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Liquid chromatography analysis of *N*-(2-mercaptopropionyl)-glycine in biological samples by ThioGlo™ 3 derivatization

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Abstract

N-(2-Mercaptopropionyl)-glycine (MPG) is a synthetic aminothiol antioxidant that is used in the treatment of cystinuria, rheumatoid arthritis, liver and skin disorders. Recent studies have shown that MPG can function as a chelating, cardioprotecting and a radioprotecting agent. Several other studies have shown that it may also act as a free radical scavenger because of its thiol group. Thiol-containing compounds have been detected in biological samples by various analytical methods such as spectrophotometric and colorimetric methods. However, these methods require several milliliters of a sample, time-consuming procedures and complicated derivatization steps, as well as having high detection limits. The present study describes a rapid, sensitive and relatively simple method for detecting MPG in biological tissues by using reverse-phase HPLC. With ThioGlo™ 3 [3H-Naphto[2,1-b] pyran, 9-acetoxy-2-(4-(2,5-dihydro-2,5-dioxo-1H-pyrrol-1-yl) phenyl-3-oxo-)] as the reagent, highly fluorescent derivatives of thiols can be obtained that are suitable for HPLC. MPG is derivatized with ThioGlo™ 3 and is then detected flourimetrically by reverse phase HPLC using a C_{18} column as the stationary phase. Acetonitrile: Water (75:25) with acetic acid and phosphoric acid (1 mL/L) is used as the mobile phase (excitation wavelength, 365 nm; emission wavelength, 445 nm). The calibration curve for MPG is linear over a range of 10–2500 nM ($r = 0.999$) and the coefficients of the variation of within-run and between-run precision were found to be 0.3 and 2.1%, respectively. The detection limit was 5.07 nM per 20 μL injection volume. Quantitative relative recovery of MPG in the biological samples (plasma, lung, liver, kidney and brain) ranged from 90.5 ± 5.3 to 106.7 ± 9.3%. Based on these results, we have concluded that this method is suitable for determining MPG in biological samples.

Keywords: Derivatization, LC; *N*-(2-Mercaptopropionyl)-glycine

1. Introduction

N-(2-Mercaptopropionyl)-glycine (MPG) is a synthetic aminothiol compound which has been proposed as an effective prophylactic treatment of cystinuria [1,2], a common, genetically inherited disorder characterized by impaired reabsorption of cystine in the renal tubuli. There is a high overall incidence of cystinuria in newborns [3]. Some studies indicate that MPG acts as a radioprotective agent by scavenging reactive oxygen species (ROS) produced by irradiation [4], and also by decreasing radiation-induced lipid peroxidation [5]. Studies have shown that MPG administration attenuates cardiac and hepatic depression caused by reactive oxygen species after severe hemorrhagic shock [6]. MPG has the ability to protect against drug-induced toxicity [7–9]. After prolonged treatment, some of the side effects observed with MPG disappear when treatment is withdrawn [10], indicating that the effects are dose-related and that the accumulation of the drug in the blood may be the reason for these effects. Therefore, drug monitoring in biological samples during treatment is advisable.

Determination of MPG in biological samples is complicated by its occurrence in multiple forms, since its free sulfhydryl group is sensitive to oxidation. MPG becomes oxidized to disulfide dimers and forms conjugates with other endogenous thiols [11]. Previous methods used for determining the quantitation of MPG include an electrochemical detection system [12], gas chromatography–mass spectrometry [13], and the HPLC method [14]. However, the time-consuming procedures, extensive manipulation of samples and complicated derivatization steps are common limitations of these detection methods.

