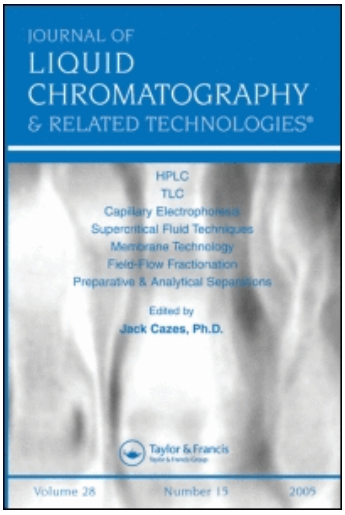

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RESOLUTION OF CHIRAL THIOL COMPOUNDS DERIVATIZED WITH N-(1-PYRENYL)-MALEIMIDE AND THIOGLO™3

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ABSTRACT

Many chiral thiols such as cysteine, homocysteine, N-acetylcysteine (NAC), and penicillamine are biologically important compounds. Among other roles, they act as antioxidants, therapeutic agents and indicators of disease. When analyzing low levels of these compounds, they are often derivatized in order to increase the sensitivity of the determination. However, it is generally an associated amine functional group that is derivatized. By selectively derivatizing only the thiol moiety with a fluorescent group, one is able to eliminate the large number of amine containing background compounds that are present in biological samples. However, there have been few reports on the enantiomeric resolution of thiol containing amino acids in which the fluorescent tag is linked exclusively through the sulfhydryl group. The first HPLC enantioresolution of N(1-pyrenyl)maleimide (NPM) and ThioGlo™3 derivatized compounds is reported on Teicoplanin and naphthylethyl-carbamate-β-cyclodextrin (NEC-β-CD) chiral stationary phases.

INTRODUCTION

The biological importance of compounds containing thiol groups has been studied extensively over the past 20 years.[1-20] Many of these compounds, such

as cysteine, homocysteine, N-acetylcysteine (NAC), and penicillamine have antioxidant properties which allow them to protect cells from oxidative stress. NAC has become very popular as a mucolytic agent used to treat chronic bronchitis,[1] an antidote for acetaminophen poisoning,[2] and has even been proposed as a therapy for HIV infection.[3,4] Cysteine has been found to play an important role in controlling nitrogen balance and maintaining body cell mass,[5] as well as regulation of mixed disulfides.[6] The concentration of homocysteine in plasma is shown to increase with cobalamin and folate deficiency, and decreases after treatment with the relevant vitamin.[7,8]. An increased concentration of homocysteine (i.e., hyperhomocysteinemia) is also a risk factor for cardio-vascular diseases such as atherosclerosis and thrombosis.[9] NAC, cysteine, and D-penicillamine have all been found to be heavy metal chelators,[10-14], and are used to treat individuals who have been exposed to copper, chromium, lead, arsenic, etc. Also, D-penicillamine has been used in the treatment of Wilson's disease, as well as many other afflictions.[14-20]

Thiol compounds have been quantitated in plasma and other biological fluids in many ways, including radioenzymatic assay,[21-23] amino acid analysis,[24,25] gas chromatography-mass spectrometry (GC-MS), GC-electron capture (GC-EC),[26-28] and high performance liquid chromatography (HPLC) followed by electrochemical, ultraviolet, or fluorescence detection.[7,26,28-42] The least expensive and most straightforward method is derivatization followed by HPLC and fluorescent detection. However, most of the common derivatizing reagents used for fluorescence detection preferentially react with amine functional groups.[36] Thus when derivatizing natural samples, a large number of fluorescent components are formed because of the large number of amino acids, amines, thiols, alcohols, etc., therein. However, there are far fewer thiol compounds in most biological systems than there are amino acids and other amines. Any derivatizing method that can be used to selectively tag thiols and ignore amine and alcohol functionalities would greatly simplify the analysis of these sulfur-containing compounds. Thiol specific derivatizing reagents were developed for this reason.

