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Journal of Chromatography B, 856 (2007) 261–266
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Quantitative and selective assay of 5-methylindirubine, an inhibitor of cyclin-dependent kinases, in murine plasma using coupled liquid chromatography and electrospray tandem mass spectrometry

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Received 3 April 2007; accepted 12 June 2007
Available online 22 June 2007

Abstract

A sensitive and rapid LC–MS/MS assay for the quantitative determination of 5-methylindirubine (5-MI) in murine plasma is described. A 50-μL-murine plasma aliquot was spiked with an internal standard, indirubine-3-monoxime (IMO), and extracted with 1.25 mL diethyl ether. Dried extracts were reconstituted in methanol–water (8:2, v/v) and 10 μL-volumes were injected onto the HPLC system. Separation was achieved on a Gemini C18 column (150 mm × 2.1 mm ID, particle size 5 μm) using an alkaline eluent (10 mM ammonium hydroxide–methanol (5:95, v/v)). Detection was performed by negative ion electrospray followed by tandem mass spectrometry. The assay quantifies 5-MI in a range from 1 to 500 ng/mL using 50 μL of murine EDTA plasma samples. Validation results demonstrate that 5-MI concentrations can be accurately and precisely quantified in murine plasma. This assay is used to support pre-clinical pharmacologic studies with 5-MI.

Keywords: 5-MI; Indirubin; Cyclin-dependent kinases; Liquid chromatography; Tandem mass spectrometry

1. Introduction

Organic colorants and their derivatives, such as indigo and indirubin, are among the most important components of historical paintings and textiles [1,2]. However, indirubin is also known as the active ingredient of Danggui Longhui Wan, a traditional Chinese antileukemia medicine, which constitutes a mixture of 11 plants [3–6]. Using oxidation and dimerization, indirubin is derived from indoxyl and isatin, which are extracted from colorless precursor conjugates indican (from *Indigofera* and *Polygonum* species among others) and isatan B (from *Isatis tinctoria*) [3,7].

Studies exploring the mechanism of action of indirubin indicated that it inhibits DNA and protein synthesis in several cell lines, in cell-free systems, and *in vivo* in rats with Walker-256 sarcoma [8–13]. A weak binding of indirubin to DNA *in vitro* has also been described [13].

Recently, indirubin and a few indirubin derivatives such as 5-methylindirubine (5-MI) have been reported to inhibit cyclin-dependant kinases (CDKs) by competing in the ATP-binding sites with high selectivity among several kinase families, which has an anti-proliferative effect on human cancer cells [3,14–16].

Indirubin and its derivatives are quite difficult to identify due to the low stability, poor solubility in water and organic solvents, and similarity in the chemical structure [17]. Moreover, indirubin is no longer used in China to treat chronic myelogenous leukemia because of its poor water solubility and gastrointestinal tract problems [18]. Studies performed by Fiebig et al., who

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doi:10.1016/j.jchromb.2007.06.006

investigated the influence of indirubin and potent analogues on the growth of human tumor xenografts *in vitro* and *in vivo*, suggested that the indirubin analogue 5-methylindirubin is very active and effective [19]. Moreover, 5-MI showed antitumor efficacy in a variety of solid tumor xenografts, e.g. colon cancer, non small cell lung cancer, mammary cancer, melanoma, prostate cancer, and renal cancer, following oral administration [20].

For the detection of indirubin and indigo, TLC, HPLC–UV [21], and fast atom bombardment mass spectrometry methods [22] have been described. Puchalska et al. [1] and Szostek et al. [23] recently used LC–MS to determine and to investigate the natural dyes (including indirubin) in textiles and paintings. Liau et al. developed an LC–APCI-MS method for detection and analysis of tryptanthrin, indigo, and indirubin in the leaves and roots of *Isatis Indigotica* and *Strobilathes cusia* [24].

However, no methods have been described so far to quantify indirubins and their derivatives in biological matrices. We successfully achieved our goal to develop an accurate and selective assay for the determination of 5-MI in murine plasma, in order to apply this assay in a pre-clinical study with 5-MI carried out in mice.