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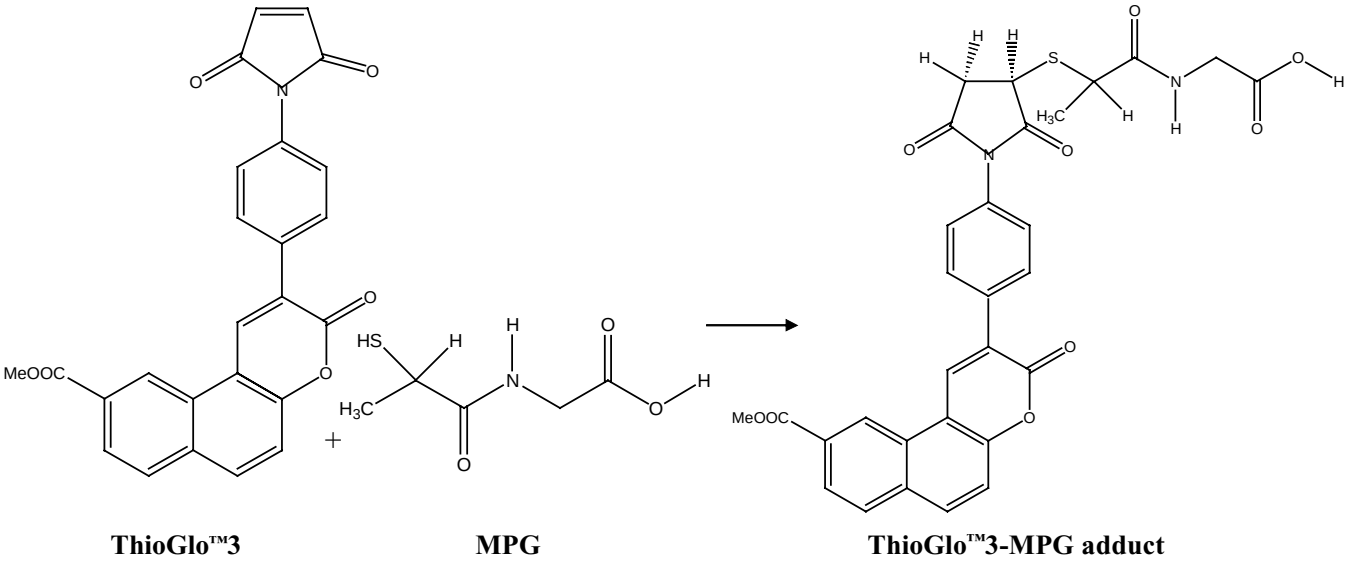

Fig. 1. Scheme of formation of fluorescent ThioGlo^{TM} 3-MPG adduct.

The present study describes a rapid, sensitive and relatively simple method of analyzing MPG in biological samples by reverse-phase HPLC. The derivatizing agent, ThioGlo^{TM} 3, has a very high affinity for thiol groups [15]. In this technique, ThioGlo^{TM} 3 reacts with the free sulfhydryl group of MPG to form highly fluorescent derivatives (Fig. 1), which are suitable for HPLC. In order to demonstrate the applicability of this simple technique to biological samples, we have quantified the MPG in liver, kidney, lung, brain and plasma samples.

2. Experimental

2.1. Reagents and chemicals

Acetonitrile, acetic acid, water, phosphoric acid (all HPLC grade) borate (boric acid) and hydrochloric acid (HCl) were purchased from Fischer Scientific (FairLawn, NewJersey, USA); L-serine, Tris–HCl (Trizma hydrochloride), and MPG were obtained from Sigma (St. Louis, MO USA), diethylenetriaminepentaacetic acid (DETAPAC) was from Aldrich (Milwaukee, WI, USA); All reagents and sorbents are reagent grade. ThioGlo^{TM} 3 was purchased from Covalent Associates Inc. (Woburn, MA, USA).

2.2. Animals

Adult male C57BL/6 mice weighing 16–20 g were obtained from Charles River Laboratories. The mice were housed in a temperature-controlled (25 °C) room equipped to maintain a 12 h light-dark cycle. Standard rat chow (Purina rat chow) and water were given ad libitum. After overnight fasting, six mice were administered 250 mg/kg body weight of MPG intraperitoneally and treated for 30 min. Then the animals were anaesthetized according to the University of Missouri Animal Care Regulations. Blood samples were collected, via intracardiac puncture, into sterile polystyrene tubes containing Heparin as an anticoagulant, and the animals were then sacrificed to obtain liver, lung, kidney, and brain samples. Plasma was obtained by centrifugation of blood and kept at –70 °C along with other tissue samples until derivatization with ThioGlo^{TM} 3.

