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A HPLC Method for the Quantitative Determination of N-(2-hydroxy-5-nitrophenylcarbamothioyl)-3,5-dimethylbenzamide in Biological Samples

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Abstract

A sensitive and simple HPLC method was developed for the determination of a novel compound, a potential anti-cancer drug, N-(2-hydroxy-5-nitrophenylcarbamothioyl)-3,5-dimethylbenzamide (DM-PIT-1), a member of the new structural class of non-phosphoinositide small molecule antagonist of phosphatidylinositol-3,4,5-trisphosphate–pleckstrin-homology domain interactions, in mouse plasma and tumor tissue homogenates. The chromatographic separation of DM-PIT-1 was achieved on C18 column using isocratic elution with acetonitrile-water (70:30) containing 0.1% formic acid (v/v). DM-PIT-1 was detected by UV absorbance at 320 nm and confirmed by LC-MS. The extraction of the DM-PIT-1 from the plasma and tumor tissue with methylene chloride resulted in its high recovery (70–80%). HPLC calibration curves for DM-PIT-1 based on the extracts from the mouse plasma and tumor tissue samples were linear over a broad concentration range of 0.25–20 µg/ml/g, with intra/inter-day accuracy of 95% and the precision of variation below 10%. The limits of detection and quantification were 0.1 ng and 0.2 ng respectively. The described method was successfully applied to study the pharmacokinetics of the DM-PIT-1 following the parenteral injections of DM-PIT-1 entrapped in 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene-glycol)-2000] (PEG-PE) micelles.

Keywords

N-(2-hydroxy-5-nitrophenylcarbamothioyl)-3,5-dimethylbenzamide (DM-PIT-1); PEG-PE micelles; HPLC; pharmacokinetics

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1. Introduction

Earlier, a selective antagonist of phosphatidylinositol-3,4,5-trisphosphate-pleckstrin-homology domains, N-(2-hydroxy-5-nitrophenylcarbamothioyl)-3,5-dimethylbenzamide (DM-PIT-1) was described [1]. This small molecule exerts a significant anti-tumor activity *in vitro* and *in vivo*, including the inhibition of tumor growth and induction of apoptosis, establishing DM-PIT-1 as a promising therapeutic agent of new structural class of small molecule for cancer treatment. However, DM-PIT-1 has poor water solubility, which limits its potential for therapeutic applications. Loading the DM-PIT-1 into polymeric micelles made of polyethylene glycol-phosphatidylethanolamine conjugates could facilitate *in vivo* bioavailability of DM-PIT-1 [2]. This approach may also result in better drug accumulation of guest molecules in tumors because of the known uptake of polymeric micelles by tumor through the “Enhanced Permeability and Retention (EPR) effect” [3–5], which is due to the disorganized and leaky blood vasculature in tumors that promotes passive accumulation of large molecules and even small particles (20 nm micelles) in the solid tumors [6].

Still, the accumulation of DM-PIT-1-micelles at the tumor site in amounts sufficient to inhibit cancer cell survival and to induce cell apoptosis by specifically inhibiting PIP3-dependent PI3K-PDK1-Akt signaling must be controlled. The optimization of the treatment protocols using the DM-PIT-1-micelles is influenced by many factors, including the dose, dose duration, injection frequency etc. Such a complex task requires the development of a rapid and sensitive analytical method for DM-PIT-1 quantification in biological samples.

A variety of analytical methods have been developed to quantify small molecule drug candidates in the blood and to study their pharmacokinetics [7–10], with high-performance liquid chromatography (HPLC) being one of the most widely applied techniques for the determination of organic compounds with anti-cancer potency in various biological samples [11–15].

The goal of our study was to develop and validate a sensitive and rapid HPLC method for the quantitative determination of DM-PIT-1 both in micellar formulations and in biological samples. To demonstrate the applicability of the developed HPLC method, we investigated the stability of DM-PIT-1 in stock solution as well as pharmacokinetics of DM-PIT-1-loaded micelles following single-dose administration to healthy and tumor-bearing mice.

