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Determination of malondialdehyde by liquid chromatography as the 2,4-dinitrophenylhydrazone derivative A marker for oxidative stress in cell cultures of human hepatoma HepG2

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Abstract

Malondialdehyde (MDA) is considered a presumptive biomarker for lipid peroxidation in live organisms and cultured cells. The present study adapts an accurate and reproducible method to measure MDA by high-performance liquid chromatography (HPLC) as its 2,4-dinitrophenylhydrazone derivative in human hepatoma HepG2 cells in culture. Since MDA is assumed to increase in conditions of cellular oxidative stress, two compounds that induce pharmacological oxidative stress in cell cultures, hydrogen peroxide (H_2O_2) and *tert*-butyl hydroperoxide (*t*-BOOH), have been used in HepG2 cells. The results report a significant increase in the content of MDA derivative after treatment with 200 and 500 μM *t*-BOOH for 3 h, while H_2O_2 in doses up to 500 μM failed to evoke a similar response, indicating a stronger lipid peroxidation of *t*-BOOH to HepG2 cells than H_2O_2. Thus, MDA can be used as a reliable biomarker for cellular oxidative stress in human hepatoma HepG2.

Keywords: Derivatization, LC; Malondialdehyde; HepG2

1. Introduction

Oxidative stress caused by reactive oxygen species (ROS) damages cellular DNA, proteins and lipids, and is widely recognised as one of the causes of the development of chronic disease [1,2]. Under normal circumstances the levels of ROS are low enough to be effectively removed by the natural defence mechanisms of the cell. There are, however, many compounds that enhance the production of ROS to such an extent that cellular defences are overwhelmed, and the cell is injured [1–3]. The study of the mechanisms involved in cell damage mediated by oxidative compounds as well as the evaluation of biomarkers of cellular stress in such conditions could greatly help to prevent appearance and development of oxidative stress-related diseases.

Lipid peroxidation is considered to be important in the development of atherosclerosis, to be involved in ageing and other clinical disorders, such as cancer or cardiovascular and liver diseases [4]. An important step in the degradation of cell membranes is the reaction of ROS with double bonds of polyunsaturated fatty acids (PUFAs) to yield lipid hydroperoxides. On breakdown of such hydroperoxides a great variety of aldehydes can be formed [5]. Malondialdehyde (MDA), a three-carbon compound formed by scission of peroxidized PUFAs, mainly arachidonic acid, is one of the main products of lipid peroxidation [6]. MDA is reactive toward amino groups of proteins and nucleic acids, it has been inferred to have mutagenic and cytotoxic effects, and possibly to participate in the onset of atherosclerosis [7]. Since MDA has been found elevated in various diseases thought to be related to free radical damage, it has been widely used as an index of lipoperoxidation in biological and medical sciences [7].

The most frequently used method to determine MDA formation in biological samples is the spectrophotometric assay of MDA after its reaction with thiobarbituric acid (TBA) [8]. However, TBA reacts not only with MDA but with many other compounds of biological origin, therefore, derivatisation of MDA with 2,4-dinitrophenylhydrazine (DNPH) and conversion into pyrazole and hydrazone derivatives has been found to allow a more specific estimation of this compound, especially if combined with its separation

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using high-performance liquid chromatography (HPLC) [9–11]. This approach and similars have been widely used to determine MDA levels in a variety of biological samples, such as rat and human plasma [5,12–17], rat liver and kidney [17,18], and other samples [19].

The study of the regulation of antioxidant defence mechanisms at the molecular level may benefit from the use of an established cell culture line. Human hepatoma HepG2 is a well-differentiated transformed cell line that has been widely used in biochemical and nutritional studies because it is considered one of the experimental models that more closely resembles the human hepatocyte in culture [20–23]. Due to the growing interest in investigating the molecular pathways of oxidative stress in cell cultures, determination of MDA in these experimental models has become an emergent topic in this field. MDA in cell cultures has been mainly determined by the TBA method [24–28], but due to its limitations, other methodology has been applied [29–31].

