SIDE COMPARATION OF TWO METHODS FOR QUANTIFYING MALONDIALDEHYDE LEVELS IN ANIMAL TISSUE EXTRACTS

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Abstract: Malondialdehyde (MDA) as an important marker used for the assessment of the oxidative level in a tissue of biological fluid. The standard assay method uses tiobarbituric acid for spectrophotometric detection but suffers from the lack of specificity. Here we show that a HPLC based method for quantitating the MDA has the advantages of increase sensitivity as well as a better specificity, allowing the detection of lower MDA levels in tissue extracts with an increased accuracy.

INTRODUCTION

Malondialdehyde (MDA) is an organic compound considered to be one of the most important indicators of lipid peroxidation of polyunsaturated fatty acids (Davey et al. 2005). MDA is the main product of the arachidonic acid conversion to prostaglandin PGH2 by cyclooxygenase 1 or cyclooxygenase 2. Prostaglandin PGH2 is further metabolized by thromboxane synthase to thromboxane A2, 12-hydroxyheptadecatrienoic acid, and MDA. Alternatively, prostaglandin PGH2 can also suffer a non-enzymatic rearrangement into a mixture of 8-cis and 8-trans isomers of 12-hydroxyeicosaheptaenoic acid and MDA (Pryor and Stanley 1975).

Reactive oxygen species degrade the polyunsaturated lipids form MDA as well. As a reactive electrophile species, MDA forms covalent adducts with important molecules such as proteins and DNA. The protein adducts are referred to as **a**dvanced **l**ipo-oxidation **e**nd-products or ALE, while the DNA adducts are mutagenic (Marnett 1999). Thereby, measuring MDA levels are used as an important marker for the oxidative level in a given tissue (Del Rio, Stewart, and Pellegrini 2005).

As a thiobarbituric reactive substance or TBARS, MDA reacts with two equivalents of tiobarbituric acid (TBA) and form a fluorescent red compound (MDA-TBA2) that can be easily quantified spectrophotometrically (Nair, O'Neil, and Wang 2008). Although alternative dyes exist such as 1-Methyl-2-phenylindole, the spectrophotometric method based on TBA is the most widely used assay method for MDA.

The main drawback of the TBA spectrophotometric method for MDA assay is its specificity. MDA is not the only compound that reacts with TBA found in a given tissue. Some other compounds, un-related with oxidative stress such as aliphatic aldehydes, metals or glyoxylic acid and sugars also react with TBA. Moreover, end-product of the MDA-TBA assay is almost identical to the end-product of the pyridine-barbiturate cyanide assay. Thereby, although the MDA-TBA spectrophotometric assay is very convenient due to its simplicity and robustness (Artenie, Ungureanu, and Negură 2008), the results must be evaluated with caution and further validated by other indicators. When an increase in specificity is needed, HPLC is the methods of choice. It is not a surprise thereby that several authors managed to put together a more specific HPLC-based assay method to measure MDA levels (Domijan et al. 2015; Moselhy et al. 2013; Lykkesfeldt 2001; Karatas, Karatepe, and Baysar 2002; Grotto et al. 2007; Khoschsorur et al. 2000). The current works focuses on adapting one of the available HPLC methods for assaying the MDA levels to the existing infrastructure at the Biology Department, UAIC Iasi and comparing it to the well-established and used spectrophotometric method in terms of specificity and ease of use.

MATERIAL AND METHODS

Chemicals. All chemicals were purchased from well-known suppliers and were of greatest purity available. As standard, 1,1,3,3-tetraethoxypropane (TEP, Sigma Aldrich) was used. Biological samples used for testing the real-life applicability of the method were clarified rat brain extracts prepared as described before. (Hritcu et al. 2013). All mobile phases were filtered through a 22 microM filter (Milipore) and degassed by applying low pressure under constant steering for 20 minutes.

MDA-TBA assay. Samples or standards up to 50 microL were mixed with 12,5 microL 3M NaOH and incubated for 30 minutes at 60^0C with constant shaking (300 rpm). 0.5 ml of H_2SO_4 98% were added, the tubes were mixed and then 0.25 ml TCA 20% was added to precipitate the proteins and DNA. The tubes were centrifuged for 10 min at 3000 rpm/min. 0.5

ml of the supernatant was further mixed with 0.25 ml 0.35% TBA and incubated at 90^0C for 40 min with constant shaking (300 rpm). Before quantification by spectrophotometry and HPLC, the samples were centrifuged for 30 min at 13000 rpm.

Spectrophotometric assay. Each sample was measured at 532 nm against a blank with water instead of the sample using a DU 730 UV/VIS Spectrophotometer (Beckman Coulter).

