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A simple automated procedure for thiol measurement in human serum samples

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Procedimento automatizado simples para determinação de tióis em amostras de soro humano

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key words

Thiol groups
Total antioxidant capacity
TBARs
Oxidative stress
Automation

abstract

Thiol groups have been described as the main responsible for antioxidative effects of plasmatic proteins. Also, thiol serum levels have shown a positive correlation with total antioxidant capacity (TAC) in many studies. Measurement of TAC by substract oxidation-based methods have been widely used as a reference to measure antioxidant status; however, in many cases these methods are inexact or imprecise, usually when performed by manual procedures. In this paper we describe a simple automated procedure for the determination of total thiols in serum, which was based on Ellman's method. It was correlated with several markers of oxidative/antioxidative status, such as TAC and thiobarbituric acid reactive substance test (TBARs). Serum thiol levels were correlated positively with TAC ($r = 0.298$, $p < 0.001$) and negatively with TBARs levels ($r = -0.330$, $p < 0.001$). The novel automated procedure for thiol groups measurement can be a great tool in estimation of antioxidant status together with TAC assay. This procedure makes the determination of total thiol groups in large scale possible in clinical chemistry or research laboratories where this approach is necessary.

resumo

Os tióis são descritos como os principais responsáveis pelos efeitos antioxidantes das proteínas plasmáticas. Além disso, diversos estudos mostram uma correlação positiva entre os níveis séricos de tióis e a capacidade antioxidante total (CAT). A medida da CAT por métodos baseados na oxidação de substratos tem sido amplamente usada como referência na estimativa da capacidade antioxidante em amostras biológicas; porém, em muitos casos esses métodos são inexatos e imprecisos, principalmente quando realizados por procedimentos não-automatizados. Neste artigo descrevemos um procedimento automatizado simples para a determinação de tióis totais no soro, com base no conhecido método de Ellman. A dosagem dos tióis foi correlacionada com diversos marcadores da capacidade oxidante/antioxidante, como a CAT, o teste das substâncias reativas ao ácido tiobarbitúrico (TBARs) e os níveis de peróxidos totais. Os tióis correlacionaram-se positivamente com a CAT ($r = 0{,}298$; $p < 0{,}001$) *e negativamente com os níveis de TBARs* ($r = -0{,}330$; $p < 0{,}001$). *O procedimento aqui descrito para a dosagem de tióis pode ser uma ferramenta importante na medida da capacidade antioxidante juntamente com a dosagem da CAT. A utilização desse novo procedimento possibilita a determinação dos tióis totais em larga escala por laboratórios clínicos e de pesquisa onde essa demanda é necessária.*

unitermos

Tióis
Capacidade antioxidante total
TBARs
Estresse oxidativo
Automação

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Introduction

Oxidative stress is a commonly used term to denote the imbalance between the concentrations of reactive oxygen and nitrogen species and the antioxidative defense mechanisms of the body[11, 13, 14, 21]. An antioxidant is a substance that is able to protect a substrate susceptible to oxidation from peroxidative injury, being itself present at fairly low concentrations in relation to the substrate[12, 29]. Oxidative stress has been associated with a number of human diseases, such as cancer, coronary heart disease, arthritis, diabetes, cataract and degenerative processes[3, 5, 9, 19]. Over time, humans are continuously exposed to oxidants of both exogenous and endogenous origins[9]. The determination of the oxidative stress status of a person not only allows comparison with the average of a population, but can also indicate the risk of suffering disorders and diseases that are product of oxidative stress[2].

Thiols are those compounds which contain the sulfhydryl group (- SH) attached to a carbon atom. They are endogenous molecules that assist aerobic cells maintaining a reducing state, despite an oxidizing environment[9, 25]. Thiols are extraordinarily efficient antioxidants protecting cells against consequences of damage induced by free radicals, due to their ability to react with the latter[3, 27]. Both intracellular and extracellular redox states of thiols play a critical role in the determination of protein structure and function, regulation of enzymatic activity of transcription factors and antioxidant protection[29].

Thiol groups are found in all body cells and are indispensable for life[3, 24]. Some sulfur-containing antioxidant compounds are cysteine, methionine, taurine, glutathione, lipoic acid, mercaptopropionylglycine, N-acetylcysteine, and the three major organosulfur compounds of Gallic oil (diallyilsulfide, diallyldisulfide and diallyltrisulfide)[3, 10]. Proteins constitute the main antioxidant component of serum and their sulfhydryl groups are mainly responsible for their antioxidative effects. In a recent study it was calculated that SH protein groups contribute 52,9% to the measured serum total antioxidant capacity in healthy subjects[17].

