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Sensitive liquid chromatographic assay for the basic DNA intercalator (*N*,*N*-dimethylaminoethyl)-9-amino-5-methylacridine-4-carboxamide and its nitroarylmethyl quaternary prodrugs in biological samples

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Abstract

Nitroarylmethyl quaternary (NMQ) ammonium salts of the basic DNA intercalator AMAC (*N*,*N*-dimethylaminoethyl-9-amino-5-methylacridine-4-carboxamide) are of interest as anticancer prodrugs. A sensitive HPLC assay has been developed for quantitation of AMAC and its NMQ prodrugs in cultured cells, plasma and tissue. Recovery of the prodrugs, without conversion to AMAC, was achieved using extraction in alkaline acetonitrile followed by immediate reneutralisation. Reversed-phase HPLC with fluorescence detection gave a detection limit of 3 fmol for AMAC, with linearity to 20 nmol (using diode array absorbance at high concentrations). This assay was used to measure cellular uptake, and hypoxic metabolism to AMAC, of three NMQ-AMAC prodrugs.

Keywords: DNA intercalators; *N*,*N*-Dimethylaminoethyl-9-amino-5-methylacridine-4-carboxamide; Nitroarylmethyl quaternary salts

1. Introduction

DNA intercalators such as doxorubicin and mitoxantrone are important anticancer agents, and many other examples of this broad class (and related prodrugs) are currently in preclinical or clinical evaluation. The avid binding of these basic polyaromatic agents to macromolecules has made them relatively challenging to extract from tissue, and current methods are often cumbersome and/or provide low absolute recoveries. For example, extraction of mitoxantrone from tissue homogenates using chloroform–methanol (2:1) followed by chloroform–30% ammonium hydroxide (10:1) provided an average recovery of only 46% [1]. A more rapid single step dichloromethane extraction using an ion-pairing reagent under alkaline conditions provided similarly low recoveries (average 38%) [2].

We encountered similar difficulties in attempting to assay the aminoacridine DNA intercalator (*N*,*N*-dimethylaminoethyl)-9-amino-5-methylacridine-4-carboxamide (AMAC, Fig. 1), which is the active cytotoxin released by a new series of nitroarylmethyl quaternary (NMQ) ammonium compounds under

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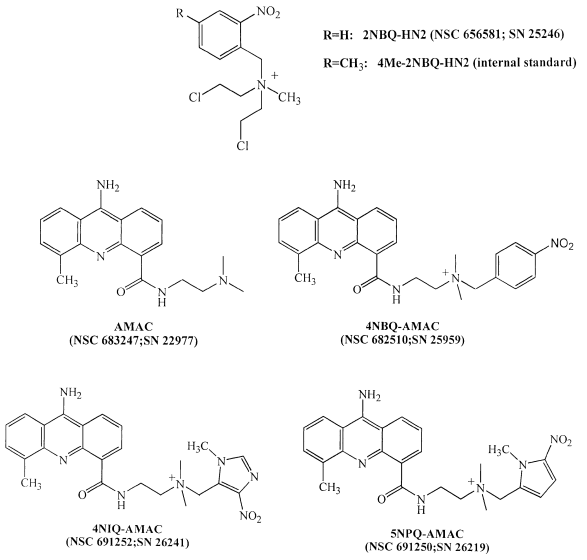

Fig. 1. Structures of NMQ prodrugs and AMAC.

development as anticancer prodrugs. The initial NMQ prodrugs were designed to release the nitrogen mustard mechlorethamine (HN2), as illustrated by the 2-nitrobenzyl quaternary salt 2NBQ-HN2 (Fig. 1) [3–6]. These compounds can be activated by enzymatic reduction of the nitro group under the hypoxic conditions known to prevail in tumours [7]; this leads to fragmentation of the benzylic C–N bond and release of the tertiary amine cytotoxin HN2. Some NMQ-HN2 compounds can also be reduced efficiently by ionising radiation under anoxic conditions [8,9], raising the possibility that prodrugs of this general design might be activated by therapeutic radiation during radiotherapy of tumours [10]. However, development of useful radiation-activated prodrugs will require release of amines with greater cytotoxic potency than nitrogen mustards in order to compensate for the low yield of reducing equivalents at clinically relevant radiation doses.

