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Determination of 2,3-dimercaptosuccinic acid in mice blood and tissues by HPLC with fluorescence detection

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ABSTRACT

2,3-Dimercaptosuccinic acid (DMSA) is an orally effective chelating agent for the treatment of heavy metal poisoning. The increasing therapeutic use of DMSA has stimulated the need for sensitive and selective methods for its determination in biological samples, as well as study on pharmacokinetics and tissue distribution. According to the previously reported method, an improved method was established for the determination of DMSA in mice blood and tissues, in which oxidized DMSA was reduced by the disulfide-reducing agent, dithiothreitol (DTT), and DMSA was converted to a highly fluorescent and stable derivative by reaction with monobromobimane (mBBr) in alkaline solution. Acetonitrile was used for deproteinization and dichloromethane was used for condensation and purification, which significantly shortened the amount of time used to process the sample. Meanwhile isocratical elution was performed and excellent separation of the DMSA derivative was obtained, this enabled a run finish within 20 min. The limits of quantitation were 0.025 μg/ml in brain and 0.1 μg/ml in blood, lung, heart, intestine, liver, spleen and kidney, respectively. The calibration curves were linear in all samples ($r^2 > 0.992$) with a range of 0.025–1.6 μg/ml for brain homogenate and 0.1–6.4 μg/ml for blood and homogenates of lung, heart, intestine, liver, spleen and kidney, respectively. Therefore, the method is simple, rapid and sensitive, and it could be applicable to the studies in an animal model to evaluate the distribution of DMSA in blood and tissues.

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1. Introduction

2,3-Dimercaptosuccinic acid (DMSA; shown in Fig. 1) is a SH-containing, water-soluble, non-toxic, orally administered metal chelator which has been in use as an antidote to heavy metal toxicity since the 1950s [1,2]. DMSA is an analogue of 2,3-dimercaptopropanol (BAL), a lipid-soluble compound also used for metal chelator. DMSA's water solubility and oral dosing create a distinct advantage over BAL, which has a small therapeutic index and must be administered in an oil solution via painful, deep intramuscular injection. Besides that, DMSA has a large therapeutic window and is the least toxic of the dithiol compounds [3]. It has been classified as orphan drug by the US Food and Drug Administration. Recent studies of men and children with high levels of lead in their blood have demonstrated the particular usefulness of DMSA as an agent to increase urinary lead excretion [1,4,5]. And in animal studies, DMSA especially combined with oligomeric procyanidins could ameliorate or reverse the lead-induced neurotoxicity [6–9]. DMSA has been shown also to increase the rate of elimination of arsenic [10,11], mercury [8,9,12,13], methylmercury and cadmium [3] in children, adults and animals intoxicated with the heavy metal.

The therapeutic use of DMSA as a heavy metal-chelating agent has generated a requirement for quantitative methods to assay it in biological media. Although a colorimetric assay using Ellman's reagent, 5,5′-dithiobis (2-nitobenzoic acid), has been reported for the quantitative determination of DMSA in whole blood [14], the sensitivity and selectivity has been unsatisfactory due to the susceptibility of the SH groups to oxidation. A polarographic procedure, quantifying DMSA in pharmaceutical preparations, has also been described elsewhere [15], but the method was also non-specific when applied to sample matrices containing many potentially electroactive components.

HPLC methods [16–22] have been employed as tools for the quantitative measurement of DMSA in biological media, *e.g.* blood, plasma and urine samples. An HPLC assay with fluorescence detec-