2. Experimental

2.1. Materials

5-Methylindirubin ($C_{17}H_{12}N_2O_2$; Fig. 1A) was kindly supplied by Oncotest GmbH (Freiburg, Germany). Indirubin-3-monoxime ($C_{16}H_{11}N_3O_2$; Fig. 1B) [25] was obtained from Sigma–Aldrich Chemie (Steinheim, Germany). Methanol (LC gradient grade) was obtained from Bissolve Ltd. (Amsterdam, The Netherlands). All other solvents or chemicals were analytical grade or better. Distilled water was used throughout the analyses. Drug-free murine EDTA plasma was obtained from Oncotest GmbH (Freiburg, Germany).

2.2. Standard, quality control, and internal standard solutions

Two sets of stock solutions of 5-MI in ethanol were prepared from two independent weighings at a target concentration of 0.5 mg/mL. The solutions were placed in the ultrasonic bath for 1 h to dissolve all the 5-MI in ethanol. These solutions were further diluted with control murine EDTA plasma to obtain working solutions. One set of working solutions was used to prepare calibration standards and the other was used to prepare quality control samples. The plasma working solutions of 5-MI used for calibration curves were further diluted in control murine EDTA plasma to yield the concentrations of 50,000, 5000, 2500, 1000, 500, 100, 50, 20, and 10 ng/mL. Calibration standards were prepared freshly at concentrations of 1, 2, 5, 10, 50, 100, 250, and 500 ng/mL by diluting 15 μL of the plasma working solutions with 135 μL of control murine EDTA plasma. Standards were processed in singlicate and analyzed in duplicate.

The plasma working solutions of 5-MI used for the preparation of the quality control samples were further diluted in control murine EDTA plasma to yield the concentrations of 50,000, 10,000, 1000, 100, and 10 ng/mL. Quality control samples for 5-MI were prepared in murine EDTA plasma at concentrations of 1, 3, 50, and 375 ng/mL, by diluting the working solutions in control murine EDTA plasma in volumetric flasks. Replicate 50 μL aliquots were transferred to 2.0 mL tubes for storage at nominally −20 °C. The calibration standards and quality control samples were vortex-mixed for approximately 30 s before processing.

A stock solution of IMO was prepared in ethanol at a concentration of 0.1 mg/mL. An internal standard working solution of IMO was prepared by transferring 50 μL of IMO stock solution to a 50.0 mL volumetric flask and adding methanol–water (8:2, v/v) to give a final concentration of 100 ng/mL. All solutions were stored at −20 °C.

2.3. Sample preparation

Sample pretreatment was performed at ambient temperatures. 5-MI and internal standard were extracted from murine EDTA plasma using liquid–liquid extraction (LLE). To 50 μL sample aliquots, 20 μL of internal standard working solution in methanol–water (8:2, v/v) and subsequently 1.25 mL of diethyl ether were added. The samples were vortexed for 10 s, followed by automatic shaking for 10 min at 1250 rpm (Labinco, Breda, The Netherlands). Samples were then centrifuged for 10 min at 23,100 × *g*, the aqueous layer was frozen in an ethanol–dry ice mixture and the organic layer was decanted into a clean 2.0 mL-eppendorf tube. The organic solvent was evaporated under a gentle stream of nitrogen gas at 30 °C. The residue was reconstituted with 50 μL of reconstitution solvent (methanol–water (8:2, v/v)) by vortex-mixing for 30 s. After shaking for 15 min and centrifuging for 5 min at 23,100 × *g*, the clean supernatant was transferred to a glass autosampler vial with insert.

