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A high-performance liquid chromatography method for the quantification of cysmethynil, an inhibitor of isoprenylcysteine carboxylmethyl transferase, in mouse plasma

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ABSTRACT

Cysmethynil, a newly identified small molecule inhibitor of isoprenylcysteine carboxylmethyl transferase (Icmt) is involved in the post-translational modification of CaaX proteins. Cysmethynil causes cell death in many human cancer cells *in vitro*, and inhibits tumor growth in the xenograft mouse model *in vivo*. A HPLC method for the quantification of cysmethynil in mouse plasma was developed and validated. The lower limit of quantification of this method was 0.01 μg/ml. Inter- and intra-day variability ranged from 0.38–8.5% and accuracy was between 86% and 98%. This sensitive method was used to quantify cysmethynil in plasma of mice after intraperitoneal dosing for preliminary pharmacokinetic studies.

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1. Introduction

Post-translational modification through prenylation pathway is required for the biological function of many important signaling proteins, one of the most well known one being the Ras family of oncoproteins [1]. The prenylation pathway has been one of the focuses in the development of novel therapeutic strategies for fighting diseases. The most advanced of these efforts are that targeting farnesyl transferase (FTase) as it is the enzyme that is involved in the modification of Ras oncoprotein. The disappointing efficacy results of FTIs in phase III clinical trials, and the discovery of alternative prenylation which helps to explain the dismal results sparked the interest in finding compounds that block both farnesylated and geranylgeranylated proteins from getting the subsequent modifications through inhibition of the distal processing steps in the pathway, i.e. Rce I or Icmt, since there is but a single enzyme for both of these steps that processes both farnesylated and geranylgeranylated proteins [2].

Cysmethynil (2-[5-(3-methylphenyl)-1-octyl-1H-indol-3-yl] acetamide) (Fig. 1) is a recently identified small molecule inhibitor of Icmt, an unique methyltransferase catalyzing the last step of prenylation pathway in the post-translational modifications of a group of important signaling proteins with a C-terminal CaaX motif (cysteine, aliphatic, aliphatic and variable residues, respectively) [3,4]. Cysmethynil was identified in a screen of a diverse chemical library of ∼10,000 compounds containing 70+ subfamilies derived from unique scaffolds using an *in vitro* assay that measures the incorporation of a [^{3}H]methyl group into an appropriately-modified CaaX protein substrate [4]. Cysmethynil then was independently synthesized, characterized to confirm identity and purity, and numerous studies have since been done including enzymatic study [5], cellular study [4] and most recently *in vivo* anti-tumor efficacy study [6]. These studies demonstrated the therapeutic potential for further preclinical development of cysmethynil as an anti-cancer chemotherapeutic agent.

Pharmacokinetic and pharmacodynamic studies are indispensable in the development process for new drugs. It is therefore critical to develop a method to determine drug concentrations in various biological samples such as plasma and tissues, and to determine the metabolism and identify metabolites of the drug. As a newly identified compound, there was no information available regarding the analysis of cysmethynil in biological samples treated with the drug. The aim of this study was to develop and validate a rapid, simple and specific bioanalytical HPLC–UV method to determine cysmethynil concentrations in mouse plasma samples. The method was utilized for preliminary pharmacokinetic investigation of cysmethynil in mice after intraperitoneal (i.p.) dosing of cysmethynil, setting the foundation for further preclinical and clinical studies. The method has been validated according to the Food

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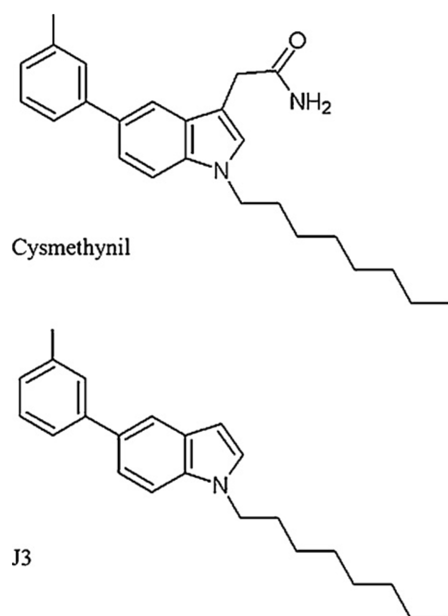

