 1–3 min via a butterfly needle pre- and post-flushed with 5 ml of 150 m*M* sodium bicarbonate. Blood samples were taken using a butterfly needle from the patient's other arm at between 3 min and 3 h after injection, the first five samples being taken within 30 min. The blood was cooled on ice and transferred to a pre-cooled (4°C) centrifuge usually within 30 min of being taken.

Plasma was obtained from blood with or without BZQ by centrifugation (1720 *g*, 7.5 min) in glass heparinised tubes. The plasma obtained was stored on ice for analysis the same day.

Extraction

Ice-cold plasma (3 ml) was loaded onto a pre-wetted [2 ml methanol, 5 ml water (pH 8.5), both ice-cold] C_{18} Sep-Pak cartridge (Millipore, Harrow, U.K.). The cartridge was washed with 5 ml ice-cold water (pH 8.5) and excess fluid was removed with 10 ml of air. BZQ was eluted slowly with ice-cold methanol. The first two drops were discarded and the next seventeen drops of eluate collected. After mixing, 250 μl of eluate were diluted with 500 μl of ice-cold ammonium acetate (0.0525 *M*) to bring it to approximately the same composition as the mobile phase. The diluted eluate (250 μl) was injected into a 200-μl Rheodyne sample loop and analysed. The volume of the remaining undiluted eluate was measured and the total volume of undiluted eluate was calculated.

A calibration curve for BZQ in plasma was prepared by the addition of known amounts of the drug to blank plasma samples followed by extraction and HPLC analysis.

Chromatography

An HPLC method has been developed using a mobile phase consisting of methanol–0.05 *M* ammonium acetate (40:60, v/v) at a flow-rate of 1 ml/min (Constametric III, LDC, Stone, U.K.) and using a Spherisorb ODS1 5-μm, 250 mm × 4.6 mm I.D. reversed-phase column (Phase Sep, Clwyd, U.K.) plus the equipment described previously [8]. Recently a diode array detector (1040A, Hewlett Packard, Winnersh, U.K.) has been employed allowing the analysis of BZQ at 385 nm. Compared with earlier work at 254 nm, this produced very much less interference from plasma components.

The BZQ solution (10 μg/ml) used for external standardisation was made by dilution with mobile phase of a 1 mg/ml solution of BZQ in 150 m*M* sodium bicarbonate and was stored at −20°C.

Data analysis

Analysis of pharmacokinetic data was conducted using the STATGRAPHICS least-squares regression analysis program on a IBM PCAT computer. The biexponential decline in plasma concentrations of BZQ was fitted to the equation $C=Ae^{-\alpha t}+Be^{-\beta t}$ where C is the concentration of BZQ in plasma in ng/ml at time t (min), A and B are parameters measured in ng/ml, and α and β are apparent first-order distribution rate constants (min^{-1}). Plasma half-lives ($t_{1/2\alpha}$ and $t_{1/2\beta}$) were calculated from these parameters, and the area under the curve (AUC) was calculated using the equation $\text{AUC}=A/\alpha+B/\beta$.

Statistical analysis of chromatographic results was performed by a Hewlett Packard Series 79994A Chemstation using the DOSTAT macro running under version 4.05 system software.

Concentration of BZQ in plasma was calculated using the equation $C=R\times A\times D$ where R is the response of the BZQ plasma standard (μg/ml/peak area), A the peak area of the BZQ plasma sample and D the dilution factor ($V\times 0.015$ μl) where V is the volume of collected eluate.

Protein binding

The binding of BZQ to human serum was determined by centrifugal ultrafiltration (Amicon centrifree micropartition systems, Amicon, Stonehouse, U.K.), each device containing an Amicon YMT membrane of 30 000 MW cut-off. Human serum (Flow Labs, Rickmansworth, U.K.) was spiked with BZQ at two concentrations (500 and 1500 ng/ml) and 1-ml aliquots were applied to the membrane. The units were centrifuged at 1760 g for 30 min at room temperature (20°C). Approximately 400 μl of protein-free ultrafiltrate were produced and two 100-μl aliquots were analysed by HPLC.

Drug stability

Although the drug stability of BZQ has been extensively investigated [7], we wished to determine whether dilute solutions of the drug could be stored frozen without the precipitation problems encountered at higher concentrations. BZQ was therefore made up at a range of concentrations (80–0.625 μg/ml), stored at −70°C for nineteen days and analysed periodically.

Drug stability was also investigated in the diluted eluate after extraction and quantification from plasma.

RESULTS

Chromatography

A calibration curve for the BZQ external standard was prepared by plotting peak area against concentration at 10, 5, 1, 0.5, 0.1 and 0.05 μg/ml.

Regression analysis of calibration data indicated a linear response over this concentration range (correlation coefficient >0.9999). For six replicate standard injections at 10 and 0.05 μg/ml the relative standard deviation for peak area was 0.67 and 2.12%, respectively. Long-term stability of the external standard was

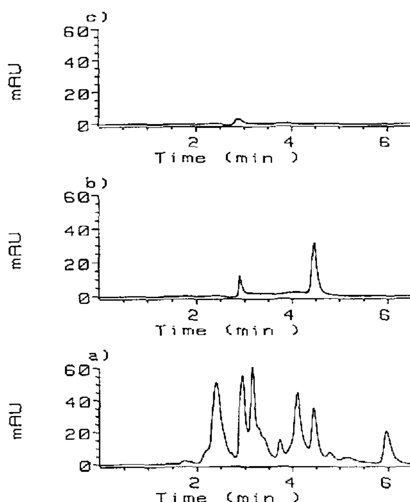

Fig. 2. Typical chromatograms of BZQ in plasma (1 μg/ml) at (a) 254 nm and (b) 385 nm. Chromatogram c is a blank plasma injection at 385 nm.

determined over a ten-day period; 27 injections produced a mean response of 0.02826 ± 0.00042 μg/ml/peak area giving a relative standard deviation of 1.49%.