2. Materials and methods

2.1 Materials

DM-PIT-1 was purchased from ChemBridge Corporation (San Diego, CA, USA). The purity of DM-PIT-1 was >90% [1]. 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene-glycol)-2000] (PEG-PE) was purchased from Avanti Polar Lipids (Birmingham, AL, USA). Acetonitrile, hexane, isopropyl ether, trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, MO, USA), chloroform and methylene chloride were purchased from Fischer Scientific (Fair Lawn, NJ, USA). All other reagents were of analytical grade, the chemicals were used without further purification. Deionized water was used in all aqueous solutions.

2.2 Chromatographic methods

DM-PIT-1 was determined by the reverse-phase HPLC using a Hitachi, Elite La Chrome (Manufect) instrument, equipped with photodiode-array detector (L-2455) and autosampler (Model L-2200). The chromatographic separation was performed on a C18 column (5 μ m, 4.6×250 mm, Hichrom, CA). The elution was performed using the isocratic mobile phase which consisted of acetonitrile-water (70:30) containing 0.1% formic acid (v/v) delivered at

the flow rate of 1 ml/min and the total run time was 8 min. Formic acid was added to the eluents as HPLC method was developed to be compatible with LC-MS analysis with positive mode ESI. The formic acid is added to improve ionization efficiency. Sample volumes of 10 or 20 μ l were injected, DM-PIT-1 was detected by the UV absorbance at 320 nm.

The chromatographic peak of DM-PIT-1 was identified by the LC-MS analysis after the appropriate fraction was collected and analyzed on Agilent HP-LC/MCD, Trap instrument, with C18 column (EclipsedPluse 4.6 $\times$ 50 mm). The mobile phase consisted of acetonitrile (0.08% TFA) and water (0.10% TFA) at volume ratio of 70:30, respectively.

2.3 Preparation of standard solutions and calibration curves of DM-PIT-1

Standard solutions of DM-PIT-1 in acetonitrile were prepared at seven concentrations of 10, 25, 50, 100, 250, 500, and 1000 ng/ml in triplicates by adding defined volumes of DM-PIT-1 from a primary stock solution (0.5 mg/ml) prepared by dissolving 20 mg of DM-PIT-1 in 40 ml of acetonitrile. For the quality control samples (QC), dilutions with concentration of 50, 250 and 1000 ng/ml were prepared in the same way.

The limit of detection (LOD) and limit of quantification (LOQ) were determined by injecting progressively low concentrations of DM-PIT-1. The LOD was defined as the lowest concentration of an analyte that can be readily detected at signal to noise ratio 3:1. The LOQ was defined as the lowest concentration of an analyte that can be quantified with acceptable precision and accuracy. The resulting LOQ was defined as a signal/noise ratio of 10:1.

2.4 Formation and characterization of DM-PIT-1-loaded micelles

PEG-PE in chloroform (20 mg/ml) stored at -80°C (solution I), was mixed with the solution of DM-PIT-1 (0.5 mg/ml) in acetonitrile (solution II), micelles with different DM-PIT-1/PEG-PE ratio were prepared by adding various volumes of solution I to solution II.

The solvent was removed by rotary evaporation and further by overnight lyophilization to form a thin film of drug/micelle material mixture on the flask wall. To form micelles, the film was hydrated in a 10 mM HEPES buffer saline (HBS), pH 7.4, and treated by ultrasonication for 7 min. The non-entrapped DM-PIT-1 formed precipitate, which was removed by filtration through a 0.22 μ m filter (Fisher Scientific, Hampton, NH, USA).