To address this requirement, a series of NMQ prodrugs of AMAC has recently been prepared in this laboratory (Fig. 1). In addition to the very potent cytotoxicity of AMAC [11], its fluorescence (which is largely retained in the prodrugs) provides very sensitive detection. Thus the NMQ-AMAC prodrugs are useful probes for investigating radiolytic activation of NMQ compounds (i.e., AMAC release) in cells and tissues. A robust and sensitive analytical method is required for this purpose, and for investigation of the pharmacokinetics and metabolism of NMQ prodrugs.

The present study reports the development of an extraction and HPLC method for quantitation of AMAC and NMQ-AMAC prodrugs in cells, blood plasma and tissue at concentrations in the picomolar — micromolar range. Given that radiolytic reduction of NMQ-AMAC compounds can be expected to give only low yields of AMAC, it was of particular importance to identify methods for extracting and detecting AMAC in the presence of a large excess of the prodrugs. This is a significant challenge because of the propensity for nucleophilic displacement of the quaternary leaving group of NMQ salts [3,5], which provides a potential route for decomposition to AMAC during extraction and sample workup.

2. Experimental

2.1. Chemicals and reagents

AMAC was synthesised as reported previously [11]. The NMQ prodrugs (Fig. 1) were synthesised in this laboratory; synthetic details and characterisation will be reported elsewhere [12]. The prodrugs were dissolved in 8% orthophosphoric acid to ca. 300 m*M* then diluted in water to 5 m*M* and stored at −20°C. These stock solutions contained traces of AMAC (typically 0.1% for 4NBQ-AMAC, 0.3% for 5NPQ-AMAC and 2NBQ-AMAC, 0.05% for 4NIQ-AMAC). The internal standard (*N*,*N*-bis(2-chloroethyl)-*N*-methyl-*N*-(4-methyl 2-nitrobenzyl) ammonium chloride, 4Me-2NBQ-HN2, Fig. 1) was synthesised as reported [6]. Acetonitrile (HiPerSolv), ammonium formate (Analar grade) and formic acid were purchased from BDH (Poole, UK). Water was purified using a MilliRO/Milli-Q system (Millipore Corporation, Bedford, MA).

2.2. Chromatography

The chromatographic system initially comprised a HP 1100 quaternary pump, autosampler and diode array detector, with a HP 1046 fluorescence detector (Hewlett-Packard, Waldbronn, Germany). HPLC separation was performed on a μBondapak C_{18}

reversed-phase radial pak column (10×0.8 cm, 10-μm diameter particles, Waters Corporation, Milford, MA) with a guard column of the same material. The mobile phase comprised a mixture of 80% acetonitrile in water (A), and 0.45 *M* ammonium formate (pH 4.5) in water, using linear gradient segments of 15–40% A for 10 min, 40–60% A for 1 min, 60–90% A for 2 min, with return to 15% A over 2 min and a post-run equilibration time of 5 min. The flow-rate was 1.8 ml/min. The eluate was monitored by UV detection at 264 nm (bandwidth 4 nm, reference wavelength 526–576 nm) and/or fluorescence detection (excitation 258 nm, emission wavelength at 465 nm). Peak areas were quantified using HP Chemstation software, version A04.01. Subsequent studies used a later model fluorescence detector (HP 1100; emission 261 nm, excitation 461 nm) and 3.2×150-mm Alltima C_8 5 μ columns (Alltech Associates, Deerfield, IL) which together provided a sensitivity increase of approximately 100-fold.

