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High-performance liquid chromatographic analysis of AQ4N, an alkylaminoanthraquinone *N*-oxide

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Abstract

A simple, highly selective and reproducible reversed-phase high-performance liquid chromatography method has been developed for the analysis of the new anti-cancer pro-drug AQ4N. The sample pre-treatment involves a simple protein precipitation protocol, using methanol. Chromatographic separations were performed using a HiChrom HIRPB (25 cm×4.6 mm I.D.) column, with mobile phase of acetonitrile–ammonium formate buffer (0.05 *M*) (22:78, v/v), with final pH adjusted to 3.6 with formic acid. The flow-rate was maintained at 1.2 ml min^{-1}. Detection was via photodiode array performed in the UV range at 242 nm and, since the compounds are an intense blue colour, in the visible range at 612 nm. The structurally related compound mitoxantrone was used as internal standard. The validated quantification range of the method was 0.05–10.0 μg ml^{-1} in mouse plasma. The inter-day relative standard deviations (RSDs) (n=5) ranged from 18.4% and 12.1% at 0.05 μg ml^{-1} to 2.9% and 3.3% at 10.0 μg ml^{-1} for AQ4N and AQ4, respectively. The intra-day RSDs for supplemented mouse plasma (n=6) ranged from 8.2% and 14.2% at 0.05 μg ml^{-1} to 7.6% and 11.5% at 10.0 μg ml^{-1} for AQ4N and AQ4, respectively. The overall recovery of the procedure for AQ4N was 89.4±1.77% and 76.1±7.26% for AQ4. The limit of detection was 50 ng ml^{-1} with a 100 μl sample volume. The method described provides a suitable technique for the future analysis of low levels of AQ4N and AQ4 in clinical samples.

Keywords: AQ4N; Alkylaminoanthraquinone *N*-oxide

1. Introduction

The search for a new and effective treatment for cancer in which selective toxicity to tumour cells can be achieved with minimal toxicity to normal tissue, is part of the continuing challenge in cancer research. This goal can potentially be aided by targeting the treatment-resistant hypoxic fraction within a tumour with cytotoxic agents that are not activated in normal oxygenated tissues. In this light, considerable interest has been shown in the rational development of the tertiary amine *N*-oxide derivatives [1,2].

The novel anticancer bioreductive compound, the alkylaminoanthraquinone *N*-oxide AQ4N (1,4-bis-{[2-(dimethylamino-*N*-oxide)ethyl]amino} 5,8-dihydroxyanthracene-9,10-dione) (Fig. 1) is a non-

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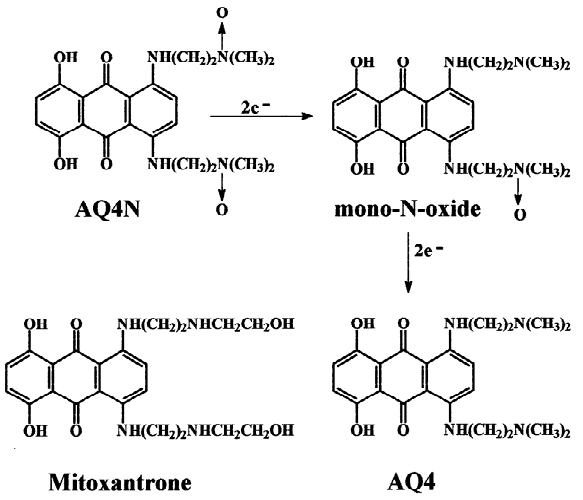

Fig. 1. Structures of compounds AQ4N, mono-*N*-oxide, AQ4 and mitoxantrone. Diagram shows the bioreductive pathway from the di-*N*-oxide prodrug AQ4N, to the two-electron reduced intermediate AQ4M (mono-*N*-oxide), and the four-electron reduced product AQ4, which is a potent cytotoxic agent. Shown for comparison is the structurally related anthracenedione derivative mitoxantrone (used as internal standard).

