CHROMBIO. 5263

Quantitation of the antitumour agent N-[2-(dimethylamino)ethyl]acridine-4-carboxamide in plasma by high-performance liquid chromatography

D. YOUNG, P.C. EVANS and J.W. PAXTON*

Department of Pharmacology and Clinical Pharmacology, University of Auckland School of Medicine, Private Bag, Auckland (New Zealand)

(First received September 26th, 1989; revised manuscript received February 7th, 1990)

ABSTRACT

N-[2-(Dimethylamino)ethyl]acridine-4-carboxamide is a new experimental antitumour agent which has excellent in vivo activity against the Lewis lung tumour in mice. A reversed-phase high-performance liquid chromatographic method is described for the measurement of this agent in plasma. The internal standard was N-[2-(diethylamino)ethyl]acridine-4-carboxamide. The compounds of interest were extracted from plasma (0.2 ml) with acetonitrile and further purified on C_{18} solid-phase extraction Bond Elut columns. After elution with acetonitrile–ammonium acetate buffer and evaporation, the final separation was carried out on a C_{18} μBondapak column with fluorimetric detection. Over the plasma concentration range 100–5000 nM, the intra- and interassay coefficients of variation were less than 4.1 and 7.7%, respectively. The accuracy of the method varied from 97 to 105% of the theoretical values. The lowest concentration which could be measured with acceptable accuracy ($\pm 10\%$) and precision (coefficient of variation $<10\%$) was 10 nM. The method was sufficiently sensitive to allow pharmacokinetic analyses of 30 μmol/kg doses for more than six half-lives ($t_{1/2}$) in rabbits ($t_{1/2}=4$ min) and mice ($t_{1/2}=1.3$ h).

INTRODUCTION

N-[2-(Dimethylamino)ethyl]acridine-4-carboxamide (AC; NSC 601316; Fig. 1) is an experimental antitumour agent which has been developed in the Cancer Research Laboratory, University of Auckland Medical School [1]. The 9-anilinoacridine precursors of this agent include amsacrine, which is now used
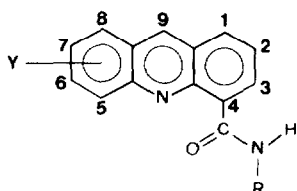
NAME		R	Y
AC		$-(CH_2)_2N(CH_3)_2$	H
IS		$-(CH_2)_2N(CH_2CH_3)_2$	H
COMPOUND	I	$-(CH_2)_2N(CH_3)_2$	7-OH
	II	$-(CH_2)_2NH_2$	H
	III	$-(CH_2)_2NHCH_3$	H
	IV	$-(CH_2)_2N(CH_3)_2$ $\downarrow$ O	H

Fig. 1. Structures of AC, I.S. and some possible 'theoretical' biotransformation products (compounds I–IV).

in the treatment of acute leukemia [2], and its 4-methyl-5-(N-methylcarbox-amide) analogue (CI-921), which has completed phase I clinical trials [3] and is now undergoing phase II evaluation against various solid tumours. Amsacrine had only marginal antitumour activity in vivo against the Lewis lung carcinoma in mice [4], whereas AC cured approximately 90% of mice when treatment was commenced five days after inoculation of the tumour [1]. AC was even more active against this tumour in vivo than CI-921 which was selected for clinical trial on the basis of its solid tumour activity [4]. If the trials of CI-921 demonstrate significant activity against human tumours, AC will be an obvious candidate for further clinical investigations. It has been suggested that the high activity of AC against solid tumours in mice could be partly explained by better distributive properties as it is more lipophilic than amsacrine and CI-921, and at physiological pH the ionisation of its acridine nitrogen is almost completely suppressed [1].

The pharmacokinetics of AC have not been studied as no analytical method has been developed for the determination of this agent in biological fluids. We report here a reversed-phase high-performance liquid chromatographic (HPLC) method for AC using solid-phase extraction and fluorimetric detection. This method was primarily developed to study the pharmacokinetics of

AC in the rabbit to allow comparison with previous kinetic studies of amsacrine and CI-921 [5,6]. However, the assay was also applicable to mouse plasma.

EXPERIMENTAL

Materials

The chloride salts of AC, the chosen internal standard (I.S.) N-[2-(diethylamino)ethyl]acridine-4-carboxamide, some possible 'theoretical' biotransformation products (compounds I–IV, Fig. 1) and other possible internal standards were kindly synthesised by Dr. Bill Denny, Cancer Research Laboratory, Auckland University Medical School.