The aim of the present study was to establish an accurate and precise method to determine MDA by HPLC as the 2,4-dinitrophenylhydrazone derivative in cultured HepG2 cells. Since MDA is assumed to increase in conditions of cellular oxidative stress, two compounds that induce pharmacological oxidative stress in cell cultures, hydrogen peroxide (H_2O_2) [32–38] and *tert*-butyl hydroperoxide (*t*-BOOH) [39–41], have been used. The results show that HPLC analysis of MDA as its 2,4-dinitrophenylhydrazone can be used as a fair and reliable biomarker for lipid peroxidation in human hepatoma HepG2 in culture.

2. Experimental

2.1. Reagents

Acetonitrile, methanol, sodium hydroxide, sodium chloride, di-sodium hydrogen phosphate anhydrous, potassium di-hydrogen phosphate, as well as formic, hydrochloric, perchloric and sulphuric acids were acquired from Panreac (Barcelona, Spain). DNPH, H_2O_2, *t*-BOOH and 1,1,3,3-tetraethoxypropane (TEP) were purchased from Sigma Chemical Co. (Madrid, Spain). All reagents were of analytical grade unless stated otherwise.

2.2. Cell culture

Human hepatoma HepG2 cells initially isolated from a liver biopsy in a 15-year-old Caucasian male [42], were a gift from Dr. Paloma Martin-Sanz (Instituto de Bioquimica, CSIC, Madrid, Spain). They were grown in DMEM F-12 medium from Biowhitaker (Innogenetics, Madrid, Spain), supplemented with 2.5% Biowhitaker foetal bovine serum (FBS) and 50 mg/l of each gentamicin, penicillin and streptomycin (all from Sigma, Madrid, Spain). The same medium deprived of serum but containing the antibiotic mixture was used in all experiments. This cell line was grown in a humidified incubator containing 5% CO_2 and 95% air at 37 °C. The culture medium was changed every other day and the cells were usually split 1:3 when they reached confluence.

2.3. Samples

Cells were grown in 100 mm diameter plates and the assay was carried out two days later when the confluence was 80% (concentration of 5×10^6 per plate approximately). On the day of the assay, plates were changed to the different experimental conditions with FBS-free medium (6 ml): control (medium without agent) and stressors (200 and 500 μM *t*-BOOH and H_2O_2) in quadruplicate. After incubation for 3 h, the culture medium was removed, cells were washed twice with PBS (0.01 M phosphate buffered saline solution, pH 7.4) and then collected. The cells from duplicate plates corresponding to a particular condition were collected in 0.25 ml of PBS and combined in a 1.5 ml Eppendorf vial. Each plate was washed again with 0.25 ml of PBS and combined with the collected cells, having a final volume of approximately 1.0 ml. After centrifugation at $220 \times g$ for 5 min at 4 °C, the supernatant was removed and the cells were resuspended in 200 μl of PBS. Cells were sonicated for 7 min at room temperature, to break down the cell membrane and release the total amount of MDA. After centrifugation at $3500 \times g$ for 15 min at 4 °C, the supernatant was transferred into a 1.5 ml Eppendorf and kept frozen at −20 °C until analysis within 12–24 h.

2.4. Sample preparation

Sample was prepared and derivatisation of MDA with DNPH performed as described by Pilz et al. [5] with some modifications.

An aliquot of 125 μl of cytoplasmatic content was placed in a 1.5 ml Eppendorf and 25 μl of 6 M aqueous sodium hydroxide added. This mixture was incubated in a 60 °C water bath for 30 min to achieve alkaline hydrolysis of protein bound MDA. Then, protein was precipitated adding 62.5 μl of 35 % (v/v) perchloric acid, and the mixture was centrifuged at $2800 \times g$ for 10 min. A 125 μl volume of supernatant was transferred to an Eppendorf vial and mixed with 12.5 μl DNPH prepared as a 5 mM solution in 2 M hydrochloric acid. Finally, this reaction mixture was incubated for 30 min at room temperature protected from light. An aliquot of 50 μl of this reaction mixture was injected onto the HPLC system.

2.5. HPLC procedure

HPLC analyses were performed on an Agilent 1100 liquid chromatographic system equipped with a diode array UV-Vis detector and a Rheodyne injection valve (50 μl loop). A Nucleosil 100 RP-18 column (4.0 × 125 mm) with a 5 μm particle size (Agilent) preceded by a Lichrospher

precolumn of the same material as the stationary phase (4.0 mm × 4.0 mm), was used. An Agilent Chemstation software system controlled all the equipment and carried out the data processing.

