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Instability of solutions of diaziquone stored at negative temperatures

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Summary

The stability of diaziquone (AZQ, NSC 182986, aziridinylbenzoquinone, 2,5-diaziridinyl-3,6-bis-(carboethoxyamino)-1,4-benzoquinone) in solution has been investigated by reversed-phase high-pressure liquid chromatography. Dissolved in dimethylacetamide and diluted with phosphate buffer (for i.v. administration), the drug was found to be most stable at 4 °C and ≤ −70 °C, but less stable between −12 °C and −50 °C. Dilution of the drug in phosphate-buffered saline resulted in a solution that was least stable at −35 °C but more stable between 4 °C and −12 °C and ≤ −86 °C. Investigation of the stability of diaziquone in a number of solutions with and without chloride showed that phosphate and chloride was the damaging combination, the aziridinyl rings opening to form chlorethylamino groups. Citrate was identified as conferring most stability on the drug at −20 °C, and a long-term study showed that diaziquone could be stored in 150 mM citrate buffer, pH 6.3, at −20 °C or −70 °C for at least 3 months.

Introduction

Both pharmacists and research scientists are becoming more interested in the stability of anticancer drugs frozen in solution. The former are interested in saving time and money by batch reconstitution and freezing. The latter are interested because they require dilute solutions of drugs to be readily available for in vitro drug sensitivity assays at short notice.

It is now known that a number of anticancer drugs can be frozen at relatively high concentrations after reconstitution without detrimental effect (Franco et al., 1984; Hoffman et al., 1979; Karlsen et al., 1983; Kirk et al., 1984; Yang and Drewinko, 1985). However, the freezing of more dilute solutions may result in either greater or less stability, the latter probably being the case for doxorubicin (Bosanquet, 1986).

We have investigated the stability of frozen solutions of melphalan (Bosanquet, 1985b), and chlorambucil (Bosanquet and Clarke, 1986), and now present stability data on the investigational agent, diaziquone (AZQ, NSC 182986, aziridinylbenzoquinone, 2,5-diaziridinyl-3,6-bis-(carboethoxyamino)-1,4-benzoquinone). This drug was identified as a potential anticancer agent by Kahn and Driscoll (1976) whilst screening a series of aziridinylbenzoquinones that it was hoped would cross the blood–brain barrier. Over the past few years, it has been investigated in many phase II trials.

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Whilst we were testing the stability of solutions of an analogue of diaziquone, BZQ (NSC 224070, 2,5-diaziridinyl-3,6-bis(2-hydroxyethylamino)-1,4-benzoquinone) (Bosanquet and McLoughlin, 1985), we found that both drugs were unstable frozen when in dilute solution in phosphate-buffered saline (PBS). As we wanted to incorporate diaziquone into the Differential Staining Cytotoxicity (DiSC) assay that we are investigating (Bird et al., 1985, 1986), we decided to further test the stability of diaziquone in an attempt to identify a method for successfully storing it frozen in solution.

Materials and Methods

Pure diaziquone (lot AJ 58.5) was kindly supplied by Dr. R.R. Engle (Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, U.S.A.). The drug was also obtained formulated for i.v. injection (prepared for the NCI by Ben Venue Laboratories, Bedford, OH, U.S.A.): 100 mg diaziquone (lot BV-82-209) to be dissolved in 0.5 ml of anhydrous *N,N*-dimethylacetamide (DMA) and diluted with 9.5 ml 0.01 M phosphate buffer, pH 6.5. Dichloro-diaziquone ($AZQCl_2$, Fig. 1) was kindly supplied by Dr. J.A. Kelley (also of the Developmental Therapeutics Program).

Diaziquone was dissolved at 20 mg/ml in DMA (the FDA approved solvent for this drug) for all experiments except those in Fig. 3 (where dimethylsulphoxide (DMSO) was used) and some in Table 1.

High-pressure liquid chromatographic (HPLC) conditions were the same as those used for previous experiments with BZQ (Bosanquet and McLoughlin, 1985): a 25 cm Spherisorb-ODS (5 μm) column and a mobile phase of 30% methanol plus 70% 0.05 M ammonium acetate (resultant pH approx. 6.6). Diaziquone peak heights were measured and compared to the peak height of standard solutions of diaziquone stored in the mobile phase at $-20\,^{\circ}$C. These standard solutions showed negligible degradation peaks over the course of individual experiments. Calibration curves of diaziquone standard solution in the mobile phase