2.3. Preparation of samples

Mouse blood was collected in heparinised microtainers (Becton-Dickinson, Franklin Lakes, NJ) to prepare plasma, samples (100 μl) of which were mixed with 0.2 *M* NaOH (15 μl) in 1.5-ml microcentrifuge tubes to adjust the pH to ca. 9–10. Protein was precipitated by addition of 1 ml of cold acetonitrile and mixed by vortexing before addition of 5 μl of internal standard (4Me-2NBQ-HN2) dissolved in DMSO to 2.5 m*M*. Samples were then centrifuged (12 000 *g*–5 min) and the supernatant removed and transferred to a tube containing 30 μl 0.2 *M* formic acid to give a pH of ca. 7. The sample was then reduced in volume to approximately 50–100 μl in a centrifugal concentrator (Savant Instruments, Farmingdale, NY) and ammonium formate buffer (0.45 *M* pH 4.5) was added to a final volume of approximately 250 μl. A 200-μl aliquot was then analysed by HPLC.

Samples of the KHT tumour, obtained by dissection of mice with subcutaneous tumours, were prepared by homogenising ca. 0.5 g tissue with two parts water by weight, using a Polytron PT 2000-Kinematica homogeniser (Lucerne, Switzerland). A 100-μl aliquot was then extracted as for plasma.

AA8 cells, grown in spinner flasks in α-minimum essential medium containing 10% foetal bovine serum, were collected by centrifugation and pellets containing 10^7 cells (total volume ca. 10 μl) were lysed by the addition of 90 μl ice-cold water. Extraction was as for plasma except only 10 μl of 0.2 *M* NaOH were added to the AA8 cell lysate and 20 μl of 0.2 *M* formic acid to the acetonitrile extract.

2.4. Method validation

Stock solutions of the analytes were prepared in DMSO and stored −80°C. Aliquots (5 μl) of these were added to 245 μl of the initial mobile phase (0.45 *M* formate buffer, pH 4.5, with 12% MeCN) in triplicate, and samples (200 μl) analysed by HPLC to determine the standard curve. Aliquots of 5 μl were similarly added to 100 μl of mouse plasma, tumour homogenate or cell lysate to give final concentrations of 0.1–100 μ*M* (plasma and tumour) and 0.05–50 μ*M* (cell lysate). These samples were extracted and analysed as above. Recovery was determined by comparing the peak areas of standards and extracted samples. Five replicates were analysed at four different concentrations to determined intra-day reproducibility. Inter-day reproducibility was tested by freezing plasma and tumour homogenates containing the prodrugs (1 μ*M*), and extracting five replicate samples (each assayed in triplicate) over five consecutive days. The precision at each concentration was calculated as the relative standard deviation (RSD). The quantitation limit was defined as that giving a peak height equal to 2.5 times baseline noise (six times the standard deviation of the linear regression) in an adjoining region of the same chromatogram.

3. Results

3.1. Standard curve: solution stability and comparison of detectors

Detector response to the key analyte AMAC, a fluorescent 9-aminoacridine (Fig. 1), and solution stability was evaluated by reversed-phase HPLC using 200-μl injections (Fig. 2). Detection was by

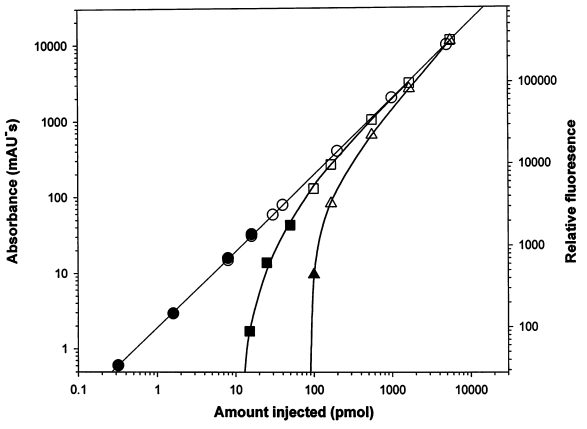

Fig. 2. HPLC detection of AMAC by absorbance (264 nm, bandwidth 4 nm; open symbols) and fluorescence (excitation 258 nm, emission 465 nm; filled symbols). AMAC solutions prepared by dilution from DMSO into water showed loss of AMAC at low concentration when assayed immediately (squares), with a more severe loss when assayed 4–6 h later (triangles). This non-linearity was prevented by dilution into HPLC mobile phase (0.45 *M* formate buffer, pH 4.5, with 12% MeCN; circles).

