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# HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF 4′-(9-ACRIDINYLAMINO)METHANESULFON-*m*-ANISIDIDE IN PLASMA

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## SUMMARY

A rapid and selective high-performance liquid chromatographic method for the measurement of 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (AMSA), a new anticancer drug, has been developed. The method employed an analogue of AMSA, 4′-(3-methyl-9-acridinylamino)methanesulphonanilide as internal standard. Plasma samples were acidified, washed with hexane, readjusted to pH 9.0 and extracted with diethyl ether. The evaporated extract was chromatographed on a Radial-Pak $C_{18}$ column using acetonitrile—water containing 0.01 mol/l triethylamine phosphate as mobile phase. Detection was by UV absorbance at 254 nm. Chromatography time for each sample was 5.5 min. Using 0.5 ml of plasma, AMSA concentrations as low as 50 nmol/l could be measured with acceptable accuracy and precision. Patients' samples remained stable when stored at —20°C for up to one month. Plasma AMSA concentrations were followed for 24 h after 200 mg/m² infusions in two patients with acute myeloid leukemia. This method appears eminently suitable for investigation of the pharmacokinetics of AMSA in patients and laboratory animals.

## INTRODUCTION

4′-(9-Acridinylamino)methanesulfon-*m*-anisidide (AMSA) (I) is an acridine derivative (Fig. 1) currently undergoing clinical evaluation as a chemotherapeutic agent for the treatment of human tumors. The drug has demonstrated significant activity against leukemias and lymphomas [1—5], with moderate activity against metastatic breast cancer [6—8] and malignant melanoma [9]. Although there is some information on the distribution of radiolabelled AMSA in rodent [10] and man [11], there is a lack of good pharmacokinetic studies in man using a specific analytical technique. A better knowledge of the kinetics of AMSA may provide a basis for its more rational use with regard to increased

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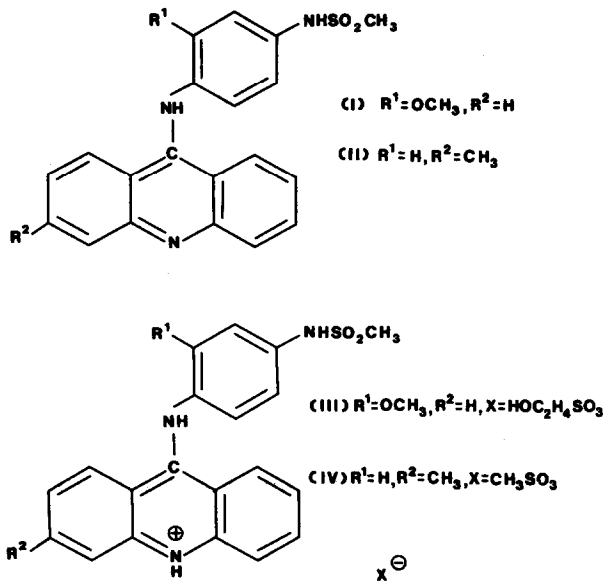


Fig. 1. Structures of (I) AMSA, (II) internal standard, and (III) and (IV) the respective salts of these compounds.

efficacy or avoidance of toxicity in particular patients such as those with hepatic or renal dysfunction who may be at risk.

Determination of AMSA in plasma has been reported using a non-specific fluorescence method [12] and gas chromatography (GC) [13]. This latter method did not use an internal standard and reported recoveries were only 85% on average. To our knowledge, with the exception of an abstract from Malspeis et al. [14], no high-performance liquid chromatographic (HPLC) method for AMSA has been published in full. We wish to report a selective and relatively rapid HPLC method using 4′-(3-methyl-9-acridinylamino)-methanesulfonanilide (II) as internal standard. This method can determine AMSA concentrations as low as 50 nmol/l in 0.5 ml of plasma with acceptable accuracy and precision.