Elution was performed isocraticaly with a mixture of 0.2% (v/v) acetic acid in deionised water and acetonitrile (62:38, v/v) at a flow rate of 0.6 ml/min at room temperature. Chromatograms were acquired at 310 nm.

2.6. Quantitative determination by HPLC

A stock solution of MDA was obtained as follows: 25 μl TEP was dissolved in 100 ml of deionised water to give a 1 mM stock solution. MDA was prepared by hydrolysis of 1 ml TEP stock solution in 50 ml 1% sulphuric acid and incubation for 2 h at room temperature [43]. The solution was stored at 4 °C and used within 4 weeks. The resulting MDA standard of 20.00 nmol/ml was further diluted with 1% sulphuric acid to yield different concentrations of 10.00, 5.00, 2.50, 1.25, 0.62, 0.31, 0.20 and 0.10 nmol/ml of MDA.

A reference curve was prepared mixing a 250 μl volume of each of the above concentrations of standard MDA with 25 μl DNPH prepared as a 5 mM solution in 2 M hydrochloric acid and this mixture was incubated for 30 min at room temperature in the dark. An aliquot of 50 μl of this mixture of reaction was injected onto the HPLC system. This reference curve allowed evaluating the actual recovery of MDA after the process.

However, since the experimental samples (i.e. cytoplasmatic contents of HepG2 cells) were resuspended in PBS, a new calibration curve was obtained to analyse the actual MDA concentration in the samples. Adequate volumes of a 40 nmol/ml MDA standard solution obtained from the TEP stock solution as described above were spiked in PBS to achieve a concentration range from 20.00 to 0.10 nmol/ml. These solutions were treated as the experimental samples (i.e. treatment with 6 M NaOH for 30 min at 60 °C followed by protein precipitation with 35% perchloric acid) before derivatisation with DNPH.

Linearity was checked by performing linear regression analysis of the peak area corresponding to the 2,4-dinitrophenylhydrazone derivative versus concentration.

Concentration in cell preparations was expressed as nmol MDA per mg protein content of HepG2 cells.

2.7. Determination of protein content

Protein content was estimated by Bradford's method [44], using a Bio-Rad protein assay kit (500–0006, Bio-Rad Ltd., München, Germany).

2.8. Statistical analysis

Results are expressed as means ± standard deviation (S.D.) of four or more determinations. One way analysis of variance (ANOVA) followed by Duncan's multiple comparison test was used to contrast groups. The method used to test the homogeneity of variances was the Cochran's test, and to discriminate among means the Fisher's least significant difference procedure was applied. With this method, there is a 5% risk of calling each pair of means significantly different when actual difference equals zero. The level of significance was $P < 0.05$. A Statgraphics Plus program version 2.1 (Statistical Graphics Corp., Rockville, MD) was used.

3. Results and discussion

Several HPLC methods have been developed for the determination of MDA in culture cells. However, these techniques generally require a long execution time [6] and prepurification of the MDA–TBA complex or elimination of interfering substances [45–47]. Here we report an adaptation of a very rapid and simple isocratic reversed-phase HPLC separation of MDA derivative as its 2,4-dinitrophenylhydrazone [5] in cell culture, without previous purification of MDA derivative.

3.1. Sample preparation for MDA analysis

The analytical conditions used for alkaline hydrolysis of protein bound MDA, i.e. protein precipitation with perchloric acid and later derivatisation with DNPH, were similar to those adopted by Pilz et al. [5] for plasma samples. However, extraction with hexane and enrichment of MDA, necessary in plasma samples, was not a requisite in cultured cell samples. A clear supernatant was obtained from cytoplasmatic contents of HepG2 cells after DNPH treatment, and it could be injected directly onto the HPLC system without further purification with hexane. An example is shown in Fig. 2b.

