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Article

Quantification of Glutathione in Plasma Samples by HPLC Using 4-Fluoro-7-nitrobenzofurazan as a Fluorescent Labeling Reagent

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A rapid and highly sensitive high-performance liquid chromatograpy method with fluorescence detection has been developed for determination of glutathione (GSH) in human plasma. A simple pre-column derivatization procedure with 7-flouro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) reagent was employed. The separation of the derivatized glutathione was performed using a mobile phase consisting of phosphate buffer (0.02 mol/L, pH 6.0)–acetonitrile (77:23, v/v) at a flow rate of 1.0 mL/min with the column temperature 2°C. The eluted derivatives were fluorometrically detected at an excitation wavelength 470 nm and an emission wavelength 530 nm. Under the optimum chromatographic conditions, the calibration curve was linear over the range of 0.1 μmol/L to 10.0 μmol/L with the correlation coefficient of 0.9988. The precision of the method was satisfactory with the intra- and inter-day coefficient of variation being 6.3%, 6.9%, respectively. This method has been used to determine glutathione concentrations in plasma samples from healthy individuals.

Introdution

Glutathione (GSH) is an important tripeptide thiol (γ-glutamyl cysteinyl glycine) antioxidant and widely distributed in biological fluids and tissues (1). It is involved in many physiological functions, such as the detoxification of xenobiotics, the transport of amino acids, the stabilization of cell membranes, and the synthesis of proteins and DNA. It plays an essential role in protecting cells from the toxic effects of oxidizing agents, ionizing agents, and free radicals. Altered glutathione concentrations may play an important role in various diseases and/or pathological conditions, such as inflammation (2), autoimmune disorders (3), hepatic damage (4), sepsis, apoptosis (5), cancer (6), and chronic diseases (7). GSH is an important biomarker of oxidative/nitrosative stress, and it has been widely used in clinics (8, 9).

Because of the key role and widespread presence of GSH in biochemical systems, a number of methods have been developed for the determination of GSH in plasma. Among the methods described in the literature, there are high-performance liquid chromatography (HPLC) with UV-visible electrochemical, or fluorescence detection (10–14), capillary electrophoresis (15), or HPLC–mass spectrometry (MS) (16, 17). These methodologies differ in sensitivity, selectivity, specificity, and susceptibility to interferences. HPLC–MS provides substantial sensitivity, selectivity, and specificity. However, MS is expensive and complicated, limiting its routine application for clinical research, particularly in studies calling for the analysis of large sample numbers. Several derivatives have been exploited for the determination of glutathione, including o-phthalaldehyde (OPA) (12), 2,3-naphthalenedialdehyde (NDA) (14, 15), 2,4-dinitrofluorobenzene (FDNB) (18), and some other fluorescent agents (19, 20). The derivative with OPA is unstable, influencing the accuracy and reproducibility of GSH analysis (12). The derivative procedure with FDNB is time-consuming (1 h), and its high limit of detection (LOD) (0.5 μmol/L) limits its use in plasma sample determination, especially in some pathological conditions when the concentration of GSH is very low (17). Other derivatives can be used for the determination of GSH, but these pre-treatment procedures are cumbersome and time-consuming.

4-Fluoro-7-nitrobenzofurazan (NBD-F) is an extremely sensitive fluorescent derivatization reagent for the determination of amino acids and some drugs (21–23). NBD-F has several advantages compared to other reagents, including convenient excitation/emission wavelengths, few reagent related interfering peaks, and mild reaction conditions. Therefore, the present study is devoted to investigate NBD-F as a derivatizing reagent for GSH. The derivatization reaction conditions were evaluated and optimized. As a result, a very simple and rapid method was developed for quantifications of GSH in plasma with fluorescence detection after pre-column derivatization with NBD-F.

Experimental

Chemicals and materials

L-glutathione was purchased from Sigma Chemical Co. (St. Louis, MO). 4-fluoro-7-nitrobenzofurazan (NBD-F) was used as the fluorescent agent, and was purchased from TCI (Tokyo, Japan). HPLC-grade acetonitrile was purchased from J.T. Baker (Phillipsburg, NJ). Analytical-grade potassium tetraborte, boric acid, dipotassium hydrogen phosphate, and potassium dihydrogen phosphate were from Sinopharm Chemical Reagent Co. (Shanghai, China). Ultrapure water was prepared using a Millipore (Bedford, MA) Milli-Q water system.