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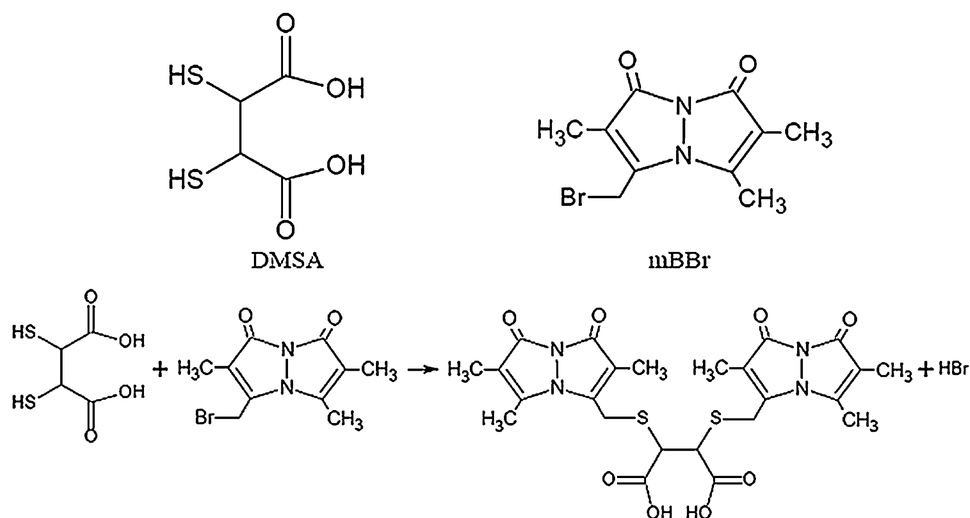

Fig. 1. Structures of DMSA and mBBr and proposed reaction of DMSA and mBBr.

tion has been developed for determination of DMSA in blood, plasma and urine and its metabolites in urine [16–19]. Unaltered DMSA (the unbound, parent compound) and total DMSA (consisting of unaltered DMSA plus oxidized DMSA determined after reduction with DTT) was detected after derivatization by mBBr. The detection limits of DMSA were 1.5 μmol/l in blood and 0.75 μmol/l in plasma (signal/noise of 2), and the linearity range was 5–80 μmol/l. The method is valid and reproducible. However, it is time-consuming because of ultrafiltration for 1 h at 23 °C. GC method has also been procedured for assay of DMSA in urine [23]. In the method, DMSA present as disulfides was first reduced electrochemically, and was derivatized with *N,O*-bis (trimethylsilyl) acetamide for GC with a flame ionization detector. The detection limit for DMSA is 1.9 nmol per 1 μl (detector sensitivity at 1×10^{-11} A/mV). But DMSA was not stable in urine and disappeared in a temperature- and pH-dependent manner. And the recovery of reduced DMSA stored at −20 °C for 24 h was poor in pH 1.0 and 7.5.

A CE method was applied to monitor of the urinary excretion of DMSA in human being [24]. The urine sample was directly injected for analysis in CE without the requirement of solid-phase extraction (SPE). Any metabolized DMSA was successfully converted to free DMSA by chemical reduction with DTT. And samples were also treated with ethylenediaminetetraacetic acid (EDTA) to transchelate any DMSA that was coordinated with metal ions present in the urine samples. The detection limit of DMSA is about 50 μmol/l, the RSD of peak area and migration time of DMSA are 4–8% and less than 1%, respectively. However, the concentrations obtained from the CE analysis were lower than that of HPLC.

In addition, the DMSA molecule contains two SH groups that are oxidized to disulfides easily both in vivo and in vitro. The oxidized species include various intermolecular disulfides with endogenous thiols, *e.g.* cysteine, glutathione and with another DMSA, and possibly even intra-molecular disulfides. To devise an assay for both DMSA parent compound and its disulfides in biological sample, it is necessary first to reduce disulfides. For this purpose, chemical techniques such as the sodium borohydride ($NaBH_4$) method [18], the DTT method [16–18,24] and electrochemical method [16,18,23] have been employed. $NaBH_4$ did not give unsatisfactory yields of reduced DMSA when used to treat samples containing DMSA disulfides [23]. Electrochemical method gave poor reproducibility and recovery. Though reduction with DTT can introduce another SH compound to the sample matrix, it has better reproducibility and recovery, and it could convert any DMSA present as disulfides into free DMSA.