Fig. 1. The chemical structure of Cysmethynil (2-[5-(3-methylphenyl)-1-octyl-1H-indol-3-yl] acetamide) and cysmethynil analog J3 (1-octyl-m-tolyl-1H-indole).

and Drug Administration (FDA) guidelines for bioanalytical method validation [7].

2. Experimental

2.1. Chemicals and reagents

The reference material cysmethynil (2-[5-(3-methylphenyl)-1-octyl-1H-indol-3- I yl]acetamide) with a purity of >99.8% was synthesized by Duke University Chemical Biology Program's chemical synthesis laboratory. The internal standard J3 (Fig. 1), a chemical analog of cysmethynil [6], was prepared at the Department of Pharmacy, National University of Singapore with a purity of 95%. All other chemicals were of HPLC grade. Milli-Q water (Millipore Corporation, Bedford, MA) was used for mobile phase preparation.

2.2. Equipment and chromatographic conditions

Analyses were performed with Agilent 1200 series HPLC system (Agilent Technologies, USA) including a binary pump, online degasser, autosampler, variable wavelength UV detector, a thermostated column compartment and the Agilent software Chemstation.

Chromatographic separations were via a Luna C18 column (150 mm × 2 mm, 5 μm, Phenomenex, Torrance, CA) coupled with a MAX-RP guard column (4 mm × 2 mm, 5 μm, Phenomenex, Torrance, CA) at room temperature. Elution of the analytes were through gradient flow of mobile phase of A (0.1% formic acid) and B (acetonitrile). Gradient elution cycle of A:B ratio was as follows: 0–4 min with 20% of A, 4–12 min reducing A to 0%, 12–18 min maintaining A at 0% and 18–28 min re-equilibrating the ratio back of 20% of A for the next cycle. The flow rate of mobile phase was maintained at 0.3 ml/min. Detection wavelength was set at UV 254 nm.

2.3. Stock and working standard solutions

The stock solution of cysmethynil (1 mg/ml) was prepared by dissolving the appropriate amount of the pure solid in methanol in an amber vial. The stock solution of the internal standard was prepared in 100% dimethyl sulfoxide (DMSO) at a concentration of 30 mM (9.6 mg/ml). Both stock solutions were stored in −80 °C freezer. The working standard solutions of cysmethynil were prepared fresh by step-wise dilutions of the above stock solution with methanol to provide calibration concentration range of 0.01–100 μg/ml when spiked into drug-free mouse plasma. The working internal standard solution was prepared from the stock by dilution with acetonitrile to 9.6 μg/ml.

Four quality control samples (QCs), were prepared from the stock solutions by spiking cysmethynil into drug-free mouse plasma to obtain final concentrations of 0.03, 0.3, 15 and 75 μg/ml. Routine calibration curves consisting of 0.01, 0.1, 1, 10, 50, and 100 μg/ml calibrators were generated together with the QCs for computation of cysmethynil concentrations in mouse samples using peak area ratios of cysmethynil and internal standard.

2.4. Animals handling, sample collection and preparation

BalbC mice weighing 20–25 g were purchased from National University of Singapore Animal Facility. They were acclimated and housed in cages at temperatures between 22 and 25 °C with free access of food and water before and during the time of experiments. All handling and experimentation were according to the protocols approved by the institutional care and use of laboratory animals committee guided by the principles for the care and use of laboratory animals.