2.3. HPLC system

The HPLC system (Shimadzu Corporation, Kyoto, Japan) consisted of a model LC-10A pump, a Rheodyne injection valve with a 20 μL filling loop, and a Model RF 535 Flourometer (Shimadzu Corporation, Kyoto, Japan), operating at an excitation wavelength of 365 nm and an emission wavelength of 445 nm. The HPLC column, from Column Engineering (Ontario, CA, USA), has 250 mm × 4.6 mm i.d. and is packed with 5 μm particles of C_{18} packing material. Quantitation of the peaks was performed with a Chromatopac Model CR601 integrator (Shimadzu). The mobile phase was acetonitrile–water (75:25, v/v) and was adjusted to a pH of 2.5 by adding of 1 mL/L of acetic acid and 1 mL/L of phosphoric acid. The ThioGlo^{TM} 3 derivatives were eluted from the column isocratically at a flow rate of 1 mL/min.

2.4. Derivatization of MPG

Tissue samples (0.14 g/mL) were homogenized in a serine borate buffer (SBB: 100 mM Tris–HCl, 10 mM Borate, 5 mM Serine, 1 mM diethylenetriamine penta acetic acid, pH = 7.00), as described [16]. Plasma and homogenates of liver, lung, kidney, and brain tissues from C57BL/6 mice

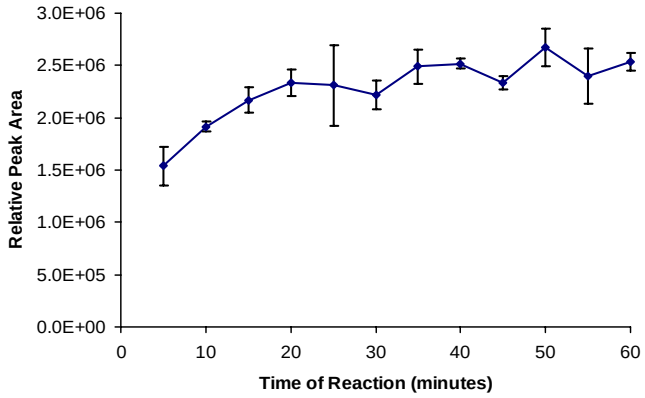

Fig. 2. Reaction time of ThioGlo^{TM} 3-MPG adduct ($n = 5$). Peak areas are reported as mean values ± standard deviations (S.D.).

were derivatized with ThioGlo^{TM} 3 to form fluorescent derivatives. The homogenized samples were subjected to appropriate dilutions (1:10 for kidney, 1:10 for plasma, 1:2 for liver, brain and lung) with a serine borate buffer before derivatizing with ThioGlo^{TM} 3. A 0.5 mM ThioGlo^{TM} 3 solution in acetonitrile (375 μL) was added to 25 μL of water (HPLC grade) and 100 μL of the diluted samples. The resulting solution was mixed and incubated at room temperature for 20 min. The reaction was completed after 20 min (Fig. 2). After incubation, 10 μL of the 2N HCl solution was added to stop the reaction. The final pH of the solution should be around 1, which is important for stabilizing the derivatives. The derivatized samples were filtered through a 0.2 μm acrodisc and then injected immediately onto a 5 μm C_{18} column in a reverse-phase HPLC system. Tissue protein concentrations were determined by the Bradford method [17].

2.5. Precision and accuracy

The between-run precision for the present method was determined by analyzing the seven replicates of the same biological sample at seven different times on each of seven different days. Within-run precision was determined by successively injecting the seven replicates of the same biological sample and comparing the peak areas of the MPG derivatives for each of the injections (Table 1). MPG-free liver tissue matrix samples, with seven replicates at multiple concentrations of 25, 125, 250, 500, 1250 and 2500 nM of MPG, were derivatized and analyzed on seven different days to determine the day-to-day CVs and the accuracy (%) (Table 2).

Table 2
Precision and accuracy of the determination of MPG in the control mouse liver tissue matrix ($n = 7$)

MPG concentration (nM)	CV (%)	Accuracy (%)
25	11.21	14.63
125	1.74	−9.43
250	4.62	−12.49
500	5.34	8.40
1250	2.50	−3.88
2500	1.87	0.90

2.6. Relative recovery

Relative recovery was determined by spiking the liver, kidney, lung, brain, and plasma samples with 500 nM of MPG in three replicates, and analytical recoveries were calculated by comparing the results to those obtained from the sample supplemented with the same concentration of MPG (Table 1).