2.4. Liquid chromatography/mass spectrometry

A HP1100 binary pump, degasser and HP1100 autosampler (Agilent Technologies, Palo Alto, CA) and an API 365

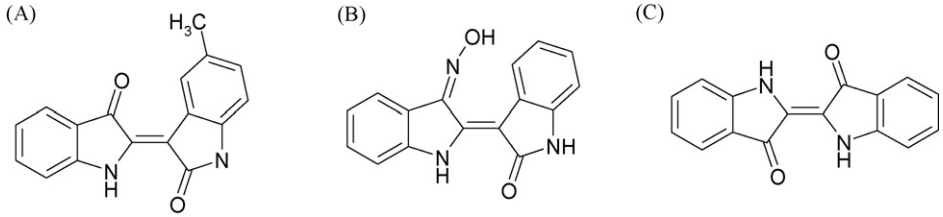

Fig. 1. Chemical structures of 5-MI (A), IMO (B), and indigo (C).

Table 1
Settings of the API365

Parameter	Setting
Run time duration	4.5 min
Ionspray voltage (negative ion mode)	−4500 V
Q1 resolution	Unit
Q3 resolution	Unit
Nebulizer gas (compressed air)	10 psi
Curtain gas (nitrogen)	6 psi
Temperature	400 °C
Collision actived dissociation gas	9 psi
5-MI	
Q1 mass	275 amu
Q2 mass	171 amu
Dwell time	150 ms
IMO	
Q1 mass	276 amu
Q2 mass	246 amu
Dwell time	150 ms

triple quadrupole MS equipped with an electrospray (ESI) ion source (Sciex, Thornhill, ON, Canada) were used. Isocratic chromatography was performed using a Gemini C18 column (150 mm × 2.1 mm ID, particle size 5 μm; Phenomenex, Torrance, CA, USA). The mobile phase of 10 mM ammonium hydroxide in water–methanol (5:95, v/v) was pumped at a flow-rate of 0.2 mL/min. Sample injections of 10 μL were carried out and the autosampler temperature was gauged at 10 °C.

Negatively charged ions were created at atmospheric pressure and the mass analyzer was operated in the multiple reaction monitoring (MRM) mode using unit resolution for the quadrupoles. The resulting MRM chromatograms were used for quantification utilizing Analyst™ software version 1.2 (Sciex). The ESI-MS/MS operating parameters used in this study are listed in Table 1.

2.5. Method validation

Partial validation of this method was performed due to the limited availability of murine plasma. To determine the linear range, calibration standards (eight non-zero standards as described in Section 2.2) were prepared in control murine EDTA plasma, processed, and analyzed in duplicate in one analytical run. The linear regression of the ratio of the areas of the analyte and internal standard peaks versus the concentration were weighed by $1/x^2$ (the reciprocal of the squared concentration). Concentrations were back-calculated from the constructed calibration curve and deviations from the nominal concentrations should be within ±20% for the lower limit of quantitation (LLOQ) and within ±15% for other concentrations with coefficient of variation (C.V.) values less than 20% and 15%, respectively [26,27].

The accuracy and precision of the analytical method were assessed by analyzing five replicates of each murine quality control EDTA plasma sample in one analytical run together with a calibration curve independently prepared from the quality control samples containing 1, 3, 50, and 375 ng/mL of 5-MI, as described in Section 2.2.

Accuracies were determined as the percentage difference of the measured concentration from the nominal concentration and the C.V. was used to report the precision.

The intra-assay accuracies (% bias) should be within ±20% at the LLOQ level and within ±15% at the other concentrations [26,27]. The intra-assay precisions should be less than 20% at the LLOQ level and less than 15% at the other concentrations [26,27].

Carry-over was tested by injecting two blank matrix samples after injecting an upper limit of quantitation (ULOQ) sample.

To test the stability of 5-MI in murine plasma after three freeze/thaw cycles, the quality control samples that had been frozen and thawed three times were compared with freshly prepared quality control samples. The stability of 5-MI in murine EDTA plasma under processing (ambient temperatures) was also evaluated. The long-term stability of 5-MI in murine plasma and the stability of stock and working solutions at −20 °C are ongoing.

