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Journal of Pharmaceutical and Biomedical Analysis 46 (2008) 386–390

JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS

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Short communication

Development and validation of bioanalytical method for the determination of asulacrine in plasma by liquid chromatography

Srinivas Ganta [a], James W. Paxton [b], Bruce C. Baguley [c], Sanjay Garg [a,*]

[a] School of Pharmacy, Faculty of Medical and Health Sciences, The University of Auckland, Private Bag 92019, Auckland, New Zealand
[b] Department of Pharmacology, The University of Auckland, Private Bag 92019, Auckland, New Zealand
[c] Auckland Cancer Society Research Centre, The University of Auckland, Private Bag 92019, Auckland, New Zealand

Received 30 August 2007; received in revised form 21 September 2007; accepted 25 September 2007
Available online 2 October 2007

Abstract

Asulacrine (9-[(2-methoxy-4-methylsulphonylamino)phenylamino]-*N*,5-dimethyl-4-acridinecarboxamide), an analogue of the antileukaemia drug amsacrine, has high antitumour activity in mice and has also shown clinical activity. A simple method is described for the quantitation of asulacrine in plasma by liquid chromatography. Chromatographic separation was achieved on a reversed phase C 18 column (250 mm × 4.6 mm, particle size 5 μm, Gemini) using isocratic elution (acetonitrile and 0.01 M sodium acetate buffer pH 4.0, 45/55, v/v) at a flow rate of 1 ml/min. Asulacrine and internal standard (the ethylsulphonanilide analogue) were measured using UV detection at 254 nm. The total chromatographic run-time was 8 min with asulacrine and internal standard eluting at ∼4.7 and ∼6.5 min, respectively. Limit of quantification was 0.1 μg/ml. The linearity range of the method was 0.1–10 μg/ml ($r^2 = 0.9995$). Mean recoveries from plasma were 100–105%. Intra-batch and inter-batch precision was 7.1 and 7.8%, respectively, and intra-batch and inter-batch accuracy (relative error) was 4.9 and 8.4%, respectively ($n = 8$ in all cases). The bench top, freeze thaw, short-term storage and stock solution stability evaluation indicated no evidence of degradation of asulacrine. The validated method is simple, selective and rapid and can be used for pharmacokinetic studies in mice.
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Keywords: Asulacrine; Amsacrine; UV detection; Pharmacokinetics

1. Introduction

Asulacrine, 9-[(2-methoxy-4-methylsulphonylamino)phenylamino]-*N*,5-dimethyl-4-acridinecarboxamide (SN 21407), is a derivative of the antileukaemia drug amsacrine first synthesised in the Auckland Cancer Society Research Centre [1,2]. Its chemical structure is shown in Fig. 1. Asulacrine, like amsacrine, is an inhibitor of topoisomerase II [3] and its antitumour action is mediated through DNA breakage [4,5]. Asulacrine (administered by intravenous infusion) underwent PhaseI/II trials and showed potential against breast and lung cancers [2,6]. Phlebitis arising from the intravenous delivery was the main dose-limiting toxicity. Considering its potential activity, development of new formulations may overcome this toxicity. Although an HPLC method has been reported for quantitation of asulacrine in plasma, this method was labour intensive with a 2-step clean up and extraction procedure. Moreover, the type of column used and high flow rate required were not suitable for analysis of large numbers of samples [7–10]. We describe here the development of a new RP-HPLC method for the determination of asulacrine in plasma, which is applicable to pharmacokinetic studies of new asulacrine formulations in mice.

2. Experimental

2.1. Chemicals

Asulacrine (SN 21407, free base) and the corresponding ethylsulphonanilide analogue (SN 23305) as an internal standard (I.S.) were provided by the Auckland Cancer Society Research Centre. Anhydrous sodium acetate was obtained from Scharlau Chemie, Spain. HPLC grade acetonitrile and methanol were obtained from Ajax Fine Chemicals (Australia). Water purified on Milli-Q system (Millipore, USA) was used. All other chemicals were of analytical grade.