Ammonium acetate, heptanesulphonic acid (sodium salt), triethylamine, glacial acetic acid (all from BDH, Poole, U.K.) and phosphoric acid (J.T. Baker, Phillipsburg, NJ, U.S.A.) were all Analar grade. The solvents used were HPLC-grade acetonitrile and methanol (both from Mallinckrodt, Paris, KY, U.S.A.). All aqueous solutions were prepared using Millipore Milli-Q water. Stock 1 M ammonium acetate buffer pH 5.0 was prepared by mixing 1 l of 1 M ammonium acetate solution with approximately 500 ml of 1 M acetic acid solution and adjusting the pH to 5.0 with the acid.

The solid-phase extraction procedure used disposable 1-ml Bond Elut columns (100 mg C_{18} sorbent) connected to a Vac-Elut system (both from Analytichem International, Harbor City, CA, U.S.A.), which allowed simultaneous extraction of ten samples. The vacuum was adjusted to allow a 5–10 ml/min flow-rate through the columns. The columns were prepared for sample extraction by solvating with 1 ml of acetonitrile and then equilibrating with 2.5 ml of 10% (v/v) acetonitrile–0.05 M ammonium acetate buffer pH 5.0. The columns were not allowed to dry out before application of the sample.

Standard solutions

Stock solutions (1 mM) of AC, I.S. and other compounds were prepared in methanol and stored at $-20°$C for four weeks without significant decomposition. The I.S. was further diluted with acetonitrile to 1 μM for use in the assay. The stock AC solution was diluted with methanol to 1 and 0.1 μM, and for the preparation of the plasma AC calibration curve, the appropriate volumes were added to glass tubes. The methanol was evaporated under a stream of oxygen-free nitrogen, and blank rabbit plasma was added to give concentrations of 5000, 2500, 1000, 500, 100, 50 and 20 nM. This calibration curve was made up fresh for each assay run. In addition, pure AC was weighed out and dissolved in three large pools of blank rabbit plasma to give final concentrations of 5000, 2300 and 116 nM. These were divided into 200-μl aliquots (5000 and 2300 nM) and 600-μl aliquots (116 nM) and stored at $-80°$C. These aliquots were used in each subsequent assay as a quality control check and as

an indicator of inter-assay precision and the stability of AC in plasma stored at $-80\,^{\circ}$C.

HPLC mobile phase

The stock solution (1 M) of triethylamine phosphate (TEAP) (pH 3.0) was prepared as previously described [7]. The mobile phase was prepared by the addition of 20 ml of 250 mM heptanesulphonic acid solution and 10 ml of stock TEAP solution to 970 ml acetonitrile–water (32:65) to give a final concentration of 5 mM heptanesulphonic acid and 10 mM TEAP. All HPLC solvents were filtered through a 0.45-μm filter (Nyaflo, Gelman Sciences, Ann Arbor, MI, U.S.A.) and thoroughly degassed before use.

Chromatographic apparatus

Chromatography was performed using a 6000A pump and a WISP 710B automatic injection system (Waters Assoc.). Compounds were separated on a Waters C$_{18}$ μBondapak stainless-steel column (30 cm$\times$0.05 cm I.D.) with a mobile phase flow-rate of 1.5 ml/min and detected with a Perkin-Elmer fluorescence spectrophotometer (650-10S) at an excitation wavelength of 358 nm and an emission wavelength of 475 nm. The spectral band widths were 15 and 20 nm for excitation and emission, respectively. Peak heights were determined manually from traces obtained from a Perkin-Elmer Model 023 chart recorder or from a data processing system CHROMCARD II on an Apple computer (Anadata Systems, Ellyn City, IL, U.S.A.).

Blood sample collection

AC (30 μmol/kg) was infused in a 10-ml solution (either sterile saline or 5% dextrose water) over 8 min into an ear vein of a New Zealand white rabbit. Blood was collected into 5-ml heparinised Venoject tubes (Terumo, Elkton, MD, U.S.A.) at various time points from a peripheral vein on the opposite ear. The samples were centrifuged (2000 g for 15 min at room temperature) immediately after collection, and the plasma was transferred to a glass tube for storage at $-20\,^{\circ}$C until analysis. All samples were analysed within three days after blood collection.