In this study, the further development of the reversed-phase HPLC method is presented for the specific and sensitive detection of DMSA in mice blood and tissues. An improvement of the early reported procedure has been made to save time and simplify sample preparation procedure. This method could be useful in the studies of DMSA distribution in various tissues of organism.

2. Experimental

2.1. Materials and reagents

2,3-Dimercaptosuccinic acid (DMSA, >98%) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Monobromobimane (mBBr, thiolyte reagent, >95%) was obtained from Fluka Chemie (Steinheim, Switzerland). Dithiothreitol (DTT, >99.5%) was purchased from Alfa Aesar (Ward Hill, MA, USA). HPLC-grade methanol and acetonitrile were obtained from J.T. Baker (Philipsburg, NJ, USA); HPLC-grade dichloromethane and acetic acid were purchased from Tedia (Fairfield, OH, USA); all other reagents were AR grade. All reagents were prepared in deionized water. Deionized water was distilled before passing through Millipore water purification system.

2.2. Preparation of stock solution and calibration standards

DMSA stock standard solution was prepared at concentration of 1.0 mg/ml in 0.20 mol/l NaOH solution. The stock solution was protected from light and stored at −20 °C. Working solutions were prepared immediately prior to use by diluting the stock solution with deionized water to appropriate concentration. Aliquots (10–50 μl) of these working solutions were added to heparinized blood and tissue homogenates for calibration curves, recovery, precision, and accuracy studies.

2.3. Sample preparation for the analysis of DMSA

Blood samples were diluted at 1:9 ratio (ml/ml) with deionized water. 125 μl of diluted blood and 25 μl of 15 mg/ml DTT (0.1 mol/l NH_4HCO_3) were added to tubes containing 350 μl of 0.1 mol/l NH_4HCO_3 solution. After purging the head-space with N_2 for 15 s, the tubes were capped, and the contents were vigorously mixed for 30 s to lyse red blood cells and incubated for 30 min in the dark at room temperature, and then 100 μl of 10 mg/ml mBBr (acetonitrile) were added and the contents were vigorously mixed. After incubation for 10 min in the dark at room temperature,

1 ml acetonitrile was added to precipitate protein. After centrifugation at $16,000 \times g$ for 5 min, the supernatants were transferred and extracted with 2 ml of dichloromethane, and then centrifuged at $2800 \times g$ for 5 min. The aqueous phases were transferred and 3 mol/l HCl were added to adjust the pH to 6–7, and 20 μl aliquots of this solution were injected into the HPLC system. The entire procedure was carried out quickly and under subdued light. The procedure to treat homogenate thereafter is the same as described above.

Heart, lung, intestine, liver and spleen tissue were homogenized at 1:4 ratio (g/ml) with 0.1 mol/l NH_4HCO_3 solution. 125 μl of homogenate was transferred into tube and processed as mentioned above. Kidney tissue was homogenized at a 1:9 ratio (g/ml) with 0.1 mol/l NH_4HCO_3 solution. 125 μl of homogenate was processed as described previously. Brain tissue was homogenized at 1:4 ratio (g/ml) with 0.1 mol/l NH_4HCO_3 solution. 500 μl of homogenate was transferred into tube and 25 μl of 15 mg/ml DTT (0.1 mol/l NH_4HCO_3) was added, and then the contents were processed as described previously.

2.4. Instruments and chromatographic conditions

The chromatographic system consisted of Shimadzu 10A Series components (Shimadzu Corp., Kyoto, Japan) including an SCL-10A_{VP} system controller, two LC-10A_{VP} pumps, a RF-10A_{XL} fluorescence detector, 7725I sampling valve with 20 μl loop and LC Solution workstation. The chromatographic analysis was achieved using a C18 analytical column (AichromBond-AQ, 5 μm, 150 × 4.6 mm, Able Industries, Miami, FL, USA) at the flow rate of 1.0 ml/min. The measurement of fluorescence intensity was carried out with excitation 356 nm and emission 450 nm. The optimized mobile phase was methanol (containing 10 mmol/l tetrabutylammonium bromide, 10 mmol/l sodium acetate)–water (containing 10 mmol/l tetrabutylammonium bromide, 10 mmol/l acetic-sodium acetate, pH 4.1) (40:60, v/v). The column temperature was 30 °C.

