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SIMULTANEOUS MEASUREMENT OF MALONDIALDEHYDE, FORMALDEHYDE, ACETALDEHYDE, AND PROPIONALDEHYDE TO MONITOR THE OXIDATIVE STRESS IN HUMANS

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Herein, a method is described for the determination of malondialdehyde, formaldehyde, acetaldehyde, and propionaldehyde in human plasma. Plasma samples were obtained from blood donors. After protein precipitation, the samples were derivatized with 2,4-dinitrophenylhydrazine and repeatedly extracted with pentane. So combined pentane extracts were evaporated to dryness, under nitrogen atmosphere, and the dried residue was re-suspended in acetonitrile. Aliquots of 20 μl in acetonitrile were injected onto a LichroCART 125-4, Purospher STAR RP-18e, 5 μm column. The derivatives were analysed with the aid of gradient elution, when using a mobile phase containing acetonitrile, water, and acetic acid. The analytical performance of this method has been found to be

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satisfact ory characterised by the intra-assay coefficients of variation below 12 % and quantitative recoveries as follows: 98.3 % (CV 1.6%) for malondialdehyde, 104.4 % (CV 4.0 %) for formaldehyde, 96.9 % (CV 3.0 %) for acetaldehyde, and 105.9 % (CV 1.7 %) for propionaldehyde.

Introduction

Aldehydes, especially malondialdehyde (MDA), have been widely used as markers of lipid peroxidation induced by oxidative stress [1-5]. Lipid peroxidation is initiated by the attack of an unsaturated fatty acid by any radical. The fatty acid radical is stabilised by molecular rearrangement to produce a conjugated diene. It undergoes reaction with the molecule of dioxygen, forming fatty acid peroxyl radical. Decomposition of the fatty acid peroxyl radical generates the secondary lipid peroxidation products, such as hydrogen gases and aldehydes [6]; the latter being highly reactive and genotoxic agents.

The degree of lipid peroxidation in plasma can be determined by several ways. The most common method for the determination of MDA in various biological samples is based on the reaction with 2-thiobarbituric acid (TBA) in acidic media [7-11]. However, this assay is non-specific in nature because other compounds can react with TBA giving rise to thiobarbituric acid reactive substances (TBARS). High-performance liquid chromatography (HPLC) with spectrophotometric or fluorescence detection improves the selectivity enhancing the sensitivity of the TBA method, too. Yet another possible method for the measurement of MDA, but also for other aldehydes and ketones, is based on the reaction with 2,4-dinitrophenylhydrazine (DNPH) [12-15]. Derivatization of MDA and other carbonyls with DNPH, as well as conversion into hydrazone and pyrazole derivatives is more specific than TBA method.

The aim of this study was to develop and validate a reliable reversed-phase HPLC method with ultraviolet (UV) detection for the measurement of MDA, formaldehyde, acetaldehyde, and propionaldehyde in human plasma.

Materials and Methods

Reagents and Chemicals

Formaldehyde (FDA), acetaldehyde (ADA), propionaldehyde (PDA), 1,1,3,3-tetramethoxypropane (TMP), hydrochloric and acetic acids together with DNPH were obtained from Sigma (St. Louis, MO, USA). HPLC-gradient grade acetonitrile and pentane were from Merck KgaA (Darmstadt, Germany). All other chemicals were of analytical grade.

Preparation of the Standards

The stock solution of MDA (≈1 mmol l^{-1}) was prepared by dissolving 83 µl TMP in 500 ml deionized water. Stock solutions of FDA, ADA and PDA (≈1 mmol l^{-1}) were then made from 38 µl FDA, 28 µl ADA and 38 µl PDA in 500 ml deionized water. All the stock solutions of aldehydes were prepared fresh daily.

Instrumentation

Chromatographic analysis was performed with a liquid chromatograph (Ecom, Prague, the Czech Republic) equipped with an LCP 4100 solvent delivery system, an AS 54 autosampler, an LCO 101 column oven, and an LCD 2084 variable wavelength spectrophotometric detector. The data were collected digitally using Clarity chromatography software (DataApex, Prague, the Czech Republic).

