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Method development and validation for monitoring in vivo oxidative stress: Evaluation of lipid peroxidation and fat-soluble vitamin status by HPLC in rat plasma

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Abstract

Monitoring in vivo oxidative stress implicates the evaluation of damage and defence parameters by well-established, validated methods. We report two optimized and validated HPLC methods for measurement of malondialdehyde (MDA) and fat-soluble vitamins in rat plasma. For the MDA method, optimization experiments of the thiobarbituric acid test resulted in the addition of 1% butylhydroxytoluene to the reaction mixture and in a heating time reduction to 40 min, ensuring inhibition of further lipid peroxidation during the test. Validation experiments showed good linearity, precision and recovery. The use of HPLC with coulometric array detection technology permits simultaneous and sensitive analysis of different fat-soluble vitamins and related compounds (tocopherols, retinoids, carotenoids and coenzyme Q_{10}), which are identified by both retention time and electrochemical characteristics. Furthermore, this method is extended to the analysis of coenzyme Q_9, the predominant homologue in rats. Validation experiments with rat plasma gave good results.

Keywords: Oxidative stress; Lipid peroxidation; Fat-soluble vitamins

1. Introduction

Over the last decade, there has been a great interest in the role of oxidative stress in diverse pathological disorders such as atherosclerosis, aging, rheumatic diseases, diabetes and cancer [1–4]. During oxidative stress, the balance between reactive oxygen species (ROS) and the antioxidant defence system is disturbed in favour of the ROS, leading to potential damage to biomolecules such as lipids, proteins and DNA [5,6]. Consequently, antioxidants could have a protective role. Supplementation or dietary intake of antioxidant compounds could have beneficial effects on the prevention or treatment of oxidative stress-mediated diseases like atherosclerosis, diabetes or cancer. Many compounds have already been tested in vitro to determine their antioxidant profile [7,8]. These tests provide an indication of their antioxidant potential, but they do not account for problems of malabsorption, metabolisation, excretion and binding to proteins. Therefore, it is absolutely necessary to investigate oxidative stress and the potency of 'promising' antioxidants in vivo. Many publications on the in vivo activity of antioxidants are hampered by the fact that they were limited to one or two assays [9–12].

However, in order to obtain a good evaluation of the antioxidant potential, a sensitive, reliable and accurate

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investigation of several biological parameters covering the different aspects of oxidative damage (e.g. lipid peroxidation, DNA-damage) and antioxidative defence (e.g. vitamins A and E, glutathione peroxidase (GSH-Px)) is required. Therefore, as a start, a battery of different, well established and validated in vivo tests is being developed. An assay for monitoring GSH-Px activity in plasma has already been reported before [13]. The present work describes optimised and validated methods to evaluate malondialdehyde (MDA), a marker of lipid peroxidation, and fat-soluble vitamins in plasma.

Lipid peroxidation is a free-radical-mediated degradative process to which the polyunsaturated fatty acids (PUFA's) are particularly subject [14]. The primary reaction site are the membrane-associated PUFA's, resulting in a reduced membrane fluidity. Lipid peroxidation has been implicated in many pathological conditions, including atherosclerosis, aging, cardiac and cerebral ischemia and rheumatic diseases [15]. MDA is known as a universal biomarker of lipid peroxidation and can be measured upon reaction with thiobarbituric acid (TBA) [16,17]. In this study, the MDA–TBA test was optimized and the resulting complex was analysed by HPLC with fluorescence detection. Optimization experiments of the MDA–TBA assay resulted in the addition of butylated hydroxytoluene (BHT) and in a heating time reduction. Furthermore, the results of a validation study have been described.

