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High-performance liquid chromatographic assay validation of Manumycin A in mouse plasma

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Abstract

Manumycin A is a natural antibiotic produced by *Streptomyces parvulus* that has antineoplastic activity against a variety of human cancers in nude mouse models. We have developed a highly sensitive reverse phase high-performance liquid chromatography (HPLC) method based on ultraviolet (UV) detection for the determination of manumycin A in mouse plasma. Manumycin A was isolated from mouse plasma by solid-phase extraction. A gradient elution of methanol and 0.05 *M* H_3PO_4 with 0.2% triethylamine mobile phase was employed and separation was achieved with a C_{18} analytical column. Manumycin A was detected by UV absorption at 345 nm. Retention time for manumycin A was 8.9±0.2 min. The manumycin A peak was baseline resolved, with the nearest peak at 1.5 min distance and no interfering peaks detected. Inter- and intra-day coefficients of variance were less than 6.1 and 5.1%, respectively. Based on an extracted manumycin A standard plasma sample of 0.25 μg/ml, the assay precision was 99.8% with a mean accuracy of 95.1%. At plasma concentrations of 0.5 and 5 μg/ml, the mean recovery rates of manumycin A were 59.64 and 60.28%, respectively. The lower limit of detection (LLD) for manumycin A was 0.1 μg/ml in mouse plasma. The lower limit of quantification (LLQ) for manumycin A was 0.125 μg/ml. Results of the stability study indicated that when frozen at −80 °C, manumycin A was stable in mouse plasma for up to 2 weeks. This method is useful in quantification of manumycin A in mouse plasma for clinical pharmacology studies in mice.

Keywords: Manumycin A

1. Introduction

Manumycin A is a natural antibiotic produced by *Streptomyces parvulus* (Fig. 1). It is known to competitively inhibit farnesyl protein transferase (FPTase) [1]. This enzyme is important in activating a variety of signaling proteins including Ras. Ras proteins are GTP-binding proteins that play important roles in signal transduction, proliferation, and malignant transformation [2,3]. Ras transduction is a

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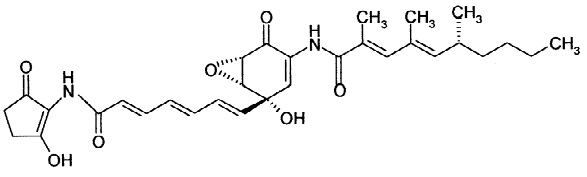

Fig. 1. Structure of manumycin A ($C_{31}H_{38}N_2O_7$); Molecular weight is 550.7 g.

key part of the signaling pathway for cell growth. Mutational activations of Ras proteins are frequently seen in human cancers, and thus are targets for cancer therapy [4].

In order for Ras proteins to function, they must be localized on the inner surface of the plasma membrane [5,6]. Post-translational modification is required for Ras to localize to the plasma membrane [6,7]. There are four steps in the post-translational modification of Ras. The first step is farnesylation (addition of a 15-carbon farnesyl group) of the cysteine residue located at the Ras COOH-terminal CAAX motif (C, cysteine; A, any aliphatic amino acid; X, any other amino acid) [8]. FPTase catalyzes this obligatory step [8–11]. However, if farnesylation is inhibited, geranylgeranylation of the cysteine residue can take place for some of the Ras proteins [12]. The second step in the post-translational modification is proteolytic cleavage of the AAX oligopeptide, followed by carboxymethylation of the farnesylcysteine [13]. Lastly, palmitoylation of cysteine residues immediately upstream of the terminal cysteine occurs [5,14,15].

The three protooncogenes, H-*ras*, K-*ras*, and N-*ras* code for four species of Ras proteins in mammals. These genes are highly conserved and preferentially expressed in different cells [16,17]. Over 90% of human pancreatic cancers have an activating mutation of the K-*ras* gene [2]. Manumycin has been shown to inhibit the growth of human pancreatic cancer cells that have a K-*ras* mutation in nude mice [18]. Manumycin A shows great promise in the treatment of many cancers including those that are difficult to treat such as anaplastic thyroid cancer (ATC), pancreatic, hepatic and colon cancer [19–23]. Additionally, Wang and Macaulay showed that manumycin A induced apoptosis in medulloblastoma cells in vitro [24]. Furthermore, in vivo studies in nude mouse xenograft models have demonstrated little toxic side effects [18,20]. Yeung et al. showed that manumycin in combination with paclitaxel induced no significant toxicity in ATC cells in nude mice [20]. Ito et al. showed that manumycin had no acute hepatotoxic effect in nude mice [18]. Xu et al. also showed that manumycin A was well tolerated by the mice with no hepatotoxicity, myelosuppression or weight loss [19]. Moreover, 40–70% of ovarian mucinous adenocarcinomas have been reported to have the K-*ras* mutation, and currently, it is undergoing pre-clinical evaluation for potential anticancer therapy in ovarian cancer cell lines [25].