Selection of Samples

Plasma samples were obtained from blood donors (n = 10, five women at the age of 21 ± 3 years and five men, 20 ± 2 years old); in all cases being the students of the University of Pardubice. All the persons involved in the study had also signed an official consent form to participate as a volunteer(s), which was approved by the Hospital Committee on Human Research, at the Regional Hospital of Pardubice, in accordance with the Helsinki Declaration.

Blood Samples Collection

Venous blood samples were obtained under standard conditions, from 7 to 8 a.m. after fasting at least 12 hours. Blood was collected into tubes (the Vacuette Detection Tube, No. 454246, Greiner Labortechnik Co., Kremsmüster, Austria) containing also EDTA. Plasma was separated from blood cells by centrifugation (at 1 700×g, for 15 min and at 8 °C) and, afterwards, immediately stored at –80 °C in 1.5-ml glass tubes.

Sample Preparation

To analyse blood plasma, 500 µl plasma were pipetted into a well-capped 1.5-ml glass tube and 500 µl cold 20 % TCA then added, the solution vortexed for 1 min, incubated 10 min at 4 °C, and centrifuged (22 000×g, 10 min, 4 °C). Then, a

volume of 500 µl supernatant was pipetted into a well-caped 5-ml extraction glass tube and 100 µl 10 mmol l^{-1} DNPH in 2 mol l^{-1} HCl added and so prepared mixture mixed vigorously using a vortex mixer for 60 s and incubated at 30 °C for 30 min. Afterward, 1 ml cold pentane was added, the solution vortexed for 20 min, and centrifuged (4 000×g, 5 min, 4 °C). The upper layer of pentane formed was transferred into 5-ml glass tube and the extraction process repeated three times. Combined pentane extracts were evaporated to dryness, under nitrogen (Linde Gas, Prague, the Czech Republic) at room temperature. The dried residue was re-suspended in 250 µl acetonitrile and vortexed for 60 s. Then, the sample was filtered through a Nylon® filter (pore size: 0.20 µm, diameter: 4 mm, Supelco, Bellefonte, USA) and transferred into a 1-ml cramped vial. The stock solutions of aldehydes were diluted with deionized water to give a series of mixed working standards. To 500 µl mixed solution of standards, 500 µl cold 20 % TCA were added and so prepared standards subjected to the same procedure as described above for plasma samples.

For recovery experiments, 20 µl mixed solution of individual aldehydes at various concentrations was added to 480 µl of plasma. The next steps were the same as above, for plasma sample preparation.

Chromatographic Analysis

The chromatography of selected aldehydes after their stoichiometric conversion to the corresponding 2,4-dinitrophenylhydrazones was accomplished using a gradient elution on a LiChroCART®, 125×4 mm i.d., PurospherSTAR® RP-18e, 5 µm analytical column fitted with a LiChroCART®, 4×4 mm i.d., PurospherSTAR® RP-18e, 5 µm guard column (Merck KgaA, Darmstadt, Germany) at 37 °C. Two mobile phases were used: A – acetonitrile + deionized water + acetic acid (30:70:0.1, v/v/v), and B – acetonitrile + deionized water + acetic acid (95:5:0.1, v/v/v). Prior to use, both phases were vacuum filtered and degassed using ultrasound. The gradient was applied in the following sequences: from 0 to 1 min … 0 % B; 1-20 min … 0 % to 20 % B (linear gradient); 20-33 min … 20 to 30 % B (linear gradient); 33-45 min … 100 % B; and 45-50 min … 0 % B. All separations were performed at 37 °C and the flow rate was kept constant at 0.5 ml min^{-1}. The optimum response of the aldehydes (measured as 2,4-dinitrophenylhydrazone derivatives) was observed, when the wavelengths were 307 nm (MDA) and 356 nm (FDA, ADA and PDA). The amount of selected aldehydes in the standard and plasma samples was evaluated and quantified from the corresponding peak area using the Clarity chromatography software. The concentration of selected aldehydes was determined from the respective calibration curve.

Statistical Treatment of the Data

Regression analysis was carried out using the least-squares method (software "QCexpert"; Trilobyte, Pardubice, the Czech Republic).

Results and Discussion

An HPLC with UV detection for the quantification of selected aldehydes in human plasma has represented the technique of choice. Lipid peroxidation — i.e., the oxidative degradation of unsaturated fatty acids — may be associated with the damaging process of cells and tissues and the resultant products are aldehydes.

