ELSEVIER

Journal of Chromatography B, 695 (1997) 401–408

JOURNAL OF CHROMATOGRAPHY B

Novel high-performance liquid chromatographic assay using fluorescence detection for the quantitation of plasma γ-methylene-10-deazaaminopterin and its major metabolite, 7-hydroxy-γ-methylene-10-deazaaminopterin, in patients with solid cancers in a phase I trial[1]

H. Su[a,2], T.-L. Chen[a,*], F.H. Hausheer[b], E.K. Rowinsky[a,3]

[a]*Division of Pharmacology and Experimental Therapeutics, The Johns Hopkins Oncology Center, 600 North Wolfe Street, Baltimore, MD 21287, USA*

[b]*BioNumerik Pharmaceuticals, Inc., San Antonio, TX 78229, USA*

Received 4 November 1996; revised 24 February 1997; accepted 24 February 1997

Abstract

γ-Methylene-10-deazaaminopterin (MDAM), a unique dihydrofolate reductase inhibitor, has demonstrated antitumor activity against a broad spectrum of human solid tumors in preclinical studies. A novel reversed-phase, ion-pair high-performance liquid chromatography (HPLC) assay that uses fluorescence detection has been developed to quantitate levels of MDAM and its major metabolite, 7-hydroxy-γ-methylene-10-deazaaminopterin (7-OH-MDAM), in human plasma. The recovery of MDAM and 7-OH-MDAM from plasma was >97% by a simple one-step deproteinization process using tetrabutylammonium bromide (TBABr) and methanol. MDAM and 7-OH-MDAM remained stable in plasma over a 28-day test period at ambient temperatures, and neither compound was light-sensitive. The limit of quantitation was 0.005 μ*M* for both MDAM and 7-OH-MDAM. This assay has been found to be simple, sensitive and reproducible in determining plasma concentrations of MDAM and 7-OH-MDAM in patients with solid cancers in a phase I trial. © 1997 Elsevier Science B.V.

Keywords: γ-Methylene-10-deazaaminopterin; 7-hydroxy-γ-methylene-10-deazaaminopterin

1. Introduction

γ-Methylene-10-deazaaminopterin (MDAM) is a novel dihydrofolate reductase inhibitor that is structurally similar to methotrexate (MTX) [1–8]. In contrast to MTX and other congeners, however, MDAM does not undergo polyglutamylation [1–8], a metabolic process that is believed to play a significant role in the therapeutic efficacy and toxicity of the drug [8]. MDAM has been shown to be more

*Corresponding author.

[1] Presented in part at the 87th Annual Meeting of the American Association for Cancer Research, 1996, Washington, DC, USA. Supported by BioNumerik Pharmaceuticals Inc., San Antonio, TX, USA.

[2] Current address: Merck and Co., Inc., Department of Drug Metabolism, P.O. Box 2000, RY57-200, Rahway, NJ 07065, USA.

[3] Current address: Cancer Therapy and Research Center, Institute for Drug Development, San Antonio, TX 78229, USA.

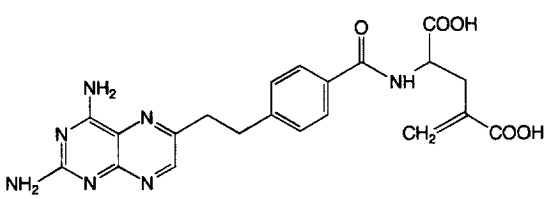
PII S0378-4347(97)00205-3

efficacious and safer than MTX in preclinical studies [6,7]. Because of these important pharmacologic properties, MDAM is currently undergoing clinical development in patients with solid cancers. In support of a phase I trial objective to define the pharmacokinetic behavior of MDAM in patients, a novel reversed-phase, ion-pair, high-performance liquid chromatography (HPLC) assay has been developed to quantitate the concentrations of MDAM and its major metabolite, 7-hydroxy-γ-methylene-10-deazaaminopterin (7-OH-MDAM) in patients treated with the agent for 30-min daily for five consecutive days every three weeks.