The mean size of the resulting micelles was determined by the dynamic light scattering (DLS) with a scattering angle of 90° at 25°C using a N4 Plus Submicron Particle System (Coulter Corporation, Miami, FL, USA). Micelles suspensions were diluted with a 10 mM HBS, pH 7.4 until a concentration providing a light scattering intensity of 5×10^4 - 1×10^6 counts was achieved. Drug content (loading %) was calculated as the weight of DM-PIT-1 in micelles divided by the PEG-PE weight used for the micelles preparation. For the quantitative determination of the DM-PIT-1 content in micelles (DM-PIT-1-micelles), 10 μ l of the micelle suspension was transferred to a glass vial, and dried under high vacuum for 30 min. The resulting DM-PIT-1/PEG-PE material was dissolved in 1.0 ml of acetonitrile, centrifuged at 14,000 g for 7 min, and clear supernatant was analyzed by HPLC.

2.5 Cell culture and animal tumor model

Murine 4T1 metastatic breast cancer cells (ATCC, Manassas, VA, USA) from the frozen stock were cultured in Dulbecco's modified Eagle's medium with 10% FBS (Life Technologies, Inc., Gaithersburg, MD, USA), 100 U/ml penicillin, and 100 μ g/ml

streptomycin. The cell culture was maintained in plastic flasks and incubated in 5% CO₂–95% air at 37 °C.

The 4T1 cells (8×10⁴) in phosphate-buffered saline (PBS) were injected subcutaneously (s.c) into the right flank of BALB/c mice (Charles River Laboratories, Wilmington, MA, USA). The tumor size (volume) was measured every two days by caliper and calculated using the formula:

$$\text{Length (mm)} \times \text{Width (mm)} \times \text{Height (mm)} / 2 \times \pi / 3 = \text{mm}^3$$

When the tumor size reached approx. 1000 mm³, the DM-PIT-1-micelles were administered to the test-mice intravenously (*i.v.*) or intraperitoneally (*i.p.*) for the pharmacokinetics study. All animal procedures were performed according to a protocols approved by the Northeastern University Animal Care and Use Committee.

2.6 Biological sample preparation for HPLC determination of DM-PIT-1

The blood from three BALB/c mice was obtained by intracardiac puncture without sodium heparin or any other anticoagulant. Tumor tissues were obtained from three 4T1 tumor-bearing BALB/c mice. Each tumor was separated from surrounding normal tissues and homogenized with a Virtis blender. Plasma or tumor tissues were spiked with 20 µl of micellar DM-PIT-1 in HBS to create a DM-PIT-1 concentrations ranging from 0.25 to 20 µg/ml. The extraction of DM-PIT-1 was performed as follows: 0.18 ml of plasma or 200 mg tumor tissue homogenate was transferred into a glass test tube, 20 µl of micellar DM-PIT-1 in HBS was added followed by 1 ml of methylene chloride. Samples were shaken for 1 hr; the lower phase was then collected and transferred into a clean glass test tube. The extraction was repeated with 3 ml methylene chloride. The combined organic phase was evaporated to dryness under nitrogen at RT, the final precipitate was dissolved in 0.2 ml of acetonitrile, transferred into conical vials (Target PP Polyspring insert, 100/PK, National Scientific, Rockwood, TN, USA), and 20 µl of the solution obtained were injected for the HPLC analysis.

2.7 Stability study of DM-PIT-1 stock solutions

Stock solutions stability, freeze and thaw stability, short-term temperature stability, and stability of the DM-PIT-1 extracted from biological samples were monitored by following changes in the chromatographic profile. Briefly, the stability of stock solutions of DM-PIT-1 (in acetonitrile) was checked by keeping the DM-PIT-1 solutions at room temperature (RT) for 6 hrs and comparing the peak area (AUC) against freshly prepared stock solution. The freeze thaw stability was characterized by subjecting low, medium, and high concentrations of DM-PIT-1 to three consecutive freeze-thaw cycles: storage at –80°C for 24 hrs and then thawing at RT. The short-term stability of samples was checked by keeping the samples at RT for 6 hrs. The low, medium, and high concentrations of DM-PIT-1 extracted from plasma were checked for stability by storing the extracts for 1 month at +4°C.

