QUANTITATIVE ANALYSIS OF MALONDIALDEHYDE IN NORMAL HUMAN PLASMA USING FLUORESCENCE AND THE STANDARD ADDITION METHOD

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Abstract

Oxidative stress has been mentioned among the toxic mechanisms of benzodiazepines at the brain level. Additionally, lipid peroxidation leads to the generation of numerous aldehydes, thus transforming them in oxidative stress markers. Malondialdehyde (MDA) was considered for many years to be a reliable marker to indicate the level of free radicals, and that is why the development of robust methods to quantify MDA in biological matrices was and is still considered a priority for clinical biochemistry. The present paper deals with a simple fluorescent method for the quantification of MDA in human plasma using the adduct of MDA with the thiobarbituric acid and the standard addition method. The method allows the quantification of MDA at concentrations of 50 nM, within the range of normal physiological amount of such products in human plasma, avoiding the interference of the matrix.

Rezumat

Stresul oxidativ a fost menţionat în literatură între mecanismele de acţiune toxică a benzodiazepinelor la nivel cerebral. De asemenea, este unanim recunoscut faptul că peroxidarea lipidică are ca efect generarea mai multor specii de aldehide, care sunt considerate printre markerii stresului oxidativ. Malondialdehida (MDA) este apreciată de mai bine de un deceniu ca un marker fidel al nivelului de radicali liberi, de aceea dezvoltarea unor metode robuste de cuantificare a MDA în matrici biologice a fost şi este considerată o prioritate în biochimia clinică. Această lucrare prezintă o metodă fluorescentă simplă de determinare cantitativă a MDA în plasmă umana, având ca principiu formarea aductului acesteia cu acidul tiobarbituric şi metoda adaosului etalon. Prin această metodă pot fi determinate concentraţii de MDA de ordinul 50 nM, în domeniul ordinului de mărime al concentraţiei acestui compus în plasma umană provenind de la subiecţi sănătoşi, evitând totodată efectul de matrice.

Keywords: oxidative stress, malondialdehyde, thiobarbituric acid, analysis, human plasma, fluorescence, standard addition method

Introduction

Oxidative stress is defined as the condition occurring when the physiological balance between oxidants and antioxidants is disrupted in favour of the former with potential damage for the organism [12]. An increased production

of free radicals and other chemical species has been demonstrated in both ischemic and hemorrhagic stroke, and oxidative stress is proposed as a fundamental mechanism of brain damage in these conditions [2,4]. Free radical species are believed to mediate prooxidative processes, and even molecular damage, in different brain regions following acute and repeated administration of diazepam. An enhancement in the thiobarbituric acid reactive substances (TBARS) formation was found in the mitochondrial fractions from cerebral cortex and brain stem after single and repeated doses of diazepam. Additionally, isoenzymes of superoxide dismutase (SOD) and glutathione reductase displayed reduced activity, a region-dependent effect, after the administration of diazepam in rats [9,10].

Of the many biological targets of oxidative stress, lipids are the most involved class of biomolecules. Lipid oxidation gives rise to a number of secondary products, mainly aldehydes, with the ability to exacerbate oxidative damage [5].

Malondialdehyde (MDA) is the principal and most studied product of polyunsaturated fatty acid peroxidation. Since the 1960s several methods have been developed to assess this molecule in order to quantify the level of oxidative stress *in vivo* and *in vitro* [1, 5-8].

The paper presents a method to quantify MDA in human plasma by using the fluorescence of its adduct with the thiobarbituric acid (TBA) and the standard addition method.

Materials and methods

Standard solutions

Malondialdehyde was prepared from malondialdehyde bis (diethyl acetal) from Merck by acid hydrolisis (1 mM solution of malondialdehyde bis in 0.01M hydrochloric acid was hydrolised for 1 hour at 50°C [9]), to a 1 mM stock standard MDA solution.

Biological samples and reagents

Normal human plasma was obtained from the "C.T.Nicolau" National Haematologic Institute and trichloroacetic acid from Merck was used for deproteinisation. Thiobarbituric acid was bought from Merck (0.67g% working solution).

Devices

The experiments were performed using the following devices: vortex mixer Genie 2 (Cole Parmer), Sigma 2-16K refrigerated centrifuge (Germany), LS 50 B (Perkin Elmer) spectrofluorimeter.

Procedure

Aliquots of 4 mL diluted (1:10 in water) human plasma samples (as such and deproteinised with 0.3 mL of concentrated trichloroacetic acid - TCA) were

spiked with MDA appropriate amounts of working solutions, then mildly vortex-mixed for 5 minutes. The samples were centrifuged at 3400 x g for 5 minutes at 4°C. Aliquots of 3mL from the supernatant were collected from each sample and an amount of 0.750 mL of 0.67 g% TBA solution was added. The samples were kept on water bath at 80°C for 15 minutes, then left still to cool for the spectrofluorimetric evaluation.

The samples were measured by recording their emission spectra between 540 – 640 nm with excitation at 530 nm, then the emission peak intensity was measured at 549 nm.

For the standard addition method, the MDA concentation in the samples was computed according to the formula (1):

$$c_{uk} = \frac{A}{B} \quad (1)$$

where c_{uk} is the unknown MDA concentration in the sample, A is the intercept and B is the slope of the regression curve plotted for the unspiked and MDA known concentration spiked plasma samples [3].