* Corresponding author. Tel.: +64 9 373 7599x82836; fax: +64 9 367 7192.
E-mail address: s.garg@auckland.ac.nz (S. Garg).

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doi:10.1016/j.jpba.2007.09.025

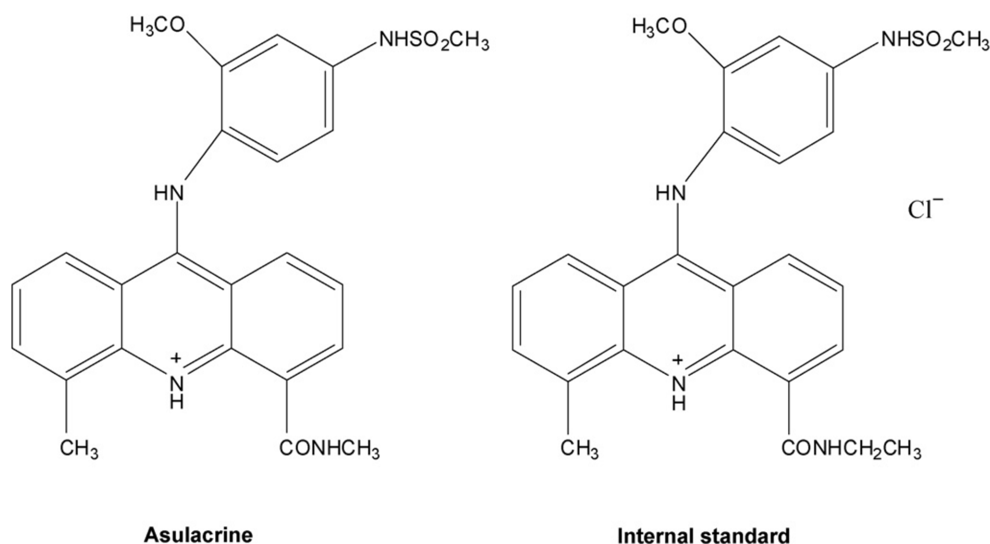

Fig. 1. Chemical structures of asulacrine and internal standard.

2.2. Instrumentation

A Waters series LC comprising of a binary pump, an autosampler, and dual wavelength detector were used with data acquisition by Breeze (Waters Corporation). Separation of compounds of interest was achieved with a Gemini C 18 analytical column (250 mm × 4.6 mm, particle size 5 μm) from Phenomenex, USA and a C 18 precolumn of the same packing (12.5 mm × 4.6 mm).

2.2.1. Chromatographic conditions

Different combinations of mobile phase consisting of organic and aqueous components were investigated. Asulacrine was not eluted when phosphate buffer was used in combination with either methanol or acetonitrile. Rapid elution was seen with 0.2% acetic acid in combination with acetonitrile. Asulacrine and I.S. were eluted using acetonitrile/0.01 M sodium acetate (pH 4.0), 60/40, v/v. This isocratic condition was further optimized to provide resolution of the drug from the I.S.

The final mobile phase consisted of 0.01 M sodium acetate buffer, pH 4.0 adjusted with acetic acid and acetonitrile (55:45, v/v). The mobile phase was filtered through 0.45 μm nylon filter (Alltech Associates, Inc., Deerfield, IL) and degassed in an ultrasonic bath (Bandelin Electronics, Berlin, Germany) before use. All samples were analysed under isocratic elution at a flow rate of 1 ml/min and UV detection at 254 nm. The autosampler temperature was maintained at 10 °C and 50 μl was injected onto the column.

2.3. Preparation of standards and quality control samples

A stock solution of asulacrine and I.S. was prepared by dissolving appropriate amounts in acidified methanol (0.1 ml of 12N hydrochloric acid added to 100 ml of methanol) and methanol respectively, to give a 1.0 mg/ml concentration. Working standard solutions of asulacrine (20, 100, 200, 1000, 1500 and 2000 μg/ml) and I.S. (1000 μg/ml) were obtained by further diluting the stock solutions with methanol.