Fat-soluble vitamins (Vitamins A and carotenoids, Vitamins E) and coenzyme Q play an important role in oxidative metabolism and free radical control [18]. Retinoids have a variety of important biological roles including signal transduction in the eye, regulation of the immune system response and proliferation and differentiation of many cell types, but their antioxidant potential is low compared to that of the carotenoids, which are effective antioxidants and free radical scavengers in vitro [6,19]. Vitamins E, a group of eight naturally occurring lipophilic forms including the tocopherols and tocotrienols, act as scavenger of peroxyl radicals and are probably the most important inhibitors of lipid peroxidation in membranes and lipoproteins [6]. α-Tocopherol is the major and most investigated form, although recent studies point out the importance of γ-tocopherol, which has an unique function in scavenging reactive nitrogen species [20–22]. Coenzyme Q plays an essential role in the mitochondrial electron-transport chain and also acts as an inhibitor of lipid peroxidation and regenerator of α-tocopherol from its radical in lipoproteins and membranes [6,18]. A global method for the analysis of fat-soluble vitamins in human plasma, as described by Gamache et al. [18], was investigated, adapted and validated for analysis in rat plasma. The use of HPLC with multi-channel coulometric detection is a relatively new, very sensitive and selective technique providing many possibilities in oxidative stress research [23]. In the study of redox biochemistry this electrochemical detector is very useful.

Since subsequent antioxidant supplementation studies will be conducted in rats, validation of the methods described below was done on pooled rat plasma.

2. Experimental

2.1. Chemicals

2-Thiobarbituric acid (TBA), 1,1,3,3-tetramethoxypropane, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), retinol, retinyl palmitate, α- and γ-tocopherol and coenzymes Q_9 and Q_{10} were purchased from Sigma–Aldrich (USA). Ethanol, ammonium acetate and sodium hydroxide were obtained from Merck (Germany). HPLC grade phosphoric acid from Acros (USA), methanol from Fisher Scientific (UK) and 1-propanol from Labscan (Ireland) were used. All compounds and reagents mentioned before were of analytical grade.

2.2. Sample preparation

Blood samples were collected in potassium EDTA tubes (Terumo, Belgium) from adult male Sprague–Dawley rats fed a normal control diet (Sniff, Germany). After centrifugation (1000 g, 10 min, 4 °C) plasma samples were stored at −70 °C. Any hemolysed samples were discarded. As reported in literature before [24], EDTA should be used as anticoagulant instead of heparine or citrate since it chelates traces of transition metals in blood and it may prevent additional MDA formation through possible platelet activation during blood processing.

2.3. Lipid peroxidation: MDA–TBA assay

The TBA test previously described by Young and Trimble [25] was optimised and validated. The TBA reaction was carried out by mixing 50 μl of rat plasma/standard, 25 μl of 1% (w/v) BHT in ethanol, 250 μl of 1.22 M phosphoric acid, 425 μl of HPLC grade water and 250 μl of a 0.67% TBA solution in clean, sealed glass tubes. Tetramethoxypropane was used as standard and is converted to MDA under the reaction conditions.

The reaction mixture was heated at 95 °C for 40 min and then cooled to 4 °C in ice. Proteins were precipitated by adding 360 μl of methanol and 40 μl of 1 M sodium hydroxide to 200 μl of the sample mixture. After centrifugation at 9500 × *g* for 5 min (Eppendorf 5415 D), samples were analysed on a complete Gilson HPLC system (322-H1 pump, 864 degasser, autosampler 234 with 20 μl loop and 832 sample cooler at 4 °C) with a Jasco FP-1520 fluorescence detector. The detector was set at excitation wavelength 532 nm and emission wavelength 553 nm. As mobile phase, solvent A: potassium phosphate buffer (25 mM, pH 4) – solvent B: methanol, 55:45 (v/v) were used. The gradient profile consisted of an 8 min isocratic elution at 45% B, after which the concentration was increased linearly up to 90% B in 3 min. This was maintained for 10 min, after which initial conditions were reinstalled in 3 min. A flow rate of 0.8 ml/min was used. A Lichrospher 100 C_{18} reversed-phase analytical column (octadecylsilane-covered spherical silica, 5 μm particle

size, 4 mm × 250 mm internal diameter) with a Lichrospher 100 C_{18} guard column (4 mm × 4 mm internal diameter) from Merck (Germany) were used for the separation of the MDA–TBA adduct.