Linearity of response with respect to plasma concentration of BZQ was determined at 5, 1, 0.5, 0.1 and 0.05 μg/ml. Regression analysis of these data showed a linear response (correlation coefficient >0.999). The average recovery of BZQ from plasma was calculated as 79%.

BZQ has two major UV absorption maxima at 230 and 385 nm. Initial work undertaken with a fixed-wavelength detector at 254 nm was compromised by interference by plasma components (Fig. 2a). The problems were lessened by using a wavelength of 360 nm, but much better results were obtained with the diode array detector at 385 nm with a bandwidth of 20 nm (Fig. 2b). The limit of detection (signal-to-noise ratio = 3) at 385 n*M* is approximately 3 ng/ml. Analysis of blank plasma samples at this wavelength revealed no peaks with a similar elution volume to BZQ (Fig. 2c). Interference from other concomitantly prescribed drugs was also checked: no interfering peaks were observed for buprenorphine, metoclopramide or ranitidine. The known degradation products monohydroxy- and dihydroxy-BZQ both elute from the column with much shorter retention times than BZQ [7].

Pharmacokinetics

Preliminary pharmacokinetic data have been determined for three patients at drug doses of 18, 22 and 33.3 mg. Fig. 3 shows the concentration versus time data for the patients in this study. In all patients the plasma decay curves were very similar, with a rapid redistribution phase followed by a slower but still quite fast elimination phase. Initial BZQ plasma concentrations of 0.7–2.8 μg/ml were measured immediately following injection. These fell to 100–120 ng/ml by 30 min and

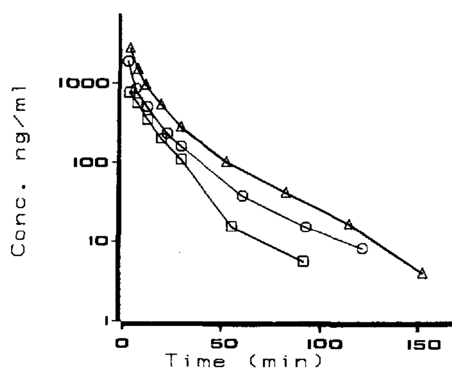

Fig. 3. Concentration versus time curve of BZQ in three patients after intravenous infusion of 18.0 mg (□), 22.0 mg (○) and 33.3 mg (△).

TABLE I

PRELIMINARY PHARMACOKINETIC DATA OF BZQ

Parameter	Patient 1	Patient 2	Patient 3
Dose (mg)	18.0	22.0	33.3
α (min^{-1})	0.09045	0.1219	0.1393
β (min^{-1})	0.02448	0.02735	0.0306
$t_{1/2\alpha}$ (min)	7.7	5.7	5.0
$t_{1/2\beta}$ (min)	27.9	25.3	22.7
AUC (min·μg/ml)	13.696	23.711	43.217
AUC/dose (min/l)	0.761	1.078	1.298

were below 20 ng/ml after 2 h. A summary of the pharmacokinetic parameters for the three patients is presented in Table I.

Protein binding

Protein binding of BZQ in human serum at 20°C was found to be negligible with recoveries of BZQ in the ultrafiltrate of 96.7±6.9% (mean ±S.D., $n=3$) and 96.3±4.5% at 500 and 1500 ng/ml, respectively.

Drug–membrane binding was measured with a solution of BZQ (10 μg/ml) in phosphate-buffered saline. BZQ recovery from the ultrafiltrate was 98.9±0.87%.

Drug stability

Rapid degradation of BZQ occurred in plasma at 37°C. The drug degraded 5% ($t_{0.95}$) in 15.5 min. At 0°C the drug degraded at a much lower rate with $t_{0.95}$ of 3.6 h.

The stability of BZQ in the diluted eluate collected during solid-phase extraction of the plasma samples was determined at −20°C. BZQ was 5% degraded in 32.6 h.

DISCUSSION

The data presented above show that this HPLC method is suitable for measuring BZQ concentrations in human patients. The extraction technique is relatively fast and the method possesses sufficient sensitivity and specificity to enable single-dose BZQ pharmacokinetic determinations.

However, as reported previously [7], BZQ solutions should not be stored for more than a few days and even then only at about 4°C. Storage of blood or plasma samples containing BZQ cannot be recommended as even with cooling to 0°C the $t_{0.95}$ is only 3.6 h. Limited storage of the diluted eluate at −20°C is possible although it is recommended that the extracted eluates be analysed sequentially immediately after dilution rather than batch extraction followed by batch analysis.

The preliminary BZQ pharmacokinetic results are quite interesting showing a possible non-linear characteristic as AUC/dose increases slightly with increasing dose. The data shows rapid removal of BZQ from plasma with $t_{1/2\alpha}$ and $t_{1/2\beta}$ of 6.1 and 26 min, respectively. Comparison with AZQ pharmacokinetics [9] shows that they have similar half-lives with $t_{1/2\alpha}$ and $t_{1/2\beta}$ for AZQ being 4.8 and 31 min, respectively. The rapid decay of BZQ is not, however, due to degradation as the calculated half-life for BZQ in plasma at 37°C is a comparatively large 209 min.

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