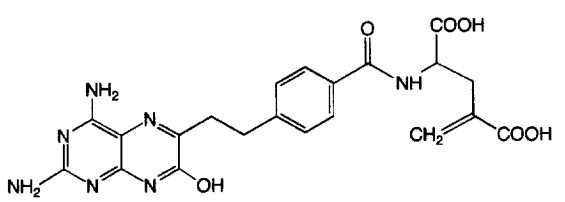
2. Experimental

2.1. Reagents and chemicals

MDAM and 7-OH-MDAM (see Fig. 1) were synthesized by Dr. M.G. Nair in the Department of Biochemistry, University of South Alabama (Mobile, AL, USA) [2,9]. Potassium phosphate monobasic (KH_2PO_4) and potassium phosphate dibasic (K_2HPO_4) of HPLC grade were obtained from Mallinckrodt (Paris, KY, USA). Tetrabutylammonium bromide (TBABr) was purchased from Sigma (St. Louis, MO, USA); HPLC-grade methanol was from EM Science (Gibbstown, NJ, USA). Human donor plasma was purchased from the Maryland Red Cross (Baltimore, MD, USA).



MADM



7-OH-MADM

Fig. 1. Chemical structures of MDAM and 7-OH-MDAM.

2.2. HPLC apparatus and chromatographic conditions

HPLC was performed on a system equipped with a P4000 pump (Thermo Separation Products, Piscataway, NJ, USA), an ISS-100 autosampler (Perkin-Elmer, Lake Success, NY, USA) and an LS-40 fluorescence detector (Perkin-Elmer). Chromatographic analyses were conducted on a Symmetry C_{18} column, 5 μm, 150×3.9 mm (Waters, Milford, MA, USA) with a Waters μBondapak CN guard-Pak precolumn insert. The mobile phase consisted of solvent A (10 m*M* potassium phosphate buffer containing 5 m*M* TBABr, pH 7.5) and solvent B (methanol). MDAM and 7-OH-MDAM were eluted by using a 19-min gradient (convex 1) that progressed from 22 to 29.5% of solvent B. This was followed by a 6-min gradient (convex 1) that progressed from 29.5 to 30% of solvent B. The flow-rate was 1 ml/min. For fluorescence detection, excitation and emission wavelengths were 375 and 460 nm, respectively.

All chromatographic data were collected electronically using a Dell Dimension XPS P60 personal computer (Dell, Austin, TX, USA), a PE Nelson 900 series intelligent interface (Perkin-Elmer, Morrisville, NC, USA) and a Chrom Perfect chromatography data system (Justice Innovations, Mountain View, CA, USA).

2.3. Standard stocks

Stock solutions (1 m*M*) of MDAM and 7-OH-MDAM were prepared by dissolving each compound in 50 m*M* KH_2PO_4 (pH 8.0); working stock solutions (25–250 μ*M*) were prepared by serially diluting the stock solution with the same solvent. Plasma standards were made by adding 0–10 μl of the working stock solutions to 250 μl of donor plasma. Calibration curves were prepared by analyzing the following ranges of concentrations: 0.005 to 10 μ*M* for MDAM, and 0.005 to 0.2 μ*M* for 7-OH-MDAM.

2.4. Plasma sample collection

Blood samples from patients receiving MDAM in a phase I trial were drawn into heparinized Vacutainers and centrifuged at 1000 *g* for 10 min. Plasma was transferred to polypropylene tubes and then stored at −20°C until HPLC analysis.

2.5. Sample preparation

MDAM and 7-OH-MDAM were isolated from plasma by methanol and ion-pairing agent deproteinization. A 250-μl volume of plasma was mixed in 1.5-ml eppendorf tubes with 50 μl of 160 m*M* TBABr and then vortex-mixed for 30 s. A 500-μl volume of methanol was then added and the solution was vortex-mixed for 10 s. After the mixture was centrifuged for 15 min at 12 000 *g*, a 300-μl volume of the supernatant was diluted with 300 μl of 10 m*M* potassium phosphate buffer solution (pH 7.5). An 50-μl aliquot was injected onto the HPLC system.

2.6. Stability study

The stability of MDAM and 7-OH-MDAM in plasma was tested by assaying plasma samples that were spiked with various concentrations of these drugs under different conditions. MDAM (1.0 μ*M*) and 7-OH-MDAM (0.1 μ*M*) were tested at ambient temperatures (22–24°C), at 4°C and at −20°C; at each of these temperatures, samples were tested either protected from light or exposed to light.