The standard method for measuring human serum thiols was originally described by Ellman[15] and further modified by Hu[22]. However, the measurement of thiols and disulfides in this sample has proven to be difficult. Most thiols are present in low concentration (0.1-10nmol/mL) and are very unstable in the isolated serum[16]. Determining a new method for thiol measurement has been the focus of many researches. High-performance liquid chromatography (HPLC) is the most popular method for measurement of isolated thiol contents. This method, however, has some disadvantages, including being time-consuming and having complicated derivatizations. Also, HPLC-based method usually consumes more reagents and is, therefore, high costly[4].

In the present paper we describe a novel automated method for determination of thiols in human serum samples, which was based on Ellman's method, and was adapted to a chemical analyzer COBAS MIRA® Plus (Roche Diagnostic Systems, Welwyn, UK). The levels of total thiol groups were directly proportional to total antioxidant capacity and inversely proportional to serum levels of malondialdehyde (MDA). That new methodology makes the determination of serum thiols in large scale possible in clinical chemistry or research laboratories.

Material and methods

Chemicals

Trolox® (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was obtained from OXIS International Inc. (USA). Xylenol orange (o-cresosulfonphthalein-3,3-bis [sodium methyliminodiacetate]), ferrous ammonium sulfate, ferric chloride, hydrogen peroxide (H_2O_2), sulfuric acid, hydrochloride acid, butylated hydroxytoluene (BHT), thiobarbituric acid (TBA), 1,1,3-3-tetramethoxypropane (malondialdehyde bis [dimethyl acetal]), ethylenediamine tetraacetic acid (EDTA) and sorbitol were purchased from Sigma Chemical Co. (St. Louis, Mo, USA) and Merck Co. (Darmstadt, Germany). All chemicals were ultrapure grade, and type I reagent grade deionized water was used.

Subjects and blood samples

A total of 244 apparently healthy volunteers (122 female and 122 male) were included in this study. The median age of the entire group was 42 years with a range of 18-91 years. All subjects gave oral informed consent and the study was approved by the local ethical committee of Universidade Federal do Amazonas (UFAM). A 5mL blood sample was collected under 12-hour fasting conditions, in blood collecting test tube (Vacuum II, Villfend Corporation), and the serum was separated by centrifugation at 2.500rpm for ten minutes and stored at -20°C until biochemical determinations.

Laboratory measurements

Biochemical analyses

The serum total concentrations of total protein (TP), albumin (ALB), uric acid (AUC), bilirubin (BIL), magnesium (Mg) and iron (Fe) were measured by using commercial kits (Labtest Diagnóstica, Minas Gerais, Brazil) on the Express Plus biochemical analyzer (Ciba-Corning Diagnostics, Medfield, USA). The intra-assay coefficient of variation was evaluated by performing 15 repetitions in a single analytical run, using serum of healthy subjects. The inter-assay coefficient of variation was evaluated in triplicate (on three different dispensing cycles) in eight different analytical runs using serum of healthy subjects. The same procedures were used for manual and novel automated assay.

Thiol groups assay

Total serum thiol concentration or sulfhydryl groups (SH) were measured by the methods originally described by Elmman[15] and modified by Hu[22]. Here, thiols interact with 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB), forming a highly colored anion with maximum peak at 412ηm (e412 = 13,600$M^{-1}cm^{-1}$). Here, for the first time this method was adapted to an automated biochemistry analyzer (COBAS MIRA® Plus). **Table 1** resumes the main parameters for COBAS MIRA® Plus programming. In manual protocol, as described by Hu[22], an aliquot of fresh serum (25µL) was mixed with 1mL Tris-EDTA buffer (0.25mmol/L Tris base, 20mmol/L EDTA, pH 8.2), and absorbance at 412ηm was read (A1). Next, a 25L aliquot of DTNB stock solution (10mmol/L in absolute methanol) was added to the solution. After 15 minutes at ambient temperature, the absorbance was read again (A2) together with a DTNB blank (B). The concentration of sulfhydryl groups was calculated by using reduced glutathione as sulfhydryl group standard and the result was expressed in mmol/L.