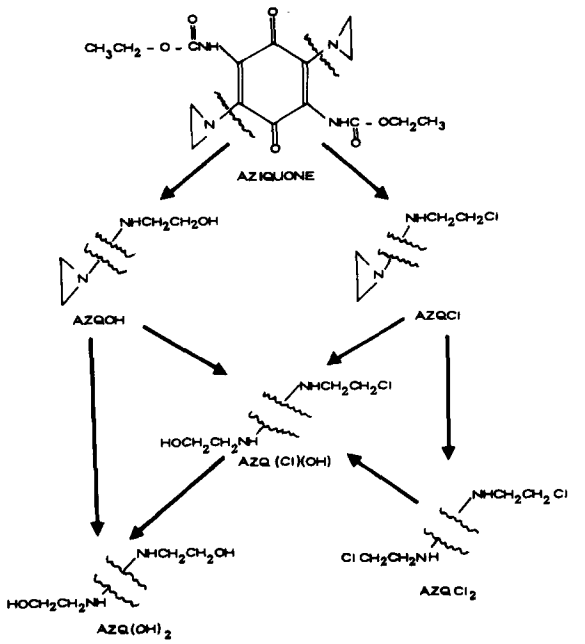

Fig. 1. Probable degradation pathways of the aziridinyl rings of diaziquone in the presence of chloride ion. The expected direction of degradation is shown: the reversibility of these reactions is not known.

were linear ($r > 0.999$) over the range 1–20 μg/ml. Replicate injections of the lower and upper limits of the standard curve produced relative standard deviations of 0.42 and 0.39%, respectively. Experiments were performed to determine that the HPLC method was stability-indicating for diaziquone in 150 mM citrate buffer (see below). Diaziquone at 1 mg/ml was partially degraded at pH 4.0 (HCl), pH 11 (NaOH) and in the citrate buffer. Chromatography of the solutions was performed using a diode array detector (Hewlett Packard) and upslope, apex and down-slope UV spectra (210–600 nm) for the diaziquone peaks compared. For all 3 solutions, a 100% match was obtained suggesting no interference by coeluting peaks.

Temperatures were maintained by water bath (50 °C and 37 °C), commercial refrigerator (4 °C) and freezers (-20, -35, -50, $-70\,^{\circ}$C), cryostat (-7, $-12\,^{\circ}$C), dry-ice ($-86\,^{\circ}$C) and liquid nitrogen ($-196\,^{\circ}$C).

Solutions (all 0.1 M) were prepared as follows: citrate, a solution of citric acid was adjusted to pH 7.0 or 6.3 with NaOH; sulphate, sodium sulphate

was dissolved in water (not adjusted for pH); bicarbonate, commercially available 0.15 M sodium bicarbonate was diluted 1 : 1.5 with water (pH approx. 9.0); Tris, a solution of Tris was adjusted to pH 7.0 with HCl; acetate, ammonium acetate was dissolved in water (pH approx. 6.5); phosphate, 0.53 g KH_2PO_4 and 0.86 g Na_2HPO_4 were dissolved in 100 ml water (pH approx. 6.6). PBS was made by dissolving one PBS tablet (Oxoid, Basingstoke, Hampshire, U.K.) in 100 ml water.

For the experiments using these solutions (see Fig. 5), they were diluted with either an equal volume of water or an equal volume of 0.1 M NaCl.

Results and Discussion

Results of initial experiments to test the stability of frozen diaziquone were very surprising. A few samples of the drug, dissolved in DMSO at 5 mg/ml and diluted to 10 μg/ml with PBS were placed at −35 °C and analysed after 2 and 20 h. At 2 h, no degradation was seen, whereas at 20 h only 13% of the original diaziquone peak height was observed and two other major peaks had appeared. These two peaks had retention times longer than diaziquone (Fig. 2B) suggesting that these were not hydrolysis products (which would

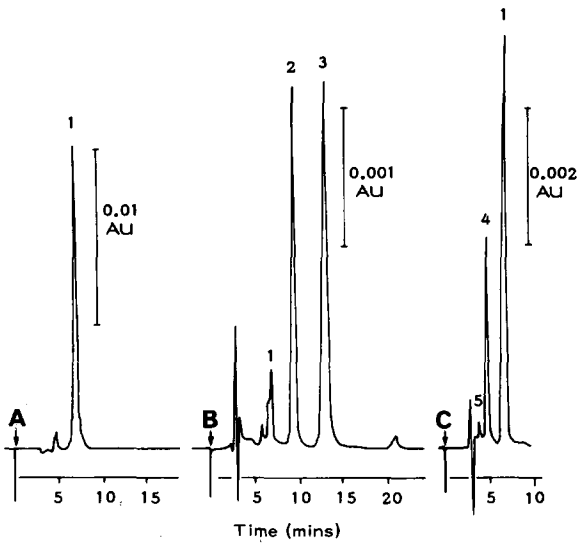

Fig. 2. HPLC of diaziquone. A: 500 ng standard diaziquone solution stored in mobile phase. B: 250 ng diaziquone (10 μg/ml) frozen for 7 days at −35 °C in PBS. C: 250 ng diaziquone (10 μg/ml) stored for 7 days at 4 °C in H_2O. Peak identification: 1, diaziquone; 2, AZQCl (tentative); 3, $AZQCl_2$; 4, AZQOH (tentative); 5, $AZQ(OH)_2$ (tentative). Arrows indicate the points of injection.