The above described stability experiments were executed in triplicate at two concentration levels (3.25 ng/mL and 406 ng/mL) for 5-MI. 5-MI is considered stable in the stock and working solutions when 90–110% of the fresh sample's ratio is found and it is considered stable in biological matrixes or extracts when 85–115% of the initial concentration is recovered [26,27].

2.6. Pre-clinical study

The analytical method described in this article has been used to support a pre-clinical study in mice, carrying the large cell lung cancer LXFL 529 as solid tumor xenograft. During the study carried out at Ontcotest GmbH, mice received a single oral dose of 150 mg/kg 5-MI, formulated as fine homogeneous suspension at 10.0 mg/mL in PEG-200. Blood samples were collected in EDTA-coated polystyrene tubes (EDTA-1000A Kabe Labortechnik, Nümbrecht-Elsenroth, Germany) from three mice per time point at 2, 8, 24, and 48 h following administration of 5-MI. After centrifugation, plasma was removed and stored at −20 °C until analysis.

3. Results and discussion

3.1. Sample pretreatment

Several methods of sample pretreatment were investigated. Recoveries were determined by comparing responses from murine plasma samples processed according to the procedures mentioned below to the response of 5-MI standard solutions in reconstitution solvent (methanol–water (8:2, v/v)), which represented a 100% recovery. Protein precipitation of murine EDTA plasma samples containing 5-MI using methanol and acetonitrile was tested. This resulted in very low recoveries (around 30%), broad peaks, and ion suppression; therefore, protein precipitation was discarded for these reasons. Liquid–liquid extraction using diethyl ether, ethyl acetate, dichloromethane,

ethyl acetate–diethyl ether (1:1, v/v), and hexane–diethyl ether (1:1, v/v) was investigated. Diethyl ether yielded highest and most reproducible recoveries for 5-MI, along with low ion suppression. The overall recovery of 5-MI from murine plasma, which corresponds to the LLE recovery plus the contribution of the ion-suppression, was 98%. This means that the effect of the ion-suppression can be neglected. Therefore, the sample pretreatment using LLE with diethyl ether was chosen.

To concentrate the analytes, the organic layer was evaporated under a stream of nitrogen gas at 30 °C. We observed that 5-MI was not stable in either a very acidic or basic environment. When 5-MI was dissolved in a solution containing more than 30% water, 5-MI was crystallized due to the very poor solubility of 5-MI in water. Due to the facts mentioned above, we chose a neutral organic solution to reconstitute 5-MI, such as methanol–water (8:2, v/v). The use of methanol resulted in better peaks than using acetonitrile.

3.2. Internal standards

Due to the absence of the labeled and/or deuterated internal standards, structurally related compounds to 5-MI have been tested. Indigo ($C_{15}H_{10}N_2O_2$; Fig. 1C) is a structural analog of 5-MI and differs from 5-MI in the absence of the methyl group and, furthermore, the molecular skeleton is arranged differently. However, the solubility of indigo and the ionization were very different from that of 5-MI. Moreover, the chromatographic properties of indigo were poor when the analytical system developed for 5-MI was applied. For these reasons, indigo was discarded and another internal standard was tested, namely indirubin-3′-monoxime (IMO). This compound appeared to be a suitable internal standard: it was ionized in the negative mode, the solubility was comparable to 5-MI, and it eluted just before the analyte with acceptable chromatographic properties.

3.3. Liquid chromatography

Thus far, no other assay has been described for the determination of 5-MI in murine EDTA plasma using HPLC coupled to the tandem MS. We tested several solvents as possible eluents, such as 1 mM ammonium acetate, 10 mM ammonium acetate, and ammonium formate buffer pH 4. An alkaline mixture of an aqueous 10 mM ammonium hydroxide solution pH 10.5 and methanol appeared to be most appropriate to elute 5-MI from the column with small peak widths at base and acceptable peak symmetries. Moreover, the highest signal to noise ratio at the LLOQ was observed using 10 mM ammonium hydroxide in the eluent.