HPLC assay. 20 microL of the supernatant prepared as depicted above were injected on a Zorbax Eclipse XDB - C18, 250 mm length, 3 microm particle size coupled to a Shimadzu Prominence HPLC system (2x LC20AD pumps, SIL20AC autosampler, CT20AC oven, SPD M20A DAD detector). As mobile phase, methanol (Carlo Erba Reagents): 30 mM KH_2PO_4, pH 6,7 35:65 was used at a flow rate of 1 ml/min for a total of 20 minutes. The MDA-TBA adduct eluted at 9,520 ± 0,2 minutes, example chromatograms being presented in figure 1.

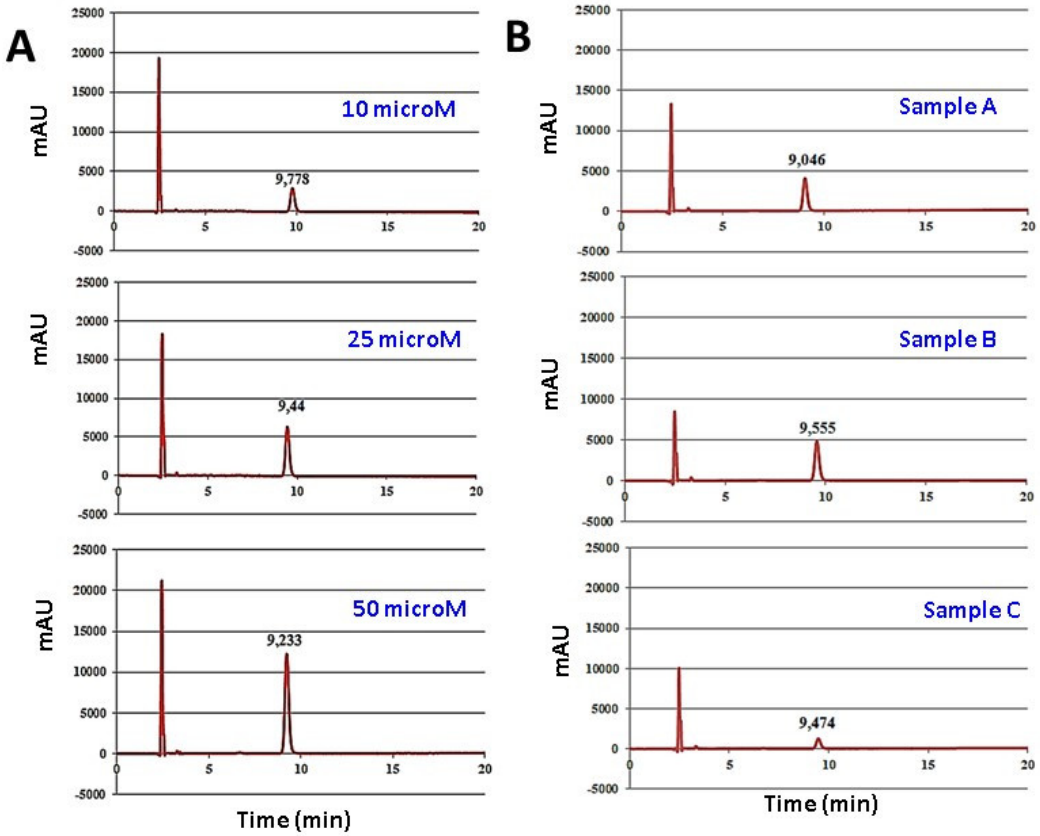

Figure 1. Typical chromatograms for (A) TEP-TBA adduct at various concentrations and (B) MDA-TBA adducts in various biological samples.

The chromatographic data was acquired using the Shimadzu LC solution Software and manually interpreted. Peak areas and peak height were measured and used. All calibration curves were built in Microsoft Execel using 3 technical replicates/point.

RESULTS AND DISCUSSIONS

Linearity and sensitivity. In order to assess the linearity of the two methods, a calibration curve using TEP was built on a very wide concentration interval 10-500 microM, while in order to evaluate the sensitivity a narrower interval was chosen, but with significantly lower

concentrations – 10-50 microM. The data obtained is depicted in Figure 2, where A are the spectrophotometric measurements, B is the HPLC data based on peak height and C is the HPLC data based on peak area.