Assay procedure

The I.S. (100 μl, 0.1 nmol) was added to a tapered glass tube. Acetonitrile (0.9 ml) and plasma (up to 200 μl) were added to the tubes which were vortexed immediately. After centrifugation (2000 g for 10 min at 10$\,^{\circ}$C) the supernatant was transferred to a 10-ml glass tube and diluted with 9 ml of 0.05 M ammonium acetate buffer pH 5.0. This was then applied to the pre-equilibrated Bond Elut columns which were then washed successively with 2.5 ml of 10% (v/v) acetonitrile–0.05 M ammonium acetate buffer pH 5.0, two 0.5-ml volumes of 20% (v/v) acetonitrile–ammonium acetate buffer pH 5.0 and 0.7

ml of 30% (v/v) acetonitrile–ammonium acetate buffer pH 5.0. The columns were allowed to dry out slightly between the wash and elution steps after sample application. AC and the I.S. were then eluted with two 0.5-ml volumes of 70% (v/v) acetonitrile–0.05 M ammonium acetate buffer pH 5.0, which were pooled. After evaporation to dryness at 30°C under a stream of oxygen-free nitrogen, the residue was reconstituted in 100 μl mobile phase, and 5–20 μl were injected into the HPLC system.

Calculations

Calibration curves were constructed by plotting peak-height ratios of AC to I.S. against the corresponding AC concentrations in the calibration plasma samples. The best-fit straight line was determined using the method of least squares. Quantitation of AC in unknown samples was achieved by calculating the peak-height ratio in the unknown sample and using the linear regression analysis to compute a concentration.

RESULTS AND DISCUSSION

Detection and chromatography

Using the chromatographic conditions described, baseline separation of AC and I.S. was achieved with retention times of 9.3 and 15.4 min, respectively (Fig. 2). AC and I.S. were quantified by measurement of either fluorescence at excitation and emission wavelengths of 358 and 475 nm, respectively, or UV
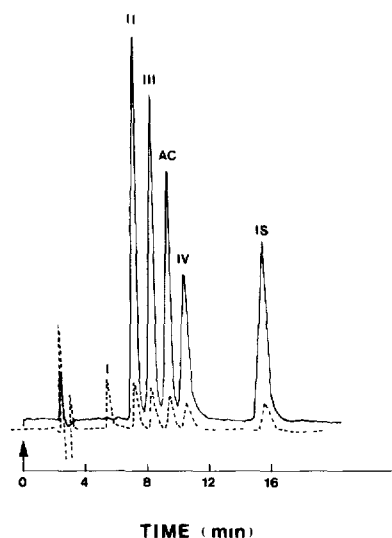

TIME (min)

Fig. 2. HPLC trace of standard solutions of AC, I.S. and compounds I–IV with fluorimetric detection (solid trace) and UV detection (broken trace).

absorption at 254 nm. The latter had the advantage that the 7-hydroxy deriv-ative (compound I) could be measured, but UV absorption was less sensitive than fluorescence for AC and I.S. Initially both systems were used simultane-ously (Fig. 2) but the sensitivity required at the later time points in our phar-macokinetic studies made fluorescence the preferred method.

The main mechanisms by which AC is removed from the body are not known. However, its structure suggests the possible occurrence of several typical drug biotransformation reactions such as aromatic hydroxylations, N-demethyla-tions and N-oxidation with the formation of biotransformation products such as those shown in Fig. 1 (compounds I–IV). The retention times for these 'theoretical' biotransformation compounds were 5.3, 7, 8.3 and 10.5 min, and they did not interfere with AC or the chosen I.S. (Fig. 2). These compounds were extractable from rabbit plasma by our method and would have been ob-served in our chromatograms if present in significant amounts in post-infusion plasma samples. Two close-running unknown 'metabolite' peaks (X and Y; Fig. 3) were observed in post-infusion rabbit samples. Their retention times did not correspond to those of compounds II–IV, nor to the acridine-4-carbox-ylic acid derivative (retention time 8.3 min). However, Y had a similar reten-tion time to compound I, but was detected by both fluorescence and UV detec-tion (in contrast to compound I which was not detectable by fluorescence emission under these conditions), suggesting a different structure to that of compound I.

Choice of internal standard

As most metabolites are usually more polar than the parent compound, they would be expected to have shorter retention times in reversed-phase chroma-tographic conditions. To avoid any interference by possible metabolites in the chromatography, the obvious choice of I.S. is a compound which is more lipo-philic than AC. A number of such compounds were available including the 5-methylacridine ring-substituted derivative of AC and compounds with addi-tional methyl groups on the carboxamide side-chain, such as N-[2-(dimeth-ylamino)propyl]acridine-4-carboxamide and N-[2-(diethylamino)ethyl]-ac-ridine-4-carboxamide. The latter was chosen as an appropriate I.S. as it was well separated from AC and possible biotransformation products (Fig. 2).