On the day of experiment, the animals were dosed according to their weight at 200 mg/kg through i.p. route. This dose has been found to be efficacious in tumor bearing mice with minimal systemic toxicity. Blood samples were taken from their tails at the time intervals of 15 min, 1, 3, 6, 10, 24, 33, 48 and 57 h. At each time point, blood samples were collected from five mice to compute the mean concentration. Since only a maximum of 3–4 samples of 50 μl each could be sampled from a mouse over a short time, three groups of five mice were needed to complete the full sampling intervals. The blood samples were collected into heparin-coated tubes and maintained on ice for a short time before spinning in a microcentrifuge at 4 °C to harvest the plasma samples, which were immediately stored at −80 °C until analysis.

2.5. Extraction preparation

10 μl plasma obtained from dosed animals, as described above, was mixed with 10 μl methanol and 30 μl of internal standard solution in acetonitrile. For calibration and quality control samples, 10 μl of their respective working standards prepared in methanol were added to each 10 μl of drug-free plasma before mixing with 30 μl of internal standard solution. Methanol and acetonitrile in the mixture precipitated the plasma proteins. The mixture was then centrifuged at 10,000 rpm at 4 °C for 5 min, and the supernatant containing the extracted cysmethynil and internal standard was then transferred to the auto-sampler of the HPLC for analyses.

2.6. Method validation

The method was validated based on the principles provided by the "Guidance for Industry" under the section of "Bioanalytical Method Validation" by Food and Drug Administration of USA [7].

2.6.1. Selectivity, linearity

To establish that endogenous substances present in mouse plasma do not co-elute with cysmethynil and the internal standard in the HPLC method, plasma samples from at least six untreated mice from both BalbC and SCID strains were used to verify selectivity of the analytical method.

To evaluate the linearity of the calibration curves, six calibration standards containing cysmethynil at nominal concentrations

of 0.01, 0.1, 1, 10, 50 and 100 μg/ml were prepared as described in Section 2.3. The standard calibration curves were constructed using cysmethynil/IS peak–area ratios vs. the nominal concentrations of cysmethynil by weighted (1/concentration) linear regression.

2.6.2. Precision, accuracy and recovery

The precision of the method was assessed by determining the coefficients of variation (CV) of the 4 QC samples (concentrations of 0.03, 0.3, 15, and 75 μg/ml) within the same analysis ($N=5$, intra-day precision) and over a series of analyses ($N=6$, inter-day precision). Both intra-day and inter-day precisions were calculated with the following formula:

$$\mathrm{CV}(\%) = \left[\frac{(\text{standard deviation})}{(\text{mean})}\right] \times 100$$

The accuracy of the method was evaluated by determining how close the mean measured results were from the nominal concentrations using the 4 QC samples (0.03, 0.3, 15, and 75 μg/ml) within the same analysis ($N=5$, intra-day accuracy) and over a series of analyses ($N=6$, inter-day accuracy). The accuracy was calculated with the following formula:

$$\text{Accuracy}\,(\%) = \left[\frac{(\text{mean measured concentration})}{(\text{nominal concentration})}\right] \times 100$$

The precision and accuracy for the first calibrator of 0.01 μg/ml, lower limit of quantification (LLOQ) were also evaluated.

The recovery experiments in this study were performed by comparing peak areas of cysmethynil and internal standard in spiked plasma samples, extracted by the method described in Section 2.5, with those where the compounds of interest at concentrations corresponding with 100% recovery were added to similarly post-extracted blank plasma.

2.6.3. Stability

To ensure the reliability of the method in analyzing the plasma samples, we assessed the stability of cysmethynil at different stages of the manipulation. Specifically, the long term stability of the samples when stored at −80 °C for up to 5 months was analyzed since this is the temperature of which the samples were stored before analysis. In addition, the stability of samples that had been subjected to repeated freeze and thaw cycles was analyzed. All stability tests were performed with either QC samples or plasma samples from cysmethynil-dosed mice. To assess the stability of the samples between the time of extraction and HPLC run, we analyzed the samples at the time of extraction, compared the values to the 10 and 20 h post-extraction values of the same samples stored at room temperature. These tests provide the validation for using the auto sampler when analyzing multiple samples.