Preparation of solutions

Glutathione standard solution

An accurately weighed amount (15.4 mg) was quantitatively transferred into a 50-mL dark volumetric flack, dissolved in distilled water, and completed to volume with the same solvent to produce stock solutions of 1.0 mmol/L. The working standard of GSH was obtained daily by diluting the stock solutions with the distilled water.

4-Flouro-7-nitrobenzofurazan (NBD-F) derivatizing reagent

An accurately weighed amount (18 mg) was quantitatively transferred into a 1-mL volumetric flack, dissolved in

acetonitrile, and completed to volume with the same solvent to produce stock solutions of 0.10 mol/L. The solution was freshly prepared and protected from light during use and stored at −20°C before use.

Buffer solution

For the borate buffer solution, a volume of 30 mL of 0.05 mol/L sodium borate ($Na_2B_4O_7 \times H_2O$) and 70 mL of 0.2 mol/L boric acid (H_2BO_3) were mixed together, and the pH of the solution was adjusted to 8.0 ± 0.1 by a calibrated pH-meter (MP220K, Mettler Toledo, Switzerland).

For the mobile phase solution, weighed amounts of potassium dihydrogen phosphate (KH_2PO_4, 2.72 g) and dipotassium hydrogen phosphate (K_2HPO_4, 0.29 g) were dissolved in 1000 mL distilled water to obtain the phosphate buffer solution (0.02 mol/L, pH 6.0).

Chromatographic system

The HPLC apparatus consisted of a Shimadzu system (Shimadzu Corporation, Tokyo, Japan) equipped with two LC-10Atvp pumps, 20 μL injection loop, HT-230A column heater, RF-10AXL fluorescence detector, and SCL-10Avp system controller. The chromatographic separations were performed on an analytical column Diamonsil C_{18} (250 mm length × 4.6 mm, 5 μm particle diameter) manufactured by Dikma Technologies, Inc. (China). The column temperature was maintained constant at 25°C. The mobile phase used for separation consisted of a mixture of an acetonitrile–phosphate buffer (0.02 mol/L, pH 6.0) (23:77, v/v) pumped at a flow rate of 1.0 mL/min. The mobile phase was filtered by a Millipore vacuum filtration system, equipped with 0.45 μm pore size filter, and degassed by ultrasonication. The fluorescence detector was set at 470 and 530 nm for excitation and emission wavelengths, respectively.

Sample collection, preparation and derivatization

Blood samples (total 36) from 16 healthy laboratory personnel and 20 healthy medical students (24 males and 12 females) were analyzed. Informed consent was obtained from all subjects after explaining the aims and risks of the study. Five milliliter of venous blood samples were obtained by venepuncture and collected in heparinized tubes. Blood samples were centrifuged immediately at 1500 rpm for 15 min at room temperature to obtain the plasma. The plasma samples were prepared immediately before analysis.

The plasma sample, in a centrifuge tube, was mixed with twice the volume of acetonitrile, vortexed for 1 min, and centrifuged at 12000 rpm for 10 min. The supernatant was collected and the pH was adjusted by adding a borate buffer for derivatization after it was filtered through a 0.45-μm pore size Millipore filter. The filtrated sample was carried out at 30°C for 10 min in dark conditions after being mixed with an equal volume of NBD-F (1 mmol/L) and borate buffer. After cooling to room temperature, 10 μL of the solution was subjected to HPLC.

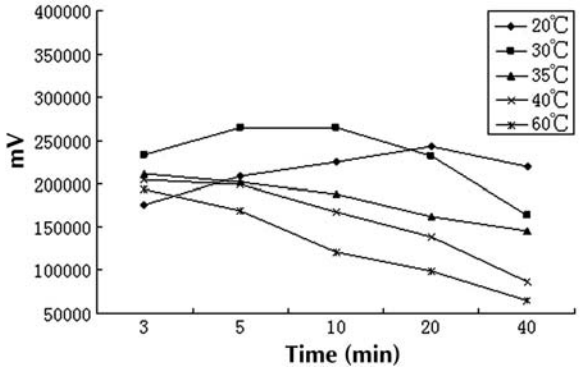

Figure 1. Effects of the temperature and time on the derivative reaction.