toxic prodrug that is reduced in hypoxic conditions to a stable oxygen-insensitive cytotoxic metabolite AQ4 (Fig. 1) capable of intercalating DNA and inhibiting topoisomerase II activity [3]. Unlike conventional bioreductive agents the products of reduced *N*-oxides remain active even if hypoxia is transient [1,4]. The stability of AQ4 coupled with the high binding affinity for DNA encourages prolonged exposure to cells where it is formed [4] which facilitates cytotoxic activity in regions of the tumour normally evasive to conventional treatments, and may actually enhance the activity of other chemotherapeutic agents such as cyclophosphamide [5,6]. Furthermore, when administered concurrently with radiation, AQ4N has been shown to reduce considerably the radiation dose required to give an anti-tumour effect equivalent to that of radiation alone [4,7]. AQ4N is thus a lead compound from a new series of anticancer agents proposed for clinical evaluation in the UK in early 2000.

The method that we have developed for the analysis of AQ4N by reversed-phase high-performance liquid chromatography (HPLC) was based on a paper by Raleigh et al. [8] and similar methods for the analysis of related compounds mitoxantrone [9] and CI-941 [10]. For the analysis of AQ4N Raleigh et al., used a Waters C_8 reversed-phase column with a mobile phase consisting of 80% aqueous ammonium formate (0.5 *M*, pH 4.2)–20% acetonitrile. While for the determination of the structurally related compound mitoxantrone (Fig. 1), Schoemaker et al., employed a C_{18} Bondapak reversed-phase column with a mobile phase consisting of acetonitrile–ammonium formate buffer (0.16 *M*) (30:70, v/v) (pH 2.7) with hexane sulphonic acid added as an ion-pair reagent [9]. However, for the analysis of CI-941, Graham et al., employed a Spherisorb C_6 analytical column and a mobile phase of acetonitrile–methanol–0.25 *M* ammonium formate (adjusted to pH 3 with 98% formic acid) (1:1:8, v/v) [10].

In this study, a sensitive and reproducible reversed-phase HPLC method is described for the quantification of AQ4N and its metabolite AQ4 in plasma, using mitoxantrone as an internal standard. The use of photodiode array detection (PDA) in the visible region at 612 nm confers a high degree of selectivity for AQ4N and its metabolites, which simplifies identification when extracting from biological samples.

The assay is appropriate for the accurate determination of the pharmacokinetic profile of the drug, both pre-clinically in rodents, and in phase I clinical trials in which the starting dose is likely to be 20 mg kg^{-1} which is 1/10 mouse maximum tolerated dose (MTD).

2. Experimental

2.1. Chemicals and reagents

AQ4N, AQ4 and the mono-*N*-oxide (Fig. 1) were synthesised as previously described [11]. Mitoxantrone was purchased from Sigma–Aldrich (Poole, UK). Stock solutions were shown to be stable prepared at 1.0 mg ml^{-1} and stored in polypropylene tubes. AQ4 (1 mg ml^{-1}) was wetted with dilute HCl (0.1 *M*), before being prepared as an aqueous solution and stored at −20°C. Stock solutions of AQ4N and mitoxantrone (1 mg ml^{-1}) were prepared in water, AQ4N was stored at 4°C and mitoxantrone at −20°C. High-purity HPLC-grade solvents (Fisher

Table 1
Extraction efficiencies for AQ4N and AQ4 from mouse plasma ($n=6$)

AQ4N concentration ($\mu g\ ml^{-1}$)	Recovery (mean±1 SD)	AQ4 concentration ($\mu g\ ml^{-1}$)	Recovery (mean±1 SD)
100	90.3±0.61	100	73.5±3.2
10	90.6±6.0	10	84.3±7.2
1.0	87.4±8.6	1.0	70.5±8.6

Scientific, Loughborough, UK), analytical grade chemicals (Sigma–Aldrich) and triple distilled water were used throughout.

Heparinised polypropylene tubes were used for collection of blood samples, polypropylene microcentrifuge tubes (Sigma–Aldrich) were used throughout for sample handling and storage, and polypropylene autosampler vials (Sigma–Aldrich) were used to load samples for HPLC analysis.

