European Online Journal of Natural and Social Sciences 2016;
Vol.5, No.1 pp. 15-21
ISSN 1805-3602

Glutathione Peroxidase Activity Assay with Colorimetric Method and Microplate Reading Format and Comparison with Chemiluminescence Method

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Received for publication: 06 September 2015.
Accepted for publication: 19 December 2015.

Abstract

Glutathione peroxidase (GPX) has a key role in the protection of organisms from oxidative damage. Many diseases and disorders are associated with changes in GPX activity. Therefore, its activity assay can be crucial in prevention, diagnosis and treatment of them. Several companies produce GPX activity assay kit but it is being imported to Iran which is expensive and time-consuming. This research has been done to investigate a simple, rapid and inexpensive method for GPX activity assay. In this study, GPX reduced cumene hydroperoxide while oxidized glutathione (GSH) to GSSG. The generated GSSG was reduced to GSH with consumption of NADPH by glutathione reductase. The decrease of NADPH absorption which was proportional to GPX activity measured at 340 nm with microplate reading format. Sensitivity, precision and accuracy have been examined. The results obtained by the colorimetric method compared with chemiluminescence method and correlation coefficient has been determined. Sensitivity of this method was 15 mU/ml. The coefficient of variation percent for intra and inter assay was less than 9.7 %. According to parallelism and recovery tests, ratio % and recovery % ranged from 91% to 112% and correlation coefficient between the two methods was 0.9898 (n=60). Data from this study showed that the method has an acceptable sensitivity, precision and accuracy and can be suitable for both clinical and research studies. Indeed, this study is the first step of domestic commercial kit production purpose.

Keywords: Glutathione peroxidase, Colorimetry, Chemiluminescence, Microplate reading format

Introduction

Glutathione peroxidase (GPX or GPx) is the original name of an enzyme family which has peroxidase activity and its standard number according to the enzyme commission is EC 1.11.1.9 (Ladenstein, Epp, Bartels, Jones, Huber, Wendel, 1979). GPX was discovered by Milles (1957) in red blood cells in order to protect them from oxidative hemolysis and to stabilize their wall and shape (Mills, 1957). GPX catalyzes the reduction of lipid peroxides to corresponding alcohols and free hydrogen peroxide to water by using reduced glutathione. This function of Glutathione peroxidase is significantly important in antioxidant defense and in maintenance of the health of cells and organisms (Gallo, Martino, 2009). Glutathione peroxidase has several isozymes which are

encoded by different genes (Brigelius-Flohé, 1999). These isozymes are located in different parts of cells and have different substrate specificities and most of them are tetrameric which have a selenocysteine site in their active site (Wendel, Fausel, Safayhi, Tiegs, Otter, 1984). Glutathione peroxidase is considered as the first line of defense against oxidizing agents (Koziorowka-Gilun, Koziorowski, Strzezek, Fraser, 2011). Obviously, any malfunction of it depends on the degree, location and causes of many irreparable failures, diseases and injuries. In fact, the changes of GPX activity or concentration can be associated with various diseases such as diabetes (Taheri, Djalali, Saedisomeoli, Malekshahi, Jazayeri, Qorbani, 2012), renal failure (De Vega, Fernandez, Mateo, Bustamante, Herrero, Munguira, 2002), rheumatoid arthritis (Aryaeian, Djalali, Shahram, Jazayeri, Chamari, Nazari, 2011), cardiovascular disease (Blankenberg, Rupprecht, Bickel, Torzewski, Hafner, Tiret, Smieja, Cambien, Meyer, Lackner, 2003), cancer (Jardim, Moschetta, Leonel, Gelaleti, Regiani, Ferreira, Lopes, Zuccari, 2013), obesity and metabolic syndrome (Ghayour-Mobarhan, Aziminezhad, Ghafouri, Kazemi, 2008).

Determining GPX activity and concentration can help to prevent, diagnose or treat some diseases. Also, it is necessary in many areas of molecular and cellular research. Measuring GPX concentration is usually done with the enzyme-linked immunosorbent assay (ELISA) (Jacobson, Yee, Ng, 2007). This *technique* is able to measure small amounts of Glutathione peroxidase through a specific reaction between antigen and antibody. But, in this method there is no difference between functional enzymes and dysfunctional ones and concentrations of all Glutathione peroxidases are measured (Jacobson, Yee, Ng, 2007). For measuring GPX activity, the conventional method is spectrophotometry or colorimetry which was presented by Paglia and Valentine in 1967 (Paglia, Valentine, 1967). The basis of the method is oxidation of glutathione (GSH) and the reduction of organic peroxided by GPX, paired with oxidized glutathione (GSSG) recovery by consumption of NADPH and glutathione reductase activity. The decrease of NADPH absorption at 340 nm is proportional to the activity of Glutathione peroxidase. Chemiluminescence method is also used to assess the activity of antioxidants (Said, Kattal, Sharma, Sikka, Thomas, Mascha, Agarwal, 2003). To evaluate the GPX activity by chemiluminescence method, first the reaction of glutathione and excess amount of an organic peroxide like tert-butyl hydroperoxide (tBuOOH) or hydrogen peroxide is done by Glutathione peroxidase. Then, the excess amount of peroxide reacts with luminol resulting to emittance of photons. These photons are recorded by luminometer and become a radix for measuring enzyme activity.