Calibration standards (0.1–10 μg/ml) were prepared by diluting stock solutions in mouse plasma. Quality control (QC) samples (0.5, 5, and 10 μg/ml) were prepared in the same way as described above from the working standard solutions. Working standards for the QC samples were made from an independently prepared stock solution (1 mg/ml). All QC samples were prepared as a single batch and stored at −20 °C.

2.4. Plasma sample processing

Blood from anaesthetised mice was collected via retro-orbital sinus into eppendorf tubes containing heparin as the anticoagulant. Plasma was immediately prepared by centrifugation (Sigma Laborzentrifugen, Germany) at 3500 rpm for 10 min. I.S. was added to plasma (0.1 ml) in 10 ml glass tubes, and protein precipitation carried out by addition of 1 ml chilled acetonitrile. After vortexing for 1 min using a VX100 Labnet vortex mixer (Labnet Int., NJ, US), the sample was kept on to ice for 30 min to precipitate any remaining protein. Samples were then centrifuged (Sigma Laborzentrifugen, Germany) at 3500 rpm for 15 min, and the supernatant transferred to clean test tubes and vacuum dried (Labconco Corporation, Kansas, US). Residues were dissolved in 1 ml of mobile phase, and 50 μl injected into the HPLC.

2.5. Bioanalytical method validation

The method was validated according to FDA guidelines for validation of bioanalytical methods [11,12]. In order to show the acceptable nature of the analytical method, the following protocol was implemented during the method evaluation.

2.5.1. Selectivity

The selectivity of the method was evaluated by processing drug-free plasma samples obtained from six mice (C57 BL/6,

male) in a similar manner to the plasma calibration standards and unknowns. Chromatograms were compared for any interference from the matrix or any of the assay reagents.

2.5.2. Sensitivity

The lowest standard (i.e., 0.1 μg/ml) on the calibration curve was identified as the lower limit of quantification (LOQ) as the analyte peak was identifiable, discrete and reproducible with a precision of less than or equal to 20% and accuracy of 80–120%.

2.5.3. Linearity

A calibration curve was prepared from a blank sample (plasma matrix sample processed without an internal standard), a zero sample (matrix sample processed with internal standard), and six calibration samples covering the range (0.1–10 μg/ml), including the lower limit of quantification (LOQ). The acceptance criterion for each back-calculated standard concentration was 15% deviation from the nominal value, except the LOQ, which was set at 20%.

2.5.4. Recovery

Recovery was performed by comparing the concentrations of three QC samples ($n = 8$ replicates) at low, medium and high with the unextracted reference standards containing the same amount of the analyte. With regard to the preparation of the unextracted reference standards, plasma was precipitated using acetonitrile, vortexed and centrifuged. Supernatant was drawn off, to which standard and internal standard were added ($n = 8$), dried and reconstituted with mobile phase for analysis.

2.5.5. Accuracy and precision

Intra-batch accuracy and precision were determined by analysis of eight replicates of the low, medium and high concentration QC samples; while inter-batch accuracy and precision were determined by the analysis of these QC samples on three separate occasions. The overall precision of the method was expressed as relative standard deviation (R.S.D.) and the accuracy of the method was expressed in terms of relative error.

2.5.6. Stability

The freeze-thaw stability of the samples was obtained over three freeze-thaw cycles, by thawing the QCs unassisted at room temperature, and then refreezing for 12–24 h followed by analysis. Short-term stability was evaluated by keeping the QCs at room temperature for 24 h and then reanalysing. The stability of the processed samples in the autosampler was tested by analysis after storage in the autosampler for 24 h at 10 ± 1 °C. For all stability studies, low and high plasma QC samples were used with five replicates of each. The concentration of asulacrine after each test condition was compared to the initial concentration added to the sample.

