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Determination of thiocyl in biological samples by liquid chromatography with ThioGlo^{TM}3 derivatization

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Abstract

Thiocyl (sodium thiosalicylate) belongs to a salicylate group of drugs, thus it has analgesic, antipyretic and anti-inflammatory effects. It possesses metal chelating function because it also belongs to a thiol-containing group of compounds which are well-known chelators. The studies of our research group showed that thiocyl is a promising chelator of lead poisoning due to its antioxidant and metal-chelating abilities. To the best of our knowledge, no methods were currently available for measuring thiocyl in biological samples. Therefore, we developed a reversed-phase HPLC method using fluorescence detection ($\lambda_{ex} = 365$ nm, $\lambda_{em} = 445$ nm) with a one-step derivatizing reaction between thiocyl and a derivatizing agent-ThioGlo^{TM}3 (9-acetoxy-2-(4-(2, 5-dihydro-2,5-dioxo-1H-pyrrol-1-yl)pyenyl)-3-oxo-3H-naphtho[2,1-b]pyran). Most biological thiols (such as *N*-acetylcysteine (NAC), cysteine (CYS), glutathione (GSH) and homocysteine (HCYS)) do not interfere with the detection of thiocyl by using this technique. The linear range of its calibration curve was determined to be 25–2500 nM, and the detection limit of thiocyl was found to be 3 nM with 20 μL injection volume. The coefficients of variation (CV) for within-run precision and between-run precision ranged from 0.93 to 7.21%. This assay proved to be a rapid, sensitive and simple method for determining thiocyl in biological samples.
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Keyword: Thiocyl

1. Introduction

Thiocyl (sodium thiosalicylate) has the similar structure to sodium salicylate and asprin (Fig. 1)—the well-known agents to suppress the symptoms of inflammation and also exert antipyretic and analgesic effect, thus it is also one of the choices to treat the patients with inflammatory diseases such as rheumatic arthritis [1]. Thiocyl contains a functional thiol group (Fig. 1) which has a high affinity for metals. It has been shown that thiocyl can function as a metal chelator for various metals, such as gold(I) and silver(I) in inorganic studies [2,3]. The studies of our research group also showed that thiocyl is a potent chelator for lead ions due to its antioxidant and metal-chelating abilities.

Thiocyl has not been a commonly used compound therefore there have not been many studies regarding the effects of thiocyl. One study showed that thiocyl has an inhibitory action on the development of tuberculosis with the suggestion that the efficacy of it may come from its sulfhydryl group [4]. The combination of fusidic acid, L-ascorbic acid and thiocyl used for treatment of human immunodeficiency virus (HIV) infections was patented as an antiviral composition in 1993 [5].

There has been an increased interest in the scientific community for using thiol compounds in various oxidative stress related disorders, such as Alzheimer's disease, multiple sclerosis, rheumatoid arthritis, ischemic-reperfusion injury, etc. Oxidative stress occurs when reactive oxygen species surpass cell's antioxidant capacity. The list of diseases caused by oxidative stress increases significantly every year. Biological thiols function as the first line of defense to scavenge free radicals when cells undergo oxidative stress. Therefore,

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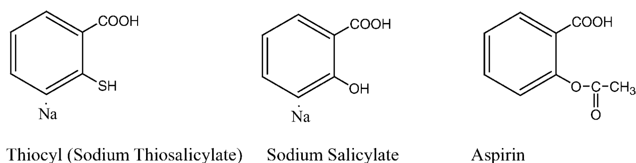

Fig. 1. Structure of thiocyl, sodium salicylate and aspirin.

natural or synthetic thiol compounds are usually given to replenish the antioxidant capacity of the cells under oxidatively challenged circumstances.

