Talanta 79 (2009) 648–656

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Second-order calibration of excitation–emission matrix fluorescence spectra for determination of glutathione in human plasma

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ARTICLE INFO

Article history:
Received 24 January 2009
Received in revised form 18 April 2009
Accepted 21 April 2009
Available online 3 May 2009

Keywords:
Glutathione
Fluorescence
Excitation–emission
Second-order calibration
Standard addition
Plasma

ABSTRACT

A rapid non-separative spectroflourimetric method based on the second-order calibration of the excitation–emission data matrix was proposed for the determination of glutathione (GSH) in human plasma. In the phosphate buffer solution of pH 8.0 GSH reacts with *ortho*-phthaldehyde (OPA) to yield a fluorescent adduct with maximum fluorescence intensity at about 420 nm. To handle the interfering effects of the OPA adducts with aminothiols other than GSH in plasma as well as intrinsic fluorescence of human plasma, parallel factor (PARAFAC) analysis as an efficient three-way calibration method was employed. In addition, to model the indirect interfering effect of the plasma matrix, PARAFAC was coupled with standard addition method. The two-component PARAFAC modeling of the excitation–emission matrix fluorescence spectra accurately resolved the excitation and emission spectra of GSH, plasma (or plasma constituents). The concentration-related PARAFAC score of GSH represented a linear correlation with the concentration of added GSH, similar to that is obtained in simple standard addition method. Using this standard addition curve, the GSH level in plasma was found to be $6.10 \pm 1.37\ \mu mol\ L^{-1}$. The accuracy of the method was investigated by analysis of the plasma samples spiked with $1.0\ \mu mol\ L^{-1}$ of GSH and a recovery of 97.5% was obtained.

1. Introduction

Glutathione (GSH) is a tri-peptide consisting of three amino acids: glutamate, cysteine and glycine. It has a key role in the defense system of many mammalian tissues. A look at the literature will introduce us to an extensive variety of functions attributed to GSH among which antioxidant activity against both endogenous and exogenous reactive species outstands [1,2]. GSH can be considered as a redox buffer [3,4]; it scavenges free radicals and detoxifies xenobiotics and is a strong defense against oxidative and nitrosative stresses [5–7]. GSH has also role in regulating cell proliferation [8] as well as cell apoptosis, the programmed death of cells [9–11]. Glutathione has two main redox forms; GSH is the reduced form of glutathione and is actually the predominant form of it over other oxidized forms [12,13]. Among the oxidized forms, GSSG is known more and is both enzymatically and non-enzymatically formed by GSH [14]. In most of the cells, GSSG accounts for only 1% of the depleted GSH whereas it comprises more than 25% of the oxidized GSH in the subcellular parts, e.g. in the mitochondria [15,16].

For some reasons, reported reference levels of glutathione seem to vary from one work to another in the literature. These differences are mainly derived from different methodologies adopted [17]. So, a good sample preparation including, for example, avoidance of unwanted hemolysis of the red blood cells (RBCs) or the storage of whole blood or plasma samples under an appropriate temperature condition [18] is of crucial importance in the assay of glutathione. In almost all of the methods for glutathione determination, a deproteinization step is necessary [14]. Apart from that, such methods mostly adopt a derivatizing agent that can be either colorimetric or fluoregenic. Sometimes when the determination of total glutathione is desired, a disulfide reduction step is also included [19]. Therefore, all these types of sample interventions can be responsible for the variations seen in the determination results. Such fluctuations from one work to another may be well recognized when blood and plasma samples are concerned; and out of these two assay environments, plasma data show more diversity; maybe because plasma undergoes more manipulations than whole blood in the preparation step [17].

There are different methodologies that are adopted in the quantification of glutathione and its analogues. These methods are classified mainly as separative and non-separative techniques. Chromatographic approaches including high performance liquid chromatography (HPLC), thin layer chromatography (TLC) and gas chromatography (GC), together with capillary electrophoresis (CE) serve as separative ones while spectrophotometric, spectrofluorimetric and electrochemical methods fall in the second category

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doi:10.1016/j.talanta.2009.04.041

[14]. Chromatographic techniques take the advantage of high specificity and selectivity for they physically neutralize the interfering effects of artifacts and when used in combination with sensitive detectors like fluorimetric ones, they become so sensitive and that is why there have been so increasing attention towards them. On the other hand, non-separative approaches are not very well reflected in the works of recent investigators mainly because of the problem of unsatisfactory specificity caused by other aminothiols present in the biological matrices. Although non-separative techniques lack enough specificity, they are very promising with respect to the matter of their simplicity and low cost if we manipulate the assay system in a way so as to somehow separate and probably recognize the interferences contributing to the final instrumental signals.

GSH and its analogues do not present any strong chromophores or fluorophores in their structures and hence, are frequently derivatized by either colorometric or fluoregenic tags to exhibit convincing sensitivity [20]. Except for the electrochemical-based detection methods which do not rely on the derivatization of glutathione, other methods lack enough sensitivity when no derivatization has been considered and therefore have either a chromophore or fluorophore introduction step in their protocols [14]. Fluoregenic labeling agents are commonly used in the assay of glutathione. They offer high sensitivity compared to UV–vis labeling agents. These fluoregenic agents are *o*-phthaldehyde (OPA) [21–25], monobromobimane (BrB) [26], fluorobenzofurazan derivatives [27,28], Rhodamine-based probes [29,30], etc., among which OPA (not fluorescent by itself) is widely used to form a highly fluorescent, stable derivative of GSH and other aminothiols. The mild laboratory conditions required for the reaction of OPA with GSH have made this reagent much attractive to be used by many investigators involved in glutathione determination [21–25,31]. The introduction of OPA as a derivatizing agent in the assay of glutathione was done by Cohn and Lyle [23] and was then modified by other authors [24]. Although OPA serves as a good labeling tag as mentioned before, it is very active towards amino acids as well as aminothiols other than GSH, putting a challenge in front of its applications in the GSH assay [17,31–35].