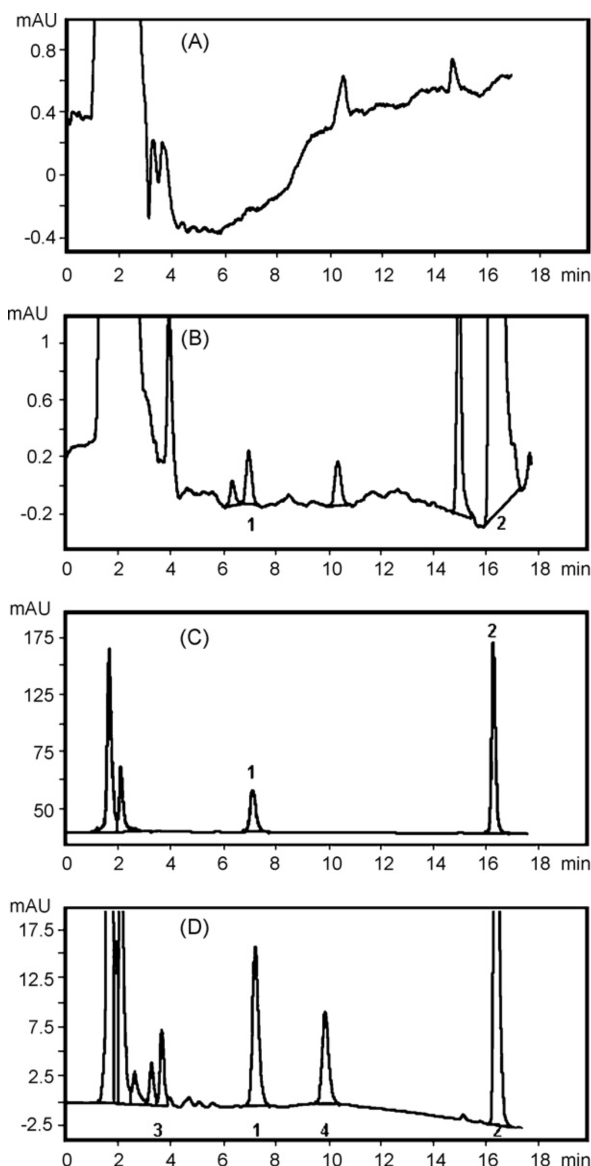

Fig. 2. HPLC chromatograms of drug-free plasma (A), QC sample spiked with cysmethynil at 0.03 μg/ml and internal standard (B), a calibrator spiked with cysmethynil at 10 μg/ml and internal standard (C), and plasma sample 33 h after i.p. administration of cysmethynil (D). Peak 1: cysmethynil at about 7.1 min; Peak 2: internal standard at about 16.2 min; Peaks 3 and 4: apparent metabolites between 3 and 4 min and at 9.8 min respectively.

3. Results

3.1. Method validation

3.1.1. Selectivity and linearity

Fig. 2 shows the representative gradient HPLC chromatograms of control, blank plasma from untreated mouse (A), low QC, blank plasma sample spiked with 0.03 μg/ml cysmethynil (B), a calibrator, 10 μg/ml (C), both with spiked internal standard and finally a blood sample obtained 33 h after i.p. injection of cysmethynil into a BalbC mouse (D). Fig. 2 B with a concentration 3 times the LLOQ shows a steady baseline, the cysmethynil peak was symmetrical and well separated from a plasma peak. Cysmethynil, and internal standard were well separated using the above described chromatographic condition with retention times of 7.07 and 16.37 min respectively. Internal standard J3 is more lipophilic than cysmethynil and eluted much later even with increase of acetonitrile in the gradient program. The mouse sample (Fig. 2D) shows additional peaks between 3 and 4 min as well as at 9.8 min. These are the likely metabolites of cysmethynil. No interfering peaks from endogenous substances in the control plasma samples of different mice or other reagents were detected under the conditions employed.