## EXPERIMENTAL

### *Materials*

The 2-hydroxyethanesulfonic acid salt of AMSA (III, Fig. 1) and the methanesulfonic acid salt of 4′-(3-methyl-9-acridinylamino)methanesulfonanilide (IV, Fig. 1) were kindly supplied by Dr. B. Baguley and associates, Cancer Chemotherapy Research Laboratory, University of Auckland School of Medicine Auckland, New Zealand. Both salts were used without further purification.

The solvents used were UV-grade acetonitrile, methanol (both from Waters Assoc., Milford, MA, U.S.A.), anhydrous diethyl ether and hexane (both from J.T. Baker, Phillipsburg, NJ, U.S.A.). Sodium tetraborate, hydrochloric acid, triethylamine (all from BDH Chemicals, Poole, Great Britain) and phosphoric acid (J.T. Baker) were all Analar grade. All aqueous solutions were prepared

using Millipore Milli-Q water. All glassware was routinely washed in chromic acid solution.

*Standard solutions*

Stock solutions of AMSA (10.0 mmol/l) and the internal standard, 4'-(3-methyl-9-acridinylamino)methanesulfonanilide (1.0 mmol/l) were prepared in methanol. The internal standard stock solution was further diluted 1:50 with methanol to 20 μmol/l for use in the assay. The stock AMSA solution was further diluted 1:500 with fresh blood bank plasma to give a plasma concentration of 20 μmol/l which was further serially diluted to yield plasma calibration concentrations of 10, 8, 6, 4, 2, 1, 0.8, 0.6, 0.4, 0.2, 0.1 μmol/l for use as the standard curve. In addition, pure AMSA was weighed out and dissolved in four large plasma pools to give concentrations of 10.0, 0.5, 1.0 and 0.5 μmol/l for the determination of the accuracy and precision of the assay. These plasma pools were aliquoted into 1-ml volumes, stored at −20°C and used in each subsequent assay as a quality control check and to determine the inter-assay precision. All standard solutions were stored at −20°C when not in use.

*Mobile phase*

The HPLC mobile phase was prepared by adding 10 ml of stock triethylamine phosphate (TEAP) solution to 990 ml of acetonitrile—water (40:60) to give a final concentration of 0.01 mol/l TEAP and pH 3.80. The stock solution of TEAP (1.0 mol/l) was made up by adding 13.9 ml triethylamine to 60 ml of water, adjusting to pH 3.0 with phosphoric acid and adjusting the final volume to 100 ml with water. All HPLC solvents were filtered through a 0.45-μm filter (Type HA) (Millipore, Bedford, MA, U.S.A.) and thoroughly degassed before use.

*Apparatus*

The chromatographic separations were performed with a Waters Assoc. Model 6000A pump, U6K injector and a radial compression system (RCM-100) containing a 10 × 0.8 cm Radial -Pak $C_{18}$ column (10 μm particle size). The mobile phase flow-rate was 7 ml/min with detection by a Model 440 UV detector at 254 nm. Peak areas were determined using a Perkin-Elmer Sigma 10 chromatography data station.

*Collection of samples*

Blood samples (5 ml) from patients were collected in heparinised Venoject tubes by venipuncture of a peripheral arm vein or from a surgically implanted arterial catheter. Blank samples were collected for each patient prior to commencement of the AMSA infusion. Immediately after collection the samples were centrifuged at 1720 *g* at 10°C for 15 min, the plasma removed and stored in capped glass tubes at −20°C until analysis.

*Assay procedure*

The internal standard (100 μl, 2 nmol) was added to 12-ml screw-cap glass culture tubes and the methanol evaporated off under nitrogen at 35°C. Plasma

(0.5 ml) was added to each tube and adjusted to pH 3.0—4.0 by addition of 120 μl of hydrochloric acid (0.5 mol/l). After gentle vortexing, 5 ml of hexane was added, the tube capped and shaken for 20 min followed by centrifugation at 1720 *g* for 10 min. The upper hexane layer was discarded. The remaining plasma was adjusted to pH 9.0 with 0.5 ml saturated sodium tetraborate solution and re-extracted for 15 min with 6 ml diethyl ether. After centrifugation at 1720 *g* for 15 min the ether layer was transferred to a tapered glass tube and evaporated at 35°C under a gentle flow of nitrogen. The residue was reconstituted in 100 μl of methanol and 20—40 μl injected into the liquid chromatograph.