Previously, we developed the techniques for thiol-containing compounds including glutathione (GSH), cysteine (CYS), homocysteine (HCYS) and *N*-acetylcysteine (NAC) [6–8]. To the best of our knowledge, determination of thiocyl in biological samples has never been investigated before. Therefore, we developed a reversed-phase HPLC method using fluorescence detection with ThioGlo^{TM}3 derivatization to measure thiocyl levels in biological samples. ThioGlo^{TM}3 has high affinity to sulfhydryl (–SH) group and it can easily react with the –SH group of thiocyl to form fluorescent derivative (Fig. 2). It is a thiol derivatizing reagent which appears to be superior to existing thiol reagents used in determination of thiol groups by fluorescence detection. Unreacted ThioGlo^{TM}3 itself has very low fluorescence but it has high fluorescence quantum yields after reaction with free –SH groups. And it is also resistant to hydrolysis in aqueous buffer and there is only one simple hydrolysis product which allows us to determine thiol groups without substantial interference. We are also investigating its role as a metal chelator and as an antioxidant in various disorders.

2. Experimental

2.1. Reagents and chemicals

Acetonitrile, acetic acid, and *o*-phosphoric acid (all HPLC grade) were purchased from Fisher Scientific (St. Louis, MO, USA). Thiocyl was purchased from TCI America (Portland, Oregon, USA). ThioGlo^{TM}3 was purchased from Covalent Associates Inc. (Woburn, MA, USA).

2.2. Animals

All experiments were performed with adult male C57BL/6 mice weighing 16–20 g which were purchased from Charles River Laboratories. They were kept 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. They were administered 100 mg/kg body weight of thiocyl intraperitoneally after overnight fasting and anaesthetized and sacrificed according to the University of Missouri Animal Care Regulations. Samples of brain, kidney, lung, liver and blood were collected 30 min later. Plasma and tissue samples were kept in −70 °C for later analysis or minced and homogenized on ice and derivatized by ThioGlo^{TM}3 immediately.

2.3. HPLC system

The HPLC system (Shimadzu) consists of a Model LC-6A pump, a SIL-6B System Controller, an auto-injector with 20 μL filling loop, a RF 535 Fluorescence HPLC monitor (λ_{ex} = 365 nm and λ_{em} = 445 nm) and a Chromatopac

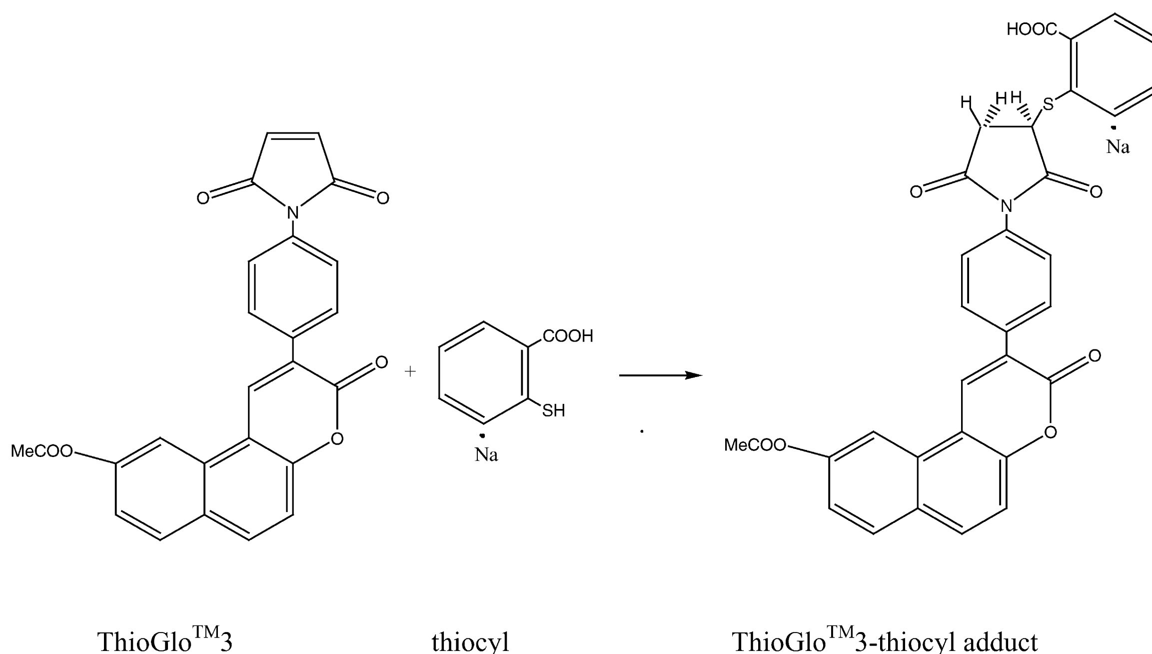

Fig. 2. Formation of fluorescent ThioGlo^{TM}3-thiocyl adduct.