2.2. Animals

Female NMRI mice aged 6–8 weeks, were obtained from B & K Universal (Hull, UK). They were fed a CRM pellet diet (CRM, Special Diets Service, Witham, UK) and water ad libitum. All animal experiments were carried out under a project licence approved by the Home Office, London, UK, and UK CCCR guidelines [12,13] were followed throughout.

2.3. Instrumentation

The chromatographic system consisted of Waters Model 510 HPLC pump, a Waters 717 autosampler and a 996 PDA system (Waters, Watford, UK). Data were collected using λ_{max} values of $\lambda_1=242$ nm and $\lambda_2=612$ nm and chromatograms were processed using Millennium Software (Waters).

2.4. Chromatographic conditions

The chromatographic separation of AQ4N was performed isocratically using a HiChrom HIRPB (25 cm×4.6 mm I.D.) column (HiChrom, Reading, UK). The mobile phase consisted of acetonitrile–ammonium formate buffer (0.05 *M*) (22:78, v/v), with the final pH adjusted to 3.6 with formic acid (the pK_a values of the compounds are unknown though similar compounds have pK_a values in the region of 10). The flow-rate was maintained at 1.2 ml min^{-1}.

2.5. Sample preparation

Mouse plasma samples used in the extraction and calibration procedures were taken by cardiac puncture from drug-free anaesthetised NMRI mice. A volume of 100 μl of blank plasma was spiked with AQ4N and AQ4 to give the appropriate final concentrations of 100.0, 10.0 and 1.0 $\mu g\ ml^{-1}$ for extraction efficiencies (Table 1), and 10.0, 5.0, 1.0, 0.5 and 0.05 $\mu g\ ml^{-1}$ for calibration purposes (Table 2). Three volumes of methanol, containing the appropriate concentration of internal standard, were added to precipitate protein. After vortex-mixing the samples were centrifuged at 7000 *g* for 3 min, the supernatant was carefully removed and 60-μl volumes injected onto the HPLC system. The extraction

Table 2
Assay performance data for AQ4N and AQ4 in mouse plasma showing accuracy and precision for inter-day variation ($n=5$)

AQ4N theoretical concentration ($\mu g\ ml^{-1}$)	Mean calculated concentration ($\mu g\ ml^{-1}$)	Relative standard deviation (%)	AQ4 theoretical concentration ($\mu g\ ml^{-1}$)	Mean calculated concentration ($\mu g\ ml^{-1}$)	Relative standard deviation (%)
10.0	9.83±0.29	2.92	10.0	11.44±0.38	3.34
5.0	4.79±0.25	5.32	5.0	5.51±0.37	6.67
1.0	1.01±0.13	12.71	1.0	1.07±0.09	8.23
0.5	0.64±0.10	15.20	0.5	0.57±0.05	8.19
0.05	0.07±0.12	18.4	0.05	0.054±0.004	12.1

efficiency ($n=6$) was calculated as a percentage of non-extracted saline controls. All samples were handled in polypropylene microcentrifuge tubes to avoid potential binding to glass.

The suitability of the method for pharmacokinetic analysis was assessed in the mouse by administering AQ4N at 200 mg kg^{-1} intravenously (i.v.). A blood sample (50 μl) was taken following 2 min and prepared for analysis as above.

2.6. Calibration

Plasma calibration standards were prepared by spiking drug-free mouse plasma to give the final concentration range of 0.05–10.0 μg ml^{-1}. The calibration curves were determined using the linear regression analysis of the ratios of peak area of the analyte versus internal standard, plotted against concentration.

Between-day variation was calculated by preparing calibration curves ($n=5$) on non-consecutive days and calculating accurate concentrations for known standards from the curves.

2.7. Protein binding

Protein binding of AQ4N in plasma was investigated using ultrafiltration with two different types of micropartitioning system; firstly a Nanospin Plus M_r cut-off 30 000 (Gelman Sciences, Ann Arbor, MI, USA) containing a cellulose acetate membrane, and secondly an Ultrafree MC 30K with a polysulphone membrane (Millipore, Watford, UK). Murine plasma samples were spiked with AQ4N and AQ4 to give a range of final concentrations of 100, 10.0 and 1.0 μg ml^{-1}. Triplicate samples were subjected to ultrafiltration at 5000 *g* for 10 min. Filtered and non-filtered saline controls were used to determine the extent of drug binding to filters. Protein binding and filter binding of AQ4N was calculated as a percentage of the filtered and non-filtered controls, respectively.