TABLE 1

Effect of diluent and solvent on the stability of diaziquone stored at −35 °C and 4 °C

Temperature/diluent	Solvent	Day 1				Day 7			
		AZQOH	AZQ	AZQCl	$AZQCl_2$	AZQOH	AZQ	AZQCl	$AZQCl_2$
−35 °C/PBS	DMA	– [a]	4 [b]	49	27	–	3	37	43
−35 °C/PBS	DMSO	–	4	54	31	–	3	40	39
−35 °C/PBS	MeOH	–	15	55	30	–	2	30	42
−35 °C/H_2O	DMA	3	100	–	–	2	97	–	–
−35 °C/H_2O	DMSO	3	98	–	–	3	100	–	–
−35 °C/H_2O	MeOH	–	95	–	–	–	99	–	–
4 °C/PBS	DMA	–	99	–	–	5	100	–	–
4 °C/PBS	DMSO	–	98	–	–	5	99	–	–
4 °C/PBS	MeOH	–	99	–	–	5	97	–	–
4 °C/H_2O	DMA	10	96	–	–	47	65	–	–
4 °C/H_2O	DMSO	9	96	–	–	44	62	–	–
4 °C/H_2O	MeOH	7	94	–	–	46	63	–	–

[a] – = less than 1% detected.

[b] All figures are percentages of initial diaziquone peak area. Some totals are more and some less than 100% because the peak area/weight of AZQ will not be identical to that of its degradation products.

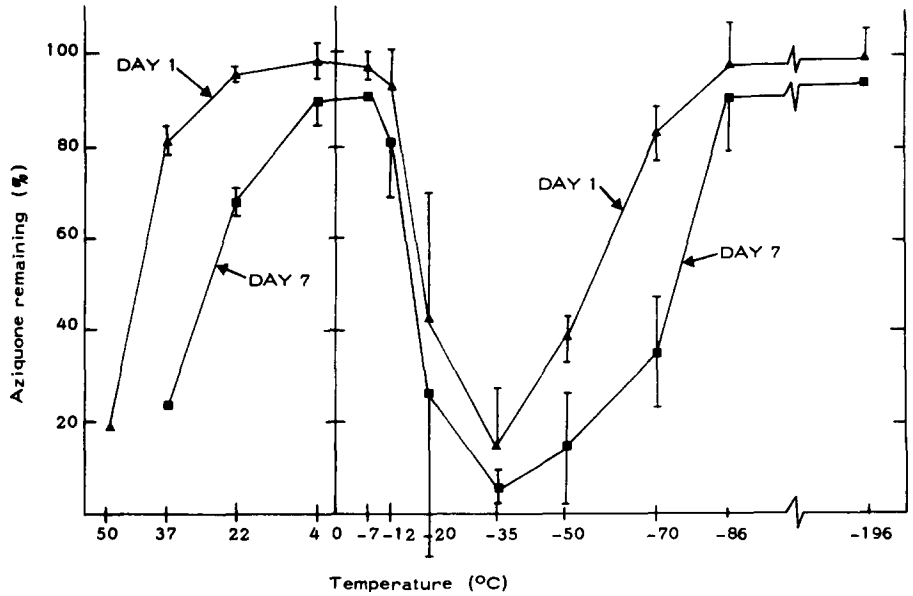

Fig. 3. Storage for 1 and 7 days of diaziquone (10 μg/ml) dissolved in DMSO and diluted with PBS. The results are combined for a number of experiments. ▲, day 1; ■, day 7. Error bars are ±1 S.D.

be expected to come out before the parent drug with reversed-phase HPLC). Fig. 2 shows the HPLC output after 7 days in PBS at −35°C showing peaks due to AZQCl and $AZQCl_2$, and after 7 days in water at 4°C showing peaks most likely due to AZQOH and $AZQ(OH)_2$.