Representative chromatograms of IMO and 5-MI in the blanks and at the LLOQ levels in murine plasma are depicted in Fig. 2. Peak shapes were excellent with the asymmetry factors of 1.2 for 5-MI and IMO and the capacity factors (k')

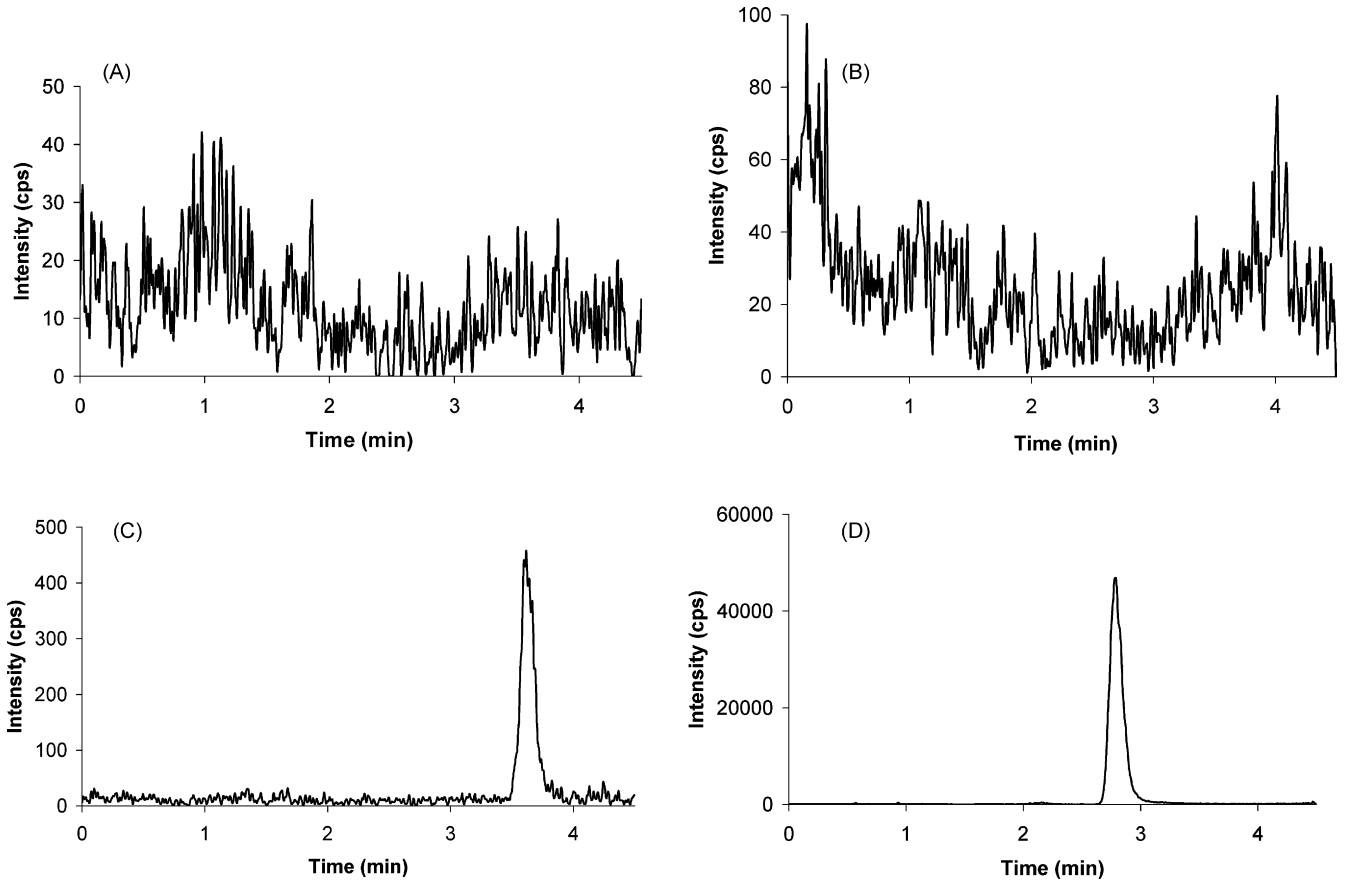

Fig. 2. Representative HPLC–MS/MS chromatogram of a blank sample for 5-MI (A) and for the internal standard IMO (B), a validation sample at the LLOQ (1 ng/mL) for 5-MI (C), and IMO (100 ng/mL; D) in murine plasma.