2.4. Analysis of fat-soluble vitamins

Fat-soluble vitamins were extracted by mixing 100 μl of standard mixture or plasma with 100 μl diluent (i.e. HPLC grade water for standard samples and ethanol containing 1% BHA for plasma samples) and 500 μl hexane for 10 min on a Micromix 5 vortex mixer (DPC). Supernatant was withdrawn after centrifugation for 10 min at 4000 × *g*. This extraction procedure was repeated twice. After evaporation of the combined extracts by vacuum centrifugation (Savant), the residue was redissolved in 100 μl of ethanol containing 1% BHA and analysed on a Gilson 321-H1 HPLC system with ESA-5600A CoulArray 8-channel Electrochemical detector with Thermal Organizer. Samples (20 μl) were injected using a Gilson 234 autosampler with 832 sample cooler set at 10 °C. Mobile phase A consisted of methanol–water–1 M ammonium acetate pH 4.4, 90:8:2 (v/v). Mobile phase B consisted of methanol–1-propanol–1 M ammonium acetate pH 4.4, 78:20:2 (v/v). The flow rate was 0.6 ml/min. The gradient profile consisted of a 21 min linear gradient from 0 to 80% B, a 10 min linear gradient from 80 to 100% B and 14 min isocratic at 100% B before returning to initial conditions. The cell potentials were 200, 400, 500, 700, 800, −1000, 200 and 500 mV versus palladium reference and the system temperature was set at 37 °C. An ESA MD-150 RP_{18} column (3 mm × 150 mm) was used for the separation of the fat-soluble vitamins.

3. Results

3.1. Lipid peroxidation assay

3.1.1. Method optimisation

Using the conditions described a single, well-separated peak was produced in both standards and plasma samples with a retention time of 5.2 min. Although the MDA–$(TBA)_2$ complex eluted under isocratic conditions, a linear gradient to 90% of organic phase was necessary to clean the column when analysing plasma samples. In Fig. 1, the resulting HPLC chromatogram from the analysis of MDA in rat plasma is shown.

As reported before [14,15] blank samples also responded in the TBA-test. The use of glass – instead of plastic – tubes and an intensive cleaning procedure were necessary to reduce the blank peak to a minimum, equating to an MDA level of 0.1 μmol/l. During this cleaning procedure the tubes were filled with TBA-phosphoric acid mixture and heated for 1 h in boiling water prior to an extensive rinse with ultra-pure water [15]. Nevertheless, several blank samples were run in every test. The blank peak is not due to the fluorescence of TBA itself as reported by Londero et al. [14], but to the reagents and solvents that are contaminated with small amounts of MDA (<0.05 μM).

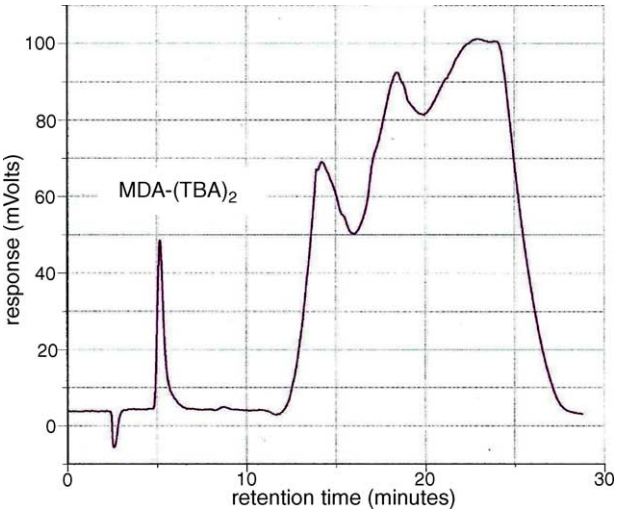

Fig. 1. Resulting chromatogram from HPLC-analysis of MDA–$(TBA)_2$ in basal rat plasma (0.49 μM).