GPX activity assay kit is produced commercially by several companies but it does not have domestic production in Iran and the importing process takes so much time and costs. Therefore, designing a simple, rapid and inexpensive method seems to be essential. Accordingly, this study was conducted in order to design a colorimetric method to assay Glutathione peroxidase activity. The secondary objectives of this study were applying human serum sample directly with no need for preparation, shortening the reaction time and speeding up the reading process with microplate reading format. The ultimate purpose of the study is producing Glutathione peroxidase activity assay kit in near future in Iran.

Materials and Methods

First, the required solutions were prepared by using chemical and enzymatic compounds with high purity (Sigma, United States of America) as follows:

Standard solutions

First, 1 mM NADPH standard solution was prepared from NADPH pill in diluent buffer (phosphate buffer 50 mM, pH 7.2 containing 5 mM ethylenediaminetetraacetic acid). Then, from this stoke solution, 0, 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50 and 100 µl volumes were poured into

respective microplate wells in the form of double and all wells were brought to 100 µl volume by diluent solution. Thus, the standard solution with concentrations of 0, 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50 and 100 mM were prepared in the wells. Absorbance readings at 340 nm become the radix to plot standard curve.

Positive control solution

10 µl positive control solution of Abcam kit which contains Glutathione peroxidase, plus 90 µl of diluent buffer were used.

Blank solution

Diluent buffer was used as a blank solution and 100 µl of it was poured in the respective well.

Reaction mixture solution

The reaction mixture was consisted of 10 µl glutathione (5 mM), 10 µl glutathione reductase solution (10 U/ml), 10 µl NADPH (40 mM), 10 µl cumene hydroperoxide solution (0.5 mM), and 10 µl diluent buffer.

Procedure

According to the aforementioned method, 100 µl standard solution was added to the wells related to standards. 10 µl of positive control, 10 µl of blank solution and 10 µl of the test samples were poured into respective microplate wells. Then, 50 µl of reaction mixture was added to all wells, except for the standard wells. In order to have the same volume as the final one of the standard wells, each well was reached to the volume of 100 µl with diluent buffer. Absorbance of wells at 340 nm was immediately read (A_1) by microplate reader (Sun Rise, TECAN, Austria). Afterwards, microplate was incubated for 10 minutes at 37°C. At the end of this time, absorbance of wells at 340 nm was measured again (A_2).

GPX activity related to test samples was calculated by ELISA program based on the standard curve and the difference of absorption of test sample at two different times (A_1-A_2).

To evaluate the introduced method, it was compared with the quantitative chemiluminescence assay (Chen , Zhao , Huang , Baeyens , Delanghe , He , Ouyang, 2005) that the procedure was followed.

First, 100 µl phosphate buffered saline solution 10 mM, pH 7.4 was poured to the microplate wells. Then, 10 µl of glutathione solution (2 mM) and 10 µl of the test samples were added. Afterward, 50 µl tert-butyl hydroperoxide was added and incubated for 60 seconds. At the end of the period, 50 µl luminal solution (0.5 µM) was added to all wells and its emittance was measured for 1 minute by luminometer (Lumistar, BMG, Austria).

In order to assess the validity of the method, its sensitivity, precision and accuracy were examined.

Sensitivity was calculated based on the mean zero standard signals plus three standard deviation (3SD).

To evaluate the precision, three samples in the range of low, medium and high concentrations were selected to determine the intra and inter assays. The reproducibility was calculated based on coefficient of variations (CV%). Replication number in intra and inter assay were 10 and 8 respectively.

The accuracy of the test was also evaluated by parallelism and recovery tests. To evaluate the parallelism test, first the GPX activity was measured in a sample with the method under study. Then, this sample was diluted by 2, 4, 8, 16 and 32 times. By using the expected and the measured data, ratio% of parallelism test was determined. In evaluating the recovery test, 0, 10, 25, 50, 100, 200 µl of the standard solution was added to a sample. Considering the expected and the measured

data, recovery% was calculated. To compare the results of the method under study with another one, quantitative chemiluminescence was used and the correlation coefficient was calculated.

Results

The sensitivity of the colorimetric method, on the basis of 3SD from the zero standard definition was 15 mU/ml.

Table 1 and 2 contain the data for intra assay and inter assay precisions. Regarding to that all coefficient of variations are under 10%, the introduced method has acceptable precision.

Table 1. Evaluation of precision by intra assay test

Concentration	Replication No.	Mean	SD	CV%
Low	10	55	4	7.3
Medium	10	174	11	6.3
High	10	312	18	5.8

Table 2. Evaluation of precision by inter assay test

Concentration	Replication No.	Mean	SD	CV%
Low	8	59	5	8.5
Medium	8	181	16	8.8
High	8	319	31	9.7

In evaluating the accuracy of the method, parallelism and recovery test results are provided in table 3 and 4. The results show the acceptable accuracy for the method. According to table 3, the ratio% of parallelism test is acceptable up to 1:16 dilution.