Model CR 601 integrator. The HPLC column was C_{18} column (5 μm packing material) with 250 mm × 4.6 mm i.d. (Column Engineering, Ont., CA, USA). The mobile phase was acetonitrile–water (70:30, v/v) adjusted to pH = 2.5 by addition of 1 mL/L of acetic acid and *o*-phosphoric acid. The ThioGlo™3 derivatives were eluted from the column isocratically at a flow rate of 1.0 ml/min.

2.4. Sample derivatization

Plasma was obtained by centrifugation of blood samples for 10 min at 1000 × *g*. Tissue samples ranging from 0.2 to 0.5 g were kept on ice and minced and homogenized in 1 mL 100 mM Tris buffer containing 10 mM borate and 5 mM serine with 1 mM diethylenetriaminepentaacetic acid (pH = 7.0) [9]. Fifty μL diluted plasma or sample homogenates were added into 150 μL 0.5 mM ThioGlo™3 solution then incubated at room temperature for 30 min, then 5 μL 2N HCl was added to stop the reaction. Derivatized plasma or tissue samples were filtered through a 0.2 μm acrodisc and injected directly into HPLC system.

2.5. Protein assay

The Bradford method was used to determine the protein content of the tissue samples [10]. The coomassie blue dye working solution was made by diluting concentrated coomassie blue solution (Bio-Rad) with distilled water (1:4, v/v). A standard curve was constructed by using 0.05 ml of bovine serum albumin (BSA) ranging from 0 to 1.0 mg/ml as the serial standard solutions with addition of 2.5 ml commassie blue working solution. The homogenated tissue samples were subjected to appropriate dilutions and 0.05 ml of each sample was added with 2.5 ml of the same commasie blue working solution as that used in standard curve construction. The mixtures were then incubated at room temperature for at least 5 min and the absorbance was measured at 595 nm by a spectrophotometer.

3. Results

3.1. Investigation of reaction time of ThioGlo™3-thiocyl adduct

The investigation of the reaction time of ThioGlo™3-thiocyl adduct was conducted by measuring the peak area of each thiocyl sample derivatized with ThioGlo™3 at different reaction times. The result (Fig. 3) shows that the maximum peak area can be obtained at 30 min reaction time, thus 30 min reaction time was used in this technique.

3.2. Calibration curve

For calibration studies, calibration standard stock solution of thiocyl (1 mM) was prepared by weighing out 0.0018 g thiocyl accurately and dissolving it into 10 ml Serine-borate buffer (100 mM Tris buffer containing 10 mM borate and 5 mM serine with 1 mM diethylenetriaminepentaacetic acid (pH = 7.0)). This stock solution was further diluted into the calibration standard working solutions with varying concentrations. These working solutions were used to prepare calibration standard solutions by adding 50 μL of these solutions to 950 μL of each of the control mice plasma or tissue sample homogenates to get the final calibration standard solutions with certain concentrations. Calibration curve (Fig. 4) was plotted by using integrated peak areas versus standard thiocyl concentrations. Linearity was achieved over a concentration range of 25–2500 nM ($r = 0.9985$).

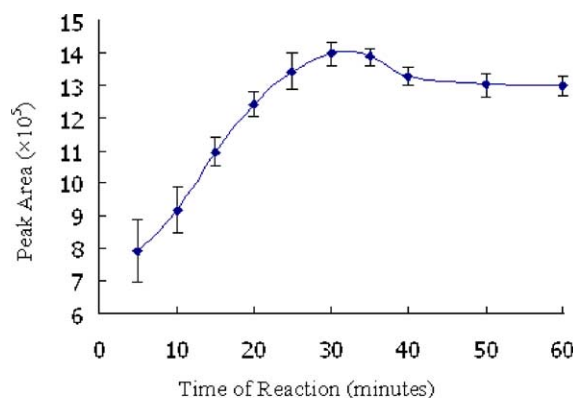

Fig. 3. Reaction time of ThioGlo™3-thiocyl adduct ($n = 3$, mean ± S.D.).