2.8 Precision and accuracy

The intra-day precision and accuracy of the replicate assay (n=6) were tested by using three different concentration of DM-PIT-1, namely 50, 250 and 1000 ng/ml. Accuracy was calculated using the formula:

$$\% \text{Recovery} = (\text{found concentration}) / (\text{nominal concentration}) \times 100$$

Precision was calculated using the formula:

$$\% \text{Relative Standard Deviation } (\% \text{Coefficient of Variation, RSD}) = \text{SD} \times 100 / \text{Mean}$$

All validation parameters met the criteria set in the international guidelines for bioanalytical methods [16, 17].

2.9 Determination of the maximum tolerated dose (MTD)

Appropriate groups of healthy Athymic Nude-Foxn1^{nu} mice (Charles River Laboratories, Wilmington, MA, USA) received a single dose of DM-PIT-1-micelles or 10 mM HBS buffer saline (HBS), pH 7.4, as a control. The DM-PIT-1-micelles were administered *i.v.* or *i.p.* at a dose of 25 mg/kg of DM-PIT-1 and 1 g/kg of PEG-PE. Mouse survival and body weight were monitored in all groups. The drug dose was assumed to be below the MTD if it induces no more than 15% weight loss *vs.* control, causes no toxic death, and is not associated with significant changes in vital signs within two weeks after administration.

2.10 Determination of the DM-PIT-1 in biological samples

A group of eighteen female BALB/c mice received a single *i.v.* injection of 12.5 mg/kg DM-PIT-1-micelles and a second set of eighteen mice received a single *i.p.* dose of an equal amount of DM-PIT-1-micelles. Mice were euthanized using carbon dioxide (CO₂) prior to cervical dislocation and blood samples were collected by an intracardiac puncture into Eppendorf-style microfuge tubes at 5, 15, 30, 60, 180, 360 min; with at least three repetitions for each time point. The plasma samples were separated by centrifugation at 1000×g for 10 min, following by immediate extraction.

For the determination of DM-PIT-1 in tumor, three female BALB/c mice with 4T1 metastatic breast cancer received a single *i.v.* or *i.p.* dose of DM-PIT-1-micelles at a target dose of 12.5 mg/kg. Mice were euthanized using carbon dioxide (CO₂) prior to cervical dislocation after 1 hr and the tumor tissues were harvested, weighed, homogenized, and extracted.

Experimental data were expressed as mean values and standard deviation (mean±SD). Differences in the mean change in DM-PIT-1 concentration between groups were analyzed with a paired Student's *t*-test. Significance was set at *P* < 0.05.

2.11 Pharmacokinetic analysis

All pharmacokinetic parameters were determined by non-compartmental analysis [18]. The peak plasma level (C_{max}) was obtained directly from the concentration-*vs.*-time data. The elimination rate constant (K_e) was calculated from the slope of the logarithm of the plasma concentration versus time using the final 4 points. The apparent elimination half life (t_{1/2}) was calculated as 0.693/K_e. The area under the plasma concentration-*vs.*-time curve (AUC) was calculated by the trapezoidal rule. The volume of distribution (V_d) was calculated as Dose/C₀ (initial concentration). The total body clearance (CL_t) was calculated as Dose/AUC.

3. Results and discussion

3.1 Detection, quantification and stability of DM-PIT-1

The HPLC method developed for the determination of DM-PIT-1 (Fig.1) based on the reverse phase chromatography with isocratic elution and UV detection results in a single sharp peak (Fig. 2) at the retention time of 6.3 ± 0.02 min. Calibration curve was constructed

by plotting peak height against known concentration of DM-PIT-1. Slope, intercept, and correlation coefficient (R) calculated from linear regression analysis. The calibration curve, as determined with standard solutions of DM-PIT-1 in acetonitrile was linear in the range of 10–1000 ng/ml ($R^2=0.999$), resulting in the regression equation, $Y=98.913x-11.201$ where Y is the peak area (arbitrary unit) and X is the concentration (ng/ml). Data were obtained at seven different concentrations, each repeated in triplicate.