The most widely used thiol derivative is the monobromobimane (mBBr) assay, developed by Fahey and Newton.[43] This method is specific for thiols but the sample preparation and derivatization reaction are complex and lengthy. In 1995, a new HPLC method developed using N-(1-pyrenyl)maleimide (NPM) as a derivatizing agent was reported.[32] A related naphthopyranone-based fluorescent thiol probe, ThioGlo™3 (9-Acetoxy-2-(4-(2,5-dihydro-2,5-dioxo-1H-pyrrol-1-yl)phenyl)-3-oxo-3H-naphtho[2,1-b]pyran), was reported in 1991,[34] but has not been used in HPLC until recently. These fluorescent derivatizing agents react quickly with thiols at room temperature, (usually less than one minute).

Recently, NPM and ThioGlo™3 have been used for the detection of biological thiols. But to our knowledge, the chiral resolution of these compounds

(when exclusively derivatized at their thiol functional group) has not been reported. This would allow a simpler and more effective separation and quantitation of many sulfur containing compounds with little or no interference from other amino acids and amine compounds. The quantitation of each enantiomer also is essential for any biologically relevant assays. In the case of penicillamine, many studies have looked at the effects of D-penicillamine,[14] but their chromatographic and detection methods do not provide for the separation of D- and L-penicillamine. The purity of the D-penicillamine used, as well as, the levels of L-penicillamine in the biological samples were not determined. We have developed a method to separate these enantiomers so that their specific effects can be considered.

EXPERIMENTAL

Three HPLC systems were used. The first system was a Shimadzu (Kyoto, Japan) auto injection HPLC system consisting of two LC 10AT pumps, an SIL-10A auto injector, an RF-535 fluorescence detector, an SCL-10A system controller, and a CR 501 Chromatopac. The second system was a Shimadzu system consisting of two LC-6A pumps, an RF-535 fluorescence detector, an SCL-6A system controller, and a C-R6A Chromatopac. The third system was a BAS (West Lafayette, IN) HPLC system consisting of a BAS PM-80 pump, a CC-5 injection port, and a Waters (Millipore, Milford, MA) 470 scanning fluorescence detector interfaced with a BAS DA-5 ChromGraph Interface to a Gateway 2000 computer (486, 66MHz). All HPLC columns were purchased from Advanced Separations Technologies, Inc. (Astec, Whippany, NJ).

The ThioGlo™3 and N(1-pyrenyl)maleimide (NPM) were purchased from Covalent Associates Inc. (Woburn, MA) and Aldrich (Milwaukee, WI) respectively. Their structures can be seen in Figure 1. Racemic mixtures as well as purified enantiomers of homocysteine, cysteine, N-acetylcysteine, and penicillamine were purchased from Sigma (St. Louis, MO). Their structures can be seen in Figure 2. The standards were dissolved in water to a concentration of 0.1 to 1m<u>M</u>. The ThioGlo™3 was dissolved in acetonitrile to a concentration of 0.5m<u>M</u>. The NPM was dissolved in acetonitrile to a concentration of 1m<u>M</u>. 5 µL of the standard was mixed with 750 µl of the derivatizing agent and 245 µL water. This solution was vortexed and parafilmed and sat at room temperature for 5 minutes. Then 5 µL of 2<u>N</u> HCL was added to stop the reaction. The solution was then filtered through a 0.2 µm syringe filter and was then ready for injection onto the HPLC system. The derivatives were stable for up to 1 month at 4°C. NPM derivatives were detected with an excitation wavelength of 330nm and an emission wavelength of 375nm. ThioGlo™3 derivatives were detected with an excitation wavelength of 365 nm and an emission wavelength of 445 nm. The derivatization reaction can be seen in Figure 3.