Selectivity of the method

No endogenous peaks which might interfere with AC or the I.S. were found in the chromatograms of plasma samples from a number of rabbits (Fig. 3). The purity of the AC and I.S. peaks from post-infusion rabbit samples was further confirmed by a stop-flow emission spectral scan. These scans were su-perimposable with similar scans from pure AC and I.S. standards indicating peak homogeneity in the unknown samples.
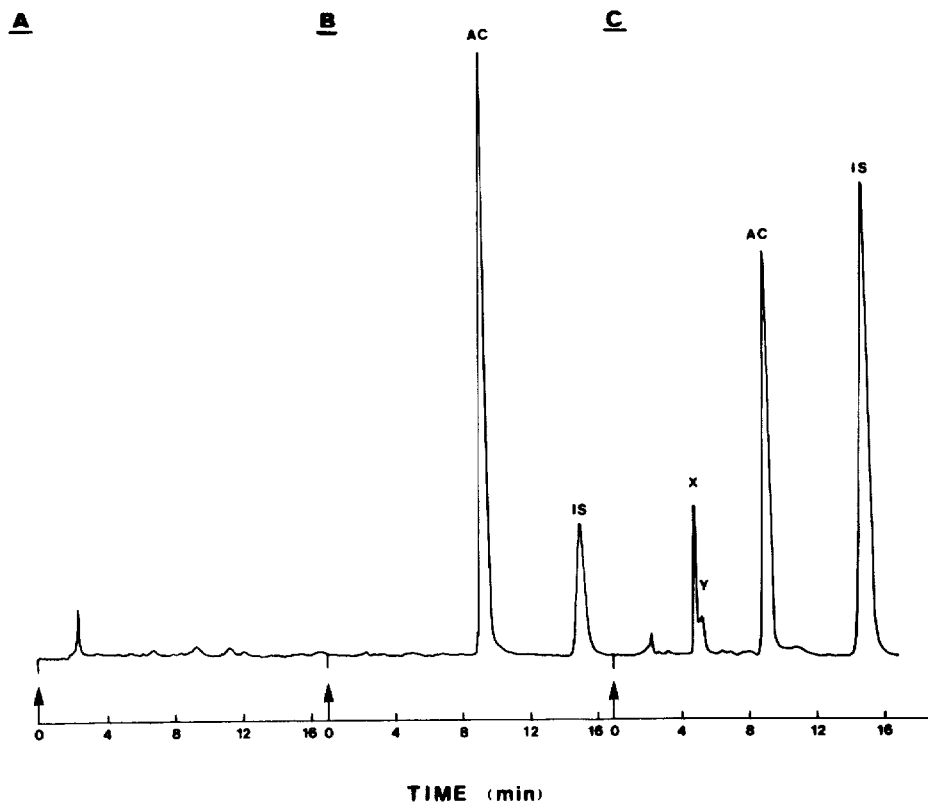
Fig. 3. HPLC trace with fluorimetric detection of (A) blank rabbit plasma, (B) plasma calibration standard (5000 nM) and (C) post-infusion rabbit sample. X and Y in the latter are possible metabolites of AC.

Limit of quantitation

A seeded plasma sample (containing a theoretical concentration of 13 nM AC) had a mean value within 10% of theoretical and a coefficient of variation (C.V.) of 8.4% after eight determinations within one assay. This suggested that the lowest concentration which could be measured with acceptable accuracy ($\pm$10% of theoretical value) and precision (C.V. $\leq$10%) was 10 nM.

Plasma calibration curve

The peak-height ratio (AC/I.S.) was directly proportional to the AC concentration in plasma over the range 20–5000 nM. Peak-height ratios from the plasma calibration standards made up fresh (beginning with the initial weighing procedure) on three separate days had acceptable reproducibility with C.V.s varying from 3.4 to 8.5% over the three days. Linear regression analysis of the standards from each day was compared with weightings of 1, $1/x$ and $1/x^2$. The linear regression with weighting $1/x$ was chosen as the most appropriate as

this gave the lowest C.V.s. for the back-calculated concentrations (3.9 to 5.6%), the smallest deviations for the mean back-calculated concentration from the theoretical value (-2 to $+8\%$) and the smallest y-intercept values ($2.4\cdot10^{-8}$ to $9.7\cdot10^{-8}$). As the intercept values were not statistically significant, the linear regressions for the calibration curves were thereafter forced through the origin.

Accuracy and precision

The accuracy and intra-assay precision of the method was assessed using the seeded quality control plasma samples. Each quality control was analysed eight times on one day. The results are given in Table I. The intra-assay precision ranged from 2.6 to 4.1% (C.V.) for the three quality controls. The quality controls were found to be within 92–100% of the theoretical values. The inter-assay precision was determined by assaying in triplicate an aliquot of each seeded quality control sample in each subsequent assay. The results are reported in Table I. The C.V.s ranged from 7.3 to 7.7% for seven assays over a period of ten weeks. The mean concentration for each quality control for this period was within 97–105% of the theoretical value. In addition, there was no trend towards a decreasing plasma AC concentration over this period, indicating that AC is stable in plasma stored at $-80\,^{\circ}$C for at least ten weeks.