Analysis of MDA-adduct showed stability of the plasma samples for up to 8 months when stored at −70 °C. No significant change in concentration of the analysed complex was observed (ANOVA, $P = 0.05$).

After TBA reaction and protein precipitation the resulting MDA-complex remained stable for up to 18 h at 4 °C, which made overnight analyses possible.

Although Draper et al. [26] reported possible co-precipitation of MDA–$(TBA)_2$ adduct with proteins, our analysis before and after protein precipitation did not show any difference in recovery.

Optimisation experiments on plasma to prevent further lipid peroxidation during TBA-reaction at 95 °C resulted in a reduction of the heating time and in the addition of BHT to the reaction mixture (Fig. 2). Different concentrations of BHT, ranging from 0 to 3.2% BHT, were tested in the reaction (0–90 min heating time). At 3.2% BHT, a strong increase of MDA–$(TBA)_2$ concentration was observed during the first 25 min of heating, followed by a plateau for up to 60 min of incubation where the observed adduct concentration remained constant. Longer heating times gave a rise in MDA–$(TBA)_2$ complex, which might be due to the diminishing activity of BHT as a chain-breaking antioxidant. In the absence of BHT a continuous increase of the complex without plateau phase was observed, indicating the constant formation of MDA artefact at any heating time. Consequently, the resulting MDA–$(TBA)_2$ concentrations were higher than those found with 3.2% BHT at the same heating time. Due to solubility problems 3.2% BHT could not be used. However, our experiments (Fig. 2) showed no significant difference in the MDA–$(TBA)_2$ concentration measured with a 1% BHT solution when heating the mixture for up to 40 min. Addition of 1% BHT to the mixture and heating time

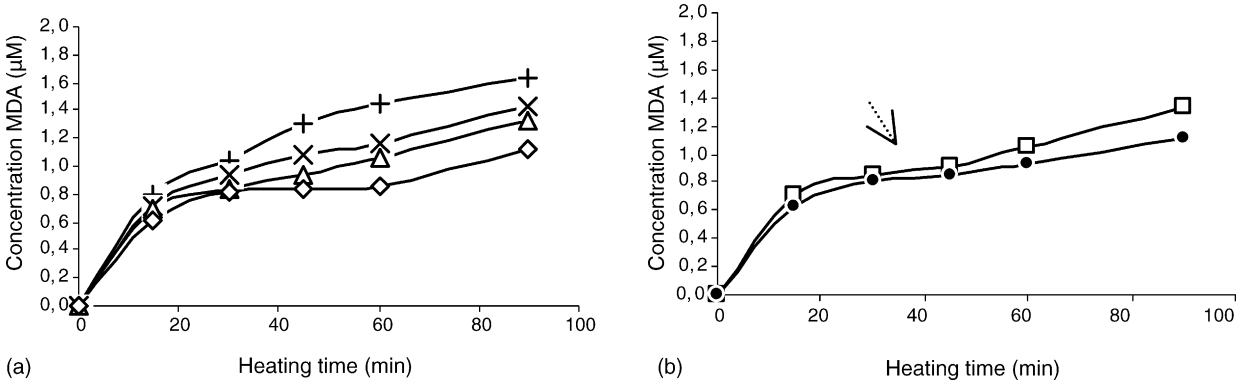

Fig. 2. Effect of heating time at 95 °C and different concentrations of BHT on measured MDA concentration in plasma. No standard was added externally. Small amounts were taken after 15, 30, 45, 60, and 90 min of heating and were analysed by HPLC. In (a) (+) no BHT; (×) 0.2% BHT; (△) 0.8% BHT; and (◊) 3.2% BHT were used in the reaction mixture. (b) Shows the results obtained with (□) 1% BHT and (●) 3% BHT.

reduction to 40 min were determined as final optimal reaction conditions.

3.1.2. Validation

For the linearity test five reference solutions were injected twice.

The assay showed good linearity up to at least 4.8 μM of MDA. The least squares line was $2052777x - 45333$ ($R = 0.9998$). *t*-Test on the intercept showed no statistical difference from zero. Following analysis of variance/lack of fit (ANOVA/LOF) and quality coefficient (QC) [27] calculation this linear model was accepted.