2.7. Protein assay

The Bradford method was used to determine the protein content of the tissue samples [17]. Concentrated Coomasie Blue (Bio-Rad) was diluted 1:5 (v/v) with distilled water; then 2.5 mL of the diluted dye was added to 50 μL of the sample. The mixture was incubated at room temperature for 5 min and absorbance was measured at 595 nm by a spectrophotometer. A standard curve was constructed by using bovine serum albumin (BSA) ranging between 0–1 mg/mL. The homogenized samples were appropriately diluted (1:20 for the tissue samples) with a serine borate buffer before the protein content was determined.

3. Results

ThioGlo^{TM} 3-MPG fluorescent derivatives formed by in situ derivatizaton of biological samples were separated by the HPLC system using this technique. Fig. 3(A) shows a chromatogram of the ThioGlo^{TM} 3 hydrolysis peak and Fig. 3(B) shows the separation of the ThioGlo^{TM} 3-MPG adduct from ThioGlo^{TM} 3 hydrolysis peaks. Chromatograms

Table 1
Precision and recovery of the analysis

Sample matrix	Liver	Lung	Kidney	Brain	Plasma	Standard
Between run precision ($n = 7$) (%)	7.84	1.59	6.07	4.45	4.54	2.16
Within run precision ($n = 7$) (%)	1.98	5.75	2.54	3.08	3.01	0.31
Percentage relative recovery	90.5 ± 5.3	92.4 ± 8.7	98.2 ± 12.4	106.7 ± 9.3	104.9 ± 1.8	NA

MPG in sample matrices and standards for within-run and between-run precision ($n = 7$). Relative recovery is reported as the average relative recovery of three samples spiked with 500 nM MPG in each sample matrix. NA = not applicable.

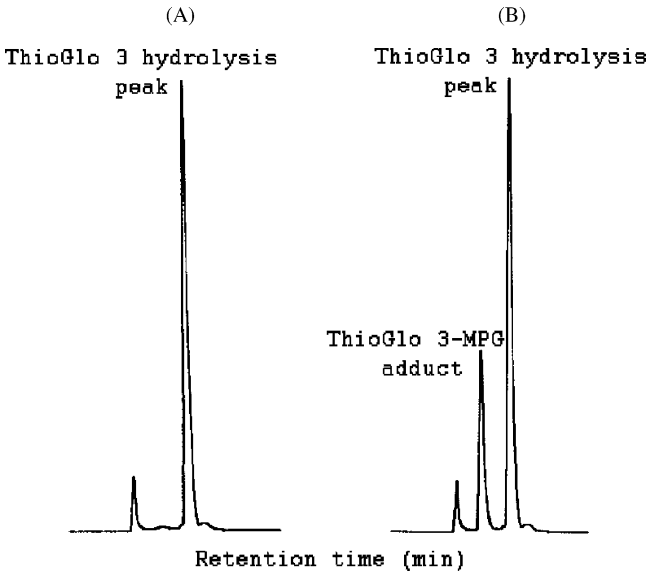

Fig. 3. (A) Chromatogram A is a blank chromatogram prepared by substituting water for a MPG containing sample which shows a ThioGlo^{TM} 3 hydrolysis peak without MPG. (B) Chromatogram B shows the separation of a ThioGlo^{TM} 3-MPG adduct (250 nM) from a ThioGlo^{TM} 3 hydrolysis peak. A 12.5 μL sample of 10 μM MPG was added to 25 μL of a serine borate buffer (SBB composition was stated in the text), followed by 375 μL of a 0.5 mM ThioGlo^{TM} 3 solution in acetonitrile. Separation was performed by using acetonitrile: water (75:25, v/v) as the mobile phase at a flow rate of 1 mL/min on a column (250 mm × 4.6 mm i.d.) packed with 5 μm particles of C_{18} packing material; the fluorescent adducts were detected flourimetrically ($\lambda_{ex} = 365$ nm, $\lambda_{em} = 445$ nm).