Hydrolysis of protein bound MDA and ulterior protein precipitation steps were still necessary in the cytosolic contents of cultured cells. To set the optimal time for alkaline hydrolysis with 6 M NaOH, samples were incubated at 60 °C for 15, 30, 45 and 60 min, and the relative amounts of MDA measured after perchloric acid precipitation and DNPH derivatisation. MDA peak area increased 11.3% when hydrolysis time was increased from 15 to 30 min. Longer incubation times did not enhance MDA peak area; on the contrary, a small, not significant reduction of 1.1% and 3.2% at 45 and 60 min, respectively, was observed. Therefore, incubation time with 6 M NaOH was set at 30 min.

On the other hand, no statistically significant differences were observed between cytoplasms analysed immediately after extraction as compared to cytoplasms kept frozen at −20 °C for 12–24 h before analysis (data not shown).

It is necessary to protect derivatised samples from light; otherwise signals are reduced by 18% of their initial magnitude within 1 h. The MDA-hydrazones were stable during the day at room temperature when protected from light.

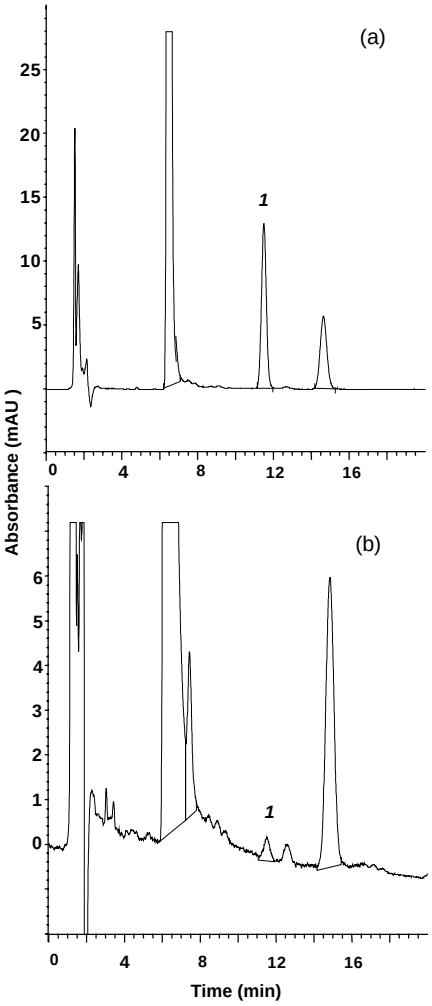

Fig. 1. HPLC chromatogram at 310 nm of MDA standard solutions spiked in PBS and treated like cell samples prior to DNPH derivatisation (see Section 2) (a) 5 nmol/ml standard MDA solution, and (b) 0.10 nmol/ml standard MDA solution used to determine the limit of quantification. Peak 1 corresponds to MDA as its 2,4-dinitrophenylhydrazone. Peaks at 6.5(7.5) and 14.5 min correspond to DNPH reagent.

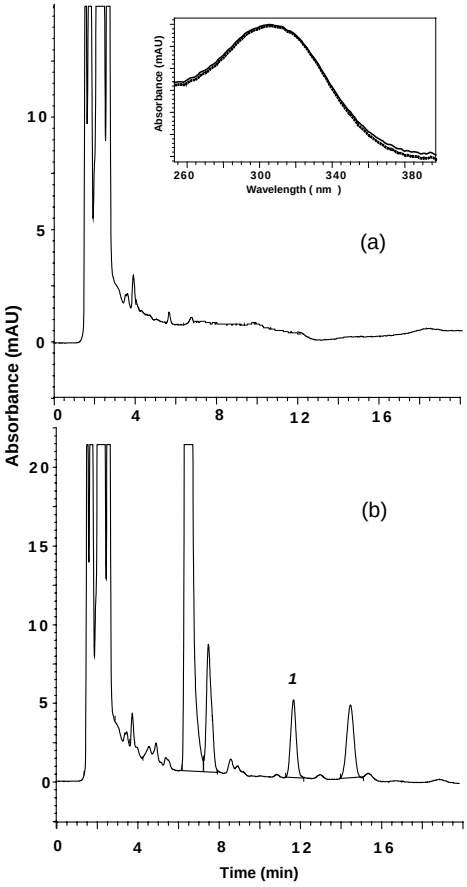

Fig. 2. HPLC chromatogram at 310 nm: (a) reagent blank of HepG2 cells (cytoplasmatic contents prepared with no DNPH), (b) cytoplasmatic content of HepG2 cells treated with 200 μM *t*-BOOH, MDA derivative (2.70 nmol/ml). Peak 1 corresponds to malondialdehyde as its 2,4-dinitrophenylhydrazone. Peaks at 6.5(7.5) and 14.5 min correspond to DNPH reagent. Insert: UV spectra obtained by diode array detection of standard (solid line) and malondialdehyde in HepG2 cells (dotted line), both as the 2,4-dinitrophenylhydrazone derivative.