2.7. Fluorescence spectra

The fluorescence spectra of MDAM were obtained using similar methods as in studies of 10-deazaaminopterin (10-DAM), 10-ethyl-10-deazaaminopterin (10-EDAM) and methotrexate [9]. Briefly, fluorescence excitation and emission spectra of MDAM were measured by a stop-flow technique. Test solutions were prepared by adding 50 μl of a 10 μ*M* MDAM stock solution to 2 ml of 50 m*M* KH_2PO_4 at the following pH values: 4, 5, 6, 7, 8 and 9.

2.8. Recovery and assay reproducibility

The recoveries of both MDAM and 7-OH-MDAM from plasma were determined by comparing the detector response (peak areas) of HPLC from the extracted samples with that of the non-extracted samples. For MDAM, three samples each were prepared at concentrations of 1.0 and 10 μ*M*; for 7-OH-MDAM, three samples each were prepared at concentrations of 0.05 and 0.2 μ*M*.

Precision parameters were determined by analyzing donor plasma spiked with drugs at the same concentrations as those used for recovery studies. Intra-day precision was determined by performing ten repetitive injections at each drug concentration in one day; inter-day precision was determined by performing ten injections during a 45-day period. The 95% confidence intervals of coefficient of variation were calculated according to Larson [10].

2.9. Protein binding

Protein binding studies of MDAM were carried out by using an ultrafiltration technique. Donor plasma samples spiked with drugs were centrifuged through a YMT membrane (Amicon, Beverly, MA, USA) with a cut-off value of 30 000 Da to obtain free-drug fractions and protein-bound fractions. The filtrates (i.e., the free-drug fractions) were collected and analyzed by HPLC. As a control, water samples spiked with drugs were processed in an identical manner and the filtrates were also analyzed by HPLC. The percentage of protein-bound drug was calculated as follows:

$$D_b\% = (1 - D_f) \times 100$$

where D_b is the protein-bound drug fraction and D_f is the free-drug fraction. Briefly, 1 ml aliquots of plasma were spiked with MDAM to make drug concentrations of 0.05, 1.0 and 10 μ*M*; 1-ml aliquots of plasma were spiked with 7-OH-MDAM to make drug concentrations of 0.01, 0.05 and 0.2 μ*M*. Samples were then transferred to sample reservoirs and the YMT membranes were centrifuged for 30 min at 2000 *g* at ambient temperatures. Aliquots of the filtrates (250 μl) were transferred to glass autovials and 40 μl of 160 m*M* TBABr and 250 μl of methanol were added. A 25-μl volume of the

mixture was analyzed on the HPLC system, as described above. Samples of each concentration were prepared and analyzed in triplicate.

3. Results and discussion

3.1. Fluorescence of MDAM

The fluorescence spectra of MDAM in aqueous solution as a function of pH are shown in Fig. 2. The level of excitation was highest at a wavelength of 375 nm; the corresponding emission intensity was highest at a wavelength of 460 nm (Fig. 2A,B). The intensity of fluorescence increased as the pH was increased from 4 to 8, and then decreased slightly at pH 9 (Fig. 2C). Intensities were comparable at pH 7 and pH 9, a finding that was consistent with intensities reported for the other antifolates, 10-DAM and 10-EDAM [11]. In order to achieve the optimal analytical conditions, a mobile phase with a pH value of 7.5, an excitation wavelength of 375 nm and an emission wavelength of 460 nm were selected.