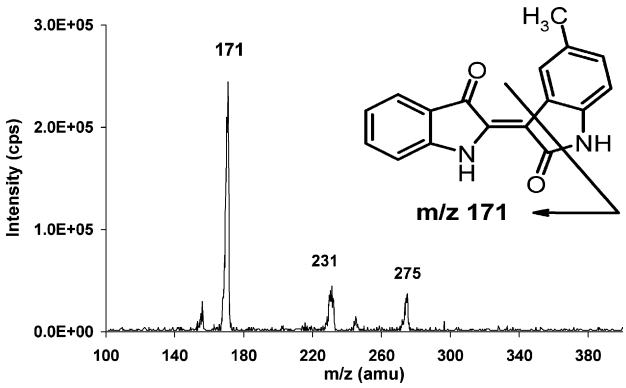

Fig. 3. MS/MS product ion scan of 5-MI (precursor ion *m/z* 275).

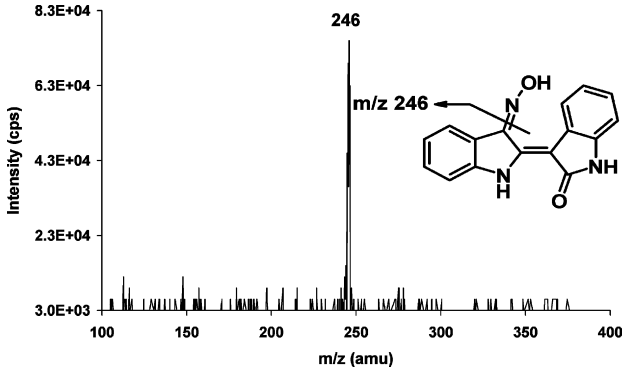

Fig. 4. MS/MS product ion scan of IMO (precursor ion *m/z* 276).

of 2.7 for 5-MI and of 2.1 for IMO. LC run time was only 4.5 min.

3.4. Mass spectrometry

No ions other than the $[M-H]^-$ ions were observed in the Q1 mass spectra of 5-MI (*m/z* 275) and IMO (*m/z* 276). In deprotonation, which is seen in our case, a proton is transferred from the analyte 5-MI and IMO to the deprotonated eluent additive (NH_3) if the proton affinity (PA) of the deprotonated eluent molecule is higher than that of the deprotonated analyte. Ammonia used as an eluent modifier is a stronger base than 5-MI and/or IMO and therefore will have higher PA, leading to the deprotonation of the analytes.

The deprotonated molecular ions of 5-MI and IMO were induced to fragment in the collision cell and the resulting product ion spectra and proposed fragmentation patterns are presented in Figs. 3 and 4, respectively. Namely, when the N atom of an amide is attached directly to an aromatic ring, which is the case in the 5-MI molecule, the fragmentation may occur which involves a four-atom cyclic intermediate. The first step in each of these eliminations is best explained by radical-site-induced migration of an H atom via a four atom intermediate. The fragment ions formed in this manner are essentially as stable as their precursor ions. They form, by an H migration that has minimal energy requirements, followed by elimination of a molecule containing a newly formed π-bond [28]. The main proposed fragment ion of 5-MI corresponds to the loss of the 1-amine-4-methylbenzene group (*m/z* 171). After optimization of the MS parameters the fragment ion was still most abundant and used for quantitative multiple reaction monitoring of 5-MI. The main fragment ion of IMO corresponds to the loss of the oxime group (*m/z* 246).

