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Determination of homocysteine thiolactone and homocysteine in cell cultures using high-performance liquid chromatography with fluorescence detection

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Abstract

A sensitive and simple method utilising fluorometric detection for the simultaneous routine monitoring of homocysteine thiolactone (HTL) and homocysteine (Hcy) in biological samples has been developed. Separation relies on isocratic ion-pairing and reversed-phase chromatography while the principle of the detection is that the lactone ring in HTL molecule is cleaved with an alkali to produce Hcy, which reacts with *ortho*-phthalaldehyde (OPA) in the absence of an added thiol reagent to form a stable fluorescent derivative. The method has a sensitivity of 200 fmol of HTL and 100 fmol for Hcy in the sample. The present method was applied to the determination of HTL and Hcy in Hep G2 cell.

Keywords: Homocysteine thiolactone; Homocysteine

1. Introduction

Homocysteine (Hcy) is a sulfur-containing amino acid generated through the demethylation of methionine. Under normal conditions, the intracellular concentration of Hcy is kept low as a result of remethylation reactions and catabolism via the transsulfuration pathway. However, several hereditary and acquired conditions can alter normal homocysteine metabolism [1–3]. A plasma Hcy concentration greater than 15 μM is termed ''hyperhomocysteinemia'' [4]. Recent clinical studies have shown an association between hyperhomocysteinemia and premature cardiovascular disease, and suggest that Hcy may impair vasomotor regulatory and thrombotic properties of vascular endothelium [5–9]. However, it is not known what aspect of Hcy metabolism is harmful to human cells and induces arteriosclerosis. Recently, the relationship between cysteine and vascular disease has also been reported by El-Khairy et al. [10].

Hcy forms a stable cyclic compound, homocysteine thiolactone (HTL). HTL can arise by a metabolic error-editing process in which Hcy is misactivated by methionyl-tRNA synthetase to form enzyme-bound homocysteinyl adenylate, from which Hcy is released as free HTL [11]. This conversion occurs excessively in mammalian, including human cancer cells, but is minimized in normal mammalian

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cells [12]. The accumulation of HTL has been suggested to be related to mechanisms of atherogenesis [13]. In cultured human cells and in human serum, HTL reacts with primary amines in the protein by nucleophilic addition, and the homocysteinylation of cellular and extracellular proteins might lead to cell damage. Ferguson [14] reported that the acylation product of HTL and ε-amino groups of apo-B lysyl residues, the homocystamide–low density lipoprotein (LDL) adduct, has been implicated in increased atherogenicity. It is therefore of great interest to study the metabolism of HTL and understand its role in atherogenesis and also to be able to modulate its level in order to decrease that risk. Moreover, there is a real interest in the simultaneous determination of HTL and Hcy when the behavior of cellular and extracellular HTL is studied. In order to study the physiological role of HTL in cardiovascular disease, a sensitive method capable of detection at n*M* levels is needed. Several analytical methods for the simultaneous determination of HTL and Hcy in biological samples have been reported such as amino acid analyzer [15], and scintillation counting of ^{35}S in combination with thin layer chromatography [12]. Although the sensitivity of the latter method is high, with a detection level of n*M*, it can be time-consuming and cumbersome to employ as a routine analysis in the laboratory.

In this paper, we developed a simple, sensitive post-column HPLC method with fluorescence detection for the simultaneous routine monitoring of HTL and Hcy in biological samples. The principle of the detection is that the lactone ring in the HTL molecule is cleaved with an alkali to produce Hcy. The Hcy is reacted with OPA in the absence of an added thiol reagent to form a stable fluorescent derivative. This method was applied to the determination of HTL and Hcy in Hep G2 cell.

2. Experimental

2.1. Chemicals

L-Homocysteine thiolactone, L-homocystine and glutathione (oxidized form) were obtained from Sigma Chemical (St. Louis, MO, USA). OPA was purchased from Nacalai Tesque (Kyoto, Japan). Tris(2-carboxyethyl)phosphine (TCEP) was obtained from Tokyo Kasei (Tokyo, Japan). All the other chemicals were of analytical grade and obtained from the usual commercial sources.