Results and Discussion

Derivatization reaction conditions

Effect of reaction temperature on the reaction time course of glutathione with NBD-F

It was reported that the derivatization reaction of amino acids with NBD-F could occur at 20°C, and the higher the reaction temperature, the shorter the time required for the reaction to be completed. The rates and yields of the derivatization reaction of GSH with NBD-F were examined at various temperatures. The effects of the temperature and the reaction time on the reaction are shown in Figure 1. To achieve the high yield of the derivative reaction and saving the reaction time, 30°C at 10 min was selected as the final reaction condition.

Optimization of pH value

As the derivatization reaction of amino acids with NBD-F is a typical nucleophilic reaction, alkaline conditions were expected to favor the reaction. Both the borate buffer and the phosphate buffer have been employed as the reaction buffer of the NBD-F with amino acids. A comparison was conducted for the reactive fluorescence intensity with different pH values in both the borate buffer and the phosphate buffer. The GSH–NBD-F peak areas were calculated at pH values ranging from 7–10. When using the borate buffer, the fluorescence intensity reached its maximum at pH 8.0.

Stability of GSH–NBD-F

The stability of NBD-derivatives of GSH were analyzed by determining the peak areas of the standard solutions of different concentrations that had been stored under dark conditions at room temperature for 1, 2, 4, 6, and 12 h after the derivatization reactions. No significant decrease in the peaks of GSH was found compared with those analyzed immediately after the preparation. When the derivative was kept at −20°C, there was no significant decrease after even 72 h.

HPLC separation

Selection of the mobile phase

The chromatographic conditions were optimized to develop a sensitive, accurate, and reproducible method for detecting GSH

in human plasma. Because the derivative was easily retained on the column, acetonitrile was chosen as the organic phase in the mobile phase, as it has better eluting ability. The retention time of NBD-F was relatively stable with the change of the organic phase percentage, but the GSH–NBD-F derivative was very sensitive to it. When the percentage of the acetonitrile in the mobile phase changed from 30% to 20%, the peak time of the GSH–NBD-F derivative drifted from 12 min to 35 min, with the NBD-F changing little.

In order to save analysis time and avoid other plasma interferences, 23% acetonitrile in the mobile phase was chosen.

Selection of the mobile phase pH and the column temperature

The pH of the mobile phase and the column temperature affected the separation of the NBD-F and the derivatives. The higher the pH of the mobile phase, the better separation achieved from the interfering the samples. When the column temperature increased, the peak of GSH could not be separated from the interferences, the derivative of GSH and NBD-F is unstable, and the peak area decreases. Finally, the 23% acetonitrile and 77% phosphate buffer (0.02 mol/L, pH 6.0) combination, at a flow rate of 1.0 mL/min and a column temperature 25°C, was chosen to achieve a good peak shape, satisfactory resolution, and relatively short analysis time.

Validation of the Method

Linearity and sensitivity

Linearity was checked by preparing the standard solution of GSH at seven different concentrations (0.1–10.0 μmol/L) using the stock solution. Each solution was injected three times. The regression equation of the calibration curve obtained was $y = 37641x + 5152$ with a correlation coefficient of 0.9988, where x and y are the GSH concentration and detector signal, respectively. The limit of quantitation (LOQ), based on a signal-to-noise ratio of 10:1, was 0.10 μmol/L. The limit of detection (LOD), as a signal-to-noise ratio of 3:1, was found to be approximately 0.03 μmol/L. These small values of LOD and LOQ confirmed the high sensitivity of the proposed method. The regression line was used to calculate the concentrations of GSH in the unknown plasma samples based on the peak area.

Precision and accuracy

Intra-day precision and accuracy were evaluated by one working day in five replicates of quality control samples at three different concentrations (0.10, 1.00, and 5.00 μmol/L) of GSH, and inter-day precision and accuracy in five replicates were evaluated in three working days (Table I). Precision was presented as the relative standard deviation (RSD), and accuracy was expressed as a relative error [(concentration found – concentration added)/concentration added] × 100(%)]. Intra- and inter-day RSD were less than 6.3% and 6.9%, respectively. The results indicate that this method is reliable, reproducible, and accurate.

Analysis of the plasma samples

The proposed method was successfully applied for the determination of GSH in the healthy human plasma samples (for the respective chromatograms, see Figure 2). The mean plasma concentrations of GSH in males and females were 4.70 ± 0.93 μmol/L ($n = 24$) and 4.72 ± 0.94 μmol/L ($n = 12$), respectively. No significant difference appeared between the male and female groups. The obtained GSH concentrations in the plasma were compared with the reported data (Table II).