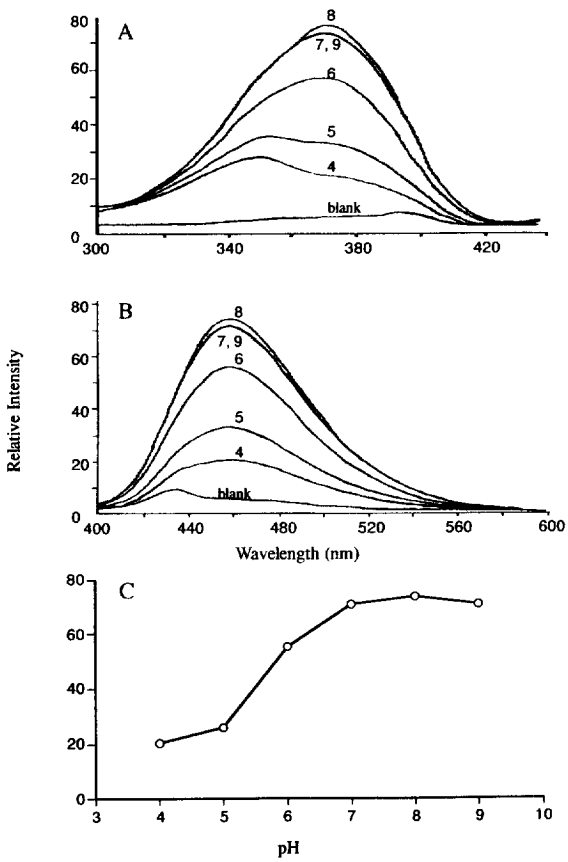


Fig. 2. Fluorescent spectra of MDAM at 0.25 μ*M* in a 50 m*M* KH_2PO_4 solution as a function of pH (4–9). (A) Excitation spectrum measured at a fixed wavelength of 460 nm. (B) Emission spectrum measured at a fixed wavelength of 375 nm. (C) Relative fluorescence–pH profile generated from spectra A and B.

3.2. Chromatography of MDAM and 7-OH-MDAM

Because MDAM and 7-OH-MDAM are structurally similar, these two compounds were, in practice, very difficult to separate chromatographically. Thus, sufficient resolution and efficiency of separation could only be achieved by optimizing both solid- and mobile-phase conditions. A Waters Symmetry column was selected for the solid-phase because it provided a high surface binding area for an increased number of theoretical plates. Resolution was also improved by means of a carefully designed gradient elution with a mixture of methanol and phosphate buffer at pH 7.5, coupled with an ion-pairing agent. Under these conditions, MDAM and 7-OH-MDAM could be well separated, with retention times of 21.3 and 22.3 min, respectively (Fig. 3). When concentrations of both MDAM and 7-OH-MDAM were above 0.3 μ*M*, or when the difference between the two concentrations was over 100-fold, the 'Set Baseline Now' function of the Chrom Perfect software was used to quantitate two peaks placed on a common baseline.

3.3. Stability study

Samples of donor plasma were spiked with MDAM and 7-OH-MDAM and peak areas were compared. A comparison of peak areas over a 28-day period showed that MDAM and 7-OH-MDAM were very stable at ambient temperatures (22–24°C) (Fig. 4). Comparable results were obtained from samples kept at 4°C and at −20°C (data not shown).

3.4. Recovery and reproducibility

MDAM and 7-OH-MDAM in plasma were separated by a one-step methanol and ion-pairing agent

