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Determination of anti-tumor agent bis(*p*-fluorobenzyl)trisulfide and its degraded compound in rat blood using reversed phase high-performance liquid chromatography

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ABSTRACT

Bis(*p*-fluorobenzyl)trisulfide (BFTS) demonstrated a broad spectrum of anti-proliferative activity and *in vivo* anti-tumor efficacy in human xenograft mice models. BFTS is rapidly degraded to its major metabolite bis(*p*-fluorobenzyl)disulfide (BFDS) in blood. In this study, we developed a reliable procedure for stable storage and treatment of blood samples containing BFTS. An HPLC assay method was developed and validated for the quantitative determination of BFTS and BFDS in rat blood. The developed procedure and method have been successfully utilized to study the concentration–time curves of BFTS and BFDS in the blood of rats after vein injection of 12.5 mg kg^{-1} BFTS.

1. Introduction

Trisulfides are a series of special compounds found in garlic (*Allium sativum* L.), onion (*Allium cepa* L.), and *Petiveria alliacea* L. [1,2]. The well-known diallyl trisulfide, one of the main active components of garlic and onion, showed antibacterial, antithrombotic, and anticancer activities [3]. Dibenzyl trisulfide (DTS), isolated from *P. alliacea* L., exhibited various pharmaceutical effects including anti-proliferative activity [4–6]. Recently, by systematically optimizing this natural product lead compound, a series of new bis-aromatic and heterocyclic trisulfide derivatives were designed and synthesized [6]. Their anti-tumor activities were thoroughly evaluated against a variety of tumor cell lines using real-time cell electronic sensing (RT-CES) cell-based assay [6]. The *in vitro* screening results indicated that the bis(*p*-fluorobenzyl)trisulfide (BFTS) (Fig. 1) exhibited the most potent and broad spectrum of anti-tumor activity with low cytotoxicity against HepG2 [6]. The advanced lead BFTS also demonstrated potent *in vivo* anti-tumor activity in human xenograft mice models with good safety profile. The therapeutic potential of BFTS encouraged its further development to an investigational new drug.

Pharmacokinetic and pharmacodynamic studies are critical parts in the new drug development process, while development of an assay to determine the drug in biological samples, such as plasma, blood, tissue, or urine, is a primary and important step. An isocratic HPLC method for the chemical analysis of BFTS has been developed for the quality control (QC) purpose in the drug development process [7]. Very few studies have been reported about the analysis of trisulfides and related organic sulfur compounds in biological samples. Lachmann et al. [8] investigated the pharmacokinetics of alliin, allicin and vinyldithiine in garlic institutes using ^{35}S-labeled compounds; however, the method could not distinguish the analytes and their metabolites. Moreover, it is very challenging to obtain the ^{35}S-labeled trisulfide. Sun et al. [9] developed a gas chromatographic method with electron capture detector to analyze diallyl trisulfide and applied the method to study the pharmacokinetics. However, this method is not suitable for the analysis of BFTS because of its low volatility. On the other hand, the existence of phenyl groups in the BFTS molecule would allow for UV detection which would improve the sensitivity during bioanalysis of this anti-tumor agent. During the preliminary studies of BFTS, we have also found that BFTS degraded to its corresponding bis(*p*-fluorobenzyl)disulfide (BFDS) in blood of rats or dogs after iv administration of BFTS. Therefore, it is also necessary to analyze the concentration of disulfide BFDS for extensive pharmacokinetic studies. Similar to diallyl trisulfide, BFTS was extremely unstable in blood or plasma under normal conditions. Sun et al. [9] acidi-

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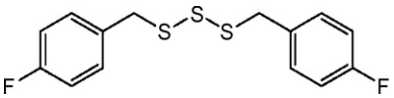

Fig. 1. The chemical structure of bis(*p*-fluorobenzyl)trisulfide (BFTS).

fied the blood sample to improve the stability of diallyl trisulfide in blood, but the effect was very limited. Therefore, it is essential to develop an analytical method to quantify this anti-tumor agent and its metabolite BFDS in various biological samples to facilitate the pharmacokinetic and pharmacodynamic studies, and further accelerate the development process of this potential anticancer drug.