Table I
Intra- and Inter-day Precision and Accuracy for the Determination of GSH in Plasma

Added concentration (μmol/L)	Measued concentration (μmol/L, mean ± SD)	RSD (%)	RE (%)
Intra-day (n = 6)			
0.20	0.19 ± 0.02	5.5	−8.3
1.00	1.06 ± 0.04	6.3	4.4
5.00	5.11 ± 0.19	2.3	3.9
Inter-day (n = 12)			
0.20	0.19 ± 0.02	6.9	−9.1
1.00	1.05 ± 0.04	5.0	4.3
5.00	5.17 ± 0.30	3.3	5.9

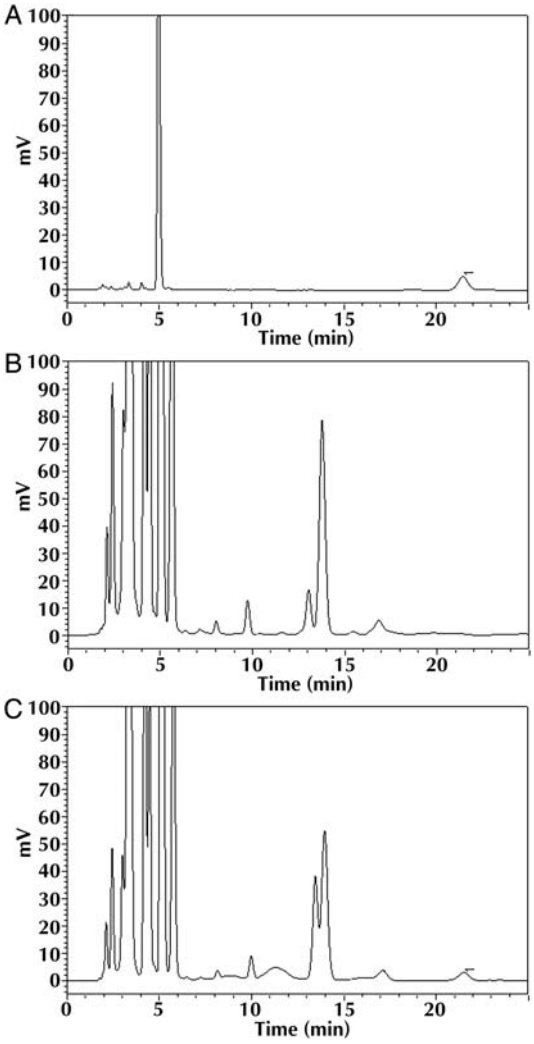

Figure 2. (A) HPLC Chromatograms standard solution of GSH (1 GSH–NBD-F 5.0 μM). (B) Blank human plasma sample. (C) Normal human plasma.

Table II
Comparison of Reported GSH Concentrations in Normal Human Plasma

Method	Glutathione concentration (μmol/L)	Reference
The current method	4.70 ± 0.92 (n = 36, aged 28.2 ± 5.0 years)	Current study
HPLC–ESI–MS–MS	2.94 ± 0.31 (n = 30)	(16)
HPLC-Fluorescence	3.39 ± 1.04 (n = 124, aged 18–73 years)	(17)
HPLC–MS	1.63 ± 0.33 (n = 10, aged 21–32 years)	(24)

The plasma GSH concentrations in physiological conditions may be affected by some life habits and different races (24). The reason that the result obtained was higher than the reported data may be because the immediate analysis after the collection of the blood samples without storage. It was found that the plasma GSH level decreased ~ 10% after it was stored at −20°C for 24 h. The plasma samples should be derivatized as soon as possible after the collection and the NBD-derivatives of glutathione can be stably stored for 72 h.

Conclusion

In summary, an optimized HPLC-fluorescence method for the determination of GSH in plasma was developed and validated. Besides the consideration of high analysis cost, MS is ideal for detecting trace levels of GSH in vivo because of its high sensitivity. The current method is more economical than MS, and compared with reported HPLC-fluorescence methods, this method has a lower limit of detection, and the derivatization procedure is finished under mild conditions without high temperatures and long derivative times, which influence the stability of GSH. In addition, only a small amount of the samples (<50 μL palsma) were needed in the analysis procedure, which indicates that it is more suitable to analyze biological and clinical samples.

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