3.3. Sensitivity, stability, reproducibility and relative recovery

The lower limit of quantitation (LLOQ) of thiocyl by this method was found to be 25 nM. Fig. 5 is the chromatogram of 25 nM of thiocyl in liver matrix. The detection limit of thiocyl by this technique was found to be 3 nM with 20 μL injection volume (S/N = 3).

Plasma and tissue samples were kept in −70 °C if analysis was not performed immediately. The data from the analysis of thiocyl in plasma and tissue samples stored in −70 °C for 1 month and from the analysis of thiocyl in sample ho-

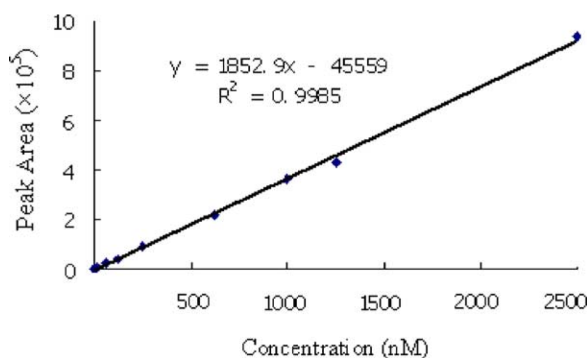

Fig. 4. Thiocyl calibration curve in liver matrix.

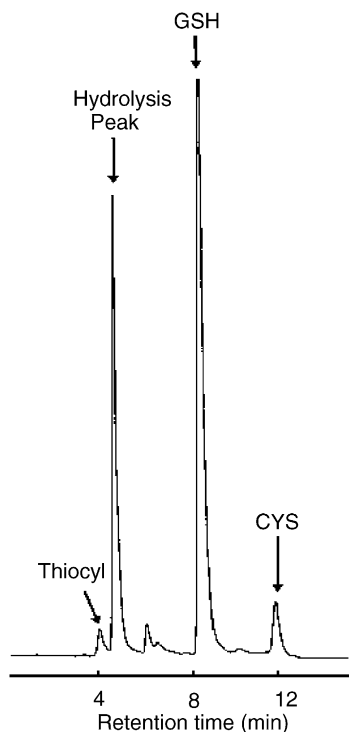

Fig. 5. Chromatogram of 25 nM thiocyl in liver matrix.

mogenates after 24 h storage at 4 °C demonstrates that thiocyl is stable under these conditions. The investigation of the stability of thiocyl in sample homogenates during freezing and thawing shows that thiocyl was stable after 2 freeze/thaw cycles. The derivatized samples kept in autosampler vials can remain stable at 4 °C for 2 weeks.

Within-run precision was determined by analyzing seven replicate thiocyl-spiked control plasma samples or tissue samples at concentration levels ranging from 50 to 1250 nM in one analytical run and comparing the thiocyl concentrations calculated from the peak areas of the seven replicates. Between-run precision was obtained by derivatizing seven replicate thiocyl-spiked control plasma samples or tissue samples at concentration levels ranging from 50 to 1250 nM in three different analytical runs. The coefficients of variation (CV) for within-run and between-run precision and relative recovery of the samples spiked with thiocyl (50, 75, 125, 500, 1250 nM) in sample matrices and standards are shown in Table 1. The CV for within-run precision and between-run precision ranged from 0.93 to 7.21% and the percentage relative recovery ranged from 90.4 to 103.3%.

