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ORIGINAL ARTICLE

A Modified d-ROMs Method for Determination of Lipid Peroxidation

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The need for simple and low costing and less time consuming methods for the determination of lipid peroxides and oxidative stress is urgent because oxidative stress is still unable to become a routine laboratory biomarker due to the sophisticated procedures and costing, a matter that made oxidative stress a research only biomarker, despite its crucial value as a risk factor and pathologic status, for many inflammatory and metabolic diseases. This work aimed at developing a modified rapid method to determine serum lipid peroxides at wavelength 492 nm and reference wavelength 630 nm. The results showed linear responses with increasing concentration and that the cheap N,N-DM-P-Phenylenediamine diHCl can be used as a chromogen under saving conditions of pH, temperatures and volume of sample.

Key words: determination of lipid peroxides, modified rapid method

Research on the complications associated with diabetes has grown over decades. The correlation of uncontrolled diabetes to other comorbidities is a point of research and oxidative stress is among the central points of investigation. As evidences are growing that reactive oxygen derivatives are predictive markers on recent health status and even future events in inflammatory disease, such as Asthma (Nakamoto *et al.*, 2016) Therefore reliable and simple methods for the determination of total lipid peroxides are of great importance, since traditional methods, such as (TBARS) is based on measurement of metabolic end products end product; malondialdehyde (MDA) (Khoubnasabjafari *et al.*, 2015) is time consuming, sophisticated and vulnerable to freeze thawing variations. While the more precise methods such as HPLC based, makes them less practical to small laboratories and time consuming, besides reagent exhaustion. The new derivatives of reactive oxygen metabolites; d-ROM methods (Janero & Burghardt, 1988) based on real-time measurement of lipid peroxides capable of creating coloured radicals from an aryl amine derivative is simpler, less costing, however a spectrophotometric method needs a large volume of buffer and less effective at saving time. Therefore, there is a need for micro-plate methods to make these methods more time saving and makes the measurement of oxidative stress more feasible in the future, especially in low income countries. Some methods developed to determine lipid peroxides by N,N-dimethyl-p-phenylenediamine by colorimetric techniques (Saha *et al.*, 2015). Our method is based on a modified d-ROMs method which utilizes the presence of iron ions in solution to enhance the formation of radicals from hydrogen peroxide and super oxide in serum by Fenton reaction, which depends on the available iron in seum. The addition of iron ions enhances the Fenton reaction to overcome inter-individual variations due to free iron availability.

Our work aimed at modifying a modified d-ROMs method from that of Fumiaki Ito et al (Ito *et al.*, 2017) to be applicable to micoplate ELISA readers and the use of the less costing chromogene; N,N-dimethyl paraphenylenediamine instaed of the more costing chromogen N,N-diethyl-paraphemylenediamine and make the method simpler and give reliable results.

MATERIALS

N,N-dimethyl-p-phenylenediamine HCL was from Avondale Laboratories, England, M W = 209.12 g. iron $^{++}$ sulphate heptahydrate from BHD, India, MW = 278.1 g

Acetic acid from BDH, India, MW= 60.02 gm, weight per ml= 1.048-1.05 g.

Sodium acetate trihydrate from Labogens, India, MW = 136.08 g of which

Reagents preparation

Chromogen:

A solution of 0.1 M concentration of N,N-dimethyl-p-phenylenediamine diHCl was prepared by dissolving 0.209 g in a 10 ml total volume of water.

Buffer:

An acetate buffer pH 5 by mixing .01 M acetic acid and acetate solution according to Hendersen-Hasselbach equation and the solution pH was confirmed by bench top pH meter (HANNA UI 110, Italy).

H_2O_2 Standards:

a series of 7 concentratios; .4 ug/ml to 4 ug/ml of Hydrogen peroxide were prepared from a 6% lab grade solution from Labogens India. The standards stock solutions : S1 0.4, S2 0.8, S3 1.2, S4 1.6, S5 2.0, S6 3.0, S7 4 µg/ml

METHODS AND RESULTS

In a plain micoplate, in each well used, we added in order; 200 µL of buffer 10 µL of Fe SO_4. 7 H_2O, 10 µL of Chromogen with mixing. In specified wells 10 µL standard was added with mixing in series from .4 to 4. Blanks and standards were added in triplicates. The plate was incubated for 5 minutes at 37 C^0.

The Instrument used: Mindray MR. 96 China at wavelength 492 as primary wavelength and 630 nm as secondary wavelength.

Readouts were printed and entered into Micosoft Excel 2010. A linear calibration curve was obtained, the R^2 obtained 0.986. The pink to red colour formed by the reaction between the peroxide and the chromogen is stable for 20-30 minutes. The test is endpoint assay.

Table 1. Readouts from Mindray MR. 96 China at wavelength 492 as primary wavelength and 630 nm as secondary wavelength

Standard curve		λ= 492 nm, Reference wavelength 630 nm	
H_2O_2 conc ug/ml	Absorbance (Abs)	Blank Abs	Corrected Abs
0.4	0.216	0.081	0.116
0.8	0.3		0.2
1.2	0.412		0.312
1.6	0.436		0.336
2	0.52		0.42
3	0.638		0.538
4	0.821		0.721

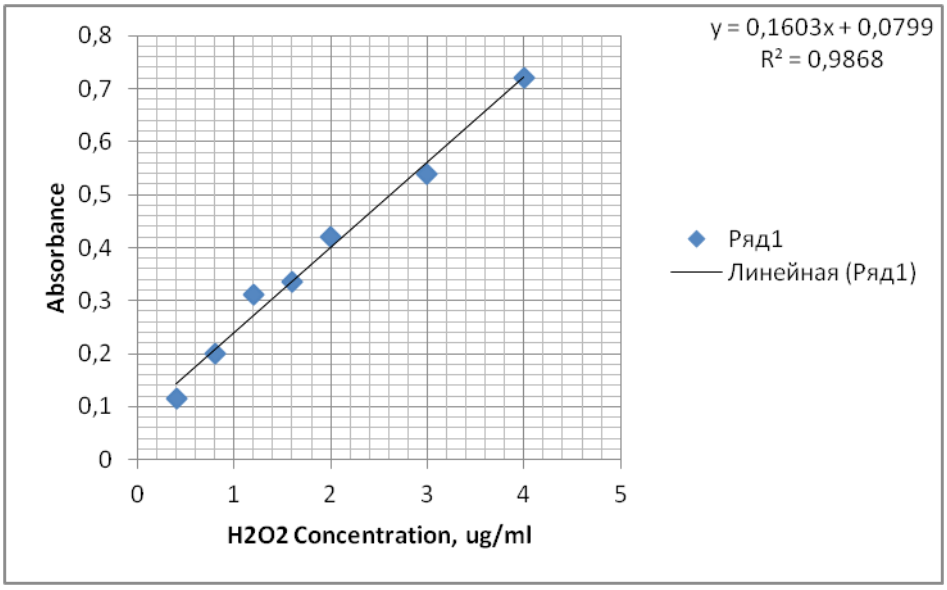

Figure 1. A linear calibration curve

CONCLUSION

N,N-dimethyl-p-phenylenediamine can be used in microplate assay to get coloured radicals with intense absorbance at 492nm for d-ROM estimation of lipid peroxidation.

CONFLICTS OF INTEREST

The authors declare that they have no potential conflicts of interest.

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