*Calculations*

Peak area ratios of AMSA to internal standard were plotted against corresponding AMSA concentrations in the calibration plasma samples and the least-squares unweighted regression line was calculated. Quantitation of AMSA in unknown samples was then achieved by calculating peak area ratio for unknown, and using the calibration curve to compute a concentration.

## RESULTS

The UV spectra of AMSA and internal standard in the HPLC mobile phase are shown in Fig. 2. Both compounds exhibited absorption maxima at 270 ± 2 nm. However, detection at 254 nm, which resulted in a 20% loss of sensitivity, was used in the assay as this was the fixed wavelength of the available detector.

Using the chromatographic conditions described, baseline separation of AMSA and internal standard was achieved (Fig. 3A), with retention times of

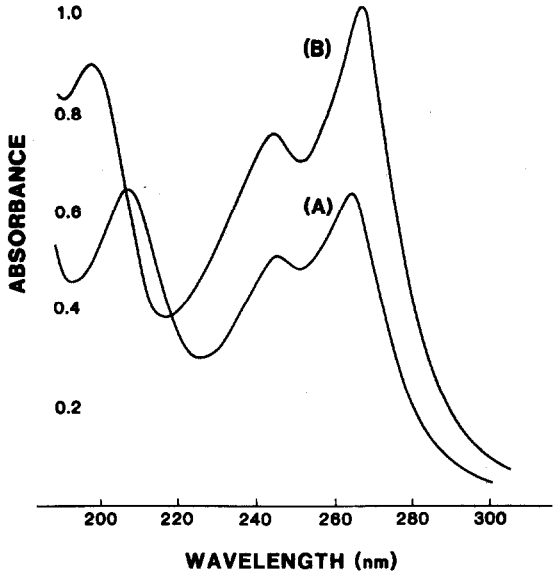


Fig. 2. UV absorption spectra of (A) AMSA and (B) internal standard in the HPLC mobile phase.

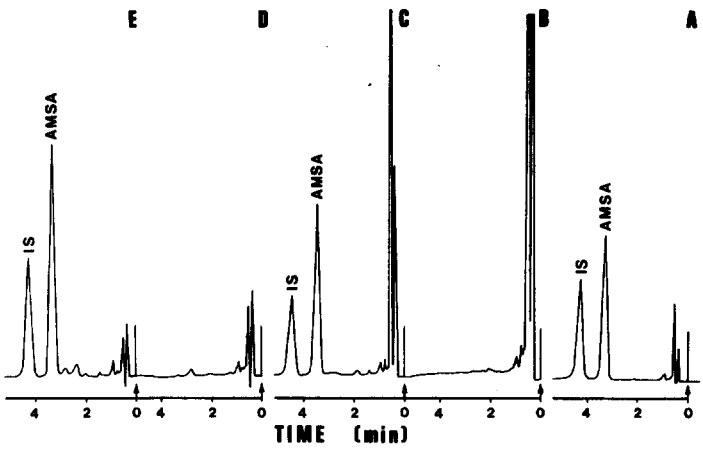


Fig. 3. Chromatograms of (A) blood bank plasma spiked with AMSA (5 μmol/l) and internal standard (IS) (4 μmol/l), (B) and (D) pre-infusion samples from the two patients, (C) and (E) correspondingly post-infusion AMSA samples from the two patients.