3.5. Method validation

The assay was linear over a concentration range of 1–500 ng/mL for 5-MI in murine plasma. Using linear regression and $1/x^2$ weighing, the lowest total bias and the most constant bias across the range were obtained. Correlation coefficient of the calibration curve was 0.9943. At all concentration levels, deviations of measured concentrations from nominal concentrations were between −9.0% and 4.7% with C.V. values less than 18.2%.

Assay performance (intra-assay accuracies and precisions) data for 5-MI is summarized in Table 2. The intra-assay accuracies (% bias) were within ±8.0% for all concentrations and found to be acceptable [26,27]. The intra-assay precisions for 5-MI were less than 10.8% for all concentrations and found to be acceptable [26,27]. Moreover, no carry-over takes place.

The stability data are summarized in Table 3. 5-MI is stable in murine plasma for at least three freeze (−20°C)/thaw cycles, and is also stable in murine plasma stored at nominally ambient temperatures for up to 6 h. Furthermore, the analytical run can be re-injected after at least 24 h of storage in the autosampler. The long-term stability testing of 5-MI in murine plasma is ongoing.

3.6. Pre-clinical study

The plasma concentration–time profile following a single oral administration of 150 mg/kg 5-MI is given in Fig. 5. The maximum 5-MI plasma concentration of 136 ng/mL was reached within 2 h after administration. Levels decreased continuously over time with concentrations of 35.1 ng/mL after 8 h and 10.9 ng/mL after 24 h, respectively. After 48 h, less than the lower limit of quantitation (<1 ng/mL) of 5-MI was present in the plasma (data not shown in Fig. 5). Whether the concentration at 2 h post administration represents the peak level of 5-MI

Table 2
Assay performance data

Nominal concentration (ng/mL)	Measured concentration (ng/mL)	Intra-assay accuracy (%)	Intra-assay precision (%)	Number of replicates
1.08	1.17	7.75	10.8	5
3.25	3.33	2.52	8.6	5
54.2	49.9	−7.96	9.72	5
406	418	2.84	7.32	5

Table 3
Stability data of 5-MI

Conditions	Matrix	Intitial concentration (ng/mL)	Found concentration (ng/mL)	Deviation (%)	C.V. (%)	Number of replicates
Three freeze (−20 °C)/thaw cycles	Murine plasma	3.31	3.46	4.54	9.45	3
		372	406	9.24	18.1	3
Ambient, 6 h	Murine plasma	3.31	3.35	1.31	10.5	3
		417	474	13.6	0.645	3
Autosampler, 10 °C, 24 h	Final extract (murine plasma)	3.46	3.74	8.29	0.408	3
		49.0	55.9	14.1	0.473	3
		450	421	−6.30	7.96	3

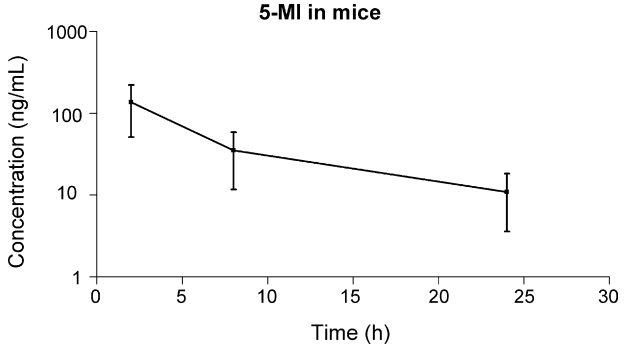

Fig. 5. 5-MI levels in the mice plasma after oral administration of 5-MI; logarithmic concentration (±SD) *vs*. time profile.

remains to be identified by analyzing further samples taken at smaller time intervals after administration.

4. Conclusions

A reproducible and selective LC–MS/MS assay has been developed for the quantification of 5-MI in murine plasma. Using 50 μL murine plasma aliquots, the assay quantifies the drug in a concentration range of 1–500 ng/mL. Validation results demonstrate that the 5-MI concentrations can be accurately quantified in murine EDTA plasma. This assay was successfully used to support a pre-clinical pharmacologic study with 5-MI.

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