2.2. Cell culture

Human cell line Hep G2 cells were incubated in 60-mm plates in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and penicillin (100 Units/ml)–streptomycin (100 μg/ml). Hep G2 cells were cultured in monolayer with a change of fresh medium every 3 days until 85% confluent. The medium was then replaced with 4 ml of methionine-free DMEM supplemented with 10% dialyzed fetal bovine serum without antibiotics, and the cultures were maintained at 37 °C in an atmosphere of 5% CO_2 for 24 h.

2.3. Cell extracts

Cell extracts were prepared from cultured cells at two time-points (4- and 24-h incubation with methionine-free medium). After collection of culture medium, the cells were washed with 1 ml of ice-cold phosphate-buffered saline (PBS). Then the cells were scraped off and extracted with 0.3 ml of lysis buffer (10 m*M* phosphate buffer (pH 6.0) containing 1% Triton X-100 and 0.1% SDS). After a 10-min incubation on ice, the extract was centrifuged for 10 min at 10 000 *g*, and the supernatant was analyzed. Protein in the extracts was determined by the BCA protein assay (Pierce, Rockford, IL) according to the manufacture's protocol.

2.4. Reduction and deproteinization

To 200 μl of cell extract or culture medium diluted two times with distilled water, 200 μl of 2.5 m*M* TCEP in 0.1 *M* phosphate buffer (pH 6.0) was added and vortex-mixed. The mixture was capped and incubated at 37 °C for 10 min. After the sample was ultracentrifuged at 5000 *g* for 20 min using Ultrafree-MC (UFC3LGC00, Millipore), 200 μl of the filtrate was transferred to an autosampler vial.

2.5. Instrumentation

The chromatographic system consisted of two L-6000 pumps (Hitachi, Tokyo, Japan), an AS 8000

autosampler (Tosoh, Tokyo, Japan), a L-7300 column oven (Hitachi), a Sic chromatocorder 12 integrator (Sic Instruments, Tokyo, Japan) and a L-7480 fluorescence detector (Hitachi). A Hitachi F-4000 fluorescence spectrophotometer was used with a 1-cm quartz cell for manual methods.

2.6. Chromatographic conditions

The ultrafiltrate was injected into a Develosil C_{30}-UG-5 column (150×4.6 mm; Nomura Chemicals, Aichi, Japan). To protect the analytical column, a Develosil C_{30}-UG-5 guard column (10×4 mm) was fitted between the analytical column and the autosampler. The column oven was kept at 30 °C. The mobile phase consisted of 50 m*M* phosphoric acid/NaOH (pH 2.0)–methanol (98:2, v/v) containing 0.5 m*M* sodium octanesulfonate. The phosphoric acid/NaOH (pH 2.0)–methanol (98:2, v/v) containing 0.5 m*M* sodium octanesulfonate was filtered through a cellulose nitrate filter (0.45 μm; Toyo Roshi, Tokyo, Japan), and degassed just prior to use. The mobile phase was run at a flow-rate at 1.0 ml/min. The eluate was mixed in a three-way tee with 6 m*M* OPA in 0.5 *M* NaOH, delivered at a flow-rate of 0.5 ml/min. The reaction coil was Teflon tubing (0.5 mm I.D.×10 m). Fluorescence intensities were monitored with excitation at 370 nm and emission at 480 nm.

2.7. Preparation of calibration standards

Stock solutions of homocystine and HTL were prepared at a concentration of 10 m*M* in 0.03 *M* hydrochloric acid and stored at −20 °C. These solutions were used to prepare working standard solutions daily for different concentrations.

3. Results and discussions

3.1. Detection of Hcy and HTL

In the present study, OPA reagent was used for detection of Hcy. This reaction was first reported by Cohn and Lyle [16] as a highly specific and sensitive fluorometric assay for tissue reduced glutathione (GSH) under conditions of about pH 10; the fluorescent product is the tricyclin compound formed by reaction of both the primary amine and the thiol moieties of GSH. Hcy scarcely reacts with OPA under the above conditions but nevertheless contain an amino and a thiol group in the molecule in analogy with GSH. On the other hand, HTL cannot be measured directly with OPA in the absence of the thiol compound. However, the lactone ring in the HTL molecule can be cleaved with an alkali to produce Hcy. Therefore, we examined the reaction conditions for the formation of the Hcy–OPA fluorescent derivative and for the opening of the lactone ring in HTL.