A high-performance liquid chromatography (HPLC) assay validation based on ultraviolet (UV) detection was developed and validated for the analysis of manumycin A in mouse plasma to support further study of this promising antineoplastic agent.

2. Experimental

2.1. Chemicals and reagents

Manumycin A was purchased from Sigma, St. Louis, MO, USA. All solvents and reagents were of analytical grade unless otherwise noted. The following chemicals were used: methanol, phosphoric acid (H_3PO_4), triethylamine (TEA), and dimethyl sulfoxide (DMSO) (Sigma). Salvaged mouse plasma was purchased from Pel-Freez, Rogers, AR, USA and stored at $-20\,^{\circ}C$ until use.

2.2. Standard stocks

Manumycin stock solution was prepared by dissolving 10 mg of drug in 1 ml DMSO to a final concentration of 10 000 μg/ml and stored in 1 ml aliquots at $-20\,^{\circ}C$ prior to further dilution with methanol to obtain working solutions of appropriate concentrations.

2.3. Sample preparation

The C_{18} 100 mg solid-phase cartridge (Varian, Harbor City, CA, USA) was conditioned with 1 ml of methanol and then conditioned with 1 ml of distilled water. Subsequently, 250 μl of plasma sample was placed on the column then washed with

1000 μl of distilled water. Then, manumycin A was eluted with 3 ml of methanol. The elution solution was vortexed and samples were allowed to dry under a gentle stream of nitrogen gas at 40 °C. Finally, the samples were reconstituted with 250 μl of mobile phase (80:20; Methanol–0.05 *M* H_3PO_4/0.2% TEA).

2.4. High-performance liquid chromatographic apparatus and chromatographic conditions

The HPLC system consisted of a Waters Model 2690 Separations Module; pump and autosampler (Waters, Milford, MA, USA) and Waters Model 2487 Dual Wavelength Absorbance Detector (Waters). The mobile phase consisted of methanol and 0.05 *M* H_3PO_4 buffer solution with 0.2% TEA. A gradient elution was used: 70–100% over 8 min at a constant flow-rate of 1 ml/min (Table 1). A 100 μl sample was injected on the column. The chromatograph run required 15 min for completion followed by a 5-min delay to allow for column re-equilibration. Manumycin A was detected and quantified by ultraviolet (UV) absorption at 345 nm at a sensitivity setting of 2.0 a.u.f.s. UV.

2.5. Separation

Separation was achieved at room temperature with a Novapak C_{18} column (3.9×150 mm with a 5 μ*M* pore size) (Waters). A pre-column treatment of the same constituents was employed.

2.6. Extraction efficiency

To determine extraction efficiency for plasma, mouse plasma samples were spiked with manumycin A to achieve a final concentration of 0.5 and 5 μg/ml then extracted. Six samples were analyzed for each concentration. Extraction efficiency was calculated with the following equation: (peak area extracted sample for *X* μg/ml/peak area neat sample for *X* μg/ml)×100=Percent Extraction Efficiency.

Table 1
Mobile phase gradient profile for Manumycin A HPLC assay

Time (min)	Flow-rate (ml/min)	Methanol (%)	0.05 *M* H_3PO_4 with 0.2% TEA (%)
1	1	70	30
4	1	85	15
8	1	100	0
13	1	70	30

2.7. Quantification

To validate this analytical method, pooled mouse plasma was spiked with manumycin A standard solutions and extracted as described above. The dynamic linear range employed for instrument calibration was 0.25 to 7.5 μg/ml. Three calibration standard curves, completed on 3 consecutive days, were obtained by analyzing five plasma standard concentrations (0.25, 0.5, 1, 5 and 7.5 μg/ml) in triplicate. These curves were deemed acceptable if the inter-day variability was less than 15%. The best-fit calibration line was determined for each curve by sum-squared linear regression analysis of the calibration data using Millennium Chromatography Manager 32 version 3.05.01 (Waters Corporation). The inter-day analysis of accuracy and precision included back calculations of concentration for all standard samples using the slope and intercept for each curve. The peak area responses from all standards were fit to each of the three curves to estimate the nominal valve for each standard concentration within <15% variability within and across each standard curve (Table 2). Intra-day analysis of assay precision and assay reproducibility was completed using concentrations of 0.5 μg/ml (n=10) and 5 μg/ml (n=25) for mouse plasma. To determine the