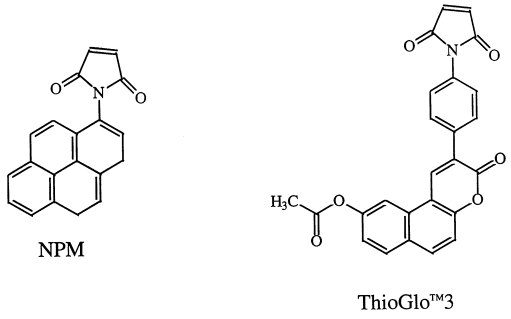

Figure 1. Structures of the derivatizing agents N-(1-pyrenyl) maleimide (NPM) and ThioGlo™3.

RESULTS AND DISCUSSION

When derivatizing biologically important compounds, the reaction often occurs with one or more of three nucleophilic moieties on the molecule of interest. They are the amine (R-NH_2), alcohol (R-OH), and thiol (R-SH) functional groups. Of these three, the amine moieties are usually the most nucleophilic and hydroxyl groups are the least nucleophilic. In this work a pH is selected that renders all amine groups and amine containing compounds to their protonated form (R-$^{+}NH_3$). Thus the amine groups are unreactive with NPM and ThioGlo™3. Also, these derivatizing agents are not sufficiently reactive to derivatize alcohol groups (-OH). Therefor only thiol groups will be derivatized under the conditions of this reaction (see Experimental). This makes NPM and ThioGlo™3 highly selective fluorescent tags for thiol compounds.

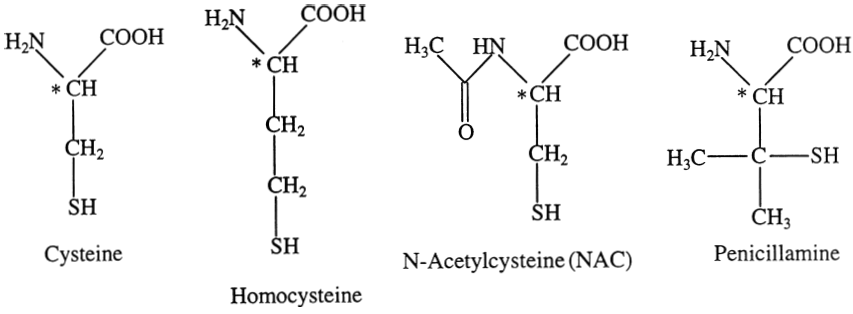

Figure 2. Structures of the thiol compounds cysteine, homocysteine, N-acetylcysteine, and penicillamine. * = chiral center.

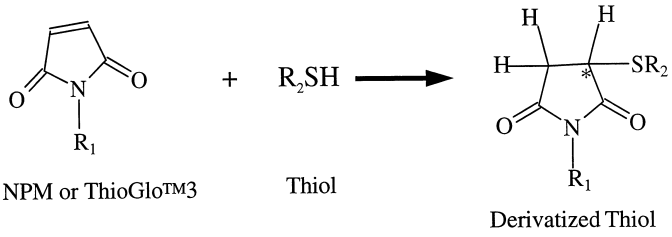

Figure 3. Derivatization reaction of NPM or ThioGlo™ 3 with thiol compounds. The asterisk (*) indicates the additional stereogenic center that is produced as a result of the derivatization reaction with the prochiral reagents.

Table 1

Chromatographic Conditions of Thiol Derivative Separations

Compound	Mobile Phase[a]	k_1[b]	α	R_s
ThioGlo™3-NAC[c]	20:80:6:.05	0.32	3.9	1.6
	20:80:6:.5	0.60	4.6	2.8
NPM[d]-NAC	20:80:6:.05	0.45	3.5	1.6
	20:80:6:.5	0.59	4.5	2.8
ThioGlo™3-homosysteine	50:50:10:.5	1.76	1.7	1.5
NPM-homosysteine	50:50:10:.5	1.55	2.0	1.5
ThioGlo™3-penicillamine	90:10:1:.5	1.67	1.7	1.5
27%MeCN in 1% TEAA pH 4.1[e]		27.1	1.1	1.6
NPM-penicillamine	15:85:.4:.1	1.40	1.6	1.5
ThioGlo™3-cysteine	7.93:15:1	5.46	1.3	1.4

[a] Mobile phases given as methanol:acetonitrile:acetic acid:triethylamine (v:v:v:v). [b] The capacity factor of the first eluting enantiomer. [c] N-acetylcysteine (NAC). [d] N(1-pyrenyl)maleimide (NPM). [e] This mobile phase is for a β-SN cyclodextrin column.