Structures of the peaks were tentatively assigned by reference to the similar results produced by Poochikian and colleagues at positive temperatures (Poochikian and Craddock, 1981; Poochikian and Kelley, 1981). The identity of the $AZQCl_2$ peak was substantiated by injecting authentic $AZQCl_2$: this precisely co-chromatographed with peak 3 of Fig. 2.

Few drugs have been shown to be less stable at lower temperatures, but in 1971 Savello and Shangraw (1971) reported that sodium ampicillin was less stable at −20°C than at 5°C in a number of solutions.

Table 1 shows the effects of solvent and diluent on this phenomenon, the former seeming to make little difference whereas the latter having a dramatic effect on drug stability. Table 1 shows that PBS produced instability at −35°C compared to H_2O, but conferred greater stability at 4°C. The results in PBS may be due to the low freezing point (down to −23.5°C) and low pH (down to 3.3) observed when solutions of mixtures of phosphates and sodium chloride were frozen (van den Berg, 1959).

A similar observation of the instability of diaziquone at −40°C (dissolved in DMSO and diluted in PBS) has recently been recorded by Hildebrand-Zanki and Kern (1986) using a bioassay to determine drug stability. They found a half-life of 2.5 days for the drug under these conditions.

A number of experiments were performed to investigate the effect of temperature on the degradation of diaziquone to AZQCl and $AZQCl_2$. The drug was dissolved in DMSO and diluted with PBS and Fig. 3 shows the combined results of all these experiments. All the samples stored at negative temperatures were visibly solid when removed for analysis, although this does not rule out the possibility of small pockets of liquid still being present. A minimum stability was found around −35°C whilst diaziquone was essentially stable at −7°C and −12°C. It is also sometimes stable but more usually unstable at −20°C, this observation resulting in the very high standard deviations calculated at this temperature.

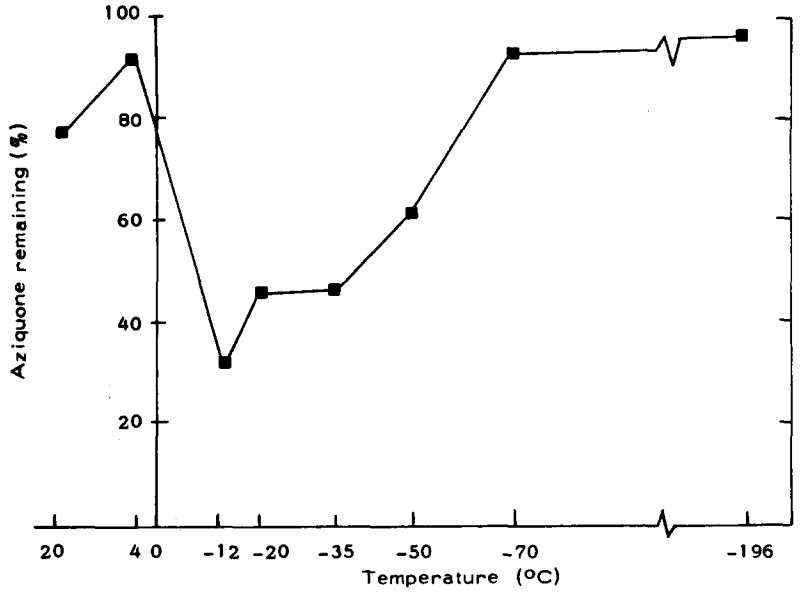

Fig. 4. Storage for 9 days of diaziquone (1 mg/ml) dissolved in DMA and diluted with the phosphate diluent supplied.

Assuming that diaziquone, AZQCl and $AZQCl_2$ gave the same response with respect to HPLC peak area per weight of material injected, almost all the reduction in diaziquone concentration at negative temperatures could be accounted for by the peaks of AZQCl and $AZQCl_2$. The greater the degradation of diaziquone, the greater the $AZQCl_2$/AZQCl peak area ratio became.

An experiment was also performed to test the stability of diaziquone made up for i.v. injection at 1 mg/ml in the phosphate buffer supplied (Fig. 4). The results indicated once again that diaziquone was unstable between $-20\,^{\circ}C$ and $-50\,^{\circ}C$, but in this case least stability was observed at $-12\,^{\circ}C$.

The results of one of a number of experiments performed to test which ions were detrimental to the stability of diaziquone are presented in Fig. 5. Consistently, phosphate and chloride was the damaging combination for diaziquone at 10 μg/ml, with chloride on its own producing 40% degradation after one week at $-20\,^{\circ}C$. Citrate was found to be the ion conferring most stability on diaziquone, and so long-term experiments were set up to see whether diaziquone could be stored in this buffer. Fig. 6, showing the results of these experiments, suggests that 0.15 M citrate buffer, pH 6.3, is a good medium in which to store the frozen drug. It is also a solution which, at pH 4.0, has been shown to be compatible with an in vitro drug sensitivity assay (Bosanquet, 1985a).