There are several methods available to quantify MDA as a suitable marker of lipid peroxidation in biological samples. Among others, the derivatisation with 2-TBA has been frequently used because of its simplicity, although the method lacks specificity. Results from this study suggest that not only MDA but also other aldehydes, such as FDA, ADA and PDA can be detected in human plasma with high specificity after derivatisation with DNPH. Other methods use direct detection or fluorescence detection of the pre-derivatised product; however, most of these methods are nonspecific or time-consuming and sophisticated and therefore, they are not suitable for routine use in clinical analysis.

Some Notes to Sample Preparation

The proper sample preparation is essential for accurate analysis. In this study, several protein precipitants were tested; namely, acetonitrile, ethanol, methanol, propan-1-ol, propan-2-ol, metaphosphoric acid, TCA, 5-sulfosalicylic acid, and perchloric acid when the respective one was carefully added to plasma. After incubation (for 10 min, 4 °C) and centrifugation (22 000×g, 10 min, 4 °C), the derivatisation agent was added to supernatant. Use of organic solvents was not possible because they contain contaminants; especially, carbonyl compounds. For example, commercial HPLC-grade methanol and/or ethanol contain relatively large amounts of C2, C3 and C4, typically accompanied by lesser amounts of longer-chain alkanals. After derivatisation, extraction of 2,4-dinitrophenyl-hydrazones with pentane was performed. As already mentioned, commercial HPLC-grade solvents contain alkanals and these solvents cannot be used before the derivatisation step. In contrast, the resulting derivatives may be extracted with an organic solvent because after an extraction the reaction is stopped. It was found that three-times repeated extraction had been sufficient for all 2,4-dinitro-phenylhydrazone derivatives, when trichloroacetic acid as a protein precipitant led to satisfactory recoveries.