Table 3. Evaluation of accuracy by parallelism test

Step	Dilution	Expected	Measured	Ratio%
1	1:1	313	313	100
2	1:2	157	145	93
3	1:4	78	71	91
4	1:8	39	43	110
5	1:16	19.6	22.0	112
6	1:32	9.8	12.1	124

Table 4. Evaluation of accuracy by recovery test

Step	Standard Addition	Expected	Measured	Recovery %
1	0+x	175	175	100
2	10+x	93	87	94
3	25+x	100	92	92
4	50+x	113	116	103
5	100+x	138	125	91
6	200+x	188	202	108

The colorimetric and chemiluminescence methods comparison is determined by using the regression analysis. The correlation coefficient between the two methods is acceptable (n = 60; r^2 = 0.9898) (Fig.1).

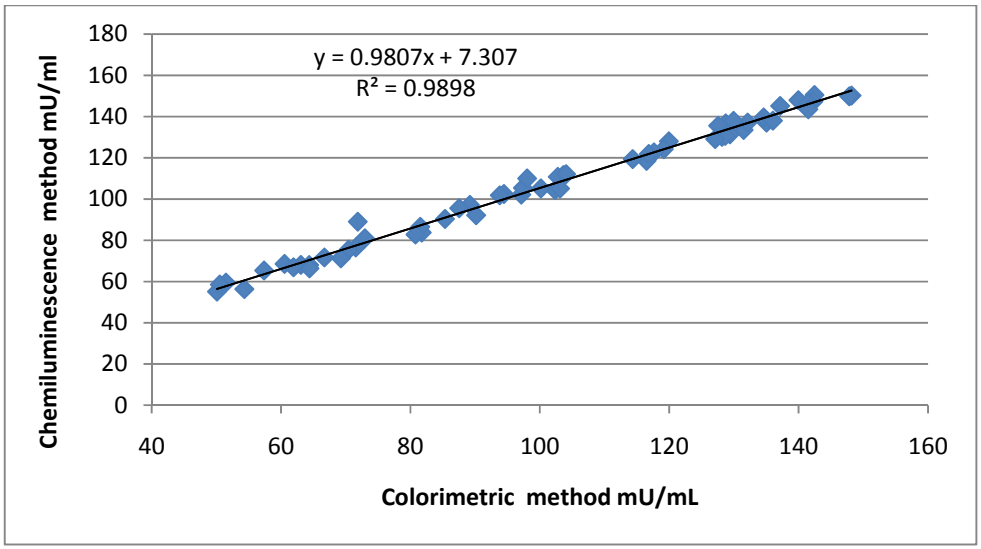

Figure 1. Evaluation of accuracy by method comparison (n=60)

Discussion

In this study, considering the importance of measuring Glutathione peroxidase activity, a simple, rapid and inexpensive method was introduced to test GPX activity in human serum samples and the results were compared with the quantitative sensitive chemiluminescence method. In fact, the basic approach of this research was using colorimetric method with microplate reading format which resulted in proper precision and accuracy.

Currently, several foreign companies produce Glutathione peroxidase activity assay kits that the basis of most of them is measuring GPX activity by colorimetric method. The basis of this method is oxidation of glutathione and reduction of an organic peroxide through the catalytic activity of GPX. GPX assay kits are made by Abcam (www.abcam.com), Cayman (www.caymanchem.com) and BioVision (www.biovision.com) companies used from the reaction between glutathione and cumene hydroperoxide to measure the activity of Glutathione peroxidase. While kit is made by BioAssay Systems (www.bioassaysys.com) company which utilizes the reaction of glutathione with hydrogen peroxide and the kit of Sigma-Aldrich (www.sigmaaldrich.com) uses the reaction between glutathione and t-butyl hydroperoxide. In this study, method of colorimetry and the reaction between glutathione and cumene hydroperoxide were used.

Measurement of GPX activity has potential clinical utility in normal and diseased populations, thus, it may be routinely measured in clinical laboratories in the future. Kits used in GPX activity measurements in laboratories and research centers in the country are completely imported and expensive. Thus, the need to design a simple, rapid and low-cost for the production of this kit in the country by Iranian researchers is essential to save time and money. This study was conducted with the same goal and that is important from the practical point of view. The proposed method is simple, rapid and inexpensive by using human serum sample directly with no need for preparation. It shortens the reaction time, speeds up the reading process and reduces the required volume of sample and reagents by microplate reading format.

The results of the validation of the method show that the method is sufficiently sensitive, precise and accurate for the determination of GPX activity. Indeed, there is no gold or consensus method for GPX activity assay and such as many other research method developments, the results of introduced method are compared with chemiluminescence which are already reported (Chen H, Zhao H, Huang L, Baeyens WR, Delanghe JR, He D, Ouyang J. 2005). The proposed method is suitable for using in clinical as well as research studies however, offering it as a ready to use kit needs more contributions.

Acknowledgments

The authors of this paper appreciate kind cooperation of Ms. Laleh Hoghooghi Rad, Ms. Hoda Golab Ghadaksaz and Dr. Mehdi Rezaeianzadeh.

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