The fluorescence excitation and emission spectra of OPA derivatives for Hcy, HTL and GSH were measured in various concentrations of NaOH (0.05–1 *M*, Fig. 1). The fluorescence spectra of Hcy and HTL showed an emission maximum at 480 nm with an excitation maximum at 370 nm, while GSH showed an emission maximum at 418 nm with an excitation maximum at 333 nm; no shifts of the maximum wavelength were observed under any alkaline conditions. The mechanism of reaction is probably the same as that of GSH with OPA, but the fluorescence spectrum of Hcy was shifted to a longer wavelength compared with GSH.

The effect of pH on the reactivity of Hcy with OPA was examined under the conditions described by Cohn and Lyle [16] (pH 10) and above pH 11,

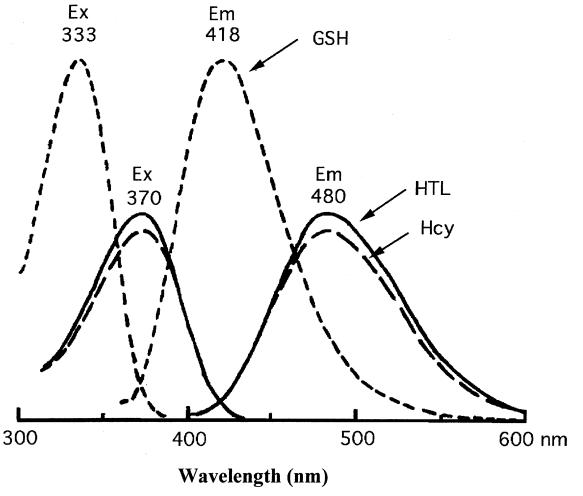

Fig. 1. Fluorescence spectra of OPA adducts for HTL, Hcy and GSH. The fluorescence spectra were measured using a HPLC system without column. Sample concentration: 1 μ*M*. HPLC conditions are given in Section2.6.

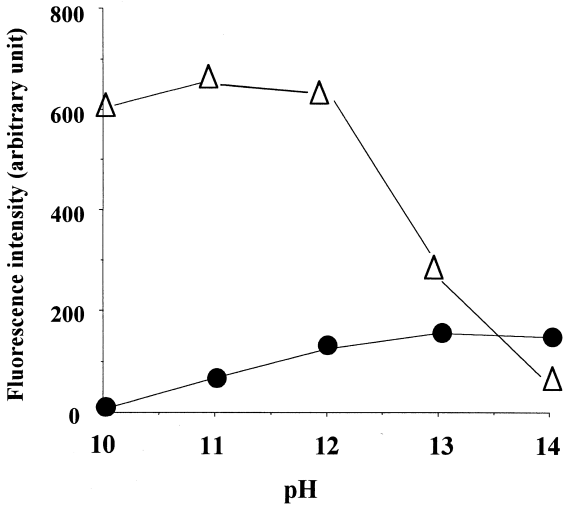

Fig. 2. Effect of pH on the reactivity of Hcy (●) and GSH (△) with OPA. Sample concentration: 1 μ*M*. Samples were adjusted to each pH indicated, OPA was added, and the fluorescence intensity was measured after 1 min at wavelengths of excitation and emission maxima of each compounds.

compared with GSH, using a fluorescence spectrophotometer at wavelengths of excitation and emission maxima of each compound (Fig. 2). When derivatization with OPA was performed at pH 10, the fluorescence response was scarcely observed for Hcy, whereas GSH produced a high response. The fluorescence intensities of Hcy increased significantly with increasing alkaline concentrations, contrary to that of GSH. The fluorescence intensity remained constant at pH 13 or above.

The rate of the lactone ring-opening reaction of HTL in NaOH (0.05–1 *M*) was measured using the HPLC system (described in Section 2) without a column (reaction time: about 1 min). In NaOH solution, HTL was completely hydrolyzed to Hcy in the reaction coil and the opening reaction rate was rapid. This result suggests that the use of OPA reagent for simultaneous determination of HTL and Hcy under alkaline conditions is suitable for a post column reaction. From the above results, we chose 0.5 *M* NaOH as the optimum alkaline condition to open the lactone ring in HTL and for the OPA reaction.