The limit of detection was 5 ng/ml (0.05 ng on column) at a signal-to-noise ratio of 3:1, and the limit of quantification was 10 ng/ml (0.1 ng on column), with a RSD value of 12.0% (n=5). This method showed good accuracy ranging from 92.0 to 103.1% ($99.6\pm4.37\%$). The repeatability of injections was evaluated by performing seven replicate injections of “in-house QCs” (see “Materials and Methods”).

The stability of the stock solutions of DM-PIT-1 was studied (Tab. 1), at storage conditions [-80°C for 6 hrs (A) or 24 hrs (B)] and at working conditions (RT for 6 hrs) as described in “Materials and Methods”. DM-PIT-1 solutions in acetonitrile were stable at -80°C and at RT with average percentage of accuracy from 97.5 ± 5.0 to 101.6 ± 4.7 for low, medium, and high concentrations QCs (see Tab.1).

3.2 Optimization of DM-PIT loading into PEG-PE micelles

To determine the optimal drug loading conditions, various micellar formulations were prepared using different DM-PIT-1/PEG-PE ratios. As expected, the amount of DM-PIT-1 (mg/ml) incorporated into PEG-PE micelles is a function of the amount of PEG-PE used, with maximal micellar concentration of the DM-PIT-1 reaching 1.892 ± 0.204 mg/ml at 100 mg/ml of PEG-PE using 3 mg/ml of DM-PIT-1 stock solution. Higher PEG-PE concentrations were not considered because of *in vivo* safety reasons. Based on our considerations, for the subsequent *in vivo* experiments, 70 mg/ml PEG-PE and 2 mg/ml DM-PIT-1 solutions were chosen to reach optimal loading efficiency of DM-PIT-1 into polymeric micelles (1.312 ± 0.147 mg/ml).

Size distribution of DM-PIT-1-loaded micelles in water and their physical properties were studied at five independent experiments. The effective diameter of DM-PIT-1-micelles was approx. 15 nm (14.5 ± 0.11) with a narrow size distribution and polydispersity index of approx. 0.05 (0.048 ± 0.03). The polymeric micelles demonstrated slightly negative surface zeta potential of 3.9 mV. The drug content in the micelles was approx. 2.0 % (wt).

3.3 Determination of DM-PIT-1 in biological samples

The extraction of DM-PIT-1 from the blood and tumor tissue based on water immiscible solvents was highly efficient. We examined three solvents with different polarities: methylene chloride, ethyl acetate, and isopropyl ether. The extraction efficiencies were determined by comparing the chromatographic peak areas of the extracted samples with areas obtained for stock solution of the drug in acetonitrile assuming 100% extraction. Methylene chloride was the preferred extraction solvent as it results in less interfering background peaks with extraction efficiency from the blood and tumor of 77.19 ± 1.59 and $67.11\pm4.72\%$, respectively. For the quantitative determination of DM-PIT-1 in biological samples, standard curves were prepared by spiking DM-PIT-1-micelles into the mouse plasma or tumor tissue homogenates to form the specified DM-PIT-1 concentrations. The standard samples were extracted and treated as specified in “Materials and Methods”. The resulting calibration curves for plasma extracts over the range of 1.0 to 20.0 $\mu\text{g/ml}$ and for tumor tissue extracts over the range of 0.25 to 10.0 $\mu\text{g/g}$ have revealed high linear response regression equation $Y=24332x-20199$ with $R^2=0.9963$ and $Y=31904x-3594.3$ with $R^2=0.9971$, respectively (Y is the peak area (arbitrary units) and X is the concentration in

µg)). The accuracy deviation was below 15% for all the data points, which is within the internationally recognized acceptance criteria for assay validations [16–17]. Data were obtained at five different concentrations, each repeated in triplicate.