3.4 and 4.3 min, respectively. The total run time for each sample was 5.5 min. No interfering peaks were observed in the areas where AMSA and internal standard elute either in normals, in blood bank plasma or in patients' samples (Fig. 3B and D). However, the sample clean-up procedure of Malspeis et al. [14] was used to remove an endogenous plasma compound with retention time 9.8 min which was detected in blank plasma after a single extraction at pH 9.0. The use of ethyl acetate instead of diethyl ether also extracted this compound plus greater quantities of other more polar endogenous compounds. A number of other anticancer drugs including adriamycin, chlorambucil, cytosine arabinoside, 5-fluorouracil, lomustine, melphalan, methotrexate, prednisolone, 6-thioguanine, vincristine and vinblastine did not interfere with the AMSA or internal standard peaks under these chromatographic conditions. The reported in vitro thiolytic cleavage products of AMSA, 4-amino-3-methoxymethanesulfonanilide, 9-aminoacridine, 9(10H)-acridone [15] had retention times of 0.60, 1.47 and 2.16 min, and also did not interfere. 9-Aminoacridine has previously been identified as a metabolite in the plasma and urine of patients receiving AMSA [16]. In the present study, a small peak with a retention time identical to that for 9-aminoacridine was observed in the chromatograms of post-infusion plasma samples obtained from patient 2. However, the small size of the peak did not permit accurate measurement or precise identification.

The relationship between the peak area ratio of AMSA to internal standard and AMSA concentration was linear from 0 to 20 μmol/l. The standard curve was set up over the range 0.1 to 10.0 μmol/l and was represented by the equation $y = 0.216x - 0.002$ ($r = 0.9998$, $p < 0.001$), where $y$ is the peak area ratio of AMSA/internal standard and $x$ is the concentration of AMSA. Using 0.5 ml of plasma, the lower limit of the assay was 50 nmol/l. This could be further reduced to approximately 20 nmol/l by increasing the volume of plasma used and by decreasing the amount of internal standard. Estimates of the accuracy and the intra-assay precision of the method are given in Table I. Each plasma

TABLE I

INTRA-ASSAY PRECISION AND RECOVERY OF AMSA ADDED TO PLASMA

| Amount added (μmol/l) | Mean conc. measured (μmol/l) | *n* | Standard deviation | Coefficient of variation (%) | Recovery (%) |
|---|---|---|---|---|---|
| 10.0 | 11.49 | 8 | 0.26 | 2.29 | 115 |
| 5.0 | 5.25 | 8 | 0.09 | 1.78 | 105 |
| 1.0 | 1.11 | 8 | 0.03 | 2.32 | 111 |
| 0.5 | 0.52 | 8 | 0.01 | 2.66 | 104 |

TABLE II

INTER-ASSAY PRECISION OF AMSA MEASUREMENT IN PLASMA

| Plasma pool | No. of consecutive assays | Mean concn. measured | Standard deviation | Coefficient of variation (%) |
|---|---|---|---|---|
| 1 (10 μmol/l) | 9 | 11.75 | 0.38 | 3.23 |
| 2 (5 μmol/l) | 9 | 5.39 | 0.13 | 2.34 |
| 3 (1 μmol/l) | 9 | 1.08 | 0.04 | 3.96 |