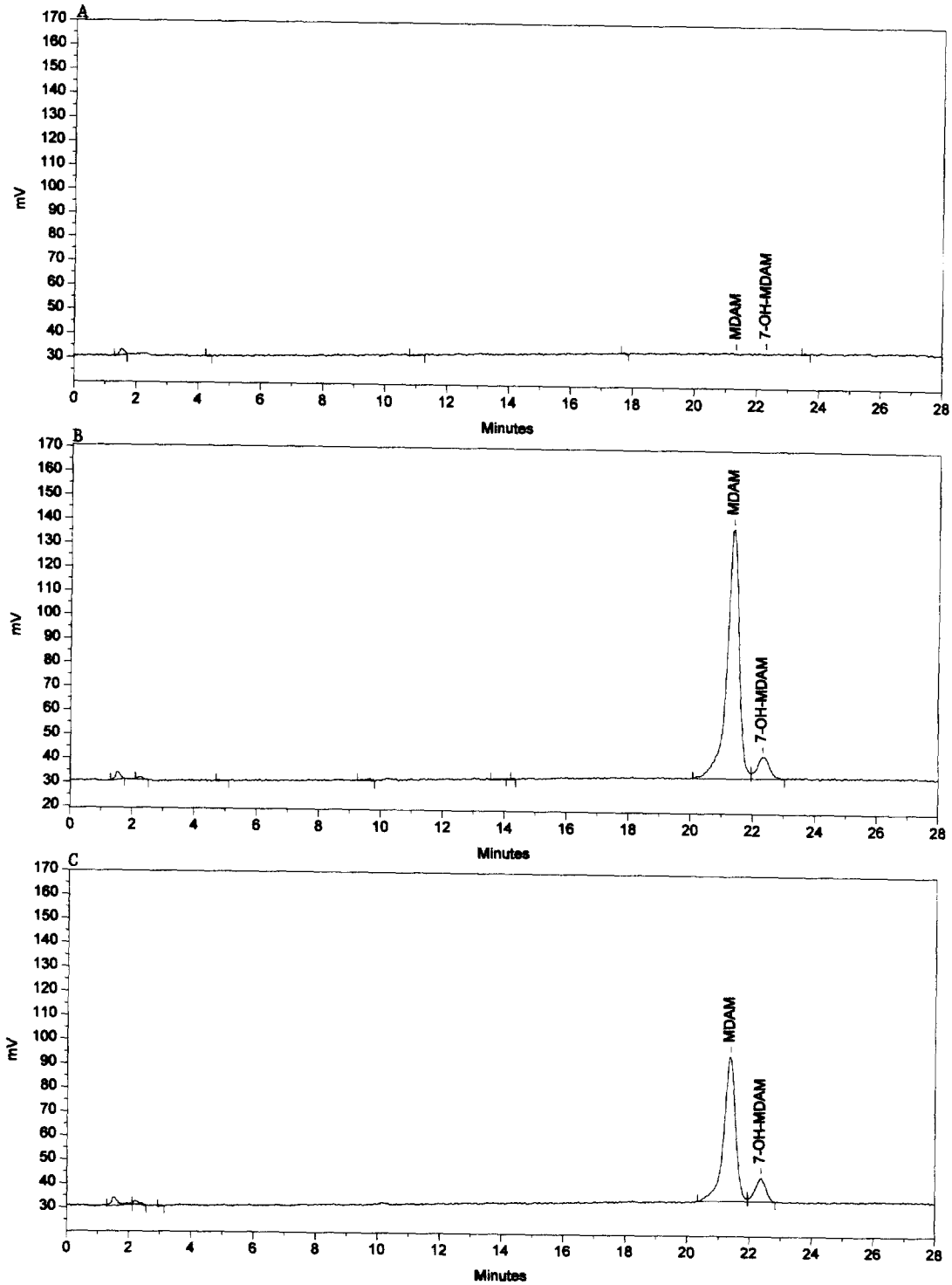


Fig. 3. (A) Chromatogram of pretreatment patient plasma. (B) Chromatogram of pretreated patient plasma spiked with MDAM and 7-OH-MDAM. (C) Chromatogram of patient's plasma 120 min following treatment with MDAM at a dose of 12 mg/m^2/day.

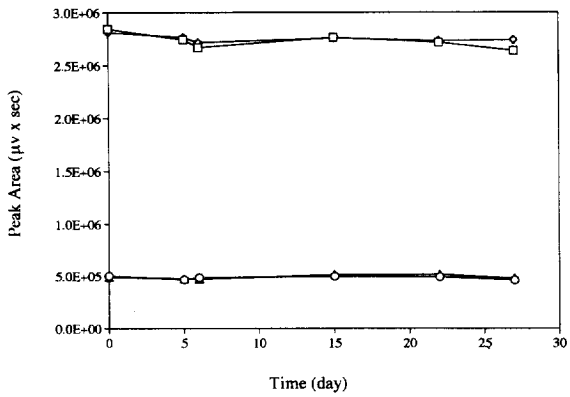


Fig. 4. Stability of MDAM and 7-OH-MDAM in plasma: (□) MDAM at ambient temperature and exposed to room light; (◇) MDAM at ambient temperature and protected from light; (○) 7-OH-MDAM at ambient temperature and exposed to room light; (△) 7-OH-MDAM at ambient temperature and protected from light.

deproteinization. This procedure was simple and reproducible, and nearly complete recovery was achieved. Table 1 lists the percentage recoveries of MDAM and 7-OH-MDAM as a function of drug concentration, as well as the intra- and inter-day coefficients of variation. Drug-spiked plasma standards were also tested by methanol deproteinization only, and these data (not shown) were compared with results from samples treated with methanol and ion-pairing agent. Recoveries from both procedures were comparable (i.e., nearly complete recoveries), suggesting that a simpler sample preparation procedure, i.e., without the ion-pairing agent, can also be used to quantitate MDAM and 7-OH-MDAM in future studies.