diode array absorbance or, for samples containing <10 pmol, by fluorescence detection. Dilution of the DMSO stock solution into water gave non-linear standard curves with under-detection at low AMAC concentration; this became more extreme when the solutions were held for 4–6 h at room temperature in the HPLC vials before analysis (Fig. 2). This loss, due to binding to glass and plastic surfaces, was completely prevented by diluting directly into the initial HPLC mobile phase (0.45 *M* formate buffer, pH 4.5, with 12% MeCN). Under these conditions, the standard curve was linear using either diode array absorbance ($r=0.999$; 8–5000 pmol) or fluorescence detection ($r=0.999$; 0.3–16 pmol), and was unchanged when samples were held for up to 24 h at room temperature. The quantitation limit for AMAC in these non-biological samples by fluorescence detection (HP 1046 detector) was 0.3 pmol, but was subsequently improved to 3 fmol by reduction of the column diameter from 8 to 3.2 mm and use of a more sensitive (HP 1100) fluorescence detector. All three NMQ-AMAC prodrugs were stable in solution and provided linear standard curves ($r>0.998$) when diluted in mobile phase, using either fluorescence (up to 8 pmol) or diode array (8–5000 pmol) detection.

3.2. Optimisation of extraction conditions: cell pellets, plasma and tumour

Preliminary studies indicated that extraction of 4NBQ-AMAC from lysed pellets of AA8 cells with 10 vol MeCN, after taking the solvent extracts to dryness and redissolving in mobile phase, gave adequate recoveries of the prodrug (80–90%). Under these conditions little conversion to AMAC was observed, with AMAC concentrations increasing from 0.15±0.02% of that of the prodrug to 0.70±0.02% after workup. However, this method was relatively ineffective in extracting AMAC itself, with recoveries in the range 35–45% only.

We tested whether deprotonation of the charged *N*,*N*-dimethylamino sidechain would diminish reversible macromolecular binding of AMAC, and hence improve recovery, by varying the pH of the lysis buffer (Fig. 3A). After MeCN extraction as above, the supernatant was taken to dryness and analysed by HPLC. Elevation of pH provided a large improvement in AMAC extraction efficiency from 20 to 30% at pH 7 and 8 to 85–95% at pH 9–11. Under the same conditions, recovery of the prodrug

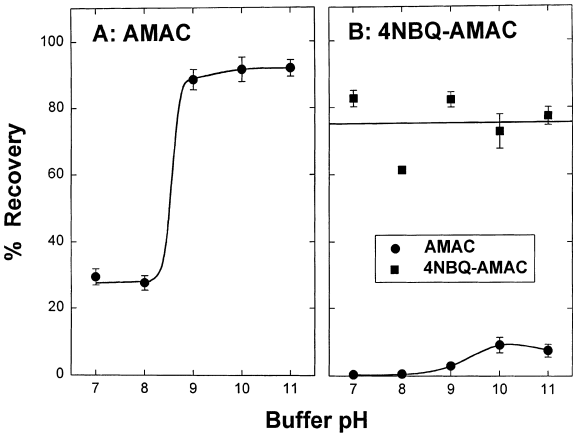

Fig. 3. pH dependence of extraction of AMAC (A) and 4NBQ-AMAC (B) from AA8 cells. Pellets of 10^7 cells were lysed with 100 μl water, followed by addition of an equal volume of 0.2 *M* phosphate buffer (pH 7, 8, 10, 11) or Tris buffer (pH 9) containing AMAC or 4NBQ-AMAC to give a final concentration in the lysate of 1 and 35 μ*M*, respectively. Lysates were extracted with 1 ml MeCN, evaporated to dryness (without reneutralisation), redissolved in mobile phase, and assayed for AMAC and 4NBQ-AMAC by HPLC.