Table 1 shows the relative fluorescence intensities for Hcy, HTL and its related compounds measured at the maximum wavelength of Hcy. The fluorescence intensity obtained with HTL and Hcy was approximately ten times higher than that of GSH.

Table 1
Relative fluorescence intensities of Hcy and related compounds on the reaction with OPA

Compounds	Relative fluorescence intensity
Homocysteine	100
Homocysteine thiolactone	100
Glutathione	9
γ-Glutamylcysteine	4
Cysteine	–
Cysteinylglycine	–

The fluorescence intensity was measured at Ex 370 nm and Em 480 nm using the HPLC system without a column. Sample concentration: 1 μ*M*. HPLC conditions are given in Section 2.6. Fluorescence intensity in homocysteine was arbitrary taken as 100.

3.2. Separation of Hcy and HTL

The conditions for the separation of Hcy and HTL were examined by ion-pair reversed-phase chromatography using the C_{30} stationary phase. The C_{30} column showed high reproducibility of retention time in the lower organic solvent concentration, compared to the C_{18} column [17]. The optimum separation was achieved by regulating the pH, sodium octanesulfonate concentration, and percentage of methanol in the mobile phase. Moreover, GSH and histidine also reacted with OPA in this alkaline condition, so the retention behavior of Hcy, HTL, GSH, and histidine was investigated. With increasing sodium octanesulfonate concentration in the range 0.25–1.0 m*M*, a moderate increase in retention was observed for both Hcy and HTL at pH 2. The complete separation of these compounds was obtained by the addition of methanol (Fig. 3). Therefore, 50 m*M* phosphoric acid/NaOH (pH 2.0)–methanol (98:2, v/v) containing 0.5 m*M* sodium octanesulfonate was chosen as a suitable mobile phase. Under these chromatographic conditions, Hcy and HTL were eluted at retention times of 13.9 and 16.2 min, respectively. Fig. 4 shows chromatograms of standards (Fig. 4A) and the cell extract from Hep G2 (Fig. 4B). No interference with constituents from endogenous substances in the cellular sample was observed because OPA reacts specifically with the compounds having both the primary amine and the

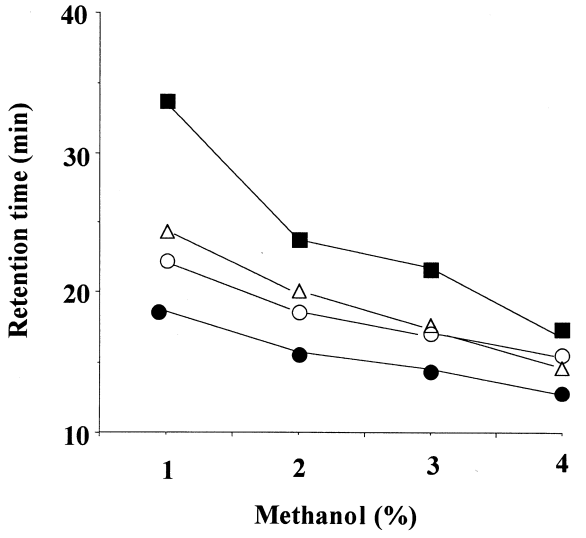

Fig. 3. Effect of the methanol concentration on the separation of Hcy (●), HTL (○), GSH (△), and histidine (■). HPLC conditions are given in Section 2.6.

thiol moieties in the molecule. Only histidine and GSH were detected as peaks separated on the chromatogram (Fig. 4B). Fig. 4C shows complete separation of Hcy, HTL, GSH and histidine in the cell extract spiked with HTL.

3.3. Sample preparation

For the determination of Hcy, the reduction of disulfides such as homocystine and homocysteine–protein mixed disulfide is required before the deproteinization. In the present method, TCEP was used as an effective reducing agent, which was not only odorless but also water-soluble. Because the lactone ring of HTL was opened at pH 8 or above, the sample preparation, including the extraction and the reduction step, was done at pH 6. Furthermore, to prevent the formation of HTL from Hcy in the highly acidic conditions, ultrafiltration was used to remove the proteins for the cell sample. Various storage conditions were examined to minimize the loss of cellular HTL and Hcy before analysis. The cell extracts could be stored at −20 °C and all samples were analyzed immediately after the sample preparation step.