2.5. Calibration curves

Calibration curves were prepared for DMSA in the blood and tissue homogenates. The absolute peak area was plotted against the different DMSA concentrations, and the curves were fitted by least square linear regression analysis. A correlation of more than 0.99 was desirable for each calibration curve.

2.6. Precision and accuracy

Validation of the HPLC method was performed by determining the intra-day and inter-day accuracy and precision under the extraction and analytical condition as described in Sections 2.3 and 2.4. Blood and tissue homogenates spiked known concentrations of DMSA working solutions were prepared on three different days and the samples were injected in triplicate. Accuracy was calculated as a percentage error, while precision was expressed as the relative standard deviation (R.S.D.) of each calculated concentration. Precision was expected to be less than 15% at all concentrations, except for the limit of quantitation, for which 20% was acceptable.

2.7. Recovery

For the determination of absolute recovery of DMSA from blood and tissue homogenates, DMSA standard solutions were prepared at three different concentrations. The samples were then treated exactly as described previously for blood and tissue homogenates. Absolute recovery was determined by comparing the peak area obtained from the blood and tissue homogenates to peak area obtained from the DMSA standard solutions.

2.8. Application

Both 18 female and 18 male Kunming mice weighted (18.7 ± 1.3) g were purchased from the Experimental Animal Center of Shandong University (Jinan, Shandong Province, China). In order to study the distribution of DMSA after administration, mice were fasted overnight, and then intragastrically administered with 0.5 ml of 2.6 mg/ml DMSA solution. At 0.5, 2, 4, 6 and 12 h after administration, three female and three male mice were sacrificed, respectively. Blood was collected in heparin-coated tubes, protected from light and stored at −20 °C immediately. Brain, heart, lung, intestine, liver, spleen and kidney were removed and washed with normal saline, and expulsion of water was done with filter paper. All tissues were protected from light and immediately stored at −20 °C until analysis.

3. Results and discussion

HPLC in conjunction with fluorescence detection has been a useful technique for the sensitive and selective determination of biogenic monothiol. Fluorogenic reagents that alkylate thiols, such as *o*-phthalaldehyde, *N*-substituted maleimides, mBBr and halogenobenzoxadiazoles, yield unique derivatives that are readily separated by reversed-phase HPLC and at the same time prevent the oxidation of thiols on the column [25–28]. A sensitive and selective method by precolumn derivatization has been developed for determination of the therapeutically useful dithiols, such as DMPS and DMSA [17]. DMPS and DMSA were first converted to highly fluorescent and stable derivative in aqueous solution at pH 8.3. The proposed reaction of DMSA and mBBr was shown in Fig. 1. DMSA molecule, as well as the derivative, contains two carboxylic groups that may be neutral or partially ionized under acidic conditions. The derivative was separated by ion-pair reversed-phase liquid chromatography.

We have made an improvement of the previously reported method [16], and developed a method for the determination of DMSA in mice blood and different tissues. In our method, oxidized DMSA was reduced with DTT, and acetonitrile was used for deproteinization. The excess mBBr, derivatized DTT, liposoluble compounds and acetonitrile was extracted with dichloromethane and the haemoglobin in blood was precipitated, thus the supernatants were condensed and purified. Therefore, the amount of time used to process the samples was significantly shortened. Tetrabutylammonium bromide was used as the ion-pair reagent in mobile phase under acidic conditions. Isocratical elution instead of gradient was performed, and we obtained excellent separation of the DMSA derivative. The derivatized endogenous thiols, hydrolysis products of mBBr and derivatized DTT elute with the solvent front. This enabled a run time for each sample to be shortened to approximately 20 min as compared to the 30-min or 35-min reported [16]. Significant time saving was achieved with this method. Therefore, the modifications of sample preparation and chromatographic conditions made the analysis of DMSA more simple and rapid.