Results and discussion

First we performed preliminary tests to seek for the quantification limits of MDA using the fluorescence of the condensation product of MDA with TBA. Examples of spectra obtained for concentrations ranging between 0.05 – 0.25 µg/mL are presented in Figure 1, thus certifying the possibility to asses normal (physiological) amounts of free MDA in plasma [11].

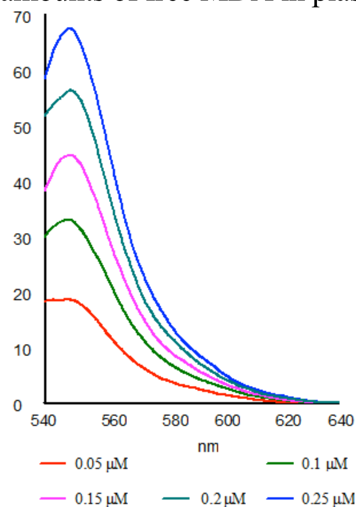

Figure 1
Emission fluorescence spectra of MDA-TBA complex in the range 0.05-0.25 µM MDA (λ_{ex}=530 nm)

In order to verify the detection limits of MDA in human plasma using the fluorescence emission intensity of the aduct of MDA with TBA and the standard addition method, we performed several experiments as follows.

We used a 1:10 dilution of human plasma in water. We splitted the sample in aliquots of 4 mL each.

To the first three samples, denoted as S1, S2 and S3, 0 μL, 2 μL and 4 μL of 0.1 μM MDA were added, respectively. The following 3 samples, denoted as S4, S5 and S6, were deproteinised as mentioned above with 0.3 mL of TCA and were added 0 μL, 2 μL and 4 μL of 0.1 μM MDA, respectively. To the last samples, denoted as S7, S8 and S9, there were first added 0 μL, 2 μL and 4 μL of 0.1 μM MDA, respectively, then they were deproteinised with 0.3 mL of TCA. Aliquots of 3 mL of each sample were complexed with TBA as described above and their emission spectra were further registered (Figure 2).

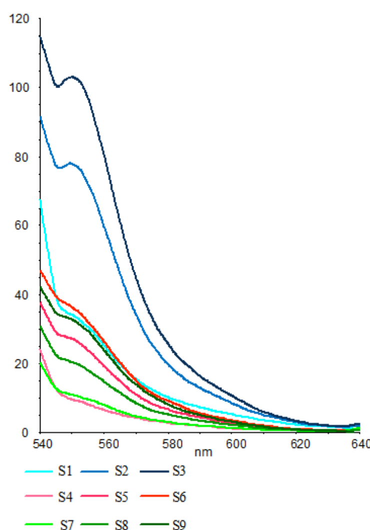

Figure 2
Emission fluorescence spectra of MDA-TBA complex in human plasma (λ_{ex}=530 nm)

As one can see, the spectra of the deproteinised samples (S1, S2 and S3) have a greater intensity than the similarily prepared deproteinised samples, suggesting that TBA is reacting also with other components of the human plasma, thus causing errors when MDA is quantified by means of a classical regression curve. An example of the regression line plotted for a deproteinised sample (corresponding to S4, S5 and S6-type samples), extrapolated to zero, is presented in Figure 3.

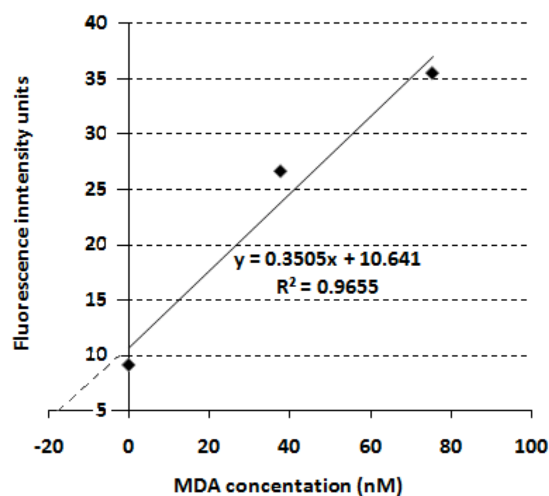

Figure 3
Exemplification of the standard addition method regression line for S4, S5 and S6-like samples

Following the procedure described above we obtained for repeated samples and corected for the dilution (1:10), the results summarised in Table I. As it can be seen, no major differences could be pointed out between deproteinised and only diluted plasma within the errors of the method. The results are in the order of magnitude of previously reported results [1,5,7].

Table I
MDA content of normal human plasma obtained using the fluorescent signal of MDA-TBA adduct and the standard addition method

Sample type	**Human plasma**	**Deproteinised human plasma**
MDA content (nM)	380.4±48.8	340.0±48.5

Conclusions

A method for evaluating the MDA content in normal human plasma is described in order to be used in oxydative stress determinations. The method is based on the fluorescence of MDA-TBA adduct measured at 530 nm excitation and 549 nm emission wavelengths and the standard addition method.

The fluorescence of the adduct in standard samples can be quantified to a lower limit of 50 nM, but the fluorescence of the adduct in human plasma can be measured up to 100 nM, due to the turbidity of the samples. However, the method can be used to quantify the MDA content in human plasma of patients treated with different benzodiazepines.

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