Herein, we describe a reliable procedure for the storage and treatment of blood samples obtained from rats administrated with bis(*p*-fluorobenzyl)trisulfide (BFTS). The procedure was used for further development of an HPLC–DAD method to quantitatively analyze BFTS as well as its major metabolite disulfide BFDS. The developed method was further validated and applied to the determination of concentration–time curves of BFTS and BFDS in biological samples obtained from rats after vein injection of BFTS at a dosage of 12.5 mg kg^{-1}.

2. Experiment

2.1. Equipment

Analyses were performed using high-performance liquid chromatographic system HP1100 (Agilent Technologies, USA) equipped with an on-line degasser, an auto-sampler, a diode array detector (DAD), and a thermostated column compartment. Blood samples were lysed using a KS-150 supersonic cell disintegrator (Ningbo Kesheng Inc., China).

2.2. Materials and reagents

The reference material bis(*p*-fluorobenzyl)trisulfide (BFTS) with a purity of >99.9% and its formulated injection samples were provided by ACEA Biosciences, Inc. The new reference material of the metabolite bis(*p*-fluorobenzyl)disulfide (BFDS) with a purity of >99.9% was also synthesized at ACEA Biosciences, Inc. The internal standard (IS) dibenzyl disulfide (DDS) (purity 99.7%) was obtained from Sigma–Aldrich Company. Acetonitrile, *n*-hexane and isopropyl alcohol (HPLC grade) were purchased from Merck Co. Ltd. Other chemicals were obtained from standard sources with the highest possible purity.

2.3. Animals

SD rats weighing 200–250 g were obtained from Experimental Animal Center of Zhejiang Academy of Medical Sciences. They were housed in cages at a temperature between 20 and 23 °C with free access to food and water. All animals were handled in accordance with the principles and guide for the care and use of laboratory animals of National Institutes of Health. Animals were fasted for 12 h before the experimentation and for 2 h after drug administration.

2.4. Chromatographic conditions

The HPLC analysis was performed using an Agilent SB C_{18} column (250 mm × 4.6 mm, 5 μm) with an SB C_{18} guard column (15 mm × 4.6 mm, 5 μm) at 30 ± 1 °C. The column was isocratically eluted with a mobile phase containing acetonitrile and water (65:35, v/v) and a flow rate of 1.0 mL min^{-1}. The DAD detector was set at a wavelength of 220 nm for eluent detection.

2.5. Stock and working standard solutions

Stock standard solutions of BFTS (1.396 mg mL^{-1}), its metabolite BFDS (1.220 mg mL^{-1}), and the internal standard DDS (1.150 mg mL^{-1}) were prepared by dissolving appropriate amounts of these reference substances in acetonitrile, respectively. The stock solutions were stable for at least 4 months at −20 °C in dark.

A series of working standard solutions of BFTS and disulfide BFDS were prepared by subsequent dilutions of the above stock standard solutions with acetonitrile to reach a concentration range of 0.05236–21.81 μg mL^{-1} for BFTS and 0.06100–40.67 μg mL^{-1} for disulfide BFDS. The working standard solution of the internal standard at the concentration of 11.5 μg mL^{-1} in acetonitrile was also prepared. The working standard solutions obtained as such were stored at −20 °C in dark and freshly re-prepared every 2 weeks.

2.6. Sample collection and preparation

Sprague-Dawley rats, fasted overnight with free access to water, were intravenously administrated with BFTS at the dosage of 12.5 mg kg^{-1}. Blood samples were collected from tail vein into a tube containing heparin pre-dose (0 min) and then at 5, 10, 20, 30, 45, 60, 90, 120, 180, 300, 420 min post-dose. Blood samples (0.20 mL each) were transferred to tubes and frozen in liquid nitrogen immediately. The frozen samples were stored at −80 °C until analysis. The quality control samples for sensitivity test, calibration curve, accuracy, and precision tests were prepared as follows: the standard working solutions of BFTS and disulfide BFDS (100 μL each) at the desired concentration were added to a new Eppendorf tube. The solvent was evaporated under vacuum. 0.20 mL of drug-free rat blood was then added to the tube, and mixed well gently. Thus prepared QC samples were frozen in liquid nitrogen immediately, and then stored at −80 °C until analysis.