4NBQ-AMAC was ca. 80% and was independent of pH (Fig. 3B). However, under these conditions there was considerable (7–10%) conversion of 4NBQ-AMAC to AMAC during extraction and workup at high pH (Fig. 3B). This problem was even more severe with the heterocyclic analogues 4NIQ-AMAC and 5NPQ-AMAC (Fig. 1), which showed variable but extensive (>20%) conversion to AMAC under these conditions (data not shown).

The stability of the NMQ-AMAC prodrugs during workup was greatly improved by mild acidification of the MeCN extract to pH 4.5–5.5 by addition of 20 μl of 0.2 *M* formic acid to the supernatant immediately after centrifugation, and was further improved by not taking the organic extracts to dryness; results for this final method are shown in Table 1. The variable residual volumes (50–100 μl) after partial drying necessitated use of an internal standard. The internal standard selected, 4Me-2NBQ-HN2 (Fig. 1), has excellent water solubility, was chromatographically well resolved from the analytes (retention time 15 min, Fig. 4), and was stable under the pH range employed. This 'pH-shift' method gave good recoveries of all analytes over a wide concentration range (0.5–500 μ*M*) with relative standard deviations generally <10% (Table 1). Importantly, conversion to AMAC was negligible for all three NMQ-AMAC prodrugs over the entire concentration range under these conditions (Table 1).

Minor modifications of this method were used to extract AMAC and its heterocyclic NMQ prodrugs from mouse plasma and from KHT tumour homogenates. Representative chromatograms showing absorbance and fluorescence detection of 5NPQ-

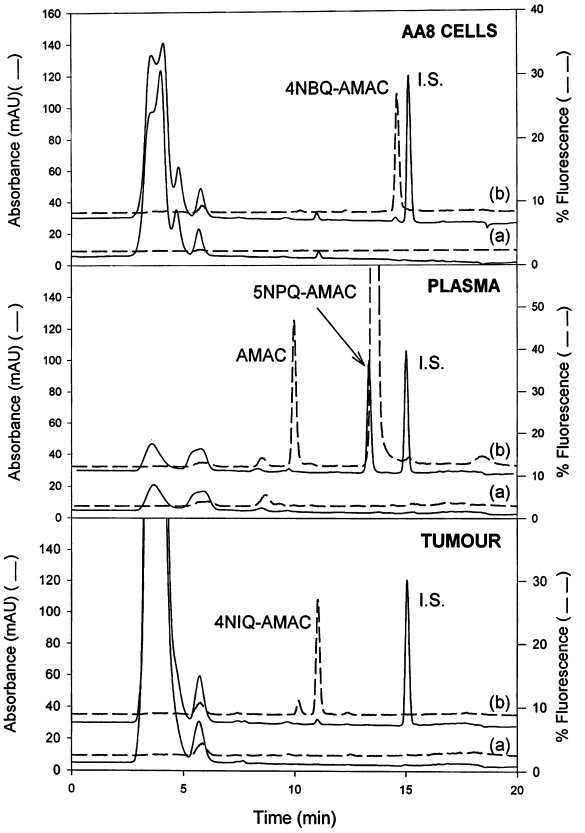

Fig. 4. Representative chromatograms for AA8 cells (top), plasma (middle) and tumour (lower) samples: (a) blanks (controls). (b) Spiked with the indicated NMQ-AMAC prodrug to the equivalent of 10 μ*M* intracellular concentration in AA8 cells (top), 10 μ*M* in plasma (middle) or the equivalent of 1 μ*M* in tumour. The internal standard (I.S.) was 4Me-2NBQ-HN2 (Fig. 1). Absorbance detection (solid lines) was used to quantify the I.S. and unknowns at high concentration, and fluorescence detection (dashed lines) for AMAC and prodrugs at low concentrations.