obtained from a biological sample (Fig. 4(A and B)] show the prominent separation of the ThioGlo^{TM} 3-MPG adduct from the ThioGlo^{TM} 3 hydrolysis peak as well as the lack of interference by the biological thiols. To demonstrate the lack of interference from the ThioGlo^{TM} 3-GSH, ThioGlo^{TM} 3-CYS, and ThioGlo^{TM} 3-HCYS peaks, chromatograms of samples containing a mixture of all four thiols (1250 nM each) were obtained [Fig. 4(C)]. Chromatograms of the kidney sample, before and after being spiked with 500 nM of MPG, are shown in Fig. 5. The retention time for the ThioGlo^{TM} 3-MPG adduct was 3.1 min.

3.1. Calibration curve

Linearity was obtained for MPG concentrations, ranging from 10 to 2500 nM, in an aqueous matrix, and calibration curves (Fig. 6) were plotted by using integrated peak areas as the *y*-axis and standard MPG concentrations as the *x*-axis. The equation for the regression line ($n = 7$) was $y = 2287.2x + 6213$ with a regression constant ($r = 0.9999$). In addition, another calibration curve was obtained by spiking liver samples from the control mice to give concentrations of 25, 125, 250, 500, 1250 and 2500 nM MPG from seven different standard curves. This was done on seven different days by injecting 20 μL of ThioGlo^{TM} 3 derivative standards containing liver tissue. Linearity in the liver tissue matrix was displayed for the same range

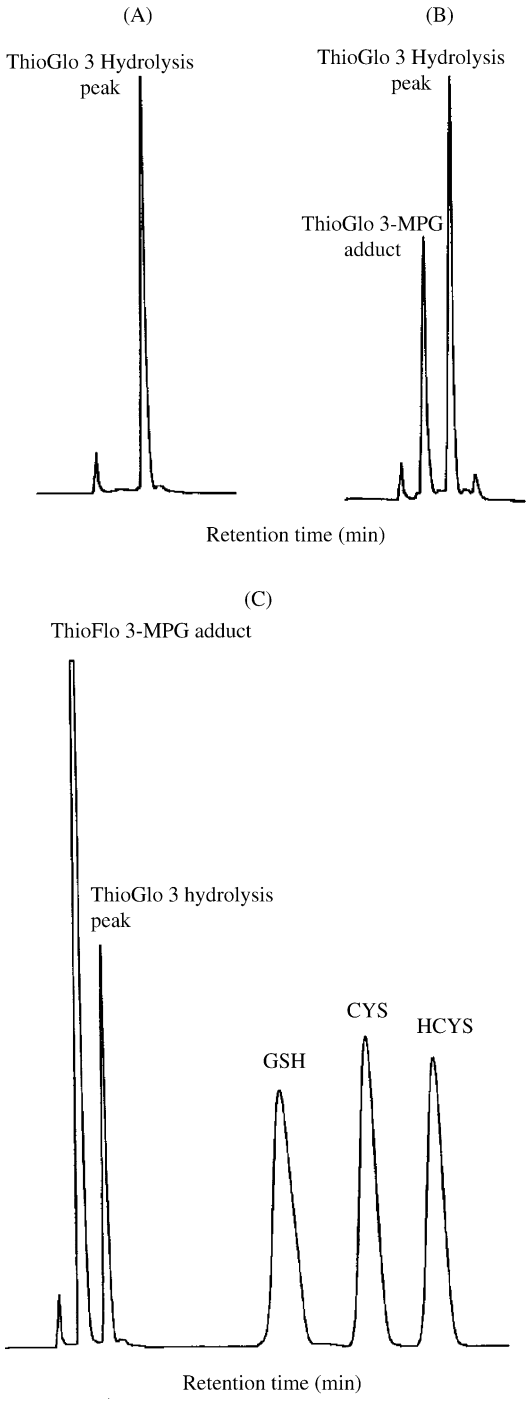

Fig. 4. (A) Chromatogram obtained from control C57BL/6 mice plasma sample (no MPG peak). (B) Chromatogram showing separation of a ThioGlo^{TM} 3-MPG adduct from a ThioGlo^{TM} 3 hydrolysis peak obtained from a C57BL/6 mice plasma sample after intraperitoneal injection of 250 mg/kg body weight of MPG. Details of the derivatization steps used for plasma samples are stated in the text. (C) A representative chromatogram of a mixture of GSH, CYS, HCYS and MPG ThioGlo^{TM} 3 derivatized samples. Peak identifications and experimental conditions were the same as those in Fig. 3. GSH: glutathione, CYS: cysteine, HCYS: Homocysteine, MPG: *N*-(2-mercaptopropionyl)-glycine.