Table 2
Results of plasma protein-binding study for MDAM and 7-OH-MDAM

Drug	Concentration (μM)	Protein binding (%)
MDAM	0.05	~100
	1.0	95.3
	10	91.5
7-OH-MDAM	0.01	~100
	0.05	89.2
	0.2	90.8

3.5. Protein binding of MDAM and 7-OH-MDAM

The results of protein binding are shown in Table 2. Although high proportions of both MDAM and 7-OH-MDAM were found to be protein-bound, the binding affinities of these compounds for plasma protein were quite weak, as indicated by the results of recovery studies in which MDAM and 7-OH-MDAM were completely recovered by only a one-step methanol deproteinization.

3.6. Linearity and detection limits

The linear range of plasma samples spiked with drugs was studied over a range of 0.005 to 10 μM for MDAM and of 0.005–1.0 μM for 7-OH-MDAM. The linear regression equation used for MDAM was $y = 2\,763\,112x - 10\,473$, $r^2 = 0.9997$ (Fig. 5), whereas that used for 7-OH-MDAM was $y = 4\,597\,662x - 3860$, $r^2 = 0.9983$ (Fig. 6). For both MDAM and 7-OH-MDAM, the minimum concentration detected, expressed as twice the signal-to-noise ratio, was 0.005 μM.

Table 1
Results of precision and recovery studies for MDAM and 7-OH-MDAM

Drug	Concentration (μM)	C.V. intra-day mean (95% CI) (%)	C.V. inter-day mean (95% CI) (%)	Recovery (%)
MDAM	0.005	11.5 (7.9–20.9)	13.1 (9.0–23.9)	
	1.0	0.4 (0.3–0.7)	2.2 (1.5–4.0)	97.0
	10	0.3 (0.2–0.6)	2.5 (1.7–4.5)	100.3
7-OH-MDAM	0.005	9.1 (6.3–16.6)	7.9 (5.5–14.5)	
	0.05	2.4 (1.6–4.3)	6.3 (4.3–11.4)	98.7
	0.2	1.7 (1.2–3.2)	3.6 (2.4–6.5)	97.6

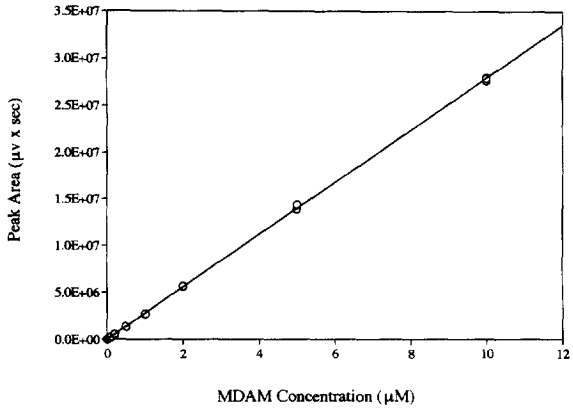


Fig. 5. Linear range of MDAM in plasma spiked with the drug. Each point represents triplicate measurements.

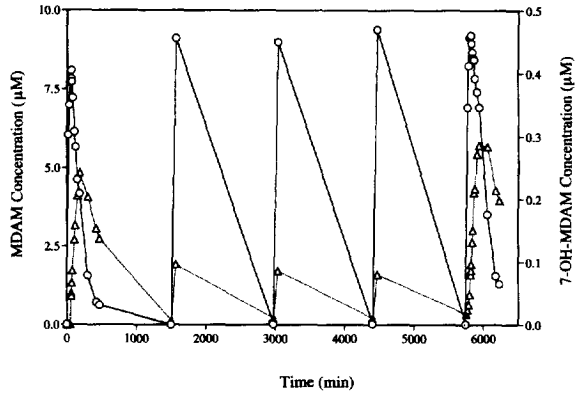


Fig. 7. Plasma concentration–time profile curves of MDAM (○) and 7-OH-MDAM (△) in a patient receiving MDAM at a dose of 80 mg/m^2/day over 30 min for five days.