The chromatographic DM-PIT-1 peak was identified by MS after collecting the corresponding peak at 6.29 min and analyzing by LC-MS as specified in “Materials and Methods”. As depicted in Fig. 3B, a peak with MW of 352.3 (calculated MW 351.76), which corresponds to DM-PIT-1, was identified. The additional peaks in the chromatogram result from the plasma as can be seen with the blank plasma extract (Fig. 3A).

PEG-PE micelles or blank plasma samples did not show any peaks which might interfere with the determination of DM-PIT-1 (Fig. 4A and B). The peak at 6.30 min in the spiked plasma (Fig. 4C) represents DM-PIT-1 as confirmed by MS (Fig. 3B). Figure 4D represents a chromatogram of plasma extract obtained 15 min after the *i.v.* injection of DM-PIT-1-micelles into the mouse, which shows the same chromatogram as the one (Fig. 4C) generated with sample of DM-PIT-1-micelles spiked into blank plasma. Finally, figure 4E and F represent chromatograms of extract blank tumor and extract of tumor obtained 15 min after the *i.v.* administration of DM-PIT-1-micelles to the mice respectively. These results approve the validation of our DM-PIT-1 analysis method for the quantitative determination of DM-PIT-1 in biological samples following parenteral injections.

3.4 Non-toxic dose level and pharmacokinetics of the DM-PIT-1-micelles

The primary goal here was to determine a non-toxic dose level of the DM-PIT-1-micelles. The DM-PIT-1-micelles were administered *i.v.* or *i.p.* at the dose of 25 mg/kg of DM-PIT-1 and 1 g/kg of PEG-PE (maximum achievable dose for the DM-PIT-1-micelles preparation). Mouse survival and body weight were monitored in all groups. No death, weight loss greater than 15% or other signs of toxicity were seen within the time period of maximum achievable dose administration, which was determined as MTD (data not shown). The dose chosen for the pharmacokinetic study was 12.5 mg/kg, which is the ½ MTD. The analysis method we developed was used to study the pharmacokinetics of DM-PIT-1-micelles in healthy and tumorbearing mice. Plasma concentration versus time was determined following an *i.v.* or *i.p.* administration of non-toxic dose of DM-PIT-1-micelles into BALB/c mice. The maximal DMPIT-1 concentration was measured at 5 min, following the parenteral administrations. Blood levels of both routes of administration of DM-PIT-1-micelles were similar (mean values of 79.7 and 114.9 µg/ml, respectively). The DM-PIT-1 concentration in the blood after the *i.v.* administration decreased consistently from the initial high concentration to less than 0.1 µg/ml after 6 hrs. Similar kinetics was found following the *i.p.* administration. The clearance of the DM-PIT-1-micelles following both *i.v.* and *i.p.* administration was relatively fast, no DM-PIT-1 could be detected by the HPLC after seven hours. The pharmacokinetic parameters of DM-PIT-1-micelles after single *i.v.* and *i.p.* administrations in healthy BALB/c mice are presented in Table 2.

The DM-PIT-1 concentration in the tumor after *i.v.* and *i.p.* administration of DM-PIT-1-micelles in tumor-bearing mice was studied, and compared to DM-PIT-1 concentrations in the blood after same routes of administration, Figure 5. In the present study, tumor concentrations of DM-PIT-1 were measured after tumors reached a size of about 1000 mm³. DM-PIT-1 levels in the blood and tumor after both routes administrations were found to be similar. Interestingly, that statistical analysis of DM-PIT-1 concentrations in tumor after *i.v.* and *i.p.* administration also did not show significant differences (0.51 ± 0.15 vs. 0.29 ± 0.03), suggesting that both routes of administration are appropriate in the used model. The two-tailed P value equals 0.067. Finally, at 6 hrs following *i.p.* and *i.v.* administration, the level of DM-PIT-1 in tumor tissues remained above 0.2 µg/ml, which is in contrast to the decrease of DM-PIT-1 in the blood to the levels below 0.1 µg/ml. This can suggest a slow turnover of

DM-PIT-1-micelles in the tumor tissue, which may lead to its accumulation upon repeated dosing.