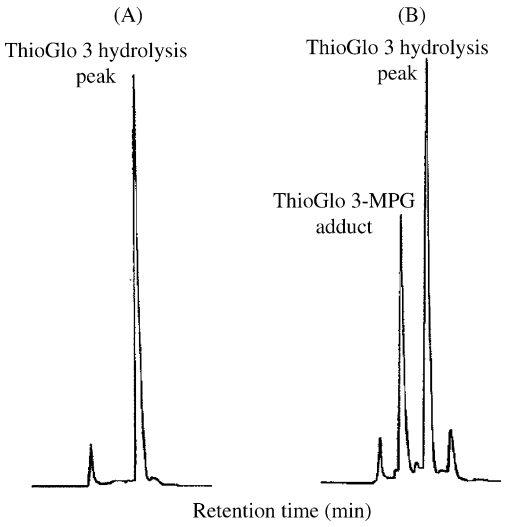

Fig. 5. (A) Chromatogram showing a ThioGlo^{TM} 3 hydrolysis peak obtained from a control C57BL/6 mouse kidney sample before spiking with MPG. (B) Chromatogram B shows the separation of a ThioGlo^{TM} 3-MPG adduct from a ThioGlo^{TM} hydrolysis peak after spiking the control C57BL/6 mouse kidney sample with 500 nM of MPG. Experimental conditions are the same as those in Fig. 4.

($r = 0.9983$) and the equation of the calibration line was $y = 1759.1x + 66207$.

3.2. Stability, sensitivity, reproducibility and relative recovery

MPG is an aminothiol compound with expanding clinical importance. In general, thiol-containing compounds are sensitive to oxidation in biological samples. In order to evaluate the stability of the thiol compound, plasma and other tissue samples containing MPG were derivatized with ThioGlo^{TM} 3 and were kept at 4 °C and −80 °C. All of the derivatized samples that were maintained at 4 °C, remained stable for 2 weeks with less than 2% deviation in all of the sample matrices. Also, the derivatized samples with ThioGlo^{TM} 3, which were kept at −80 °C for several weeks, did not show any significant change in the peak areas in our studies (data not shown). The lower limit for detection of MPG was identified as 5.07 nM ($S/N = 3$). The precision was determined by calculating the coefficients of variation for within-run and between-run precisions in the standards and biological samples; the data is shown in Table 1. The mean relative recoveries of MPG from different tissue samples are also reported in Table 1.

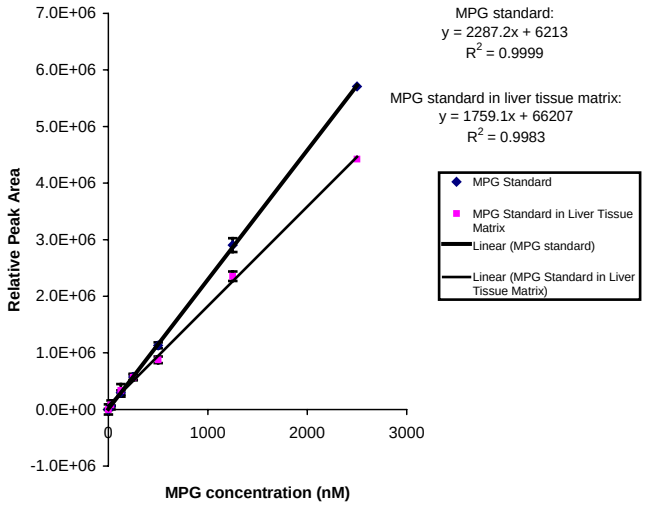

Fig. 6. Standard curves obtained from MPG and MPG in a liver tissue matrix ($n = 7$). Calibration curves were plotted using the integrated peak areas as the y-axis and the standard concentration as the x-axis. Peak areas are reported as mean values ± standard deviations (S.D.).