Since the introduction of chemometrics methods in analytical chemistry the problem of spectral overlapping has been diminished thanks to the resolving power of various multivariate calibration methods. In contrast to univariate calibration, measuring of multivariate signals per sample enables one to compensate for contributions of interferences in an unknown sample. While the first-order multivariate calibration methods are able to handle the spectral interferences of the compounds whose variations are taking into account in the calibration process [36–41], the second- or higher order data analyses methods can compensate for potential interferences not included in the calibration set [42–45]. This is universally recognized as the second-order advantage. The second-order methods need a matrix of response data per sample and thus a three-way array of data is obtained by staking the data matrices of different samples under each other. These types of data can be provided by hyphenated instruments such as HPLC-DAD, GC–MS and LC–MS, excitation–emission fluorescence spectra and spectroscopic monitoring of the reaction kinetic. Parallel factor (PARAFAC) analysis is one of the second-order calibration methods, in which the trilinearity of the measured analytical data is a necessary condition. For a detailed discussion on PARAFAC and its basis and applications, the reader is referred to the literature [42–47]. The excitation–emission matrix of fluorescence spectra is a kind of trilinear data and such data have been extensively used in the recent years to achieve second-order advantage.

In this work, we developed a non-separative spectrofluorimetric-based method, with OPA as the labeling agent for the determination of plasma GSH using PARAFAC as a second-order data analysis method. We adopted a second-order standard addition method to compensate both the contributions of other aminothiols, potentially presented in plasma, and the effect of plasma matrix on the spectrofluorimetric determination of GSH. The experimental conditions were optimized to achieve the best sensitivity for the direct determination of GSH in plasma.

2. Experimental

2.1. Reagents

o-Phthaldehyde (OPA), trichloroacetic acid (TCA), Na_2HPO_4 and EDTA were obtained from Merck Co. (Germany). GSH was purchased from Sigma Chemical Company (St. Louis, MO). Doubly distilled water was used to make disodium hydrogen phosphate buffer solution.

The buffer pH was set at 8.0 by dissolving 1.780 g of disodium hydrogen phosphate in doubly distilled water and the final volume of it was made to 100.0 mL. Apart from disodium hydrogen phosphate, 0.1 g of EDTA was also dissolved in the buffer solution to inhibit the autoxidation phenomenon of GSH which is actually common and problematic in its assay.

The mother OPA solution used for derivatization was prepared by dissolving 0.025 g OPA in 25.0 mL methanol (reagent grade, Merck) to yield a concentration of 0.1% (w/v) of OPA. This solution seemed to maintain its activity for several weeks when kept in the refrigerator. GSH stock solution was prepared by dissolving 0.015 g in 100.0 mL of phosphate buffer solution to yield a concentration of 500.0 $\mu mol\,L^{-1}$ and stored at 4 °C until used. The 10% (w/v) TCA solution was prepared by dissolving 10.0 g of cold TCA crystals in doubly distilled water and made to 100.0 mL.

2.2. Instrumentation

A Perkin-Elmer LS 50B Luminescence Spectrophotometer was used for the fluorimetric measurements. A refrigerator-equipped centrifuge model SIGMA 3K30 was used for precipitating plasma proteins at high ($10{,}000 \times g$) revolution. Data manipulation was performed employing Microsoft Excel (2003) and MATLAB 7.0.

2.3. Plasma samples and sample preparation

Daily based fresh frozen plasma (FFP) samples prepared from the venous blood of random healthy male and female blood donors of the Central Blood Transfusion Organization (Shiraz, Iran) were gathered. The process of RBC removal and other necessary steps in preparing plasma samples were done and checked by the staff of that organization. Frozen plasma samples with a temperature lower than −80 °C were put into standard cold boxes and quickly transported to the laboratory freezers until the day of experiment.

As suggested by many authors that have worked on the glutathione determination, one of the necessary steps in plasma preparation is protein removal that is done by different strategies including the use of acids (trichloroacetic acid (TCA), perchloric acid (PCA), 5-sulfosalicylic acid (5-SSA) and metaphosphoric acid (MPA)), organic solvents (methanol, acetonitrile, etc.) and ultrafiltration, among which acidic reagents are, by far, more popular than others [14]. TCA with a concentration of 10% (w/v), served as the protein precipitant in our study. Before adding appropriate volumes of acid to plasma samples, it was given enough time for the frozen plasma to gradually melt at the room temperature (25 °C). After centrifugation of acidic plasma samples at $10{,}000 \times g$ (twice for each sample) in a refrigerator-equipped centrifuge maintained at 4 °C, the supernatant liquid was carefully pipetted into laboratory tubes and frozen at −80 °C before the spectrofluorimetric measurements. Such frozen supernatants are supposed to contain GSH and