The peak area ratio (cysmethynil/internal standard) as a function of the nominal cysmethynil concentrations over the wide range of 0.01–100 μg/ml was linear. The regression coefficient R^2 was 0.999. The weighted linear regression equation was:

$$y = 0.0302101x + 0.0000185$$

3.1.2. Precision, accuracy and recovery

The intra-day and inter-day precision and accuracy for the plasma cysmethynil measurement using this HPLC method are summarized in Table 1. The intra-day and inter-day variability for

Table 1
Intra-day and Inter-day Assays Precision and Accuracy for Cysmethynil.

Nominal concentration (μg/ml)	N	Measured concentration (μg/ml)	SD	Precision CV (%)	Accuracy *(%)*
Intra-assay					
0.03	5	0.0258	0.001	3.24	*86*
0.3	5	0.272	0.013	4.79	*90.7*
15	5	13.5	0.158	1.17	*90*
75	5	72.1	0.227	0.38	*96.2*
Inter-assay					
0.03	6	0.295	0.0025	8.51	*98.3*
0.3	6	0.26	0.004	1.56	*87.2*
15	6	13.1	0.2229	1.7	*87.2*
75	*6*	*70.4*	*1.39*	*1.98*	*93.9*

Table 2
Long term stability at −80 °C.

Date of analysis	28 Jan. (μg/ml)	3 July (μg/ml)	% Change
Animal number			
1	0.40	0.44	+8.9%
2	0.70	0.71	+1.4%
3	0.72	0.70	−2.9%
4	1.15	1.22	+6.1%

the different QCs were minimal, ranging from 0.38% to 8.5%, indicating excellent precision. Accuracies using the same QCs ranged from 86% to 98.3%. All these variability were well within acceptable limits. Precision and accuracy for the LLOQ of 0.01 μg/ml were 17.6% and 98%, respectively.

Recoveries were good. This was expected because simple direct protein precipitation was employed for sample extraction. The mean recoveries for cysmethynil in 3 QC samples (0.03, 15, and 75 μg/ml) were 73%, 92% and 99%, respectively. Recovery of the internal standard was 104%. The lower recovery for the 0.03 μg/ml QC was probably due to the low concentration and loss of cysmethynil through non-specific binding to precipitated protein.

3.1.3. Stability

Cysmethynil samples were stable when stored in −80 °C for up to 5 months (Table 2). There were no significant changes in concentrations when the same samples were analyzed at 5 months intervals. Importantly samples were not affected by repeat freeze-thaw cycles, making going back to the same samples for repeat analysis reliable (Table 3). Additionally, the extracted samples were stable at room temperature for the periods under which determinations were made, which were 10 and 20 h later (not shown). This is important because samples can be stored in the auto-sampler at room temperature till measurement without compromising the concentration of cysmethynil.

3.2. Application of the method

3.2.1. The cysmethynil log-concentration vs. time curves

The established method was applied to obtain plasma concentrations of cysmethynil at different time intervals after i.p. dosing of 200 mg/kg of cysmethynil to 10–12 week-old BalbC mice. The log-concentration vs. time curve of cysmethynil through i.p. route is shown in Fig. 3. The curve represents a composite picture of absorption through the peritoneum, superimposed on to the distribution and finally the log-linear metabolism/elimination phase. The elimination half-life ($T1/2$) as estimated from log-linear elimination of Fig. 3 was about 5 h. Computed $T1/2$ using Noncompartmental analysis (WinNonlin, Pharsight Corp.) was 4.67 h.