Under the experimental conditions, retention times of DMSA derivative was 15.85 ± 0.36 min. Representative chromatograms of the blank blood sample, standard of DMSA, a blood sample spiked with DMSA and a blood sample at 2 h after an oral administration of DMSA are shown in Fig. 2. The chromatograms obtained from different tissue samples showed similar results. The DMSA derivative peak was well separated and showed no interference from any endogenous compounds of blood and the tissues, even the derivatized DTT.

The calibration curve was linear in the range from 0.025 to 1.6 μg/ml with mean values ($n = 3$) in brain, and the curves were linear over the concentration range from 0.1 to 6.4 μg/ml in blood, lung, heart, intestine, liver, spleen and kidney. The slope, intercept

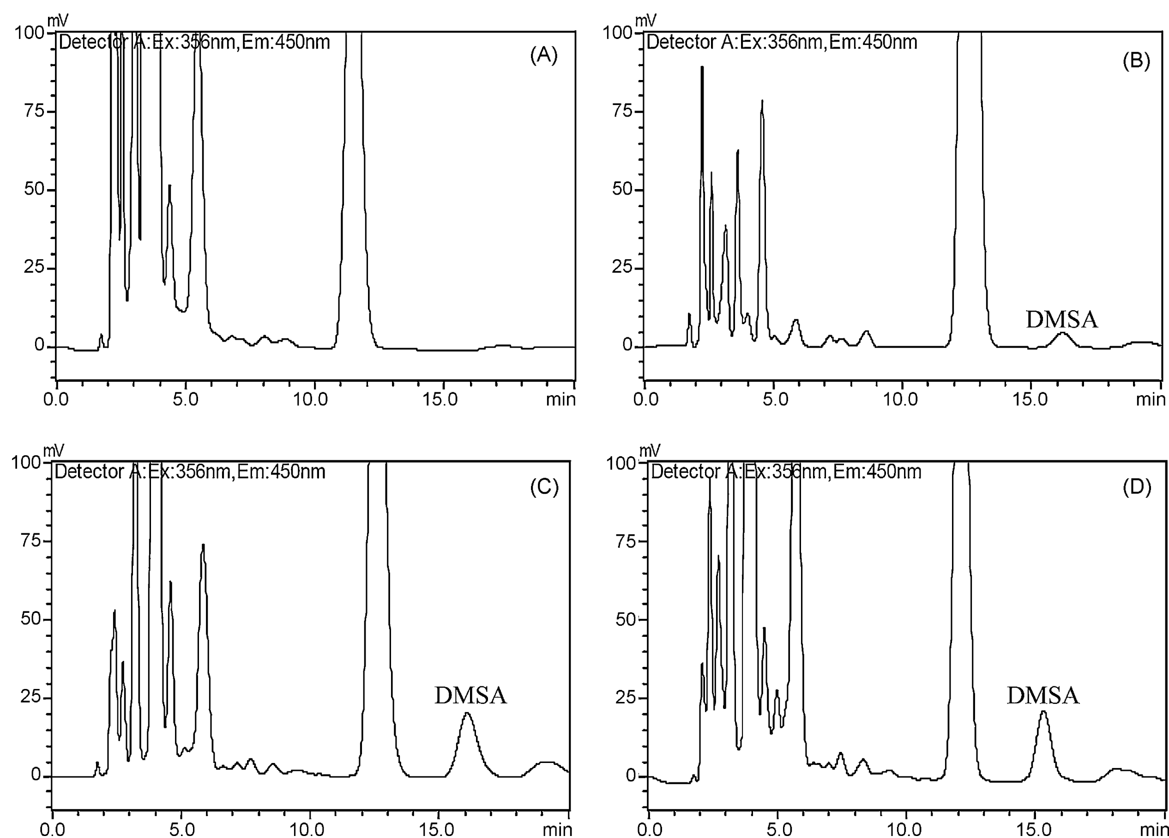

Fig. 2. Typical chromatograms of (A) blank blood, (B) standard of DMSA, (C) blank plasma spiked with DMSA and (D) blood obtained from mice at 2 h after an oral administration of DMSA.