The sample treatment procedure is briefly described as follows: 20 μL of internal standard working solution (11.5 μg mL^{-1}) and 5.0 mL of extraction solvent (*n*-hexane:isopropyl alcohol = 95:5, v/v) were added into a frozen sample successively, which was then sonicated for 15 s by a supersonic cell disintegrator in order to release the drug from blood cells. The liquid–liquid extraction was subsequently carried out by vortexing the mixture for 5 min. After the mixture was centrifuged at a speed of 4000 × *g* for 10 min, 4.0 mL of supernatant was carefully transferred into another clean tube. The solvent was evaporated to dryness in the vacuum desiccator at room temperature. The resulting residue was reconstituted with 150 μL of mobile phase. The mixture was centrifuged for 10 min at 15,500 × *g*, and 100 μL of the supernatant was injected into the HPLC apparatus for analysis.

2.7. Method validation

The method was validated according to the "Guidance for preclinical pharmacokinetic study of chemical drug" recommended by the State Food and Drug Administration (SFDA) of China.

2.7.1. Selectivity

Drug-free blood samples from at least six individual rats (gender matched), QC samples and blood samples obtained from rat after an iv administration of BFTS were processed and assayed as described in Section 2.6.

Table 1
Extract recovery of BFTS and BFDS by different cell lysis methods ($n = 3$)

Methods	Recovery (%)	
	BFTS	BFDS
Lysed by acetonitile	<60	NT[a]
Lysed by water (water:blood = 5:1, v/v)	68.7	NT[a]
Lysed by extraction solvent and sonication	104.8	103.6

[a] Not tested.

2.7.2. Linearity, range and sensitivity

The calibration curves were generated from eight concentration levels of 0.02618, 0.05236, 0.1571, 1.452, 5.235, 6.980, 8.727, 10.905 μg mL^{-1} for BFTS and eight concentration levels of 0.03050, 0.06100, 0.1830, 2.440, 9.760, 13.02, 16.27, 20.34 μg mL^{-1} for standard metabolite BFDS as well as their corresponding peak areas. These QC samples were processed and analyzed as described in Section 2.6. The linearity of the method was estimated by regression analysis based on the ratio (Y) of peak areas of BFTS or disulfide BFDS to that of the IS versus the spiked concentration (C, μg mL^{-1}) in QC samples.

2.7.3. Accuracy and precision

The QC samples with BFTS and disulfide BFDS at low, medium, and high concentrations (0.05235, 5.235 and 8.725 μg mL^{-1} for BFTS; 0.06100, 9.760 and 16.63 μg mL^{-1} for BFDS) were used for accuracy and precision studies. Five replicates for each concentration were processed and analyzed as described above for accuracy study. The method recovery and extract recovery were calculated. The intra-die and inter-die precisions (relative standard deviations, R.S.D.) were evaluated by analyzing homogeneous samples in five replicates, in 1 or successive 5 days.

2.7.4. Stability

In order to establish a reliable method for the quantitative analyses of the drug BFTS and its metabolite BFDS in blood, we performed the stability studies utilizing different procedures. All stability tests were performed with QC samples at low and high concentrations (0.1571 and 8.725 μg mL^{-1} for BFTS; 0.1830 and 16.63 μg mL^{-1} for BFDS) in triplicates for each concentration. The QC samples, without frozen in liquid nitrogen, were analyzed after they were stored at room temperature for 2 and 24 h to assess their stability at room temperature. To verify the essentiality of the frozen step in liquid nitrogen, the stability of the prepared QC samples stored at −80 °C directly or frozen with liquid nitrogen followed by storing at −80 °C was compared. The stability of liquid nitrogen frozen QC samples stored at −80 °C for 7, 15, and 30 days was also studied. After the extracted samples were dried and reconstituted in 150 μL of mobile phase, they were analyzed by HPLC at 0, 6, and 24 h to test their stability during the storage in the auto-sampler at room temperature.