Two further experiments were performed in an attempt to elucidate the cause of the degradation of diaziquone in PBS. In one, diaziquone at 1, 10 and 100 μg/ml was frozen in PBS at $-25\,^{\circ}C$: the

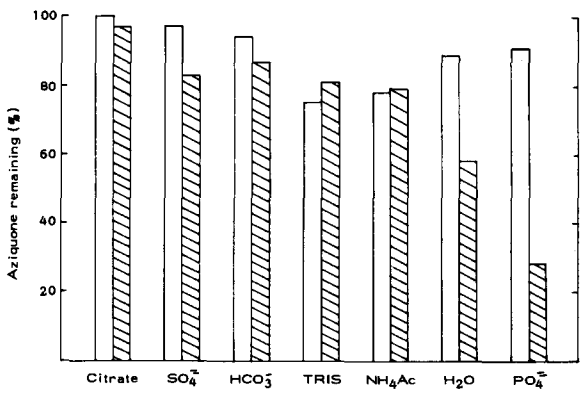

Fig. 5. Storage for 7 days at $-20\,^{\circ}C$ of diaziquone (10 μg/ml) dissolved in DMA and diluted in various solutions with (shaded) and without chloride present. A repeat experiment gave very similar results.

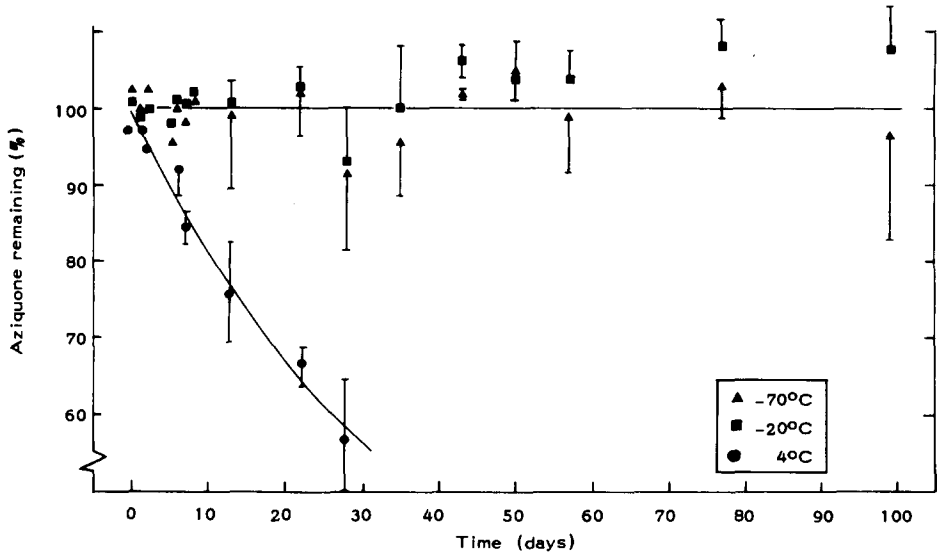

Fig. 6. Storage of diaziquone (10 μg/ml) dissolved in DMA and diluted with 0.15 M citrate buffer pH 6.3. ●, 4 °C; ■, −20 °C; ▲, −70 °C. Error bars (±1 S.D.) are not included on points above 93% on days 0–10 to reduce overcrowding.

results showed diaziquone had greater stability at the highest concentration. In the other experiment, solutions of diaziquone at 10 μg/ml in PBS were frozen in liquid nitrogen (−196 °C) and then stored at 4, −10, −25, −50 and −196 °C for 1 and 7 days. At both time points, minimum stability was observed at −50 °C with samples at −10 °C degrading more, and samples at −20 °C less, than that recorded in Fig. 3.

The final working hypothesis for the cause of diaziquone's degradation in PBS was as follows: diaziquone at 10 μg/ml is attacked by chloride ions at temperatures below about −20 °C forming first AZQCl and then $AZQCl_2$; the reaction is catalysed by phosphate ions; diaziquone is protected at −7 °C, −12 °C and sometimes −20 °C by being in pockets of solution which are not frozen; some sort of self-association may protect the drug at higher concentrations. This last point is in direct contrast to results obtained with ampicillin where the drug was found to catalyse its own degradation (Savello and Shangraw, 1971).

In conclusion, diaziquone should be stored frozen in solution in citrate buffer, pH 6.3, for the most extended solution shelf-life. At negative temperatures, chloride and phosphate are the most damaging ions that were tested.

Acknowledgements

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