Table 1
Recovery of AMAC and NMQ-AMAC prodrugs from AA8 cells

Conc. μ*M*[c]	4NBQ-AMAC			4NIQ-AMAC			5NPQ-AMAC			AMAC	
	Recovery (%)	RSD[a] (%)	AMAC[b] (%)	Recovery (%)	RSD (%)	AMAC (%)	Recovery (%)	RSD (%)	AMAC (%)	Recovery (%)	RSD (%)
0.5	97.2	6.6	nd[d]	77.6	13.2	nd	96.9	9.1	nd	88.7	4.7
5	96.4	3.7	0.048–0.10	88.9	5.4	nd	94.4	5.1	0.47–0.68	101.5	7.4
50	90.1	2.3	0.059–0.062	91.1	2.8	0.09–0.11	92.0	3.1	0.30–0.54	97.9	7.1
500	92.1	2.9	0.069–0.071	92.3	2.0	0.08–0.15	101.5	1.9	0.25–0.94	100.2	11.4

[a] Relative standard deviation of recovery. $n=5$.
[b] Percentage conversion to AMAC during sample workup (range).
[c] Intracellular concentration.
[d] AMAC not detected.

AMAC in plasma and 4NIQ-AMAC in tumour are shown in Fig. 4. This assay gave excellent recoveries and low intra-assay and inter-assay variance for all three compounds, over the concentration range 0.1–100 μ*M* (Table 2). The limits of quantitation, using the signal/noise ratio defined in Section 2.4, were 0.72 pmol for the prodrugs and 0.24 pmol for the more intensely fluorescent AMAC. This corresponds to concentrations of prodrugs and AMAC, respectively, of 90 and 30 n*M* in AA8 cells, 27 and 9 n*M* in tumour tissue, and 9 and 3 n*M* in plasma. Conversion to AMAC during workup of the plasma and tumour samples (approximately 2%; Table 2) was greater than for extraction from cells in dilute lysates. However, this extent of conversion is acceptable since pharmacokinetic studies (F.B. Pruijn and D.M. Ferry, unpublished data) indicate extensive metabolism of these compounds to AMAC in vivo resulting in substantially higher AMAC/prodrug ratios that can be accounted for by conversion during workup.

3.3. Determination of cellular uptake and metabolism of NMQ-AMAC prodrugs

The above pH-shift extraction method was used to measure cellular uptake of NMQ-AMAC prodrugs and metabolic release of AMAC. AA8 cells were exposed to the prodrugs as stirred single cell suspensions under aerobic or hypoxic conditions and samples of 10^7 cells were recovered by centrifugation and assayed for prodrug and AMAC at various times (Fig. 5). Extracellular concentrations were determined by processing 100 μl samples of the extracellular medium as for cell lysates. Uptake was biphasic, with rapid accumulation to 5–10 times extracellular concentration followed by a slower ca. 3-fold increase over the following 2 h. At 2 h, the ratio of intracellular/extracellular prodrug, based on a mean intracellular water content of 1.09 pl for AA8 cells [13], was 64±3 for 4NBQ-AMAC, 26±3 for 4NIQ-AMAC and 45±6 for 5NPQ-AMAC. Whether this strong uptake by cells reflects active transport, or passive diffusion with entrapment in the cells, has not yet been determined although initial experiments showing ca. 30% inhibition of cellular uptake by 50 m*M* ammonium chloride suggest that accumulation in acidic endosomes may contribute (B.G. Siim, unpublished data).