3. Results

3.1. Optimization of the cell lysis method

Three cell lysis methods were studied to ensure the high recovery (Table 1). The cells were first lysed by organic solvent. One volume of acetonitrile was added into one volume of the QC sample containing 1.117 μg mL^{-1} of BFTS. The resulting mixture was vortexed for 5 min, followed by centrifugation at 15,500 × g for 10 min. The supernatant was injected into the HPLC apparatus directly. The result revealed that the recovery of BFTS was less than 60%. For the second lysis method, 5-fold volume of water was added into one volume of blood, and then the liquid–liquid extraction was performed as described in Section 2.6. The recovery of 68.7% by this method was still not satisfactory though improved in some extent. Almost quantitative recovery was ultimately obtained by lysing the cells with extraction solvent, followed by sonication. Therefore, the sonicating lysis method utilizing extraction solvent was applied for further studies during the method development.

3.2. Method validation

3.2.1. Selectivity

Fig. 2 shows the representative chromatograms of blank sample (A), blank blood sample spiked with 0.5585 μg mL^{-1} of BFTS, 1.220 μg mL^{-1} of disulfide BFDS, and internal standard (IS) DDS (B) as well as blood sample obtained after iv administration of BFTS (C). BFTS, disulfide BFDS, and IS were all well separated under the current chromatographic condition. The retention times were approximately 17.7, 18.9, and 26.3 min for disulfide BFDS, IS, and BFTS, respectively. No interference peaks of endogenous constituents from blood and reagents were observed at the retention time range of these compounds. Therefore, the current method was proven to be selective and specific for the analyses of BFTS and BFDS at the same time using DDS as an internal standard.

3.2.2. Linearity, range and sensitivity

Three calibration curves of BFTS and metabolite BFDS in blood were performed in method validation. The results indicated that the curves were linear in the range of 0.02618–10.90 μg mL^{-1} and 0.03050–20.34 μg mL^{-1} for BFTS and BFDS, respectively. The correlation coefficients (R^2) for the regression equations of BFTS and BFDS were 0.996–0.999 and 0.992–1.000, respectively. The limits of quantifications (LOQs) for BFTS and BFDS were found to be 0.02618 (R.S.D. = 5.4%, $n = 5$) and 0.03050 μg mL^{-1} (R.S.D. = 13.6%, $n = 5$), respectively. The limits of detection (LODs, S/N = 3) for BFTS and BFDS were 0.01169 and 0.01031 μg mL^{-1}, respectively.

3.2.3. Accuracy and precision

The accuracy and precision data are presented in Table 2. The mean extract recoveries for BFTS and BFDS were found to be in the ranges of 94.2–104.8% and 90.3–101.8% with the relative standard deviations of 1.7–3.6% and 0.95–3.7%, respectively. The mean method recoveries of BFTS and BFDS were in the ranges of 101.5–105.5% and 95.2–102.6% with the relative standard deviations of 0.9–3.1% and 0.8–3.6%, respectively. The extract recoveries of IS were in the range of 97.0%–102.8% (Detail data not shown). Therefore, the current method demonstrated the satisfactory accuracy for the analyses of BFTS and its metabolite BFDS.

The R.S.D. values of intra-die precision were less than 3.7%, while the R.S.D. values of inter-die precision were less than 9.8%. Therefore, the current method demonstrated good repeatability for the quantitative analysis of BFTS and BFDS in rat blood samples.

3.2.4. Stability

The stability results of blood samples stored at room temperature indicated that both of these compounds degraded extensively even after 2 h (Table 3). The stability result in Table 4 revealed that 82.5–105.0% and 91.1–111.3% of added content of BFTS and disulfide BFDS were recovered after the liquid nitrogen frozen samples were stored at −80 °C for 30 days. However, only 0–7.3% and 75.6–77.4% of BFTS and disulfide BFDS were recovered when the samples were stored at −80 °C for 8 h without being pre-frozen in liquid nitrogen. These results indicated that it is extremely critical to freeze the blood samples by liquid nitrogen before storing at −80 °C. Additionally, the treated samples were stable within 24 h at room temperature after being reconstituted in mobile phase (Table 5).

