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Smartphone-based colorimetric detection of glutathione

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Abstract

OBJECTIVES: Glutathione belongs to the family of small-molecular weight antioxidants like ascorbic acid, cysteine, α-tocopherol, uric acid, etc. These molecules play important role in the neutralization of free radicals and reactive oxygen species (ROS). Oxidative stress may lead to ageing and the development of large scale of pathological states of organism. This low molecular weight antioxidant´s level can alter under pathological conditions from reduced (GSH, thiols) to oxidized (oxidized glutathione – GSSG, disulfides) form. A GSSG-to-GSH ratio is indicative marker of oxidative stress. There is a large scale of methods how to determine this biomarker. The trend of the analysis is to minimalize the instrument equipment, sample application volume and analysis cost.

DESIGN: Reduced glutathione (GSH) solutions were prepared in water in the concentration 0-16 mmol/L. Other small-molecular weight antioxidants like 0.25 mmol/L ascorbic acid, 0.15 mmol/L TROLOX and 0.02 mmol/L N-acetyl-cysteine (NAcCys) were studied as possible interferents. The samples were mixed with 5,5´-dithiobis-(2-nitrobenzoic) acid (DTNB) resulting in yellow colored drops forming. Coloration was assayed using camera integrated in a smartphone and color channels analysis. The total volume of 10 µl of sample was applied for one analysis. The smartphone-based data were compared with the reference Ellman assay.

RESULTS: The calibration of glutathione was evaluated. The blue channel intensity data were decreasing according to the increasing glutathione concentration. Red and green channel intensities were stagnating during the whole concentration scale of glutathione. Limits of detection were 0.4 mmol/l for glutathione. Addition of 0.25 mmol/L of ascorbic acid, 0.15 mmol/L of TROLOX and 0.02 mmol/L of N-acetylcysteine to GSH in final concentration 0-16 mmol/L had minimal influence on the assay. The results from smartphone-based analysis correlate with the standard Ellman method. The detection limit for GSH was 0.03 mmol/L.

CONCLUSION: The smartphone-based assay seems to be promising because of simplicity, reliability, robustness and low cost. In spite of the fact that there is a large scale for approaches for the glutathione determination, the main advantage of our colorimetric method is portability and easibility to perform the assay in the field and publically availability of smartphones for home applications.

ORIGINAL ARTICLE

Abbreviations:

DTNB	- 5,5´-dithiobis-(2-nitrobenzoic) acid
GSH	- reduced glutathione
GSSG	- oxidized glutathione
NAcCys	- N-acetyl-cysteine
ox	- oxidized
PBS	- phosphate buffer saline
RGB	- red green blue
ROS	- reactive oxygen species
red	- reduced
Trolox	- 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

INTRODUCTION

Glutathione belongs to the family of small-molecular weight antioxidants like ascorbic acid, cysteine, α-tocopherol, uric acid, etc. This tripeptide (γ-L-glutamyl-L-cysteinyl-glycine) plays an important role especially in the neutralization of free radicals and reactive oxygen species (ROS) (Thomas, Girotti 1989), xenobiotic metabolism, signal transduction, transportation or elimination of metal ions (Ouyang *et al.* 2014), immune modulation (Błońska-Sikora *et al.* 2012). Reduced (GSH) and oxidized (GSSG) forms of glutathione occurs ant the both can influence enzyme activity, protein conformation, ligand-receptor bound, protein-protein and protein-DNA interactions (Isokawa *et al.* 2014). In the humans, the GSH/GSSG ratio correspond with ageing or some pathological states of the organism (Kuśmierek *et al.* 2011). People suffered from some diseases like schizophrenia (Yao *et al.* 2006), Alzheimer's disease (Lasierra-Cirujeda *et al.* 2013; Pocernicha & Butterfielda 2012), amyotrophic lateral sclerosis (Chili *et al.* 1998, D'Alessandro *et al.* 2011), Parkinson's disease (Isokawa *et al.* 2014), Huntington's disease (Ribeiro *et al.* 2012), cardiovascular diseases e.g. atherosclerosis (Toyo'oka 2009), leukemia (Isokawa *et al.* 2014), Diamond-Blackfan anemia (Utsugisawa *et al.* 2016), pulmonary diseases e.g. asthma, idiopathic pulmonary fibrosis, cystic fibrosis (Rahman & MacNee 2000), emphysema (Engelen 2000), chronical obstructive pulmonary disease (Biljak *et al.* 2010), adult respiratory distress syndrome, reoxygenation injury (Michelet *et al.* 1995). AIDS (Toyo'oka 2009), cancer, Diabetes Mellitus (Isokawa *et al.* 2014), alcoholic liver disease (Altomare *et al.* 1988), cataract (Sun *et al.* 2015), rheumatoid arthritis (Quiñonez-Flores 2016; Michelet *et al.* 1995), and periodontitis (Bains & Bains 2015) have typically altered GSH/GSSH ratio shift. Due to the fact that the glutathione level is variable according to the age or illness, it is important to develop suitable method for GSH determination.