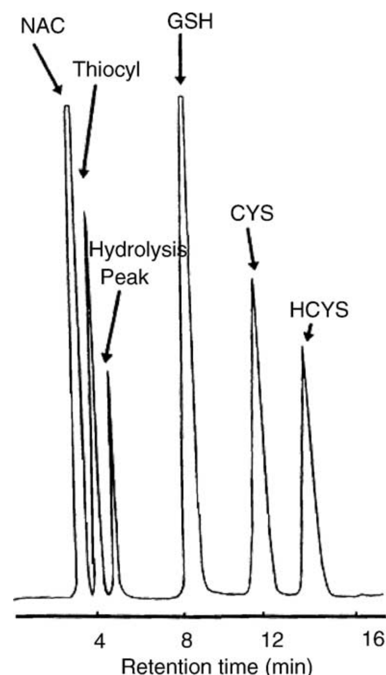

Fig. 6. Chromatogram of a mixture of 2500 nM NAC, thiocyl, GSH, CYS, and HCYS with ThioGlo^{TM}3 derivatization. Separation conditions: a C_{18} column (5 μm packing material) with 250 mm × 4.6 mm (i.d.) was used for the separation. Flow rate was 1.0 ml/min with an isocratic program of mobile phase: acetonitrile–water (70:30, v/v) adjusted to pH = 2.5 by addition of 1 mL/L of acetic acid and *o*-phosphoric acid. The ThioGlo^{TM}3 derivatives were measured by a fluorescence detector (λ_{ex} = 365 and λ_{em} = 445 nm).

3.4. Investigation of interferences

Fig. 6 shows the chromatogram of a mixture of 2500 nM NAC, thiocyl, GSH, CYS, and CYS with ThioGlo^{TM}3 derivatization. The retention time of thiocyl was 4.1 min. As we can see, those biological thiols do not interfere with the detection of thiocyl using this technology.

3.5. Results of biological samples

Fig. 7(a) shows the chromatogram of the control plasma sample from C57BL/6 mice administrated with saline solution, and no thiocyl peak appeared. Fig. 7(b) shows the chromatogram of plasma sample obtained from the animals 30 min after the administration of 100 mg/kg body weight thiocyl, and there was a thiocyl peak right before the ThioGlo^{TM}3 hydrolysis peak. Fig. 8 shows the chromatograms of liver samples obtained from C57BL/6 mice, the

Table 1
Between-run and within-run precision and relative recovery of seven sample replicates spiked with thiocyl (50, 75, 125, 500, 1250 nM) in sample matrices and standards

Sample matrix	Plasma	Kidney	Lung	Brain	Liver	Standard
Between-run precision (*n* = 7)	2.23–4.96%	1.67–6.87%	2.38–3.63%	3.86–4.56%	0.93–2.36%	1.39–5.12%
Within-run precision (*n* = 7)	2.38–6.29%	3.65–6.99%	4.12–7.21%	2.36–5.29%	1.47–4.12%	1.06–3.59%
Percentage relative recovery (*n* = 3)	98.2 ± 7.4%	94.5 ± 5.9%	90.4 ± 8.3%	91.2 ± 5.2%	103.3 ± 4.2%	N/A

Percentage relative recovery is reported as the average relative recovery (± standard deviation) of the samples spiked with 50–1250 nM thiocyl in each sample matrix. N/A = not applicable.

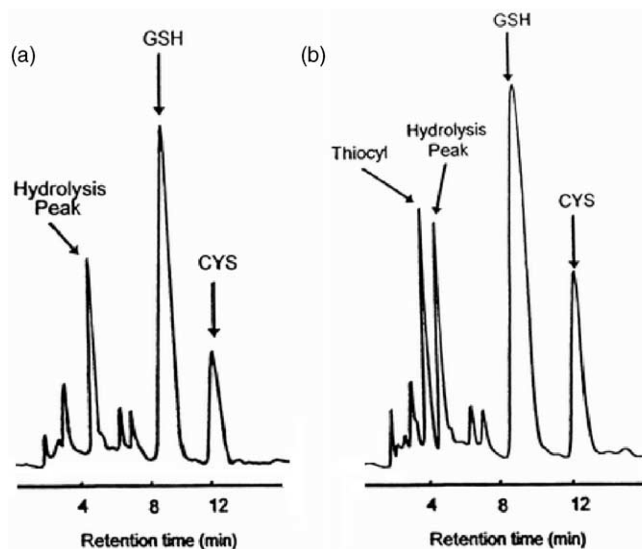

Fig. 7. Chromatograms of plasma samples obtained from C57BL/6 mice. Separation conditions are same as those of Fig. 5(a). Control plasma sample administration with saline solution (no thiocyl peak). (b) Plasma sample obtained from animals 30 min after the administration of 100 mg/kg body weight thiocyl.

chromatogram of the control liver sample (a) shows no thiocyl peak and the chromatogram of the liver sample taken after intraperitoneal administration with 100 mg/kg body weight thiocyl had a significant thiocyl peak at 4.1 min.