Total antioxidant capacity assay

The TAC of serum was measured by a method based on 2, 2-azinobis 3-ethylbenzothiazoline-6-sulfonate (ABTS)[17]. In this assay, ABTS is incubated with potassium persulfate to produce oxidation of ABTS. Briefly, an amount of 10mg of ABTS was dissolved in 10mL of an aqueous solution containing 2.5mmol/L potassium persulfate and allowing the mixture to stand in the dark at room temperature for one to four hours before use. For the study of samples, ABTS oxidized stock solution was diluted with deionized water to an absorbance of 0.70 at 734m. After addition of 1mL of diluted ABTS oxidized to 10µL of serum, the absorbance reading was taken ten minutes after initial mixing. The TAC was calculated using Trolox® as standard and the result was expressed as mEq Trolox®/L.

Ferric-xylenol orange (FOX_1) assay

The total peroxide concentrations were determined using the FOX_1 method[19]. The FOX_1 test system is based on the oxidation of ferrous iron to ferric iron by the various types of peroxides contained in plasma samples in the presence of xylenol orange, which produces a colored ferric-xylenol orange complex whose absorbance can be measured. FOX_1 reagent was prepared with 100µmol/L xylenol orange, 4mmol/L butylated hydroxytoluene, 25mmol/L sulfuric acid and 250µmol/L ammonium ferrous sulfate in a HPLC grade methanol:water (9:1 v/v) solution. An aliquot of 200µL of serum was mixed with 2mL of FOX_1 reagent, vortexed and incubated at room temperature for 30 minutes. The tubes were than centrifuged at 2.500rpm for ten minutes. Absorbance of the supernatant was measured at 560ηm. The total peroxide content of the plasma samples was determined by the difference in absorbance between test and blank samples using a solution of H_2O_2 as standard. The results were expressed in µmol H_20_2/L.

Table 1 Main parameters for thiol groups measurement using the automated biochemical analyzer COBAS MIRA® *Plus*

Measurement mode	Absorbance
Reaction mode	R-S-SR1
Calibration mode	Slope AVG
Wavelength	405nm
Calc. step A	Endpoint
Sample volume	5µL
Reagent 1	200µL (Tris-EDTA buffer [0.25mM Tris base, 20mM EDTA, pH 8.2])
Reagent 2 (start)	2.5µL ([DTNB solution [10mM in absolute methanol])

Thiobarbituric acid reactive substance (TBARs) assay

Malondialdehyde (MDA) serum levels were determined as described previously by TBARs spectrophotometric test[3]. A portion of serum (200µL) was mixed with 2mL of a solution containing 15% (w/v) trichloroacetic acid, 0.38% (w/v) thiobarbituric acid and 0.25N of hydrochloric acid (HCl). The mixture was heated at 100°C for 30 minutes and, after centrifugation, the absorbance was measured at 535ηm. The total MDA content of the serum samples was determined by the difference in absorbance between test and standard samples using a solution of MDA as standard. The results were expressed as µmol TBARs/L.

Statistical analysis

All parameters were given as mean ± standard deviation (SD). A paired sample *t* test was used to estimate differences between male and female subjects. The Mann-Whitney rank sum test was also used for analysis when the data were not normally distributed. Correlations between different measured parameters were also examined by Pearson correlation test. The tests were performed using the SigmaStat 2.03 software (Jandel Scientific, Chicago). Statistical significance for all tests was accepted at $p < 0.05$.

Results

Table 2 shows the main parameters as age and oxidative stress index of 244 healthy subjects of this study. It also shows that when separated by sex no variation was shown for the majority of the analyzed parameters, except for albumin, uric acid and iron, as was already believed (t test, $p < 0.05$). The average levels were also in accordance with those described in literature.

Table 3 shows Spearman correlation indices between serum markers of oxidative stress. There we can see that there was a statistically significant positive correlation between serum levels of thiol groups and TAC, total peroxides (LOOH) and ALB. The serum levels of thiols were also negatively correlated with the levels of TBARs and Mg. Thiol serum levels were positively correlated with TAC ($r = 0.298$, $p < 0.001$) and negatively correlated with malondialdehyde levels, an oxidant status marker ($r = - 0.33$, $p < 0.001$). The intra-assay and inter-assay coefficients of variation to novel automated thiol measurement were 3.8% and 6.7%, respectively. When manual procedure was used, the intra-assay coefficient of variation was 8.5% and the inter-assay, 10,2%. The detection limit for automated procedure was 0.05mmol/L.