Repeatability was tested by analysing five true replicates of different concentrations covering the entire range. As indicated in Table 1 control plasma samples (0.5 μM MDA–$(TBA)_2$) gave a relative standard deviation (R.S.D.) of 11%, whereas this was less than 5% for higher concentrations of MDA adduct.

For the intermediate precision, analysis of five true plasma replicates on 3 different days resulted in a R.S.D. of 10%. The results obtained on 3 different days were not significantly different (ANOVA).

The overall recovery of standard at concentrations of 0.60, 1.92 and 3.84 μM from spiked plasma samples averaged 103% (R.S.D. 6.19%). A recovery *t*-test at each concentration level did not show any significant difference ($P = 0.05$) from 100% recovery (Table 2).

Table 1
Validation of the lipid peroxidation assay in rat plasma: precision

Repeatability		Intermediate precision	
MDA (μM)	R.S.D. (%)	Analysis of five true control plasma replicates (0.5 μM) on 3 days	
0.37	10.78	R.S.D. day 1 (%)	11.55
0.48	10.65	R.S.D. day 2 (%)	6.83
1.12	3.78	R.S.D. day 3 (%)	12.78
2.40	4.38	$R.S.D._{within\ day}$ (%)	10.78
4.37	4.60	$R.S.D._{between\ days}$ (%)	10.02

Table 2
Validation of the lipid peroxidation assay in rat plasma: accuracy

TMP added (μM)	%Recovery[a]	R.S.D. (%)	Recovery test
0.60	107.0	6.55	$t < t_{tab}$
1.92	100.0	5.55	$t < t_{tab}$
3.84	101.3	5.12	$t < t_{tab}$

[a] Overall recovery of TMP added to 15 samples = 102.8% (R.S.D. = 6.19%), *t* (calculated *t*-value), t_{tab} (critical *t*-value), $P = 0.05$.

The limit of detection (signal to noise ratio 3:1) was 0.78 pmol of MDA–$(TBA)_2$ per injection, corresponding to 0.039 μM of MDA. The limit of quantification (signal to noise ratio 10:1) corresponded to a concentration of 0.129 μM of MDA.

3.2. Fat soluble vitamin assay

3.2.1. Method optimisation

The optimised method permits simultaneous analysis of retinoids, α- and γ-tocopherol, carotenoids and coenzyme Q_{10} in plasma. Within one run these vitamin peaks are well separated and identified by both retention time and electrochemical characteristics, as listed in Fig. 3.

Standard stock solutions remained stable for up to 5 months when stored in the dark at −20 °C (−70 °C for retinol).

The resulting chromatogram from rat plasma differed from human plasma, as shown in Fig. 4. Carotenoids like β-carotene and coenzyme Q_{10} were not detectable. The absence of carotenoids was due to a deficiency in the synthetic control diet. On the other hand, for coenzyme Q_{10}, a species-related difference occurred. As reported in literature before [28,29] coenzyme Q_9 is the predominant homologue in rats and mice. Indeed, analysis of rat plasma showed a peak with the same electrochemical characteristics but eluting six minutes earlier than coenzyme Q_{10}. This peak was identified as coenzyme Q_9 by comparison with pure standard of coenzyme Q_9.