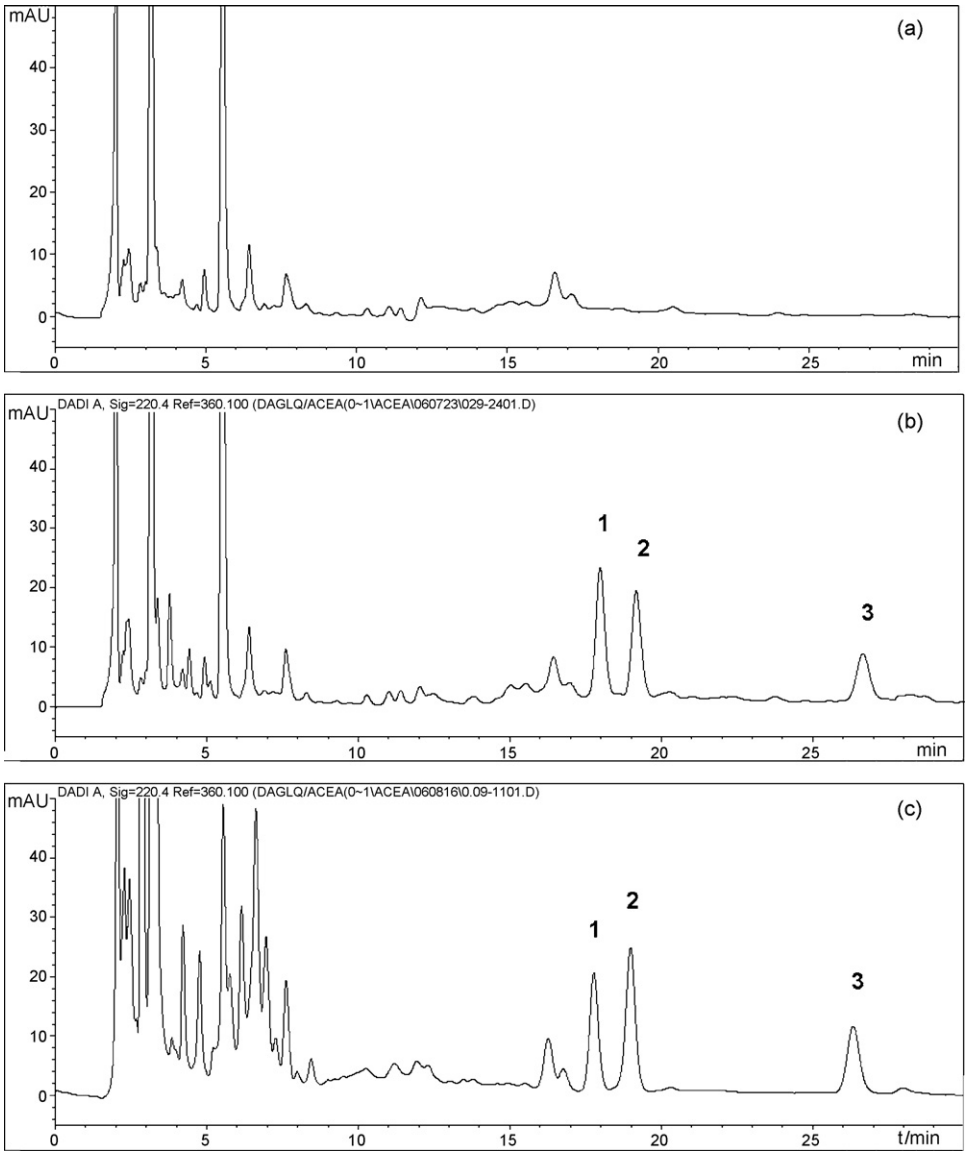

Fig. 2. HPLC chromatograms of blank sample (a), QC sample spiked with bis(*p*-fluorobenzyl)trisulfide (BFTS) (0.5585 μg mL^{-1}) and the metabolite bis(*p*-fluorobenzyl)disulfide (BFDS) (1.220 μg mL^{-1}) (b), and blood sample after iv administration of BFTS (12.5 mg kg^{-1}) for 10 min (c). Peak 1: disulfide BFDS (1.007 μg mL^{-1}), 17.7 min; peak 2: IS, 18.9 min; peak 3: BFTS (0.6702 μg mL^{-1}), 26.3 min.