4. Conclusions

In this study, a rapid and reliable HPLC assay was established and validated for the determination of DM-PIT-1 in biological samples. The sensitivity, specificity, precision and accuracy of this method were acceptable for the pharmacokinetic study of the DM-PIT-1-micelles in healthy and tumor-bearing mice.

Despite relatively fast clearance of the DM-PIT-1-micelles from the systemic circulation, the level of DM-PI-1 in tumor tissue remained above 0.2 µg/g over 6 hrs. This may suggest that repeated dosing of the DM-PIT-1-micelles may promote its accumulation in the tumor while maintaining low circulating concentrations and, thus, maximizing therapeutic index of this novel small molecule drug candidate with anti-cancer activity.

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Fig. 1.
Chemical structure of N-(2-hydroxy-5-nitrophenylcarbamothioyl)-3,5-dimethylbenzamide (DM-PIT-1); MW 351.76.

Fig. 2.
Representative chromatogram of the standard solution of DM-PIT-1 (250 ng/ml) in acetonitrile.

Fig. 3. Mass spectra of blank plasma extract (A), and plasma spiked with DM-PIT-1-micelles (B), where X axis represents the mass to charge ratio (m/z) and Y axis represents the signal intensity (cps, counts of ions per second detected).

Fig. 4.

Chromatograms of: (A) blank PEG-PE micelles in HEPES; (B) blank plasma; (C) spiked plasma with DM-PIT-1 (100 ng/ml); (D) plasma extract obtained 15 min after *i.v.* injection of DM-PIT-1-micelles to the mouse (E); blank tumor extract (F); and tumor extract obtained 15 min after the *i.v.* injection of the DM-PIT-1-micelles to the mouse.

Fig. 5.

Quantitative determination of the DM-PIT-1 (DM-PIT-Mic) concentration in plasma (µg/ml) and in tumor (µg/g). Appropriate groups of healthy and tumor-bearing BALB/c mice received a single *i.v.* or *i.p.* dose of the DM-PIT-1-micelles at a target dose of 12.5 mg/kg. Blood and tumor tissue samples were collected at 60 min and analyzed by the HPLC (n=3).

Table 1

The stability of stock solutions of DM-PIT-1 in acetonitrile expressed as percentage accuracy

Sample	DM-PIT-1concentration (ng/ml)	Freeze - thaw stability (n=6)*		
		Temperature		Short-term stability ~ RT °C (n=4)
		− 80 °C (A)	− 80 °C (B)	
Low Conc. QC	50	99.2 ± 5.3	99.8 ± 1.3	101.6 ± 4.7
Medium Conc. QC	250	100.0 ± 2.9	97.5 ± 2.6	99.7 ± 1.9
High Conc. QC	1000	100.6 ± 1.6	99.7 ± 5.0	97.5 ± 1.9

* Two repetitive freezing-thawing cycles. The freeze-thaw stability was characterized by subjecting low, medium, and high concentrations of DM-PIT-1 to three (3) consecutive freeze-thaw cycles (see "Materials and Methods"). Data represents the mean value ± SD from four to six independent experiments.

Pharmacokinetic parameters of DM-PIT-1-micelles after single *i.v.* and *i.p.* administrations in healthy BALB/c mice

Table 2

Route	Dose (mg kg ⁻¹)	K _e (hr ⁻¹)	t _{1/2} (hrs)	AUC _{0-t_{last}} (µg h ml ⁻¹)	V _d (ml)	CL _T (ml h hr ⁻¹)
<i>i.v.</i>	12.5	1.27	0.55	17.83	4.52	20.19
<i>i.p.</i>	12.5	1.29	0.54	21.55	3.13	16.71

i.v. – intravenous administration; *i.p.* – intraperitoneal administration; K_e – elimination rate constant; t_{1/2} – half-life at the terminal phase; AUC – area under the curve; CL_T – total body clearance; V_d – volume of distribution. Parameters were calculated from the mean value of three mice by non-compartmental analysis.