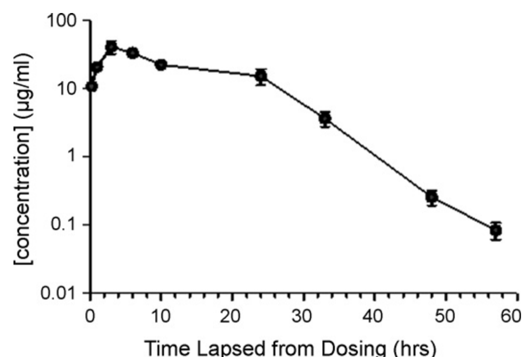

Fig. 3. Mean plasma log-concentration vs. time profile of cysmethynil in healthy BalbC mice after intraperitoneal injection of a single dose of 200 mg/kg of cysmethynil. Each time point is the mean from 5 mouse samples. The first collection time was at 15 min post-administration of drug.

3.2.2. Metabolites of cysmethynil

Chromatogram (Fig. 2D) from plasma samples of treated mice seemed to show that cysmethynil may be metabolized to a few more polar metabolites (eluting between 3 and 4 min) and a significant one that is less polar than cysmethynil eluting at around 9.8 min in the HPLC conditions.Further elucidation of these peaks is necessary to identify and confirm them as cysmethynil metabolites.

4. Discussion and conclusions

Cysmethynil is a new small molecule inhibitor of isoprenylcysteine carboxylmethyl transferase, a unique methyltransferase that catalyzes the last step of the three step post-translational modification of CaaX proteins. We have found in *in-vitro* studies that cysmethynil has mechanism-based ability to inhibit tumor cell growth and induce tumor cell death in multiple human tumor cell lines. Subsequent *in vivo* preclinical studies also demonstrated efficacy of cysmethynil in inhibiting growth of xenograft human tumors in experimental mouse model [6]. Given these promising preliminary results, it is necessary to understand the pharmacoki-

Table 3
Stability with Multiple Freeze-Thaw (F-T) Cycle.

Concentrations QCs + 100 μg/ml (F-T) Cycle	0.03 (μg/ml)	0.3 (μg/ml)	15 (μg/ml)	75 (μg/ml)	100 (μg/ml)
0 (Fresh samples)	0.033	0.35	17.0	83.6	109.2
1	0.033 (100%)	0.33 (94.3%)	17.2 (101.2%)	80.7 (96.5%)	112.5 (103%)
2	0.0325 (98.5%)	0.36 (102.9%)	17.6 (103.5%)	81.2 (97.1%)	112.5 (103%)
3	0.033 (100%)	0.35 (100%)	17.0 (100%)	81.8 (97.9%)	102.6 (94%)

netics and pharmacodynamics to further develop this potential new anti-cancer agent. Important components are to evaluate different dosing regimen in achieving persistent blood and tissue drug levels; and to evaluate the effective tissue concentrations of cysmethynil required for efficacy. Better formulations to ensure optimal delivery of cysmethynil are also critical. It is also important to understand the tumor drug levels in comparison to other tissue drug levels in order to begin to consider the complex issue of tumor tissue vascularization and its role in cysmethynil delivery and its impact on the efficacy in inhibiting tumor growth. The development of biomarkers to indicate the adequate drug penetration into tumor cells at various blood/tissue samples is also important; in this regard we have begun to profile the specific signaling proteins impacted by cysmethynil treatment. All these studies will be facilitated by the development of a simple, reliable, cost effective and expedient method of analyzing blood/tissue samples for cysmethynil levels.

We report here a reliable method in dosing, collecting and storing plasma samples from experimental mice that were dosed with this new small molecule inhibitor of Icmt–cysmethynil. We have also developed and validated a simple, efficient and reliable method for HPLC measurement of plasma samples containing cysmethynil to high sensitivity, the LLOQ being 0.01 μg/ml. This method is applicable to the plasma samples from cysmethynil-dosed mice to provide pharmacokinetic information. The $T1/2$ of about 4.7 h in mice dosed through the intraperitoneal route indicates favorable pharmacokinetics for further study of the drug as a therapeutic agent. Furthermore, some apparent metabolite peaks were clearly detected and well separated and can be further investigated. This finding opens the door to the study of the metabolism of this experimental drug. Further application of the method should provide useful pharmacokinetic and pharmacodynamic information on cysmethynil administration *in vivo*.

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