Table 2
Precision and accuracy of Manumycin A in mouse plasma

Manumycin A nominal conc. (μg/ml)	Manumycin A measured conc. (μg/ml)	%RSD (%)	Accuracy (%)
Intra-day assay[a]			
0.5	0.46±0.01	2.2	92.2
5	5.41±0.28	5.1	91.8
Inter-day assay[b]			
0.25	0.25±0.01	4	95.0
1	1.03±0.06	5.8	93.9
7.5	7.53±0.11	1.5	98.6

%RSD=relative standard deviation.
[a] Mean±SD (n=10).
[b] Mean±SD (n=9).

assay accuracy reproducibility, the mean and coefficient of variance was calculated for the set of samples. The same coefficient of variance (<15%) was set as the threshold for acceptance of intra-day reproducibility (Table 2). For standards, the inter-day (between-day) precision was 93.9% or better for each of the 3 days. Intra-day (within-day) precision was 92.2% for 0.5 μg/ml (low concentration) and 91.8% for 5 μg/ml (high concentration). Lower limit of detection, or LLD, was defined as the lowest concentration with a peak area response greater than the acceptable signal-to-noise ratio of 3:1. The lower limit of quantification, or LLQ, was the lowest concentration which could reliably and repeatedly be measured with <15% variability.

2.8. Stability study

The stability of manumycin A in mouse plasma stored at −80 °C for up to 1 month was evaluated at concentrations of 0.5 and 5 μg/ml (Table 3). In addition, the effect of freezing and thawing on the stability of manumycin A was evaluated in mouse plasma at these same concentrations for three freeze–thaw cycles. For the freeze–thaw studies, samples of mouse plasma were spiked with manumycin A and frozen at −80 °C and analyzed at 24 and 72 h then 1 and 2 weeks, and also at 30 days from the day of preparation (Day 1). Peak areas from these samples were then compared to results obtained from identical samples prepared fresh, extracted and analyzed on Day 1.

3. Results and discussion

We have developed and validated an HPLC method using UV detection for the determination of manumycin A concentrations in mouse plasma. Retention time for manumycin A was 8.9±0.2 min. The manumycin A peak was baseline resolved, with nearest peak at 1.5 min distance and with no interfering peaks detected (Fig. 2).

The linear regression equation for manumycin A mouse plasma concentration was: manumycin A plasma concentration=$(9.17\times10^{2}+Y/2.13\times10^{4})$, with a correlation coefficient (r^{2}) of 0.999. Throughout the concentration dynamic range, the coefficient of variation ranged from 0.59 to 7.27%. Relative standard deviations (RSD) of calculated values for the manumycin A calibration standards ranged from 0.9 to 7.27%.

Table 3
Stability of Manumycin A in mouse plasma

Storage condition	Nominal concentration (μg/ml)	Mean% of nominal concentration (%)	RSD (%)	C.V. (%)
Continuous storage[a]				
−80 °C (*n*=3)	0.5	65.62	35.6	5.14
−80 °C (*n*=3)	5	75.83	25.8	8.06
Freeze–thaw[b]				
1 week				
−80 °C×3 cycles (*n*=3)	0.5	103.46	3.58	6.46
−80 °C×3 cycles (*n*=3)	5	97.32	−2.67	10.84
2 weeks				
−80 °C×3 cycles (*n*=3)	0.5	99.7	−0.18	0.89
−80 °C×3 cycles (*n*=3)	5	97.32	−13.01	2.16

[a] Continuous storage was for up to 1 month.
[b] Freeze–thaw cycles consisted of thawing, analysis, and re-freezing at 24 and 72 h then 1 and 2 weeks from the initial freezing.