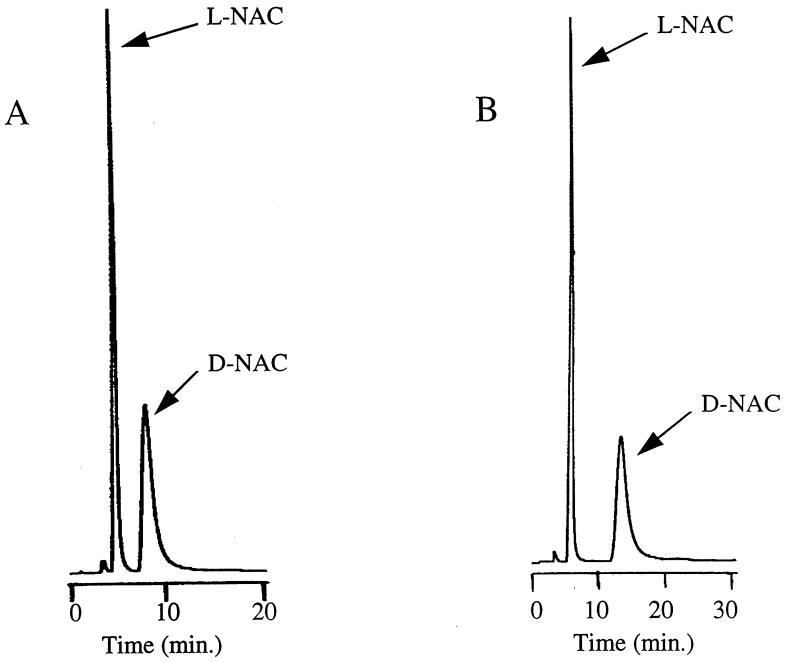

Figure 4. ThioGlo™ 3 derivatized NAC separated under different mobile phase conditions on a Teicoplanin column. A) Mobile phase was methanol:acetonitrile:acetic acid:triethylamine (20:80:6:0.05) (v:v:v:v). B) Mobile phase was methanol:acetonitrile:acetic acid:triethylamine (20:80:6:0.5) (v:v:v:v).

As can be seen in Figure 3, the derivatization reaction generates an additional stereogenic center. For this reason, only native compounds containing a single stereogenic center were studied. The generation of a second chiral center was not found to be an obstacle to the separation of enantiomers, as will be shown.

A number of cyclodextrin and antibiotic columns were screened to see which ones best resolved enantiomers of the thiol derivatized cysteine, homocysteine, penicillamine, and NAC. With the exception of cysteine, the best separations were found to all be on the Teicoplanin column in various polar organic mobile phases (mixtures of methanol, acetonitrile, acetic acid, and triethylamine). Optimum resolution for cysteine was achieved on an NEC-β-CD column in the reversed phase mode. Chromatographic conditions are listed in Table 1. Baseline or near baseline resolution was achieved for most of the derivatized compounds on the Teicoplanin stationary phase. As can be seen in