This newly developed procedure was used for the treatment and handling of all samples for further assay development.

3.3. Application of assay

Sprague-Dawley rats (n = 6, gender and weight matched, body weight: 200–220 g), fasted overnight with free access to water, were intravenously injected with BFTS at the dosage of 12.5 mg kg^{-1}. Blood samples were collected as described above in Section 2.6. BFTS and metabolite BFDS were analyzed by the developed method. During the routine analysis, one set of calibration standards and three sets (low, medium and high concentrations) of QC samples were assayed along with the testing samples. The concentrations of testing samples and QC samples were calculated according to the

Table 2
Accuracy and precision of the method (n = 5)

Compound	Final concentration in blood (μg mL^{-1})	Extract recovery		Method recovery		Precision (R.S.D., %)	
		Mean (%)	R.S.D. (%)	Mean (%)	R.S.D. (%)	Intra-day	Inter-day
BFTS	0.05235	96.1	3.6	105.5	3.1	3.1	2.0
	5.235	94.2	1.7	101.5	0.9	0.9	0.75
	8.725	104.8	2.2	103.6	1.1	1.2	0.40
BFDS	0.06100	96.4	3.7	95.2	3.6	3.7	9.8
	9.760	90.3	2.4	99.7	0.8	0.8	1.9
	16.27	101.8	0.95	102.6	2.4	2.5	2.9

Table 3
Stability results of BFTS and BFDS stored in rat blood at room temperature for 2 and 24 h ($n = 3$)

Compound	Concentration spiked ($\mu g\,mL^{-1}$)	Recovery (%)	
		2 h	24 h
BFTS	8.725	1.78	ND[b]
	0.1571	NT[a]	ND[b]
BFDS	16.625	11.54	ND[b]
	0.1830	NT[a]	ND[b]

[a] Not tested.
[b] Not detected.

Table 5
Stability results of treated samples during the storage in the auto-sampler

Compound	Concentration spiked ($\mu g\,mL^{-1}$)	Recovery (% of 0 h)		
		0 h	6 h	24 h
BFTS	8.725	100.0	100.3	100.1
	0.1571	100.0	92.0	108.1
BFDS	16.625	100.0	100.2	99.7
	0.1830	100.0	94.7	92.5

standard curve. The assay result of QC samples meets the requirements of the "Guidance of preclinical pharmacokinetic study for chemical drug" of SFDA of China (Data not shown). The mean concentration–time curves of BFTS and BFDS were shown in Fig. 3. The top concentration ($0.9578 \pm 0.098\ \mu g/mL$) of metabolite BFDS was obtained at the first collection point, which indicated that BFTS converted to BFDS very quickly. This result indicated hepatic microsome metabolism was not the main pathway for the elimination of BFTS. The concentrations of BFTS and BFDS in blood decreased rapidly, suggesting that they were metabolized and/or distributed to tissues quickly.

4. Discussion

Plasma samples are more frequently utilized in pharmacokinetic studies instead of the whole blood samples [10–12] because the concentration of drug in plasma is generally similar to the concentration of drug in whole blood, and plasma is cleaner with less endogenous impurities, which would minimize the interference from such impurities. However, the non-polar compound BFTS has high permeability to cell membrane, and it gets into the cells easily based on our preliminary experiment. Moreover, both BFTS and its metabolite BFDS are unstable in blood without special treatment. Our preliminary studies indicated that it is extremely challenging to separate the plasma from blood and also recover all the compounds out of blood cells to ensure the highly reliable quantitative determination of these compounds within the required time frame during pharmacokinetic studies. Therefore, in the present study, the whole blood was directly used for the pharmacokinetic studies of the anti-tumor agent BFTS.