and correlation coefficient (r^2) are shown in Table 1. The correlation coefficients (r^2) in blood and tissue homogenates were found to be greater than 0.992. The sensitivity of the method is satisfactory since concentrations of DMSA in prepared blood and tissues usually ranges. The limits of quantification were 0.025 μg/ml in brain and 0.1 μg/ml in blood, lung, heart, intestine, liver, spleen and kidney at signal-to-noise level of 10:1. This method is more sensitive in term of limit quantification compared to previously reported methods [16].

The extraction recoveries in blood and tissue homogenates were more than 75% for DMSA at all concentration levels (Table 2). Table 2 also summarizes the intra- and inter-day precision and accuracy for DMSA in blood and tissue homogenates. The validation of the sample preparation and HPLC procedure in the different tissues demonstrate that the method is accurate and precise. Intra-day (R.S.D. %) of and inter-day precisions (R.S.D. %) of the HPLC determinations are below 6% for blood and tissue homogenates at all concentration levels. The accuracy of DMSA ranged from 98 to 103% for blood and 97 to 106% for tissue homogenates, respectively.

It should be noted that no internal standard was used in the studies, although DMPS was reported as an internal standard previously [16]. The difficulty in using an internal standard in the studies is that it would compete with DMSA for the mBBr, which could present a potential problem especially in the analysis of unknown samples [29]. Moreover, an internal standard, though desirable, is not essential in the method as described. Since external standards could be prepared along with the unknown samples and subsequently treated in parallel with the latter, an internal standard may not be necessary. Based on our experiments, the correlation was satisfactory and there appeared to be no need for an internal standard. Therefore, the method for the determination of DMSA in mice blood and different tissues is valid, accuracy and reproducible.

The administration of DMSA resulted in a different tissue distribution as shown in Table 3. After an oral administration of DMSA, it was rapidly uptaken in intestine and distributed into blood and tissues. The concentration peaks appeared at 0.5 h in intestine, blood, liver and heart, which might be due to the fact that DMSA is uptaken in intestine [30,31] and firstly distributed into liver and

Table 1
Equations of calibration curves for the analysis of DMSA in blood and tissue homogenates ($n = 3$).

Sample	Concentrations (mg/ml)	Equation[a]	Correlation (r^2)
Blood	0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 5.6, 6.4	$y = 189{,}734x - 8450.123$	0.996
Brain	0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1.4, 1.6	$y = 548{,}963x - 6161.035$	0.992
Lung	0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 5.6, 6.4	$y = 183{,}302x - 982.424$	0.998
Heart	0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 5.6, 6.4	$y = 200{,}419x - 3896.753$	0.998
Intestine	0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 5.6, 6.4	$y = 170{,}678x - 186.247$	0.996
Liver	0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 5.6, 6.4	$y = 179{,}728x - 635.999$	0.998
Spleen	0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 5.6, 6.4	$y = 185{,}779x - 16915.899$	0.998
Kidney	0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 5.6, 6.4	$y = 262{,}254x - 2091.029$	0.998

[a] x and y are the DMSA concentration and peak area in a sample, respectively.

Table 2
Precision, accuracy and recovery of DMSA in blood and tissue homogenates ($n = 3$ days, triplicate per day).