Table 3
MPG concentration in mouse tissues and plasma 30 min after intraperitoneal injection of MPG at a dose of 250 mg/kg (NA = not applicable)

Sample	Mean ± S.D. ($n = 3$)	Control
Plasma	1487.6 ± 129.07 nmol/L	NA
Liver	0.60 ± 0.13 nmol/mg protein	NA
Lung	0.40 ± 0.14 nmol/mg protein	NA
Kidney	62.0 ± 7.00 nmol/mg protein	NA
Brain	1.80 ± 0.19 nmol/mg protein	NA

3.3. Results of biological samples

The MPG concentrations in plasma, liver, lung, kidney, and brain tissue samples are reported in Table 3. These tissue samples were taken from C57BL/6 mice. The mice were sacrificed and the samples were obtained 30 min after intraperitoneal injections of 250 mg/kg body weight of MPG. Concentrations of MPG in plasma samples were larger than those in the tissue samples. Accumulation of MPG in the kidney tissue was the largest after 30 min of exposure to 250 mg/kg body weight of MPG while the smallest amount was in the lung tissue.

4. Discussion

MPG is noted for its versatile functionality, and has been widely used as a drug in the practice of medicine. Because of this extensive use, different methods have been developed and described for detecting this compound in biological samples, including the electrochemical detection system [12], and gas chromatography–mass spectrometry [13]. HPLC, coupled with electrospray tandem mass spectrometry, has been used lately to quantitate MPG levels and other sulfhydryl-containing compounds in biological samples [18–21]. Springolo et al. [22] reported a method for determining MPG by assessing its reaction with 2-furoyl chloride. This method is suitable for determining MPG in urine. Denneberg et al. [23] also proposed a method for determining MPG by using an aminoacid analyzer. Applicability of this method to a plasma sample was not shown. Kok et al. [12] provided a method for determining MPG by using

an electrochemical detection system on a gold/mercury amalgamated electrode. Although this method offers high selectivity and sensitivity, the response is so unstable that it is not practical for clinical use. Berti Kagedal et al. [14] published a HPLC method for determining MPG by derivatizing with *N*-(7-dimethylamino-4-methyl-3-coumrinyl)-maleimide; however, this method requires such conditions as 37 °C for 16 h for the preparation of a standard solution. In addition, thiols were purified by affinity chromatography and ion exchange chromatography to detect MPG in plasma samples. Recently, Kenji Matsuura et al. coupled HPLC with electrospray mass spectrometry to determine MPG and other thiol compounds. In this method, methyl acrylate was used to stabilize the thiol group and MPG concentrations in the blood samples were analyzed using LC–ESI–MS technique [18]. Wang et al. [21] also reported a HPLC–APCI–MS–MS method for the quantitative determination of MPG in plasma samples by derivatizing with methyl acrylate. In this method, phenylmethylsulfonyl fluoride was used to stabilize the thiol compound in the rat plasma samples.

Despite the fact that there are several methods for determining MPG in biological samples, the method we describe in the present study is simple, rapid, reproducible, sensitive and specific for the analysis of MPG in various tissue and plasma samples. The derivatizing agent, ThioGlo^{TM} 3, that we used in this method has little or no fluorescence before reacting with thiols and gives highly fluorescent derivatives after reacting with the thiols [15]. Another advantage of using this ThioGlo^{TM} 3 derivatization method is that there is less hydrolysis interference. As the hydrolysis products are non-fluorescent, fewer ThioGlo^{TM} 3 hydrolysis peaks are seen in the chromatograms (Fig. 4). This method also has a lower detection limit (5.07 nM) and a linearity that ranges from 10 to 2500 nM. The samples can be subjected to long-term injections without degradation. Simple derivatization steps and ambient temperature conditions are additional advantages of this method as compared to other time-consuming and complicated derivatization methods. Each sample requires only 8 min run time and a retention time of 3.1 min for MPG.

Based on preliminary data obtained from relative recoveries of MPG in biological samples (plasma, liver, lung, kidney, and brain), we have demonstrated that this method is suitable for determining MPG in biological samples.

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