As described above, anti-tumor agent BFTS is not stable in blood or plasma at room temperature. The same phenomenon was discovered for diallyl trisulfide. Sun et al. [9] tried to stabilize the diallyl trisulfide in blood by acidifying the blood samples, however, diallyl trisulfide still degraded more than 87% when the sample was stored at −20 °C for 5 days or after one freeze–thaw cycle. It is assumed that some enzymes or reducing agents caused the degradation of trisulfide and disulfide derivatives. In order to avoid the undesirable degradation of the tested compounds BFTS and BFDS in blood, we froze the blood samples by liquid nitrogen immediately after they were collected. The frozen blood samples were then stored at −80 °C. We were then able to successfully keep the compounds in blood samples stable for more than 30 days when stored at −80 °C.

Organic solvents, such as acetonitrile, or water are generally used to disintegrate the blood cells to extract the compounds out of blood for further pharmacokinetic studies [13]. In most cases, the frozen sample is thawed before analysis. However, because of the low stability of BFTS and its metabolite BFDS, they would be degraded in blood and in thaw and extraction procedure. Therefore, in the present study, the extract solvent was directly added to the un-thawed sample to avoid the possible effect of enzymes or reducing agent on the tested compounds. As such, BFTS and BFDS were transferred into organic phase with the minimum influence from reducing agent or enzymes in aqueous phase. This new procedure minimized the degradation of BFTS and BFDS in blood and during the treating procedure.

Table 4
Stability results of BFTS and BFDS in rat blood stored at −80 °C following liquid nitrogen frozen or without liquid nitrogen frozen ($n = 3$)

Compound	Concentration spiked ($\mu g\,mL^{-1}$)	Liquid nitrogen frozen recovery (%)				Without frozen recovery (%)
		0 days (h)	7 days	15 days	30 days	8 h
BFTS	8.725	99.2	101.8	98.6	105.0	7.3
	0.1571	91.7	90.5	98.0	82.5	ND[a]
BFDS	16.625	97.4	100.2	100.6	105.0	75.6
	0.1830	91.1	111.3	98.3	94.8	77.4

[a] Not detected.

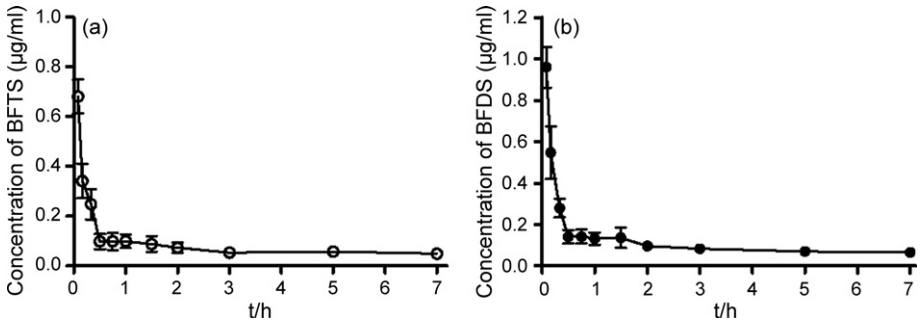

Fig. 3. Mean (±S.E.) blood concentration–time profile of BFTS (a) and the disulfide BFDS (b) in the whole blood of healthy rats ($n = 6$) after intravenous injection of a single dose of 12.5 mg kg $^{-1}$ BFTS (S.E.: standard error).

5. Conclusion

We have developed a reliable procedure for the treatment and storage of blood samples containing trisulfide BFTS and its metabolite disulfide BFDS to increase their stability and ensure their accurate determination in blood. A simple, selective, and accurate HPLC–DAD assay method was established and validated for the quantitative determination of the anti-tumor agent bis(*p*-fluorobenzyl)trisulfide (BFTS) and its major metabolite bis(*p*-fluorobenzyl)disulfide (BFDS) in blood samples. The assay method has been successfully applied to the pharmacokinetic study of BFTS and BFDS in rats following a single iv administration of BFTS. The developed procedure and assay method could be potentially utilized for the determination of other di-, tri- or tetra-sulfide derivatives in biological samples.

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