3.7. Clinical application

The assay described in this report was developed to characterize the pharmacokinetic behavior of both MDAM and 7-OH-MDAM in patients treated with MDAM doses ranging from 12 to 80 mg/m^2 as on a 30-min intravenous infusion schedule given daily for five consecutive days every three weeks in a phase I trial. A total of 200 plasma samples have been analyzed. Fig. 7 shows representative plasma concentration–time profile curves for MDAM and 7-OH-MDAM. The peak concentrations were 0.8–12.7 μ*M* for MDAM at the end of infusion, and 0.019–0.5 μ*M* for 7-OH-MDAM, 2.5 h after the end of infusion. The ratio of the highest concentration of 7-OH-MDAM to the highest concentration of MDAM was less than 4% over a five-day drug administration period. Further dose escalation is ongoing.

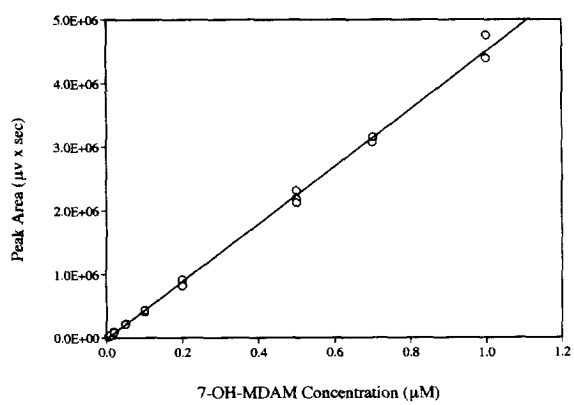


Fig. 6. Linear range of 7-OH-MDAM in plasma spiked with the drug. Each point represents triplicate measurements.

4. Conclusion

A novel HPLC method for the determination of both MDAM and its major metabolite, 7-OH-MDAM, was developed. This method is simple, sensitive and reproducible, and can be used to characterize the pharmacokinetic behavior of MDAM in patients participating in clinical studies.

Acknowledgments

We are grateful to Drs. M.G. Nair, Dennis A. Noe and John Hilton for many helpful and scientific discussions, and to Ms. Rosemary Clark for her excellent secretarial support.

References

[1] M.G. Nair and A. Abraham, U.S. Pat., 4,996,207 (1991).
[2] A. Abraham, J.J. McGuire, J. Galivan, R.L. Kisliuk, Y. Gaumont, M.G. Nair, J. Med. Chem. 34 (1991) 222.

[3] M.G. Nair, US Pat. 5 (1991) 073554.
[4] A. Abraham, M.G. Nair, F.H. Hausheer, Proc. Am. Assoc. Cancer Res. 35 (1994) 301.
[5] A. Abraham, M.G. Nair, J.J. McGuire, R.L. Kisliuk, J. Galivan, B.R. Vishnuvajjala, Adv. Exp. Med. Biol. 338 (1993) 663.
[6] M.G. Nair, R. Pati, R.L. Kisliuk, M.S. Rhee, J. Galivan, Proc. Am. Assoc. Cancer Res. 36 (1995) 380.
[7] A. Abraham, M.G. Nair, J.J. McGuire, J. Galivan, R.L. Kisliuk, B.R. Vishnuvajjala, Adv. Exp. Med. Biol. 338 (1993) 663.
[8] S. Cao, A. Abraham, M.G. Nair, R. Pati, J.H. Galivan, F.H. Hausheer, Y.M. Rustum, Clin. Cancer Res. 2 (1996) 702.
[9] A. Abraham, R. Pati, J.H. Galivan, M.S. Rhee, J.J. McGuire, R.L. Kisliuk, F.H. Hausheer, M.G. Nair, Cell. Pharmacol. 3 (1996) 29.
[10] H.J. Larson, Introduction to Probability and Statistical Inference, 3rd ed., John Wiley, New York, 1982, p. 387.
[11] J.J. Kinahan, L.L. Samuels, F. Farag, M.P. Fanucchi, P.M. Vidal, F.M. Sirotnak, C.W. Young, Anal. Biochem. 150 (1985) 203.