Since AQ4 was found to bind strongly to both types of micropartitioning filter, a further technique using ultracentrifugation (Beckman Optima TL ultracentrifuge) was employed to investigate protein binding. For this method murine plasma samples (200 μl) spiked with AQ4 to give a final concentration of 100, 10.0 and 1.0 μg ml^{-1} were centrifuged at 250 000 *g* for 16 h and compared to saline controls.

3. Results

The mobile phase for AQ4N was optimised to improve the separation of the analytes and to create good peak symmetry. Initially, the formate buffer concentration was decreased from a suggested 500 m*M* to 50 m*M*. We then decreased the pH to 3.6 with formic acid. With our initial choice of C_{18} column peak tailing, which is commonly seen with this class of compound, was observed. However, by combining the above mobile phase with a base deactivated reversed-phase HiChrom HIRPB column the peak tailing was eliminated. The organic content of the mobile phase was then altered to provide the desired separation and retention times of AQ4N and metabolites (Fig. 2).

The spectrum for AQ4N (Fig. 3) has four peak regions of absorbance; these are in the ultraviolet region at 242 and 276 nm, and in the visible region at 612 and 662 nm. The spectrum for AQ4 is similar to AQ4N but with a slight spectral shift in absorbance maxima from 276 nm to 275 nm, a shift can also be seen from 612 nm to 609 nm and at 662 nm to 659 nm (Fig. 3). Calibration curves ($n=6$) produced for AQ4N and AQ4 were linear (r values >0.993) between 0.05 and 10 μg ml^{-1} with mean gradients of 2.84 ± 0.27 and 2.97 ± 0.11 and relative standard deviations (RSDs) of 9.44% and 3.67% for AQ4N and AQ4, respectively. Intercepts were not appreciably different from zero. The inter-day RSDs ($n=5$) ranged from 18.4% and 12.1% at 0.05 μg ml^{-1} to 2.9% and 3.3% at 10.0 μg ml^{-1} for AQ4N and AQ4, respectively. The intra-day RSDs for supplemented mouse plasma ($n=6$) ranged from 8.2% and 14.2% at 0.05 μg ml^{-1} to 7.6% and 11.5% at 10.0 μg ml^{-1} for AQ4N and AQ4, respectively.

A simple and reproducible extraction technique with methanol gave high extraction efficiencies for both AQ4N and AQ4, the details of which are given in Table 1. Extraction efficiencies from plasma over the range of 1.0–100 μg ml^{-1} were >87% for AQ4N and >70% for AQ4, with RSDs <10% and <12%, respectively (Table 1). Only limited protein binding data were obtained from the techniques that

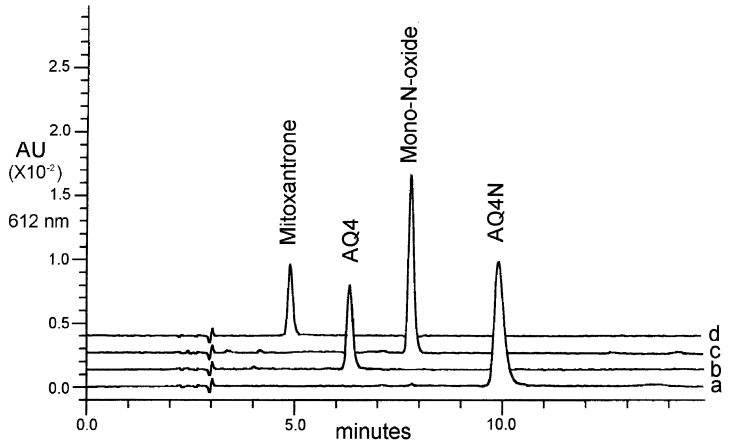

Fig. 2. Chromatographic traces demonstrating the separation achieved with a HIRPB column and a mobile phase consisting of acetonitrile–ammonium formate buffer (0.05 *M*) (22:78, v/v), pH 3.6 and a flow-rate of 1.2 ml min^{-1}. Traces shown are (a) AQ4N (2.5 μg ml^{-1}) retention time of 10.0 min, (b) AQ4 (2.0 μg ml^{-1}) retention time 6.4 min, (c) mono-*N*-oxide (3.5 μg ml^{-1}) retention time 8.0 min and (d) mitoxantrone – used as internal standard (1.5 μg ml^{-1}), retention time of 5.0 min.