Generally, a lot of analytical approaches for glutathione and thiols determination has been developed so far. GSH level in biological samples can be analyzed with liquid chtomatography (Buchberger & Winsauer 1987; Guan X *et al.* 2003; Kuśmierek *et al.* 2011; Xie *et al.* 2013). or gas chromatography (Tsikas *et al.* 2016), capillary electrophoresis (Kuśmierek *et al.* 2011; Soga *et al.* 2006, Hodáková *et al.* 2015). UV (Buchberger & Winsauer 1987), fluorescence (McMenamin *et al.* 2009), mass spectrometry (Guan *et al.* 2003), liquid chromatography – mass spectrometry (Xie *et al.* 2013), capillary electrophoresis – mass spectrometry (Soga *et al.* 2006), capillary chromatography-laser induced fluorescence (Hodáková *et al.* 2015), or electrochemical detector (Mitton Trevithick 1994) are used as well.

Thiol group of GSH interacts strongly with some metal ions (Ag, Au, Hg) or aldehyde group. Colorimetric, chemiluminiscent and electrochemical assays are based on this principle (Ouyang *et al.* 2014). Red/ox characteristics of glutathione are used in electrochemical techniques e.g. square wave voltammetry (Pohanka *et al.* 2011), cyclic voltammetry (Arteaga *et al.* 2012), differential pulse voltammetry (Hai *et al.* 2015).

Cappiello *et al.* used colorimetric methods based on the specific reaction catalyzed by γ-glutamyltransferase to form γ-glutamyl derivative and cysteinylglycine. Second product is a substrate for leucyl aminopeptidase to form glycine and cysteine that is measured spectrophotometrically (Cappiello *et al.* 2013). Other authors choose 5,5'-dithiobis-(2-nitrobenzoic acid) (Owens & Belcher 1965) to form yellow colored product according to Ellman method from 1950s (Ellman 1959; Pohanka *et al.* 2016).

In this paper, we utilized the yellow colored product from Ellman reaction (Ellman 1959) and combined it with the smartphone (Pohanka 2015). Due to the fact the smartphones are widespread in nowadays population, this assay can be also applicable at home or in the field. The sample amount can be minimized. RGB parameters were evaluated and blue channel signal was the best one for the glutathione determination.

MATERIAL&METHODS

Chemicals

All chemical were at least of analytical grade. GSH, 5,5´-dithiobis-(2-nitrobenzoic) acid (DTNB; 4 mg/mL), NAcCys, TROLOX, phosphate buffer saline (PBS; 10 mmol/L, pH 7.4) were purchased from Sigma-Aldrich, Saint Louis, Missouri, USA. Ascorbic acid was bought from Penta, Prague, Czech Republic. Solutions of GSH, NACCys, TROLOX and ascorbic acid were prepared in demineralized water (Aqua Osmotic Device, Tisnov, Czech Republic). DTNB was dissolved in PBS.

Smartphone-based assay

Prior to the analysis, the paper strips were prepared from the filter paper (Whatman, Little Chalfont, UK). 10 μL of the sample of glutathione was pipetted on the paper target and mixed with 10 μL of DTNB. After 15 minutes, the yellow drops were detected with the camera in automatic option using the smartphone XPERIA, SONY Ericsson, Tokyo, Japan. The smartphone was placed on the telephone holder designed and made with three-dimensional printing technology from

acrylonitrile butadiene styrene using 3D printer Prusa i3 (Prusa Research; Prague, Czech Republic) in our lab. The instrumental arrangement is shown in Figure 1.