Discussion

In the last years, several methods have been developed to assess the total antioxidant capacity of human serum or plasma because of the difficulty in measuring each antioxidant component separately, and the interactions among different antioxidant components in serum or plasma[1, 20, 28, 30]. The total peroxyl radical trapping parameter assay of Wayner *et al.*[28] was the assay of

Table 2 Age and oxidative stress index of healthy subjects

Parameter	Male (n = 122)	Female (n = 122)	p
Age (y)	29 ± 12	27 ± 14	0.544
Thiols (mmol/L)	0.48 ± 0.18	0.49 ± 0.17	0.655
Total antioxidant capacity (mEq Trolox®/L)	1.39 ± 0.3	1.42 ± 0.4	0.465
Total peroxides (µmol/L)	50.4 ± 43	53.8 ± 48	0.749
TBARs (µmol/L)	5.41 ± 5.4	5.32 ± 6.7	0.103
Total proteins (mg/dL)	6.6 ± 1	6.8 ± 0.8	0.203
Albumin (mg/dL)	3.9 ± 0.9	4.3 ± 0.9	0.037
Uric acid (mg/dL)	5.3 ± 2.4	4.9 ± 2.8	0.036
Bilirubin (mg/dL)	1.5 ± 2.8	1.2 ± 2.1	0.688
Magnesium (mg/dL)	1.8 ± 0.5	1.8 ± 0.6	0.539
Iron (mg/dL)	94 ± 47	81 ± 37	0.028

Table 3 **Spearman correlation indices between serum markers of oxidative stress**

	Thiols	TAC	LOOH	TBARs	TP	ALB	UAC	BIL	Mg	Fe
Thiols	-	0.298**	0.16*	-0.33**	0.111	0.133*	-0.066	0.119	-0.289**	-0.035
TAC		-	0.2*	0.252**	0.083	0.049	0.021	-0.079	-0.216**	-0.072
LOOH			-	-0.225**	-0.005	-0.105	-0.098	0.022	0.046	0.048
TBARs				-	-0.131	-0.264**	-0.255**	0.15*	0.242**	-0.094
TP					-	0.611**	0.177**	-0.054	0.098	0.319
ALB						-	0.201	-0.099	0.105	0.347**
UAC							-	-0.082	0.03	0.206*
Mg							-	-	0.061	
Fe									-	

*p < 0.05; **p < 0.001.

antioxidant capacity most widely used during the last decade. As Rice-Evans and Miller[26] pointed out, the major problem with the original total peroxyl radical trapping parameter assay lies in the oxygen electrode endpoint: an oxygen electrode will not maintain its stability over the period of time required. More recently, the Trolox® equivalent antioxidant capacity (TEAC) assay was developed by several authors[6-8, 17, 23]. The TEAC assay is based on the inhibition by antioxidants of the absorbance of the radical cation of 2,29-azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS). In case of determination of serum total antioxidant capacity, the main problem in this assay is the instability of oxidized ABTS, turning it difficult to be measured in large scale[17].

An important piece of data presented in this article was the use of an automated procedure for serum total thiol measurement as one more tool in the determination of antioxidant status marker together with the use of traditional markers of this parameter, such as TAC. The results of this study demonstrate, firstly, that the procedure for dosage of serum thiols can be easily automated in the chemical analyzer COBAS MIRA® Plus, an analyzer currently used in laboratories of clinical chemistry. On the other hand, this novel procedure significantly reduces the costs of the assay, because the volume of reagent used in the automated assay is about three times smaller than that in the manual procedure. Another advantage of the automated procedure is the reduction of analytical errors when an automated analyzer is used. In the present study, there was a reduction of the variation intra-assay coefficient of 8.5% in the manual procedure to 3.5% using the automated method. Here, after analysis of 244 samples, the serum levels of total thiol groups were correlated to TAC. Using the Pearson test, there was a positive correlation between serum levels of thiol groups and the TAC ($r = 0.298$, $p < 0.001$). Similar results have been shown in recent publications[13, 17].

In conclusion, the present study showed that the measure of serum thiols can be easily adapted to the chemical analyzer COBAS MIRA® Plus, diminishing costs and increasing the efficiency of the method, what makes the use of this procedure in large scale possible for population studies. The measurement of thiol groups also showed a positive correlation to the dosage of TAC, which indicates that this measurement can be used as an auxiliary or substitute to TAC assay in studies or physiopathological conditions in which it is needed to evaluate the total antioxidant status individually.

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