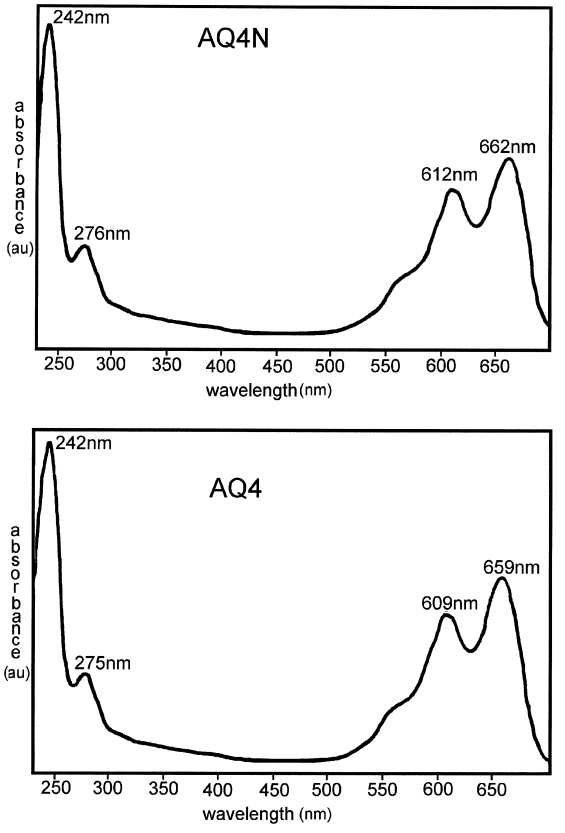

Fig. 3. Absorbance spectrum (range 225–700 nm) for AQ4N and AQ4. Absorbance peaks occur at 662 nm, 612 nm, 276 and 242 nm for AQ4N, and 659 nm, 609 nm, 275 nm and 242 nm for AQ4.

we employed. This was due primarily to the strong binding to filters and micropartitioning devices that was observed for both AQ4N and AQ4 in control (protein-free) samples. The results (not shown) indicated that AQ4 was >90% filter bound between 1.0 and 100 μg ml^{-1} and AQ4N was 6%, 38% and >90% bound to the filter at 100, 10.0 and 1.0 μg ml^{-1}, respectively. The ultracentrifugation technique was not conclusive, and data cited here is an approximation, we suggest that at 100 μg ml^{-1} AQ4N is 15% protein bound whereas at the same concentration AQ4 is approximately 80% protein bound.

The result of the preliminary in vivo metabolism investigation in the mouse, following AQ4N administration at 200 mg kg^{-1} i.v. is presented in Fig. 4. The trace shows good peak resolution with baseline separation of AQ4N, the mono-*N*-oxide, AQ4 and two unidentified metabolite peaks, possibly the mono dealkylated products. This investigation produced a level of efficiency appropriate for further detailed metabolism and pharmacokinetic studies in the forthcoming clinical trials.

4. Discussion

AQ4N is essentially a non-toxic anti-cancer prodrug that is reduced to a potently cytotoxic metabolite AQ4 in the hypoxic region of tumours. When