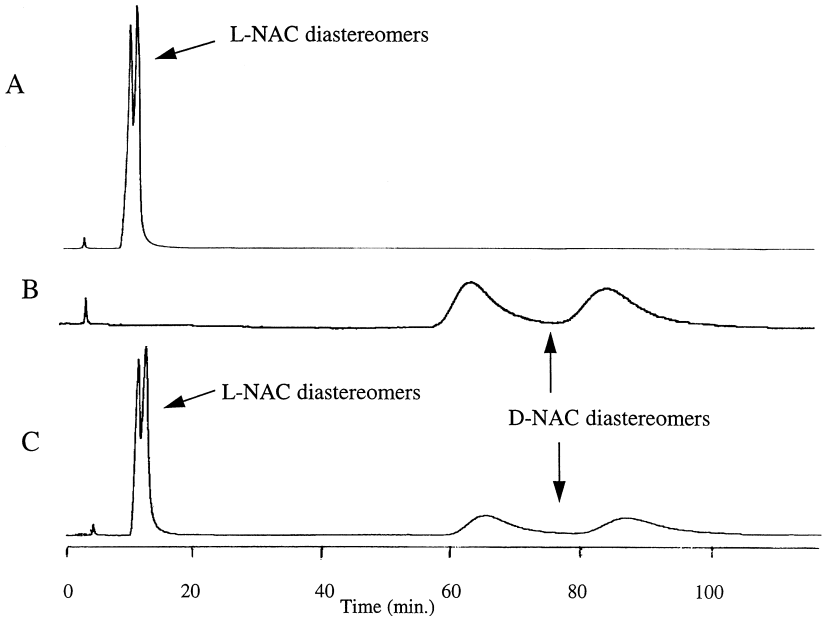

Figure 5. Separation of all four ThioGlo™ 3 derivatized NAC isomers on Teicoplanin column; mobile phase methanol:acetic acid:triethylamine (100:1:1) (v:v:v). A) L-NAC derivatized with ThioGlo™ 3; B) D-NAC; C) D/L-NAC.

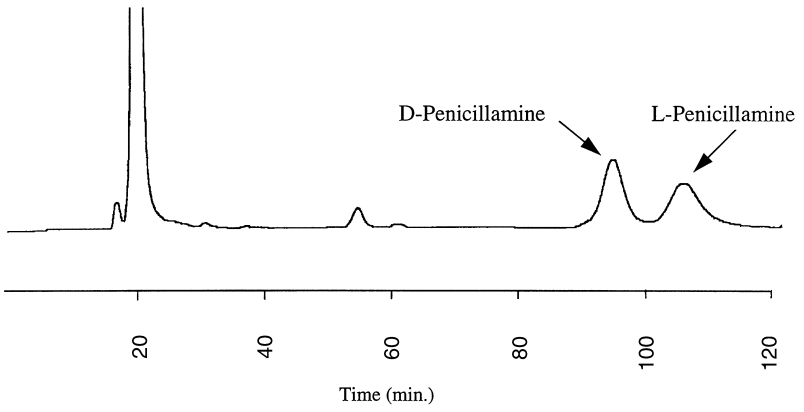

Figure 6. Separation of ThioGlo™ 3 derivatives of penicillamine on β-SN cyclodextrin column. Mobile phase is 27% acetonitrile in 1% triethylammonium acetate buffer, pH 4.1.