Metabolic conversion to AMAC was very slow under aerobic conditions (Fig. 5), but AMAC concentrations were significantly higher than in standards spiked with the prodrugs. For example in the case of 4NIQ-AMAC, the average concentration of

Table 2
Recovery of AMAC and NMQ-AMAC prodrugs from mouse plasma and KHT tumours

	Conc (μ*M*)[c]	4NIQ-AMAC			5NPQ-AMAC			AMAC	
		Recovery (%)	RSD[a] (%)	AMAC[b] (%)	Recovery (%)	RSD (%)	AMAC (%)	Recovery (%)	RSD (%)
Plasma:									
Intra-day	0.1	100.8	4.7	1.1–3.8	98.9	8.7	1.5–3.2	101.5	1.9
	1	100.6	3.5	1.6–2.4	99.9	1.5	1.4–2.7	95.6	1.6
	10	89.8	2.2	1.3–1.7	99.4	2.0	1.6–2.9	99.6	2.4
	100	97.1	1.7	1.3–1.5	98.7	2.4	2.0–2.6	102.3	3.5
Inter-day[d]	1	99.9	1.2	1.6–2.5	99.6	2.3	1.4–2.8	97.0	1.6
Tumour									
Intra-day	0.1	88.0	6.4	1.1–6.7	98.9	5.3	1.3–2.4	102.2	3.3
	1	97.9	5.8	1.3–2.9	98.0	7.8	0.4–2.2	102.4	4.2
	10	99.2	3.6	1.0–3.1	99.7	2.4	1.3–2.2	98.4	2.5
	100	99.3	1.6	0.2–2.8	97.3	0.3	1.7–2.2	99.1	2.8
Inter-day[d]	1	95.9	4.2	0.4–2.2	97.5	1.7	1.8–2.1	95.9	1.0

[a] Relative standard deviation of recovery. $n=5$.
[b] Percentage conversion to AMAC during sample workup (range).
[c] Concentration of drug in plasma and tumour (not lysate).
[d] Determined in triplicate samples on five different days.

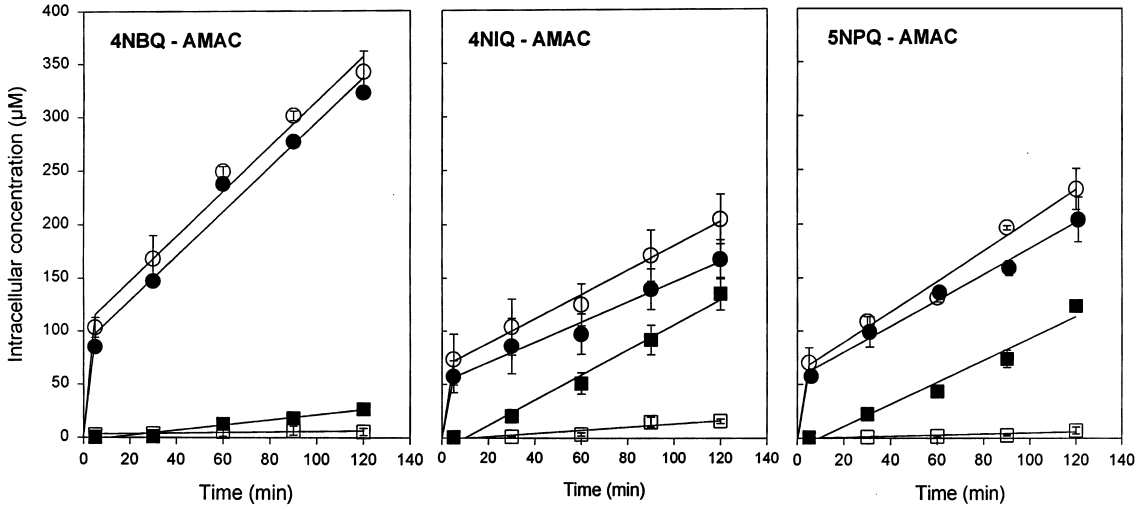

Fig. 5. Kinetics of cellular uptake, and metabolism to AMAC, of NMQ-AMAC prodrugs. Stirred suspensions of AA8 cells (10^7 cells/ml in culture medium with 5% FCS) were gassed with 5% CO_2 in air (open symbols) or N_2 (filled symbols) for 1 h at 37°C before addition of NMQ-AMAC prodrugs to 10 μ*M*. Intracellular concentrations of prodrugs (circles) and AMAC (squares) were analysed at intervals using the pH-shift organic extraction method.