The levels of thiocyl in samples of plasma, liver, lung, kidney and brain tissues taken from C57BL/6 mice are reported in Table 2. The blood and tissue samples were collected after 30 min intraperitoneal injection of 100 mg/kg body weight thiocyl. The levels of thiocyl in tissue samples were calculated by using the thiocyl concentrations (nM) of homogenated tis-

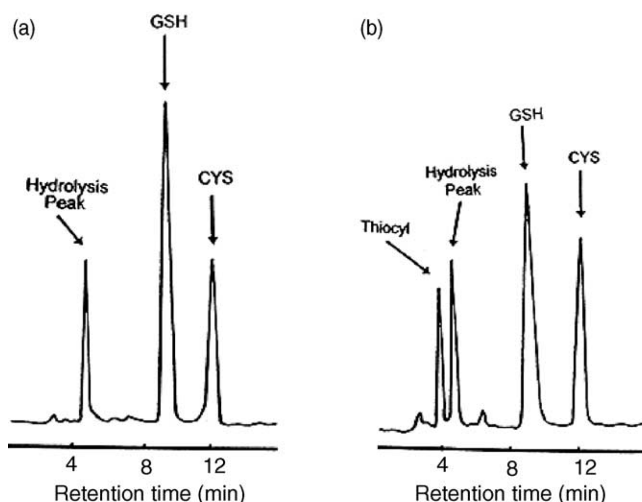

Fig. 8. Chromatograms of liver samples obtained from C57BL/6 mice. Separation conditions are same as those of Fig. 5. (a) Chromatogram of the control liver sample showing no thiocyl peak. (b) Chromatogram of the liver sample taken after intraperitoneal administration with 100 mg/kg body weight thiocyl.

Table 2
Thiocyl levels in biological samples after 30 min intraperitoneal administration of 100 mg/kg body weight thiocyl

Samples	Thiocyl concentration	
	Control (n = 3)	Mean ± S.D. (n = 3)
Plasma	N/D	12.50 ± 0.83 μM
Kidney	N/D	12.85 ± 2.38 nmol/mg protein
Lung	N/D	15.64 ± 3.15 nmol/mg protein
Brain	N/D	33.09 ± 4.09 nmol/mg protein
Liver	N/D	20.30 ± 2.78 nmol/mg protein

S.D.: standard deviation. N/D= not detectable.

sue solutions over the protein content in the solutions (mg protein/ml). The concentrations of thiocyl in plasma samples were larger than those in the tissue samples. Thiocyl with the concentrations ranging from 12.85 to 33.09 nmol/mg protein were also found in kidney, lung, liver and brain samples.

4. Conclusion

A new HPLC method has been developed for determining thiocyl in biological samples by using ThioGlo^{TM}3 as a derivatizing agent. This assay provides a sensitive, rapid and simple method for analysis of thiocyl in biological samples. The high sensitivity and resistance to hydrolysis make ThioGlo^{TM}3 a better derivatizing agent. The application of this method to biological samples such as liver, plasma, lung, kidney and brain provided satisfactory results.

Since GSH and CYS are endogenous thiol-containing compounds and their elution time are around 8 and 12 min, respectively, the running time for each biological sample should be no less than 15 min to eliminate any interference coming from the previous sample. And since thiols play an important role in the biological system and thiol status has been associated with several oxidative stress related diseases, this ThioGlo^{TM}3 method can also provide a powerful tool for monitoring the levels of various thiols such as GSH, CYS and HCYS in biological samples simultaneously.

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