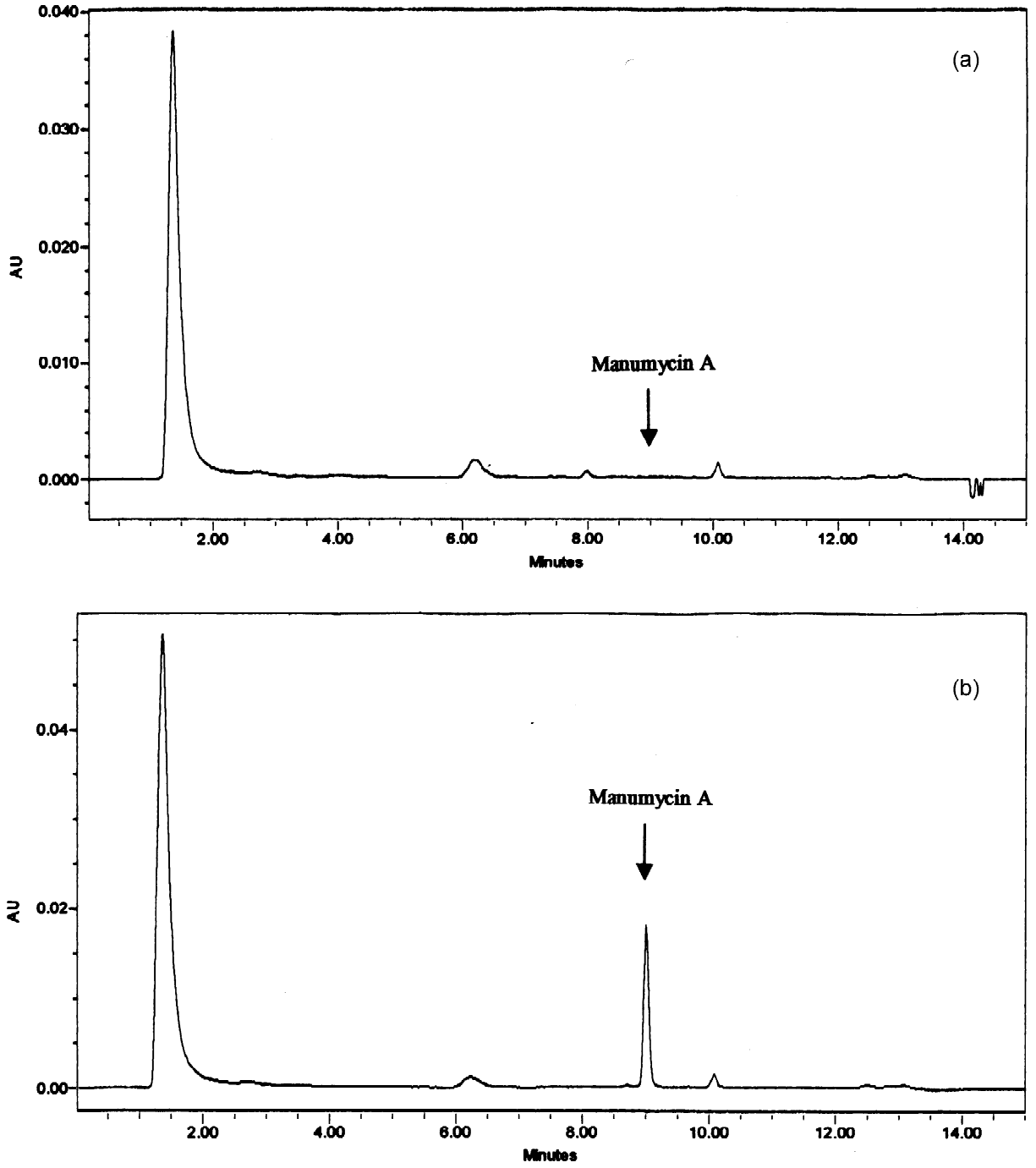

Fig. 2. Chromatograms of (a) pooled mouse plasma blank and (b) pooled mouse plasma spiked with 5 μg/ml manumycin A obtained with separation achieved by gradient elution with a mobile phase consisting of a mixture of methanol and 0.05 *M* H_3PO_4 with 0.2% triethylamine, on an ODS analytical column. The manumycin A peak was positively identified from other peaks using ultraviolet absorption at 345 nm. Retention time for manumycin A was 8.9±0.2 min.

Manumycin A peak areas were proportional over the mouse plasma concentration ranges from 0.25 to 7.5 μg/ml. Mean recoveries of manumycin A from mouse plasma at concentrations of 0.5 and 5 μg/ml were 59.64 and 60.28%, respectively.

Assay precision, based on an extracted manumycin A standard plasma samples of 0.5 and 5 μg/ml, was 92.2±7.8% with a mean accuracy of 97.3% and 94.0±6.4% with a mean accuracy of 94.9%, respectively. The lower limit of detection (LLD) for manumycin A, defined as three times the detector noise, was 0.1 μg/ml in mouse plasma. The lower limit of quantification (LLQ) for manumycin A, defined as four times the detector noise with <15% variability for repeated measures, was 0.125 μg/ml for an extracted 0.25 ml aliquot of mouse plasma.

Results of the stability study indicated that when frozen at −80 °C, manumycin A was stable in mouse plasma for up to 2 weeks. Additional freeze–thaw experiments demonstrated manumycin A was stable for up to three freeze–thaw cycles. In each instance the overall reduction in manumycin A concentration was less than the co-efficient of variation for the assay (<15%). The rates of degradation between the high and low concentrations were similar.

In conclusion, we have reported a highly sensitive, liquid chromatographic method based on UV detection for the determination of manumycin A in mouse plasma. No mouse plasma components were observed at the retention time of manumycin A. This assay method has been validated in mouse plasma and will be useful for the analysis of samples in the pre-clinical evaluation for potential anticancer therapy.

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