2.5.7. Stock solution stability

The working solutions of asulacrine (5 μg/ml) and I.S. (5 μg/ml) were determined immediately after preparation (time 0) and at 3, 6, 12 and 24 h at room temperature and 4 °C, and concentrations were compared.

2.6. Application of the method to pharmacokinetic study

C57 BL/6 male mice were administered 30 mg/kg of asulacrine via tail vein injection. Blood was collected at different time intervals via the retro-orbital sinus into eppendorf tubes containing 10 μl of 500 units/ml of heparin and plasma was separated and stored at −20 °C until analysis. Asulacrine was extracted from the plasma samples using the method described above and analyzed. The asulacrine plasma concentration–time profile was prepared and non-compartmental pharmacokinetic parameters were estimated using WinNonlin version 5.0 (Pharsight Corporation, USA). Pharmacokinetic parameters were calculated using the log trapezoidal rule with extrapolation of the terminal slope to infinity by log-linear regression.

3. Results and discussion

3.1. Chromatography method

The chromatographic conditions were optimized to provide acceptable resolution of the analytes present in the plasma matrix. Mobile phase selection was based on the peak parameters, run time and ease of preparation. The isocratic condition of 0.01 M sodium acetate buffer (pH 4.0) and acetonitrile in the proportion of 55/45 (v/v) provided good resolution of asulacrine (RT ∼ 4.7 min) and I.S. (RT ∼ 6.5). Fig. 2a–c shows representative chromatograms of blank plasma, blank plasma with I.S. and a calibration plasma with asulacrine and I.S. No interfering peaks were observed in drug-free plasma from six different mice. The limit of quantification of asulacrine was 0.1 μg/ml. The precision and accuracy for the LOQ were 7.1 and 11.7%, respectively. The calibration curve of asulacrine was linear over the concentrations range from 0.1 to 10 μg/ml with mean $r^2 = 0.9995$, $n = 3$.

3.2. Recovery

The mean relative recoveries of asulacrine from the plasma were 100 to 105% with the coefficient of variation of 2.3–7.1% at three different concentrations (0.5, 5 and 10 μg/ml) (Table 1).

3.3. Accuracy and precision

Intra- and inter-batch precision and accuracy were evaluated by assaying the three QC samples. Intra- and inter-batch precision (% CV) was less than 7.1 and 7.8, respectively. Intra- and inter-day accuracy (relative errors, %) were less than 4.9 and 8.4%, respectively (Table 2).

Table 1
Extraction recovery of asulacrine from plasma ($n = 8$)

Concentration added (μg/ml)	Concentration measured (mean ± S.D.) (μg/ml)	Recovery (%)	CV (%)
0.5	0.5 ± 0.01	100.1	7.1
5	5.2 ± 0.1	105.0	2.3
10	10.3 ± 0.4	103.3	4.2