Spectrophotometry – Ellman technique

400 µL of DTNB in concentration of 0.4 mg/mL was added to reference samples of glutathione and ascorbic acid, TROLOX and N-Ac-cys . The yellow colored samples in cuvettes were inserted into the spectrophotometer Genesys 10 S from Thermo Scientific (Waltham, Massachusets, USA) and measured at the wavelength 412nm.

Data processing

The snapshots in jpg format taken with the camera in the smartphone were evaluated according to the color intensity for red, green and blue (RGB) channels. The values of the intensity were in the range of 0–255. For one sample the combination of three values of the color intensity were obtained. The principle of multivariate image analysis was described previously by JM Prats-Montalbán (Prats-Montalbán *et al.* 2011; Pohanka 2015).

For one concentration of glutathione 10 samples were dropped on the filtration paper. From one drop three combination of RGB data were obtained. The data from the graphical software were farther processed in Origin 9.1 (OriginLab Corporation, Northampton, MA, USA). The absorbance values from Ellman technique were processed in Origin 9.1 as well.

The limit of detection was calculated from signal *to* noise ratio (*S/N*=3).

RESULTS

Calibration was made as described in methodic part. While the blue channel intensity data were decreasing with the increasing glutathione concentration, red and green channel intensities were similar for particular concentrations of glutathione. Limit of detection in blue channel was equal to 0.4 mmol/L for GSH. An example of calibration of glutathione is depicted in Figure 2.

Fig. 1. Instrumental arrangement of the smartphone and our designed and made holder for taking snapshots of the samples.

Influence of 0.25 mmol/L of ascorbic acid, 0.15 mmol/L of TROLOX and 0.02 mmol/L of N-acetylcysteine were also studied in combination with the concentration of GSH equal 0, 0.25, 0.5, 1, 2, 4, 8, 16 mmol/L. Ascorbic acid, TROLOX, N-acetylcysteine were used as standard small molecular weight antioxidants and had minimal influence on the determination of GSH as shown in Figure 3.

Spectrometric Ellman determination of GSH served as reference method for validation purposes. The calibration curve was prepared from the concentration of GSH 0, 0.03125 0.0625, 0.25, 0.5, 1, 2 mmol/L. The yellow colored solution in cuvettes of glutathione was measured immediately at the wavelength 412 nm. Possible interferents like ascorbic acid, TROLOX, N-acetylcysteine had minimal influence on the analysis of GSH as can be seen from Figure 4. Moreover, presence

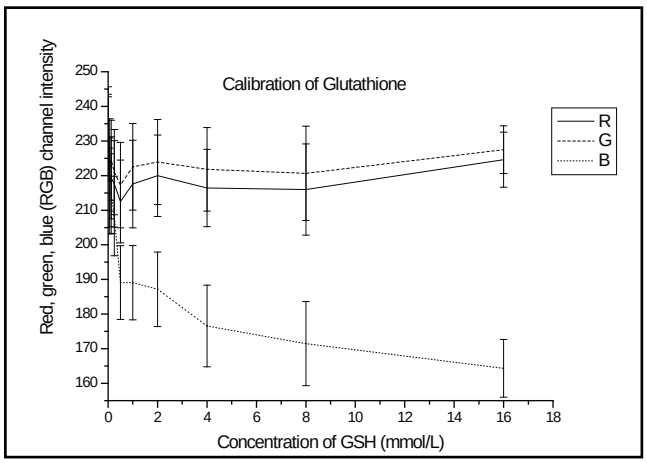

Fig. 2. Calibration of glutathione. Red, green, blue (RGB) channel intensity data were evaluated in silico. Detection limit of blue channel intensity was 0.4 mmol/L.