Sample	Added (μg/ml)	Found[a] (μg/ml)	Accuracy[b] (%)	Precision[c] (%)		Recovery[d] (%)
				Intra-day	Inter-day	
Blood	0.2	0.204	102.18	2.06	3.28	82.41
	0.8	0.791	98.87	1.76	2.62	87.24
	5.6	5.540	98.93	1.88	1.95	97.47
Brain	0.05	0.051	102.23	2.27	5.53	78.24
	0.2	0.203	101.43	1.82	3.22	77.76
	1.4	1.421	101.50	3.23	3.46	81.55
Lung	0.2	0.198	99.00	1.16	2.89	86.12
	0.8	0.812	101.51	1.78	2.51	86.99
	5.6	5.672	101.29	1.31	1.76	95.42
Heart	0.2	0.195	97.50	1.42	2.75	86.59
	0.8	0.811	101.41	1.38	2.51	97.10
	5.6	5.689	101.59	1.51	2.41	98.28
Intestine	0.2	0.202	101.22	1.66	2.93	82.87
	0.8	0.808	101.03	1.27	1.32	86.63
	5.6	5.677	101.37	1.49	2.38	88.65
Liver	0.2	0.210	105.12	1.89	2.30	86.83
	0.8	0.821	102.68	1.50	2.36	90.39
	5.6	5.666	101.18	1.49	2.18	95.03
Spleen	0.2	0.202	101.12	2.16	2.68	82.36
	0.8	0.810	101.19	1.16	2.25	85.14
	5.6	5.679	101.41	1.16	2.00	95.64
Kidney	0.2	0.204	102.20	2.66	5.22	87.35
	0.8	0.812	101.45	2.43	4.28	93.57
	5.6	5.658	101.03	1.11	2.72	97.94

[a] Results are mean of three runs.
[b] Accuracy (%) = (found concentration)/(added concentration) × 100%.
[c] Relative standard deviation (R.S.D.).
[d] Recovery (%) = (peak area obtained from sample spiked with DMSA)/(peak area obtained from the DMSA standard solution) × 100%, results are mean of three runs.

Table 3
Quantitation of DMSA in blood, brain, lung, heart, intestine, liver, spleen and kidney samples of mice by HPLC.

Sample	0.5 h	2 h	4 h	6 h	12 h
Blood	30.241 ± 6.318	17.020 ± 3.059	8.867 ± 0.865	5.128 ± 0.406	3.317 ± 0.806
Brain	1.135 ± 0.678	1.594 ± 0.452	1.152 ± 0.445	0.952 ± 0.186	0.419 ± 0.244
Lung	19.757 ± 2.026	21.704 ± 2.710	9.565 ± 1.032	5.841 ± 0.945	1.598 ± 1.312
Heart	4.848 ± 1.280	3.677 ± 0.972	2.789 ± 0.688	1.782 ± 0.735	1.588 ± 0.244
Intestine	22.741 ± 4.234	20.748 ± 7.567	12.388 ± 1.968	2.814 ± 1.625	2.346 ± 1.147
Liver	15.327 ± 3.541	11.486 ± 3.093	11.446 ± 0.917	9.055 ± 1.016	6.689 ± 2.024
Spleen	4.921 ± 1.397	5.799 ± 0.833	4.793 ± 0.505	3.623 ± 0.528	3.548 ± 0.202
Kidney	36.767 ± 9.314	54.550 ± 17.251	19.061 ± 9.868	4.934 ± 3.069	3.812 ± 1.974

DMSA was measured in the samples 0.5, 2, 4, 6 and 12 h after an oral administration of DMSA to mice (1.3 mg). The units of the DMSA concentration are μg/ml in blood and μg/g in brain, lung, heart, intestine, liver, spleen and kidney. Values represent mean ± S.D. of six mice.

heart. In brain, lung, spleen and kidney, the maximum concentrations reached at 2 h after an oral administration of DMSA. Most of DMSA was uptaken in 4 h, rapidly excreted via kidney to urine [22,31]. The highest concentration was found in kidney, which may cause acute toxic effects. Furthermore, the concentrations in liver were very high within 12 h, it could cause toxic effects on liver by increasing the mean retention time (MRT) in liver. The problem calls for further study in the above field.

4. Conclusions

The method established in the present study is simple, rapid and sensitive for the determination of DMSA in biological samples including tissues. It could be shown that this method is applicable for studies in an animal model to describe the distribution of DMSA in different tissues.

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