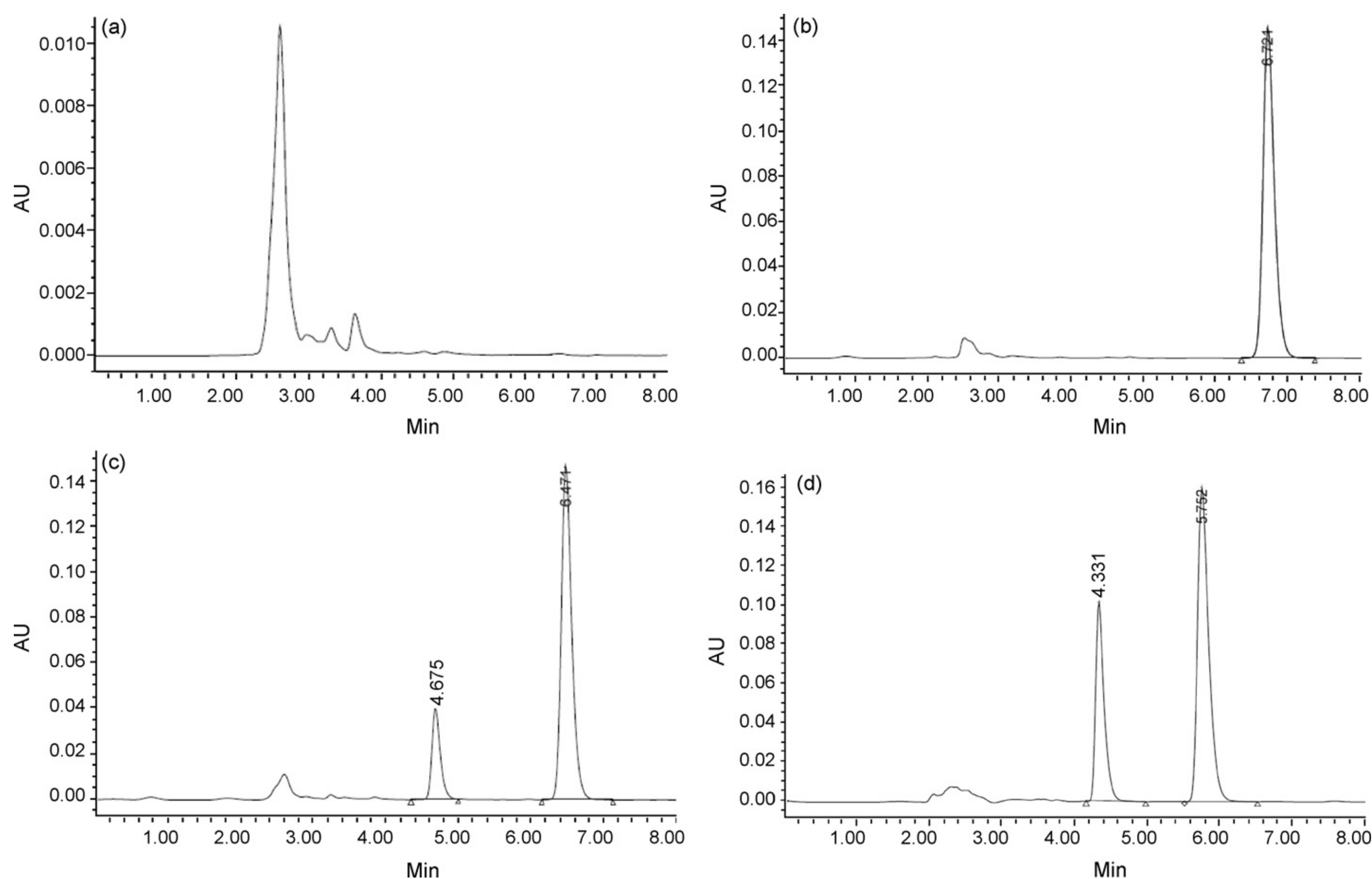

Fig. 2. (a) Blank plasma; (b) blank plasma with I.S. (5 μg/ml); (c) a representative chromatogram of asulacrine (0.5 μg/ml) and I.S. (5 μg/ml) added to plasma; (d) representative chromatogram of asulacrine and I.S. in pharmacokinetic sample.

3.4. Stability

Stability results are presented in Table 3. Short-term storage at room temperature and freeze-thaw cycles for low and high QC samples indicated that asulacrine was stable in mice plasma under experimental condition. Similarly the results indicated that both asulacrine and I.S. are stable when kept in the autosampler for up to 24 h.

The stock solution stability of asulacrine and I.S. stored at 4 °C and room temperature over 24 h showed no evidence of degradation. Asulacrine and I.S. were recovered 100% at both storage conditions indicating their stability under laboratory working conditions.