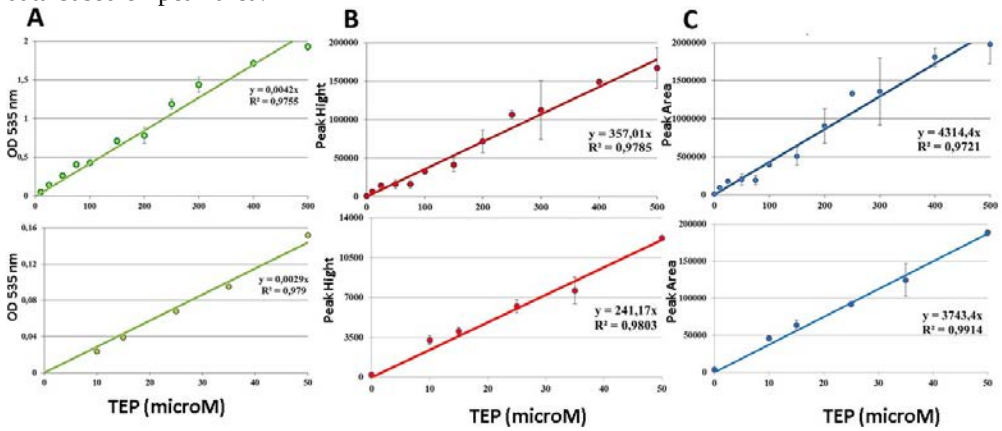

Figure 2. Linearity and sensitivity of the two MDA quantitation methods. **A**. spectrophotometric **B**. HPLC, using peak height for quantitation **C**. HPLC, using peak area for quantitation.

A quick evaluation of the regression coefficient R^2 for all the calibration curves depicted in figure 2 indicates that both methods have the same linearity, both methods providing good, but not perfect calibration curves on the 10-500 microM TEP interval with an R^2 around 0.97. In the lower concentration interval, the HPLC quantitation method based on the peak area outperforms the spectrophotometric method with a near perfect R^2 of 0.99. There is no clear difference between the performance of the spectrophotometric method and the HPLC method based on peak height. Overall, although similar in linearity, the HPLC method for quantitation of MDA based on peak height is apparently much sensible. Indeed, most of the methods we could find in the literature are used to detect low concentrations of MDA as fallows: 0.28 - 6.6 microM (Karatas, Karatepe, and Baysar 2002), 0 – 24,3 microM (Moselhy et al. 2013) and 0,15 – 3,0 microM (Domijan et al. 2015).

Real-life biological samples. In order to assess the real-life application of the method, cleared, cell-free rat brain lysates from an ongoing experiment were used to compare the MDA levels reported by the two methods. Each of the 4 samples labeled A, B, C and D were processed as stated in the Materials and methods section and the supernatant from the same tube was consecutively measured using both methods. The values were then converted into MDA concentrations using the calibration curves from figure 2. As one can see from the data plotted in figure 3, the spectrophotometric method always reported higher values of MDA comparing with the HPLC methods, independent of the parameter used for quantitation (area or height). A close inspection of the typical chromatograms from figure 1 indicates the presence several peaks at 532 nm close to the void-volume peak in the case of the biological samples. These peaks are compounds from the samples that reacted with TBA, compounds that are quantified as MDA by the spectrophotometric method and not by the HPLC method.

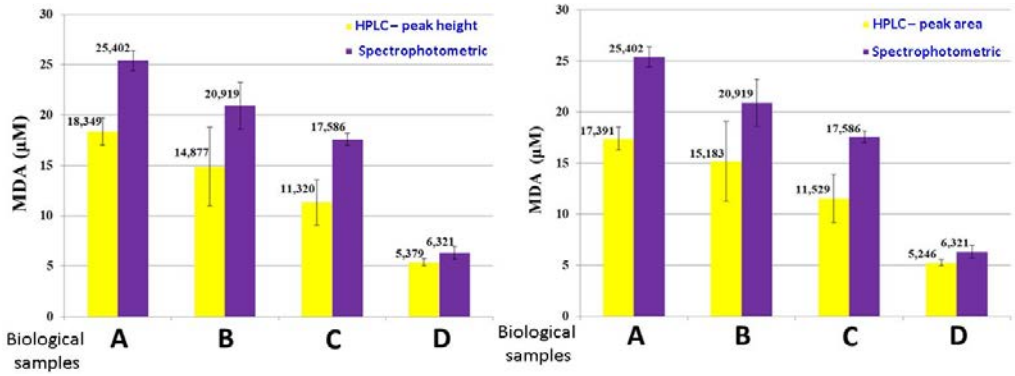

Figure 3. Data reported by analyzing real-life biological samples using the two methods described here.

CONCLUSIONS

A HPLC based method to assay for the MDA levels in biological extracts was established in the lab. Although requiring more skilled manpower, the methodology offers better sensibility for measuring lower amounts of MDA and increased specificity.

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