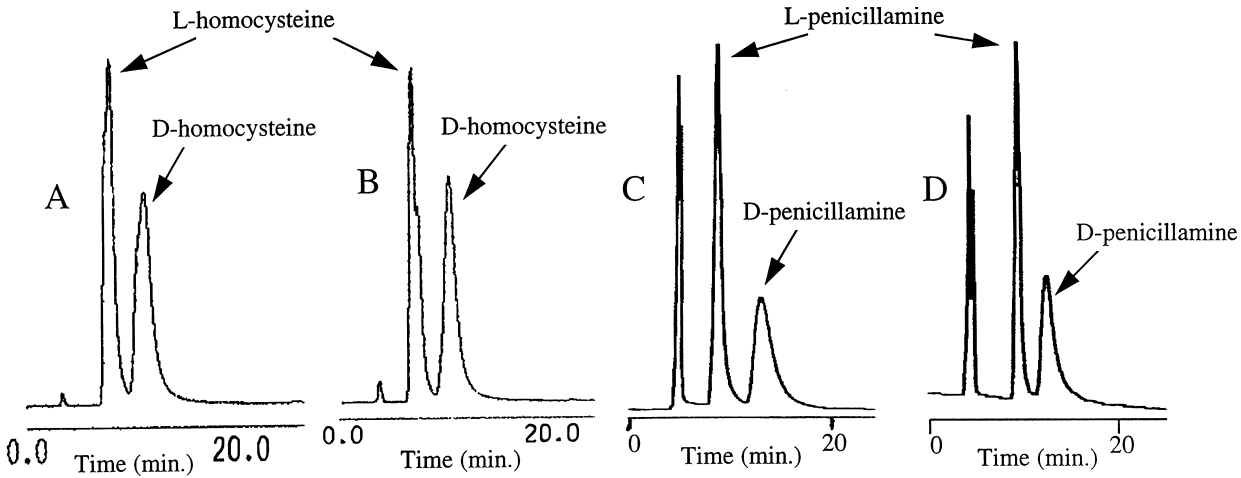

Figure 7. A) Separation of ThioGlo™ 3 derivatized homocysteine. B) Separation of NPM derivatized homocysteine. Mobile phase for A) and B) was methanol:acetonitrile:acetic acid:triethylamine (50:50:10:5) (v:v:v:v). C) Separation of ThioGlo™ 3 derivatized penicillamine. Mobile phase was MeOH:MeCN:HOAc:TEA (90:10:1:0.5) (v:v:v:v). D) Separation of NPM derivatized penicillamine. Mobile phase was MeOH: MeCN:HOAc:TEA (15:85:4:0.1) (v:v:v:v). All separations are on a Teicoplanin column.

Figure 4, with slight changes in the mobile phase, L- and D-NAC can either be separated with R_s=1.5 in under 15 minutes or if greater separation is desired, with R_s=2.75 in under 25 min. Even higher resolution can be achieved for NAC if time is not an issue. Penicillamine could also be baseline resolved. This was achieved on a β-SN cyclodextrin column, requiring a buffered aqueous mobile phase and almost two hours for elution (see Figure 6). Interestingly though the elution order was reversed with the D enantiomer eluting before the L enantiomer. For all separations on the Teicoplanin column the L enantiomer eluted before the D enantiomer.

As noted earlier, there are four stereoisomers after derivatization. With manipulation of the mobile phase, we were able to separate all four isomers (see Figure 5), or we could coelute the L isomers and coelute the D isomers thereby focusing only on the original stereochemistry of the chiral thiol compound (see Figure 6).

Interestingly, the choice of derivatizing agent did not make a difference in the separation of the stereoisomers of NAC as well as homocysteine. As can be seen in Figure 7 A and B, and Table 1, nearly identical chromatographic conditions were effective regardless of the derivatizing agent used. This was not found to be the case for cysteine and penicillamine. In fact, quite different mobile phase conditions were required for optimum separation of penicillamine derivatized with ThioGlo™3 and NPM. ThioGlo™3 derivatized penicillamine required high methanol (90%) and relatively high acid/base concentration, while NPM derivatized penicillamine required high acetonitrile(85%) and relatively lower acid/base concentration (see Figure 7 and Table 1). Likewise, ThioGlo™3 derivatized cysteine required high acetonitrile (93%) and very high acid/base concentration but NPM derivatized cysteine could not be satisfactorily separated.

CONCLUSIONS

NPM and ThioGlo™3 are highly selective fluorescent derivatizing agents for thiol compounds. Their derivatization reaction conditions are almost identical with the working concentration of the derivatizing agent being the only difference (see Experimental). The reaction is less complex and time consuming and also produces more stable derivatives than other thiol derivatizing reagents. The enantiomeric composition of biological thiols can now be quantitated and the specific effects of each enantiomer can now be evaluated in various biological environments.

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