3.5. Pharmacokinetic study

The described method has been successfully employed to study the pharmacokinetics of asulacrine in mice. An intravenous dose of 30 mg/kg of asulacrine was chosen basing on its curative activity in Lewis lung-tumour bearing mice [2]. A representative chromatogram of a mouse plasma sample after intravenous dose of 30 mg/kg of asulacrine is shown in Fig. 2d.

Table 2
Precision and accuracy for the determination of asulacrine in plasma at low, medium and high concentrations

Concentration added (μg/ml)	Concentration measured (mean ± S.D.) (μg/ml)	Precision (%)	Accuracy (%)
Intra-batch precision ($n = 8$)			
0.5	0.5 ± 0.04	7.1	0.1
5	5.2 ± 0.1	2.2	4.9
10	10.3 ± 0.4	4.2	3.3
Inter-batch precision ($n = 5$)			
0.5	0.5 ± 0.04	7.8	1.7
5	5.4 ± 0.3	4.9	8.4
10	10.5 ± 0.4	3.6	5.0

Table 3
Stability of the asulacrine

Concentration added (μg/ml)	Concentration measured (mean ± S.D.) (μg/ml)	Precision (%)	Accuracy (%)
Freeze-thaw stability ($n = 5$)			
0.5	0.5 ± 0.01	2.1	−1.9
10	10.4 ± 0.01	1.1	3.5
Short term stability for 24 h in plasma ($n = 5$)			
0.5	0.5 ± 0.003	5.4	−4.1
10	10.0 ± 0.06	5.3	0.0
Autosampler stability ($n = 5$)			
0.5	0.5 ± 0.002	2.9	5.0
10	10.3 ± 0.1	8.7	2.5

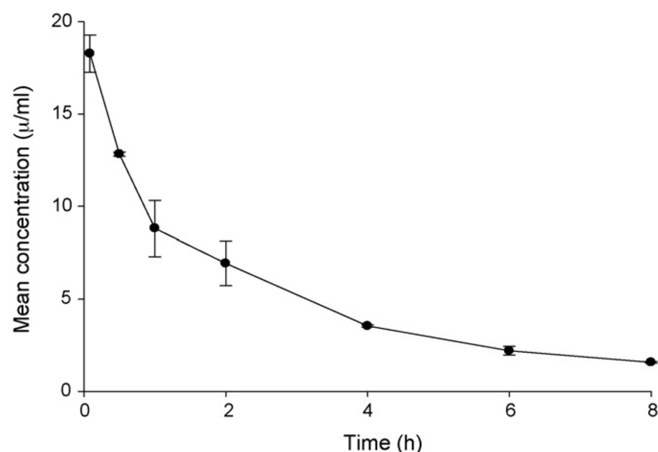

Fig. 3. Plasma concentration–time profile after i.v. administration of 30 mg/kg asulacrine. Bars represent standard deviations of the mean ($n = 3$).

The corresponding plasma mean concentration–time profile is shown in Fig. 3. The elimination half-life, volume of distribution and clearance of asulacrine were calculated to be 2.7 ± 0.2 h, 2.5 ± 0.1 l/kg, and 0.63 ± 0.04 l/(h kg), respectively.

4. Conclusion

A simple, selective and rapid HPLC method was developed for the determination of asulacrine in plasma samples. Validation was performed as per guidelines and all parameters met the criteria acceptable for routine analysis. The method was effectively employed for the study of the plasma pharmacokinetics of asulacrine in mice. The elimination half-life for asulacrine in this study with C57 BL/6 mice (2.7 h) was somewhat longer than that previously reported for asulacrine in B6 D2F1 mice [10]. In the latter study, the pharmacokinetics were observed to be non-linear with the half-life increasing from 0.6 to 1.2 h over the dose range 7–26 mg/kg. The longer half-life in the present study may be partially due to the greater dose used and also perhaps to strain differences.

Acknowledgement

The authors gratefully acknowledge the financial support provided by the Uniservices, The University of Auckland, New Zealand.

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