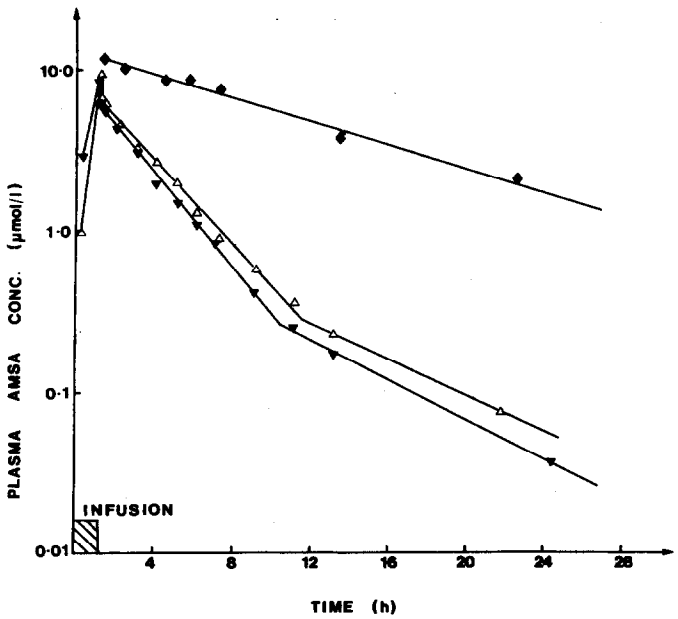


Fig. 4. Plasma concentrations of AMSA as a function of time in two patients after a 1-h infusion of 200 mg/m² AMSA. (♦) patient 1, (▼, △) two infusions 24 h apart in patient 2. Each point is the mean of duplicate determinations.

pool with added AMSA was analysed eight times in one run. Mean recoveries ranged from 104—115% with mean coefficients of variation from 1.78—2.66%. Inclusion of aliquots of three of these pools in eight subsequent assays gave acceptable estimates of inter-assay precision with coefficients of variation ranging from 2.34—3.96% (Table II).

Application of this method for measuring plasma AMSA concentrations in two patients receiving 200 mg/m$^2$ AMSA infusions (a single infusion in one patient and a double infusion 24 h apart in the other) as part of their treatment for acute myeloid leukemia is illustrated in Fig. 4. Each point was the mean of duplicate determinations. Although both patients had apparently normal hepatic and renal function, very different elimination profiles were observed. One patient exhibited a slow mono-exponential decline with a elimination half-life of 8.4 h, whereas in the other, AMSA elimination appeared to follow a bi-exponential curve with initial and terminal half-life values of 2.06, 2.32 and 4.9, 5.4 h respectively for the first and second infusion. Further information on the stability of AMSA in patients' plasma samples stored at −20°C was obtained by repeated assaying of four samples over a one-month period. These results are shown in Table III. No significant alterations in concentration were observed over this period.

TABLE III

STABILITY OF AMSA IN PATIENTS' SAMPLES STORED AT −20°C

Each measured value is the mean of duplicate extractions.

| Time after sampling (weeks) | Concn. of AMSA measured (μmol/l) | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| 1 | 6.24 | 5.64 | 2.01 | 1.54 |
| 2 | 6.25 | 5.82 | 1.93 | 1.51 |
| 4 | 6.25 | 5.75 | 1.99 | 1.52 |
| Mean | 6.25 | 5.74 | 1.98 | 1.52 |
| Standard deviation | 0.01 | 0.09 | 0.04 | 0.01 |
| Coefficient of variation (%) | 0.09 | 1.58 | 2.10 | 1.00 |

Haemolysis of the blood sample during collection led to an apparent reduction in plasma AMSA concentration. Further in vitro studies with grossly haemolysed blood indicated significant reductions in the absolute amounts of AMSA and internal standard as measured by the HPLC method. Careful collection of blood samples overcame the problem of haemolysis.

## DISCUSSION

A relatively rapid and selective HPLC method for AMSA has been developed which allows the measurement of AMSA in plasma as low as 50 nmol/l in 0.5 ml of plasma with acceptable accuracy and precision. In addition, patients' samples do not deteriorate when stored at −20°C for up to one month.

Significant reductions in AMSA levels were observed in plasma obtained from haemolysed blood samples. The reason for this is not known. However, in vitro studies [17] have shown that a reaction may occur between AMSA and plasma proteins in human blood. Nucleophilic displacement of the anilino substituent by protein thiol groups results in covalent adducts. This displace-

ment reaction was found to occur at a greater rate in the presence of red blood cells. The effect of haemolysis on AMSA levels has not been previously reported, but would probably also result in lower AMSA concentrations by the other published methods for determination of AMSA [12—14].

The HPLC method described appears eminently suitable for further investigation of the pharmacokinetics of AMSA in patients and in laboratory animals such as the rabbit, with the one restraint that sample collection must be accomplished without causing haemolysis.

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