3.2. HPLC procedure

To study the influence of solvent composition and elution gradient on the resulting chromatograms, a standard solution of MDA (5 nmol/ml) was used. Peak resolution was assessed by changing the flow (0.5–1.0 ml/min) and elution gradient of solvents in different proofs. The best chromatographic separation was obtained using a flow of 0.6 ml/min and isocratic elution that consisted of 38% acetonitrile and 62% deionised water acidified with 0.2% (v/v) acetic acid in a reversed-phase column C18 (Nucleosil; 4.0 mm × 125 mm) with a 5 μm particle size. A typical chromatogram is depicted in Fig. 1a. MDA corresponds to the peak at 11.5 min (peak 1 in Fig. 1a). As it can be seen, the chromatographic peaks resolution was very good, better than that reported by other authors [5,7] and free from interfering peaks, allowing the determination of MDA derivative without further sample purification with hexane. The good resolution enabled detection of MDA concentrations as low as 0.10 nmol/ml as shown in Fig. 1b.

Chromatograms were acquired at 310 nm at which the absorbance of MDA presents a maximum. The UV spectra (from 250 to 400 nm) of a MDA standard and the corresponding peak in HepG2 cytoplasmic contents is shown in Fig. 2 (insert). Peak identification in cell samples was carried out by comparison of the retention time and UV spectra with those of a MDA standard. It has to be noted that MDA standards showed the presence of two peeks at 6.5 and 14.5 min (Fig. 1). These peaks appeared in the MDA standards prepared in 1% sulphuric acid and PBS, as well as in samples from cytoplasmatic contents treated with DNPH (Fig. 2b) but not in those cell contents not submitted to DNPH derivatisation (Fig. 2a). These two peaks had similar UV spectra, with λ_{max} at 265 and 360 nm, different from that of the MDA derivative (310 nm). A reagent blank prepared with an aqueous TEP solution derivatised with DNPH prior

to MDA formation showed the presence of these two peaks, confirming they originate from the reagents used (data not shown). In some cases, the peak at 6.5 min also showed a smaller, not resolved peak at 7.5 min of identical spectrum corresponding also to DNPH (Fig. 2b). None of these peaks interfered in the quantification of MDA in the samples.

3.3. Quantitative determination by HPLC

The response of MDA derivative was checked by linear regression analysis in the concentration range assayed in the analytical procedure. The least-squares method of MDA derivative peak area was used. The response was linear in the range of concentrations evaluated, from 0.10 to 20.00 nmol/ml, giving an equation of $y = 48.298x + 0.723$ ($n = 54$), where y is the peak area and x is the concentration of MDA. A regression coefficient of $r^2 = 0.9999$ was obtained. As cytoplasmatic contents were dissolved in phosphate buffered saline (PBS), a curve was obtained from spiking known amounts of MDA in PBS and submitted to the overall treatment. The regression equation obtained was $y = 47.651x + 0.679$ ($n = 54$), where y is the peak area and x the concentration of MDA. A regression coefficient of $r^2 = 0.9998$ was obtained. This equation was used to quantify MDA derivative in the samples.

The precision expressed as the coefficient of variation (CV) ranged from 1.4 to 4.3%, at seven different concentrations ranging from 0.10 to 20.00 nmol/ml, indicating that the analytical method is repeatable. The values obtained are shown in Table 1.

The accuracy was determined for the overall assay by measuring the percentage of recovery after the addition of known amount of standard MDA to the PBS solution used to resuspend the cytoplasmic contents of HepG2 cells. Recovery ranged from 96.27 to 99.91% at nine different concentrations (Table 1). These values indicate that MDA as the 2,4-dinitrophenylhydrazone is quantitatively analysed using this method.