AMAC (as a percentage of prodrug concentration) was 0.12% in spiked cell lysates, 1.2% in the 5-min samples and 7.9% in the 2-h samples. The rate of metabolism to AMAC was 8–27 times higher under hypoxia. Hypoxic metabolism was fastest for 4NIQ-AMAC, almost as rapid for 5NPQ-AMAC, and slowest for 4NBQ-AMAC. Under these conditions the extracellular AMAC concentrations were in the range 0.01–0.08 μ*M* at 2 h. Although it is not clear that steady state conditions have been achieved, the very high intracellular/extracellular concentration ratios for AMAC (2700±400, mean and sem for three experiments) after hypoxic incubation with 4NIQ-AMAC for 2 h suggests that AMAC is more strongly taken up by AA8 cells than are the quaternary prodrugs.

4. Discussion

The analytical problem which occasioned this study was the need to quantify a tightly tissue bound basic DNA intercalator, AMAC, in the presence of a large excess of prodrug forms of the same compound. Elevation of pH during organic precipitation of macromolecules was effective in improving AMAC recovery, presumably because of decreased macromolecular binding (and higher organic solvent solubility) of the free base relative to the dicationic form of this compound. However, the NMQ-AMAC prodrugs are susceptible to hydrolysis to AMAC under alkaline conditions. Efficient extraction of pre-existing AMAC was achieved, with minimal conversion of the prodrugs to AMAC, by reneutralising the organic extract prior to concentration of the sample. AMAC formation was decreased further by reducing the volume of the organic extracts by about 90% in the centrifugal evaporator, rather than drying down completely. This lowered the MeCN concentration enough to allow large volume injections without interfering with the chromatography. The variable volumes following the partial concentration step necessitated the use of an internal standard to correct for these differences. The internal standard chosen (4Me-2NBQ-HN2; Fig. 1) is an NMQ prodrug of mechlorethamine with high solubility and stability. We avoided using an AMAC prodrug for this purpose to preclude any possibility of release of AMAC from the internal standard.

This pH shift assay showed good accuracy and precision, with recoveries from biological matrices close to 100% and relative standard deviations generally less than 10%. Conversion to AMAC during sample processing was minimal, and did not

compromise the intended applications. Thus for the least stable of the prodrugs, 5NPQ-AMAC, the post-workup AMAC concentrations were <1% of prodrug concentrations (Table 1). These AMAC concentrations were lower than measured in cells containing 5NPQ-AMAC, even under aerobic conditions. Thus this assay can be used to quantify metabolism of the NMQ-AMAC prodrugs to AMAC in culture, and was employed in this study to show that all three compounds are metabolised selectively under hypoxia in AA8 cells to release AMAC. The extent of conversion to AMAC was slightly higher during workup of plasma or tumour samples (Table 2), but preliminary experiments show that AMAC concentrations in plasma and tumour samples from mice injected with NMQ-AMAC compounds are much higher than can be accounted for by conversion to AMAC during sample preparation. Thus the assay reported here is useful for quantifying NMQ-AMAC prodrugs in cells, plasma and tumour tissue and for measuring AMAC derived from these prodrugs in biological materials.

The general approach used here may have application in extraction of other basic DNA intercalators from tissue. Attempted application to tightly tissue bound dihydroxyanthracenedione DNA intercalators was not successful due to very low solubility at high pH, but we have recently used the method successfully to improve extraction from cells of novel basic DNA intercalators linked to bioreductive drugs (F.B. Pruijn and W.R. Wilson, unpublished data).

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