Method application

The method was applied to the measurement of AC in rabbit plasma after an 8-min infusion of 30 μmol/kg (approximately 10 mg/kg) in 10 ml sterile saline (or 5% dextrose water). This dose was calculated by extrapolating to rabbit from the optimal intravenous dose (40 mg/kg) required to cure Lewis

TABLE I

ASSAY PRECISION AND ACCURACY IN PLASMA

Theoretical concentration (nM)	Measured concentration (mean $\pm$ S.D.) (nM)	n	C.V. (%)	D^a (%)
Intra-assay				
5000	4580 ± 190	8	4.1	−8
2340	2340 ± 70	8	3.0	0
117	116 ± 3	7	2.6	−1
Inter-assay				
5000	4830 ± 370	5	7.7	−3
2340	2470 ± 180	7	7.3	+5
117	123 ± 9	7	7.5	+5

^aD = percentage difference from theoretical.

lung tumour in vivo in 20-g mice using the ratio of their weights raised to the power 0.75. The method had sufficient sensitivity to allow pharmacokinetic analyses of this dose for more than six half-lives. The concentration–time profiles observed in two rabbits are illustrated in Fig. 4. These concentration–time profiles were very different from those observed for amsacrine or CI-921 [5,6]. After infusions of amsacrine or CI-921 ($12.7\,\mu$mol/kg), maximum plasma concentrations achieved were approximately 12 and 35 μM for amsacrine and CI-921, respectively, and concentrations were measurable for up to 12 h post-infusion. For AC after a 30 μmol/kg infusion, the maximum concentration was approximately 5 μM, which fell below the limit of quantitation by 30 min, indicating that AC is very swiftly removed from the systemic circulation.

The assay has also been validated in plasma from BDF1 mice and preliminary pharmacokinetic studies are under way in this species. A longer elimination half-life ($t_{1/2}$) was observed after intravenous administration in the mouse ($t_{1/2} = 1.3$ h) compared to the rabbit ($t_{1/2} = 4$ min).

In summary, a reliable and selective assay has been developed to allow the determination of a new anticancer agent, AC, in plasma. This method is rela-
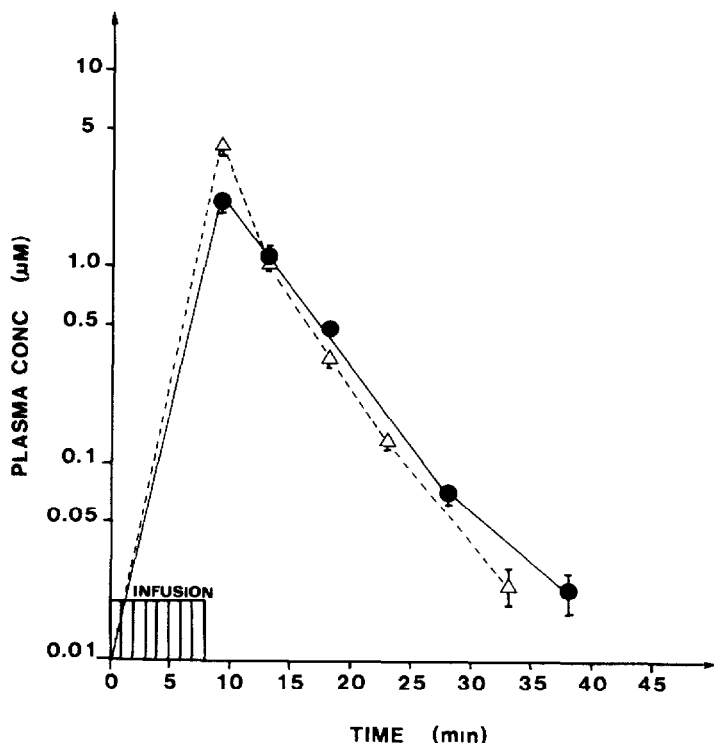

Fig. 4. Concentration–time profiles of AC after 30 μmol/kg infusions in two rabbits. Each point is the mean of triplicate determinations. The bars represent 1 S.D.

tively rapid, reproducible and allows the agent to be quantitated with acceptable accuracy and precision down to a concentration of 10 nM in 0.2 ml of plasma. This is sufficient for pharmacokinetic studies in rabbits and mice at non-toxic and effective doses.

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