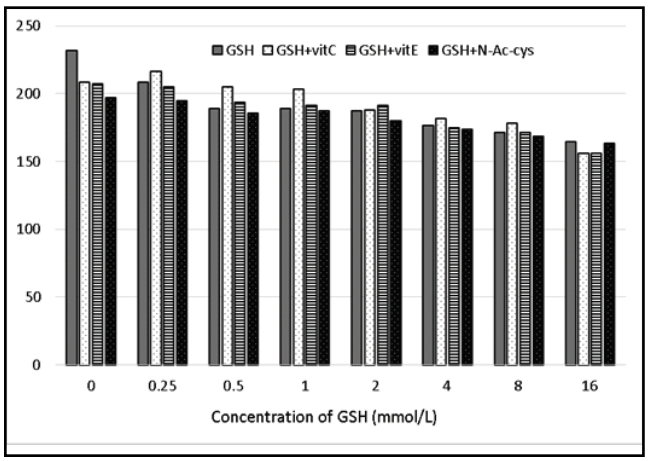

Fig. 3. Interference of ascorbic acid, TROLOX and N-Ac-Cys (color intensity assayed with the smartphone vs concentration of GSH is expressed here).

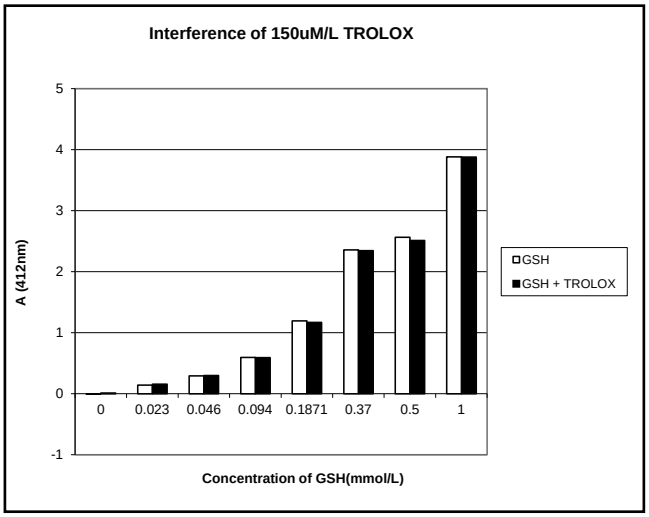

Fig. 4. Interference of TROLOX to the GSH dermination using the standard Ellman technique.

of the aforementioned compounds had no effect on limit of detection which was retained on the same level like in the case of the standard calibration performed in Figure 3.

DISCUSSION

Nowadays the multivariate image analysis is going up. The pictures are characterized with three color (RGB) channels per each pixel. Thanks to the multivariate image analysis it is possible to extract important information from the snapshot. We evaluated the intensity of yellow color of the solution of glutathione with DTNB in particular points of glutathione calibration. Generally the particular glutathione concentration in every point of calibration is characterized with three characteristics – R, G, B channel intensities. The multivariate image analysis helps us to better understand instrumental conditions. For glutathione determination the blue channel intensity was the best. With the increasing glutathione concentration the blue channel intensity decreased. The intensity of two other channels was similar for all concentrations of glutathione.

We observed minimal influence of small-molecular weight antioxidants like ascorbic acid, TROLOX or N-Ac-Cys on the glutathione measurement with both colorimetric assays. Moreover, the detection limit is lower for spectrophotometric than for the smartphone-based detection. The amount of the sample was forty times smaller for smartphone-based method when compared to the standard method.

Both methods are sensitive enough to determine GSH level in tissues (1–10 mmol/L) as reported by Tipple and Rogers (Tiple, Rogers 2012). The limitation of both methods is insufficient for assessment of low concentration of GSH in plasma (1–10 μmol/L). On the other hand, GSH has minimal physiological role in the plasma and its major task is to protect intracellular milieu where huge concentration of the both GSH and GSSG is presented. Hence the smartphone based assay presented in this paper could be suitable for the whole blood or tissue samples rather than plasma or serum.

CONCLUSIONS

In the future, the smartphone-based assay seems to be promising in medical research,Because the fact that a large scale of chronic diseases are associated with glutathione concentration. It is simple to get results of glutathione level in a few minutes with minimal computational and instrumental equipment. Nextadvantages of this technique are simplicity, reliability, and robustness. In spite of the fact that there are methods for the glutathione determination, the main advantage of our colorimetric method is portability and simplicity in necessary equipment. The proposed assay can be easily performed under field conditions or at home care.

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