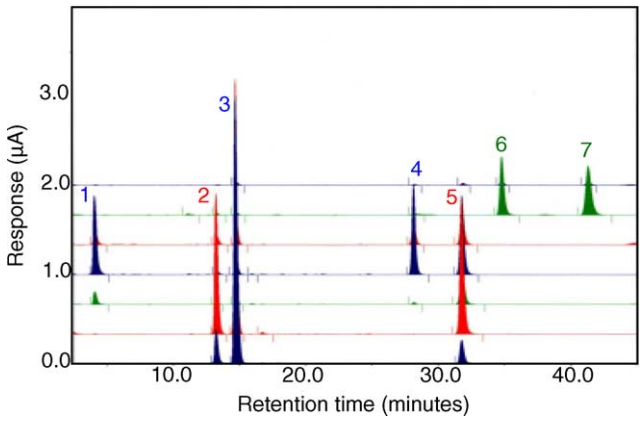

Fig. 3. Resulting chromatogram from HPLC-coulometric array analysis of fat-soluble vitamins (standards). (1) Retinol (1.80 μg/ml); (2) γ-tocopherol (2.29 μg/ml); (3) α-tocopherol (7.28 μg/ml); (4) retinyl palmitate (2.79 μg/ml); (5) β-carotene (2.30 μg/ml); (6) coenzyme Q_9 (2.26 μg/ml); and (7) coenzyme Q_{10} (2.42 μg/ml). (Channel 6 (−1000 mV) is not shown).

3.2.2. Validation

Linearity was tested by injecting five reference solutions, containing retinol, α- and γ-tocopherol and coenzyme Q_9, twice. Results are listed in Table 3.

Tables 4 and 5, respectively, show precision and accuracy data obtained for each of the vitamins and for coenzyme Q_9 in rat plasma. We evaluated within day and between days precision by analysing six true pooled rat plasma replicates on three different days. The accuracy was determined by assessing the recovery of three different standard mixture concentrations in spiked rat plasma samples. For the vitamins the recovery was not significantly different from 100% (recovery *t*-test). The overall recovery of coenzyme Q_9 averaged 112.8% (R.S.D. 9.3%).

The limits of detection (signal to noise ratio 3:1) for coenzyme Q_9, α-tocopherol, γ-tocopherol and retinol were, respectively, 0.4, 0.9, 0.2 and 6.3 pmol per injection. The limits of quantification (signal-to-noise ratio 10:1) for coenzyme Q_9, α- and γ-tocopherol and retinol corresponded to a concentration of respectively 0.056, 0.063, 0.015, and 0.3 μg/ml.

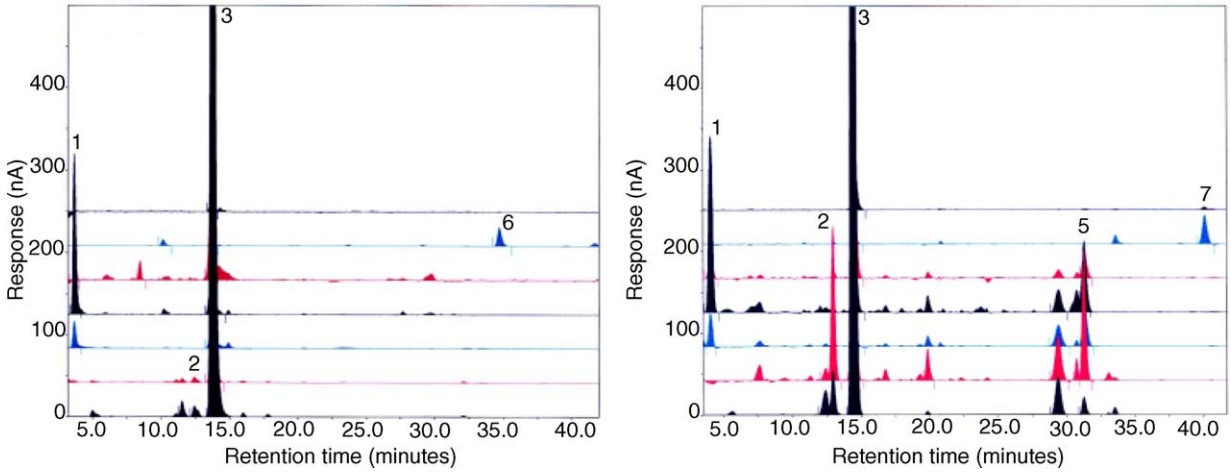

Fig. 4. Resulting chromatogram from HPLC-coulometric array analysis of fat-soluble vitamins in basal rat (left), and human (right) plasma. Each vitamin is numbered and concentrations in plasma are mentioned (r: rat; h: human): (1) retinol (r: 0.95 μg/ml; h: 1.0 μg/ml); (2) γ-tocopherol (r: 47 ng/ml; h: 713 ng/ml); (3) α-tocopherol (r: 13.0 μg/ml; h: 11.3 μg/ml); (5) β-carotene (h: 484 ng/ml); (6) coenzyme Q_9 (r: 284 ng/ml); and (7) coenzyme Q_{10} (h: 292 ng/ml). (Channel 6 (−1000 mV) is not shown).