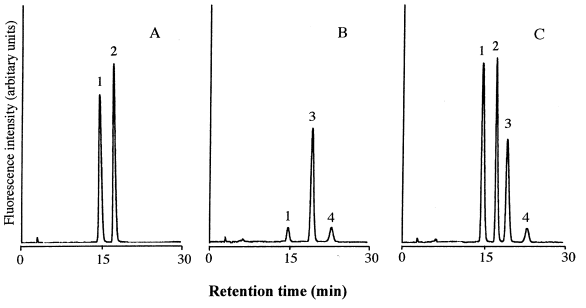

Fig. 4. Chromatograms of Hep G2 cell extracts. (A) 1 μ*M* HTL and Hcy; (B) normal cell extract; (C) cell extract spiked with 1 μ*M* HTL and Hcy each. Peak 1: Hcy; peak 2: HTL; peak 3: GSH; peak 4: histidine. The experimental conditions are described in Sections 2.3–2.4.

3.4. Calibration curves and precision

The calibration curves for Hcy and HTL were linear over the concentration range of 0.01–10 μ*M*. Under these conditions, the detection limits were 100 fmol for Hcy and 200 fmol for HTL with a signal-to-noise ratio of 3, per 20 μl of injection volume. The detection limit for Hcy in the present method was at least five times higher than fluorometric HPLC methods by using bimanes and halogenosulfonylbenzofurazans as pre-labeling reagents [18,19].

The within-run precision (C.V., $n=5$) of the assay of HTL and Hcy in the cell extracts, spiked with HTL (at 1 μ*M*), was 3.7 and 4.2%, respectively. The between-day precision were C.V.<7.7% for HTL and Hcy with analysis of the cell extract spiked with HTL at 1 μ*M*, repeated for three different days. Recovery experiments were carried out by adding known amounts of HTL and Hcy to cell extracts before the reduction step. The recoveries of HTL and Hcy from the cell extract were 95.2±3.5 and 103.4±3.4% ($n=5$, at 1 μ*M* each), respectively.

3.5. Determination of HTL and Hcy in the Hep G2 cell and culture medium

To investigate the role of HTL in a mechanism of homocysteine toxicity, the present method was applied to the determination of HTL and Hcy in the cultured cells. Jakubowski [11,20] has reported that Hcy is metabolized to HTL by methionyl-tRNA synthetase in all cell types from bacterial to human. It was also shown that HTL is secreted from cells

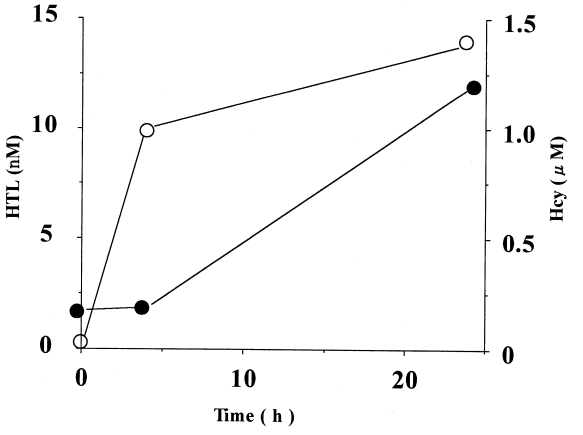

Fig. 5. HTL (○) and Hcy (●) levels in culture medium of Hep G2 cell. Culture conditions are given in Section 2.2.

and accumulates in culture medium [12]. Furthermore, Jakubowski [21] showed that human cells efficiently convert Hcy to HTL and the extent of the conversion is inversely proportional to methionine concentration. On the basis of these reports, we measured the formation of HTL in Hep G2 cells by the present method. As shown in Fig. 5, HTL was found in the medium of Hep G2 cells cultured under methionine-free condition and at increased concentration with incubation time. HTL was also detected in the Hep G2 cells extract but it was near the detection limit (data not shown).

In conclusion, the developed post-column HPLC conditions coupled with the OPA derivatization seems the most desirable method for the sensitive and selective determination of intracellular HTL and Hcy. The simultaneous determination of HTL and Hcy will provide a better understanding of their dynamic relationship in cells. The results obtained in this study show that the present method appears to be useful for determining the role of HTL and Hcy in atherogenesis.

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