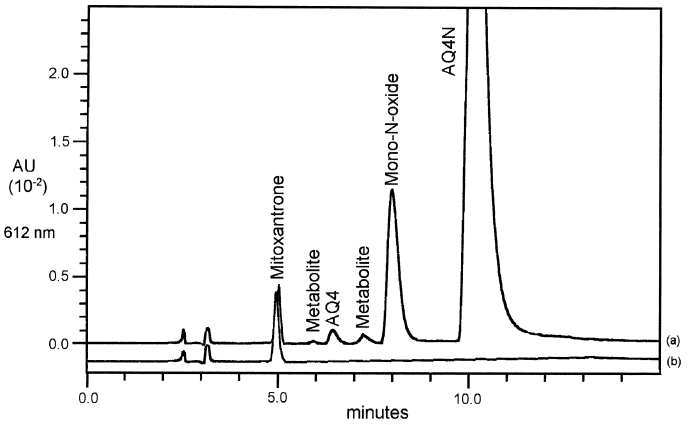

Fig. 4. Overlay chromatogram of samples taken at (a) 2 min and (b) 24 h following i.v. administration of AQ4N in mice at 200 mg kg^{-1}. Trace (a) shows AQ4N (retention time of 10.0 min), mono-*N*-oxide (retention time of 8.0 min), AQ4 (retention time of 6.4 min) and two minor metabolites (retention time of 6.0 min and 7.6 min). Trace (b), after 24 h there were no detectable peaks from parent compound or metabolites evident, the trace also demonstrates lack of interference from endogenous compounds.

AQ4 is administered alone it has been shown to be at least five-times more toxic in mice than the parent compound. The pre-clinical anti-tumour data for AQ4N have enabled the compound to progress to the clinical trial stage. A method to analyse accurately both parent compound and metabolite is therefore an essential requirement for the forthcoming trial. The method described here has been shown to be suitable for the analysis of both AQ4N and AQ4 in murine plasma. It is sensitive, highly selective and reproducible and is appropriate for measuring low levels of AQ4N and AQ4 in human samples.

For extraction of samples from biological materials a simple protein precipitation method, using three volumes of methanol, proved sufficient. Acetonitrile was tried as a protein precipitant although this did affect the separation efficiency especially when larger sample volumes (>60 μl) were injected. The use of methanol for the relatively crude precipitation of proteins gave very good recoveries and no interfering compounds were detected. We therefore felt that it was unnecessary to develop a more specific solid-phase extraction method at this point.

Both AQ4N and AQ4 are an intense blue colour and therefore the selective visible light wavelength of 612 nm can be used for detection. Endogenous peaks could be seen at 242 nm, and sensitivity was lower at 662 nm. The limit of detection for a 100 μl mouse plasma sample was 50 ng ml^{-1} at 612 nm.

K' ratios for the AQ4N/mono-*N*-oxide and mono-*N*-oxide/AQ4 were calculated and found to be the same at 1.41, which indicates that both *N*-oxides contribute to the chromatographic process equally.

Protein binding studies gave variable results. The combined basic and hydrophobic nature of the metabolite AQ4 ensured that it bound highly to membrane filters (>90%). Subsequently we attempted ultracentrifugation using polycarbonate tubes but again the data were variable. Mitoxantrone has been reported to bind to glass, which suggests that the related AQ4N, or more particularly AQ4, may also bind to glass or even polycarbonate tubes [14], which would explain the variability of our results. Reproducibility was not a problem during routine extractions using polypropylene microcentrifuge tubes, and so the potential for both AQ4N and AQ4 binding to glass or plastics may be the subject of future studies. It was clear however, even from the variable data, that AQ4 is more highly protein bound than AQ4N. This could be explained in light of the fact that *N*-oxides of alkylaminoanthraquinones such as AQ4N are electrically neutral and less basic than the tertiary amines such as AQ4 [1].

Administration of AQ4N to mice revealed that this agent is stable to reduction, with only low concentrations of both the two-electron reduction product AQ4 mono-*N*-oxide and the four-electron reduction product AQ4 detected in plasma. However, two

unknown metabolites were also resolved by the HPLC method the identity of which is currently under investigation.

With the current limit of detection at 50 ng ml^{-1} the assay is sufficiently sensitive to enable the plasma measurement of AQ4N at 1/10 of its LD10 in mice (20 mg kg^{-1}). These are likely to be the concentrations seen in patient samples at the starting dose of the clinical trial. Therefore, in summary, we have developed a simple, selective and reproducible assay for the analysis of both AQ4N and AQ4, which is suitable for future clinical and experimental studies.

Acknowledgements

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