Table 1
Reproducibility and recuperation by HPLC of increasing amounts of MDA spiked in PBS, expressed as recovered MDA ± standard deviation, CV and percentage recuperation.

MDA spiked (nmol/ml)	Recovered MDA[a] (nmol/ml)	CV (%)	Recovery (%)
20.00	19.70 ± 0.58	2.9	98.52
10.00	9.97 ± 0.17	1.8	99.72
5.00	4.82 ± 0.21	4.3	96.37
2.50	2.49 ± 0.03	1.4	99.52
1.25	1.22 ± 0.04	3.3	97.98
0.62	0.60 ± 0.01	1.4	96.27
0.31	0.31 ± 0.01	1.6	99.74
0.20	0.20 ± 0.01	2.6	99.71
0.10	0.10 ± 0.00	1.5	99.91

[a] Recovered MDA was calculated using the calibration curve obtained from MDA in 1% sulphuric acid ($y = 48.298x + 0.723$).

The limit of detection (LOD) was 0.05 nmol/ml on the basis of a signal-to-noise ratio of 3. The limit of quantification (LOQ), based on the standard curve in PBS, was 0.10 nmol/ml (Fig. 1b). Both LOD and LOQ were defined in the MDA standard solution in PBS and not in the sample matrix since cytoplasms initially contained a high amount of MDA as shown later. Rapid separation and highly reproducible data were obtained, and no diminution of peak resolution was observed even after 100 analyses.

The procedure described here allows routine analysis of the MDA content of cultured cells. Confluent cells treated with oxidizing agents for three hours can be collected and cytoplasms isolated and kept frozen until analysis the following day, when derivatised samples (i.e. MDA hydrazones), stable within the working day when protected from light, can be analysed by HPLC in a rapid, sensitive, precise and accurate way.

3.4. Validation of the method

Since MDA is assumed to increase in conditions of cellular oxidative stress, two oxidative stressors in cell cultures, H_2O_2 [32–38] and *t*-BOOH [39–41] have been used in HepG2 cells in culture at two different concentrations (200 and 500 μM). MDA concentrations analysed in cytoplamatic contents are shown in Table 2. Protein and MDA concentrations varied between assays due to differences in cell number, rendering average values with considerably high standard deviations. However, when MDA concentration (nmol/ml) was corrected by protein content to normalize for cell number variations in each assay and expressed as nmol MDA per mg protein, these variations were largely reduced, allowing reliable statistical analysis of the results.

A 3 h treatment with 200 or 500 μM *t*-BOOH, doses commonly used in the literature, significantly enhanced MDA generation in cell cultures as can be seen in Table 2. This indicates an unequivocal increase in lipid peroxidation and cell damage in HepG2, and suggests a dose-response relationship, with higher MDA concentrations in cells treated with the highest *t*-BOOH dose. On the contrary, H_2O_2 in doses

Table 2
Protein content and quantitative analysis of MDA by HPLC in cytoplasmatic contents of HepG2 cells treated with vehicle (control), *tert*-butyl hydroperoxide (*t*-BOOH) or hydrogen peroxide (H_2O_2) for 3 h

		Samples		
		Protein (mg/ml)	MDA (nmol/ml)	MDA* (nmol/mg protein)
Control		2.06 ± 0.69	2.31 ± 0.76	1.12 ± 0.10 a
t-BOOH	200 μM	2.09 ± 0.43	2.74 ± 0.49	1.31 ± 0.06 b
	500 μM	1.91 ± 0.95	2.96 ± 1.16	1.60 ± 0.16 c
H_2O_2	200 μM	1.44 ± 0.33	1.83 ± 0.52	1.20 ± 0.17 a
	500 μM	1.48 ± 0.47	1.82 ± 0.57	1.23 ± 0.09 a

Values are means ± S.D. ($n = 4$).

* Different letters indicate statistically significant differences ($P < 0.05$).