Table 3
Validation of the linearity for retinol, α- and γ-tocopherol and coenzyme Q_9 in the fat-soluble vitamin assay

	Retinol	α-Tocopherol	γ-Tocopherol	Coenzyme Q_9
Least squares line	$y = 6.262x - 0.076$	$y = 6.365x - 0.121$	$y = 7.021x - 0.004$	$y = 3.212x - 0.007$
Range (μg/ml)	0.3–4.54	0.06–16.8	0.015–4.85	0.06–3.055
Correlation coefficient	0.999	0.9998	0.9998	0.9998
t (intercept)	$-0.526 < t_{tab}$ (0,0) included	$0.399 < t_{tab}$ (0,0) included	$0.034 < t_{tab}$ (0,0) included	$-0.0279 < t_{tab}$ (0,0) included
t (slope)	$92.96 > t_{tab}$	$165.47 > t_{tab}$	$151.74 > t_{tab}$	$188.21 > t_{tab}$
ANOVA-LOF/QC	$3.6 < F_{tab}$	$F > F_{tab}$ QC < 2.5%	$F > F_{tab}$ QC < 2.5%	$F > F_{tab}$ QC < 2.5%
Accept linear model	Yes	Yes	Yes	Yes

Linearity experiments were conducted on standard mixtures of different concentrations. *t* (calculated *t*-value), t_{tab} (critical *t*-value), *F* (calculated *F*-value), F_{tab} (critical *F*-value), $P = 0.05$.

Table 4
Validation of the fat-soluble vitamin assay in rat plasma: precision of the analysis of retinol, α- and γ-tocopherol and coenzyme Q_9

	Retinol	α-Tocopherol	γ-Tocopherol	Coenzyme Q_9
R.S.D. day 1 (%)	11.41	5.68	4.92	8.82
R.S.D. day 2 (%)	11.19	5.35	4.91	7.86
R.S.D. day 3 (%)	9.15	2.71	2.49	5.53
$R.S.D._{\cdot within\ day}$ (%)	10.59	4.74	4.27	7.36
$R.S.D._{\cdot between\ days}$ (%)	10.46	7.39	5.78	11.49

Table 5
Validation of the fat-soluble vitamin assay in rat plasma[a]: accuracy of the analysis of retinol, α- and γ-tocopherol and coenzyme Q_9

Recovery concentration	Retinol	α-Tocopherol	γ-Tocopherol	Coenzyme Q_9
1 (%)	103.8 (6.14)[b] (0.5 μg/ml)[c]	87.4 (25.38)[b] (1.1 μg/ml)[c]	96.5 (14.50)[b] (0.1 μg/ml)[c]	125.3 (1.90)[b] (0.17 μg/ml)[c]
2 (%)	96.6 (3.81) (1 μg/ml)	97.2 (3.49) (8.16 μg/ml)	99.3 (1.80) (1 μg/ml)	109.6 (4.15) (0.42 μg/ml)
3 (%)	101.1 (2.39) (2.37 μg/ml)	98.1 (3.14) (15.2 μg/ml)	101.8 (2.23) (2.12 μg/ml)	103.4 (5.72) (0.92 μg/ml)

[a] Fat-soluble vitamin concentrations in rat plasma were 9.6 μg/ml α-tocopherol, 0.24 μg/ml γ-tocopherol, 0.45 μg/ml retinol, 0.19 μg/ml coenzyme Q_9.
[b] R.S.D. (%).
[c] Concentration of standard added to plasma samples.