Table 3
MDA content in culture media and plain cytosols directly treated for 3 h with vehicle (control), *tert*-butyl hydroperoxide (*t*-BOOH) or hydrogen peroxide (H_2O_2)

	Samples	
	MDA (nmol/ml)	MDA* (nmol/mg protein)
Cytoplasmatic content		
Control	1.27 ± 0.06	1.11 ± 0.05 a
500 μM *t*-BOOH	1.22 ± 0.02	1.07 ± 0.02 a
500 μM H_2O_2	1.09 ± 0.02	0.94 ± 0.02 a
Culture medium		
Control	3.40 ± 0.08	
500 μM *t*-BOOH	2.38 ± 0.11	
500 μM H_2O_2	2.50 ± 0.06	

Values are means ± S.D. ($n = 3$).
* MDA levels normalized for the protein concentration in the combined cytoplasmatic contents (protein concentration was 1.15 ± 0.02 mg/ml).

up to 500 μM evoked no changes in this parameter in similar experimental conditions (Table 2). These differences might be due to a better capacity of the cellular antioxidant defence system against H_2O_2 damage, with the presence of a specific intracellular detoxifying enzyme, catalase, whilst the cell has not evolved specific defences against *t*-BOOH. This might account for the stronger oxidative damage caused by *t*-BOOH as reflected by the increased MDA concentrations observed. This result is in agreement with unpublished observations from our laboratory, which show specific changes in cellular biomarkers of oxidative stress, such as concentration of reduced glutathione and activity of antioxidant enzymes in cultures of HepG2 treated with *t*-BOOH but not when treated with H_2O_2 (Alia et al. article in preparation).

It is noteworthy the high MDA levels observed in control cells not submitted to any oxidative insult (1.12 nmol/mg protein). Values of up to 0.1 nmol MDA/mg protein have been reported for Caco-2 cells in culture [48], although the analytical procedure used to measure MDA was not specified. Therefore, this value, 10 times lower than that found in our experiment with control HepG2 cells, cannot be directly compared with ours due also to differences in the cell line and culture media used.

Interestingly, when culture media was used to analyse MDA (after 6 M NaOH and perchloric acid treatment followed by DNPH derivatisation), a significant amount of MDA derivative was found (3.4 nmol/ml, Table 3). This high formation of MDA was mostly due to reaction of glucose (present in the culture medium at a concentration of 3.15 g/l) with DNPH as checked with a pure glucose solution. No other major component of the culture medium in the concentration range present in it reacted with DNPH (data not shown). Nevertheless, since cells were collected in PBS after thoroughly removing the culture medium, interference of the later in the results obtained with HepG2 cells can be ruled out.

To investigate a possible increase of MDA in cell lysates caused by indirect factors other than lipid peroxidation, such as sample processing or activation of some compounds in cytoplasm, two control experiments were carried out: (1) incubation of culture medium with *t*-BOOH and H_2O_2, and (2) incubation of plain cytoplasm contents with the two oxidizing agents. In both experiments the highest concentration of stressors (500 μM) was used, and samples prepared as described in the Experimental section.

Surprisingly, when culture medium was incubated with *t*-BOOH or H_2O_2 a significant decrease in MDA levels was observed (Table 3). This reduction might be due to direct interaction of the assayed stressors with medium components like glucose resulting in a decreased MDA formation, although this point was not checked. As to the cytoplasm contents, when these were directly treated with *t*-BOOH or H_2O_2, MDA levels were similar to the controls (Table 3), with concentrations comparable to those obtained in control HepG2 cells (Table 2) after normalization for protein content. This indicates that the assayed stressors do not directly affect cytosol components, probably due to their reduction by antioxidant defence systems present in the cytoplasm contents (e.g. catalase, gluthatione). Also, these results show that the increased MDA values in *t*-BOOH treated cells reported in Table 2 were not due to interferences caused by the culture medium nor by the processing of the cell lysates but by a direct effect of *t*-BOOH on the cell components, probably through peroxidation of membrane lipids.

In conclusion, to test MDA as a biomarker for lipid peroxidation in human hepatoma HepG2 in culture, a simple, precise, sensitive, reproducible, and reliable method has been developed by assaying a MDA derivative, 2,4-dinitrophenylhydrazone, by HPLC. The results of this work suggest that t-BOOH evokes a higher level of lipid peroxidation than H_2O_2 in HepG2 in culture.

Finally, this study establishes MDA as a good experimental parameter to investigate the potential protective effect of natural dietary antioxidants in cell cultures under oxidative stress conditions, studies that are currently being undertaken.

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