4. Discussion

In the current study, methods for monitoring lipid peroxidation and fat-soluble vitamins in rat plasma were optimised and fully validated. Validation of the assays received much attention, since it is often neglected or very limited in the method development of bio-assays.

The TBA-test, for the determination of MDA, is widely used to measure lipid peroxidation, although it has been criticised to have low specificity due to the reaction of many other substances with TBA [30,31]. This interference is a particular problem when the complex is measured spectrophotometrically [32,33]. We have described an HPLC method combined with fluorescence detection, which is a very sensitive and specific technique to analyse the MDA–TBA adduct. Furthermore, it has been shown in this study that further lipid peroxidation during the heating stage of the reaction can be prevented by the addition of BHT and by the reduction of the heating time to 40 min. The use of high quality reagents and solvents and carefully cleaned glass tubes reduces the MDA-peak in blank samples. Linearity of the assay was demonstrated by analysis of variance (lack-of-fit test). Other previously reported validation studies [14,25] only mentioned the correlation coefficient, but this should not be used to prove the linearity of the model [34]. For the accuracy and precision, previously reported data [14,15,25] are restricted to listing recoveries and coefficients of variation, respectively, without mentioning any results of statistical analysis. In this study, accuracy of the method over the entire range was shown by a recovery test at three different concentration levels. Tests on repeatability gave a R.S.D. of 11% for the lowest MDA-concentration analysed and for the intermediate precision, results obtained on 3 different days were not significantly different (ANOVA). The limit of quantification corresponded to a concentration of 0.129 μM MDA, which is substantially lower than the basal rat plasma concentration (0.4–0.6 μM).

This method allows easy, reliable assessment of MDA to evaluate lipid peroxidation. In addition to MDA, isoprostanes, especially F_2-isoprostanes, which are oxidation products of arachidonic acid, have been proposed as biomarkers of lipid peroxidation [35]. Although no doubt important markers, inclusion in an oxidative status screening battery is hampered by a complex sample preparation procedure and sophisticated quantification techniques (GC–MS or LC–MS/MS). Immunoassays also have been developed, but suffer a lack of reliability and specificity [36].

A method for the simultaneous analysis of fat-soluble vitamins in rat plasma has been optimised and validated. The use of HPLC with multi-channel coulometric detection is very useful for the analysis of different compounds in one run providing not only the retention time but also the electrochemical characteristics as unambiguous identification. This very specific and sensitive technique permits analysis of redox-sensitive compounds in complex biological matrices like plasma. Validation studies showed good linearity (ANOVA/LOF) for the different vitamins. The recovery was tested at three different concentrations, covering the entire range, and was statistically not significantly different from 100% ($P = 0.05$) for the fat-soluble vitamins. The overall recovery of coenzyme Q_9 averaged 112.8%. Tests on repeatability gave a R.S.D. of 7.4% for coenzyme Q_9, 10.6% for retinol and less than 5% for the tocopherols. Between day R.S.D. were 11.5% for coenzyme Q_9, 10.5% for retinol, 7.4 and 5.8% for α- and γ-tocopherol respectively, which are acceptable for this biological method. The limits of detection for coenzyme Q_9, α-tocopherol, γ-tocopherol and retinol corresponded to a concentration of respectively 17 ng/ml, 19 ng/ml, 4.5 ng/ml and 0.09 μg/ml.

In conclusion, these methods can be used for the evaluation of in vivo oxidative stress and promising antioxidants. In our opinion, the use of different in vivo assays, to assess the damage to endogenous biomolecules together with the evaluation of the body's defence systems, is essential to draw valuable conclusions concerning in vivo oxidative stress status and antioxidant potential. Validated analytical methods are a prerequisite to evaluate the in vivo oxidative stress status. In this study methods for the evaluation of MDA, a marker of lipid peroxidation, and fat-soluble vitamins have been optimised and validated. Both methods can be included in a battery of different tests to study in vivo oxidative stress.

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