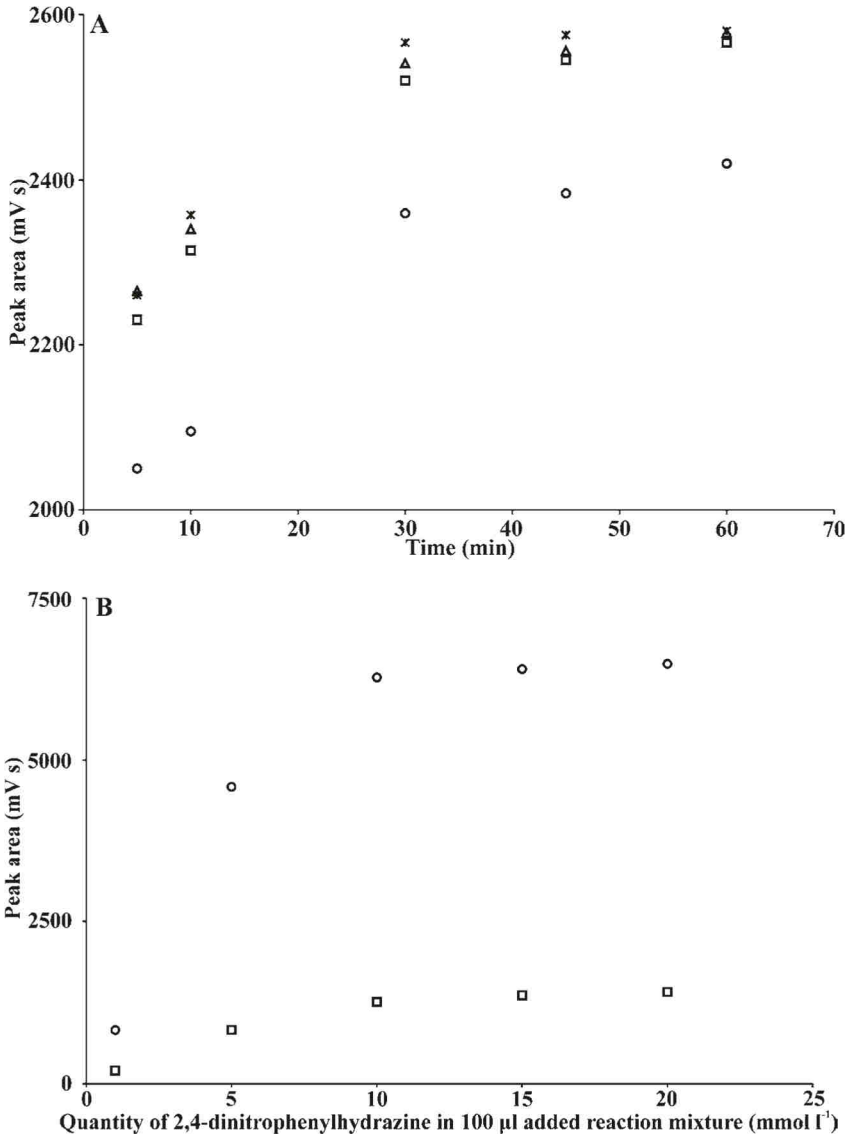


Fig. 1 Optimisation of derivatisation procedure. Effect of temperature and time on the derivatisation recovery (A); malondialdehyde standard solution, 2 µmol l^{-1} (○ 25 °C, □ 30 °C, △ 45 °C, * 60 °C) and the effect of 2,4-dinitrophenylhydrazine concentration on the derivatisation recovery (B); malondialdehyde standard solution (○ 5 µmol l–1, □ 1 µmol l^{-1}). For optimisation of derivatisation procedure, malondialdehyde was chosen because it reacts with 2,4-dinitrophenylhydrazine least readily

Derivatization procedure was optimised in order to achieve the maximum derivative signal for 2,4-dinitrophenylhydrazone with the lowest interferences from the derivatising reagent DNPH. Various temperatures (from 25 to 60 °C) and various concentration of DNPH solution (from 1 to 20 mmol l^{-1}) were tested. We reduced the concentration of the derivatisation reagent in order to minimise artificial signals from an excess of DNPH reagent. The optimum results of the derivatisation method were obtained using a DNPH solution with the concentration of 10 mmol l^{-1} (and the final concentration of 1.67 mmol l^{-1} after mixing with supernatant) at 30 °C for 30 min. The results are summarised in Fig. 1.

Aldehydes after their derivatisation with DNPH were separated as the 2,4-dinitrophenylhydrazone derivatives on a reversed-phase column using the gradient system with acetonitrile, deionized water, and acetic acid. The mobile phase was optimised in an effort to obtain the best separation of the analytes in the shortest time. Standard solutions of selected aldehydes, as well as pooled plasma samples were used for studying the mobile phase composition, when assessing several eluents in the form of mixtures of organic solvents (e.g., acetonitrile, ethanol, and methanol with acidified deionized water) at various gradients.

Table I Results of determination of malondialdehyde, formaldehyde, acetaldehyde and propionaldehyde in human plasma

Precision (within day)	MDA Mean ± *S.D.* μmol l^{-1}	CV %	FDA Mean ± *S.D.* μmol l^{-1}	CV %
10	1.80 ± 0.21	11.7	3.12 ± 0.35	11.2
10	4.52 ± 0.33	7.3	8.54 ± 0.59	6.9
Precision (within day)	ADA Mean ± *S.D.* μmol l^{-1}	CV %	PDA Mean ± *S.D.* μmol l^{-1}	CV %
10	3.55 ± 0.29	8.2	1.73 ± 0.13	7.5
10	8.21 ± 0.49	6	5.22 ± 0.27	5.1

MDA – malondialdehyde; FDA – formaldehyde; ADA – acetaldehyde; PDA – propionaldehyde

The best results were obtained for the set of conditions described in section Chromatographic Analysis. In this case, column temperature was changed from 25 to 45 °C, the optimum temperature interval from 35 to 40 °C and the main criteria were the resolution, stability of the absorbance and time of analysis. Two different columns, both of C18 type, were assayed in the study: a LiChroCART® and a Discovery (Supelco, Bellefonte, PA, USA), when the first one was selected for further experiments. Two lengths (150 and 250 mm) were tested and that of 150 mm length yielded the best resolution with shortest retention time for selected aldehydes in human plasma. The same column also provided the proper separation between aldehyde-2,4-dinitrophenylhydrazone derivatives from potentially interfering substances. Typical chromatograms of selected aldehydes in the standard solution and human plasma are shown in Fig. 2.

The precision of MDA, FDA, ADA, and PDA analysis in the plasma samples is shown in Table I. To determine such a precision, the plasma samples were analysed ten times within the same day and under the same conditions. In all

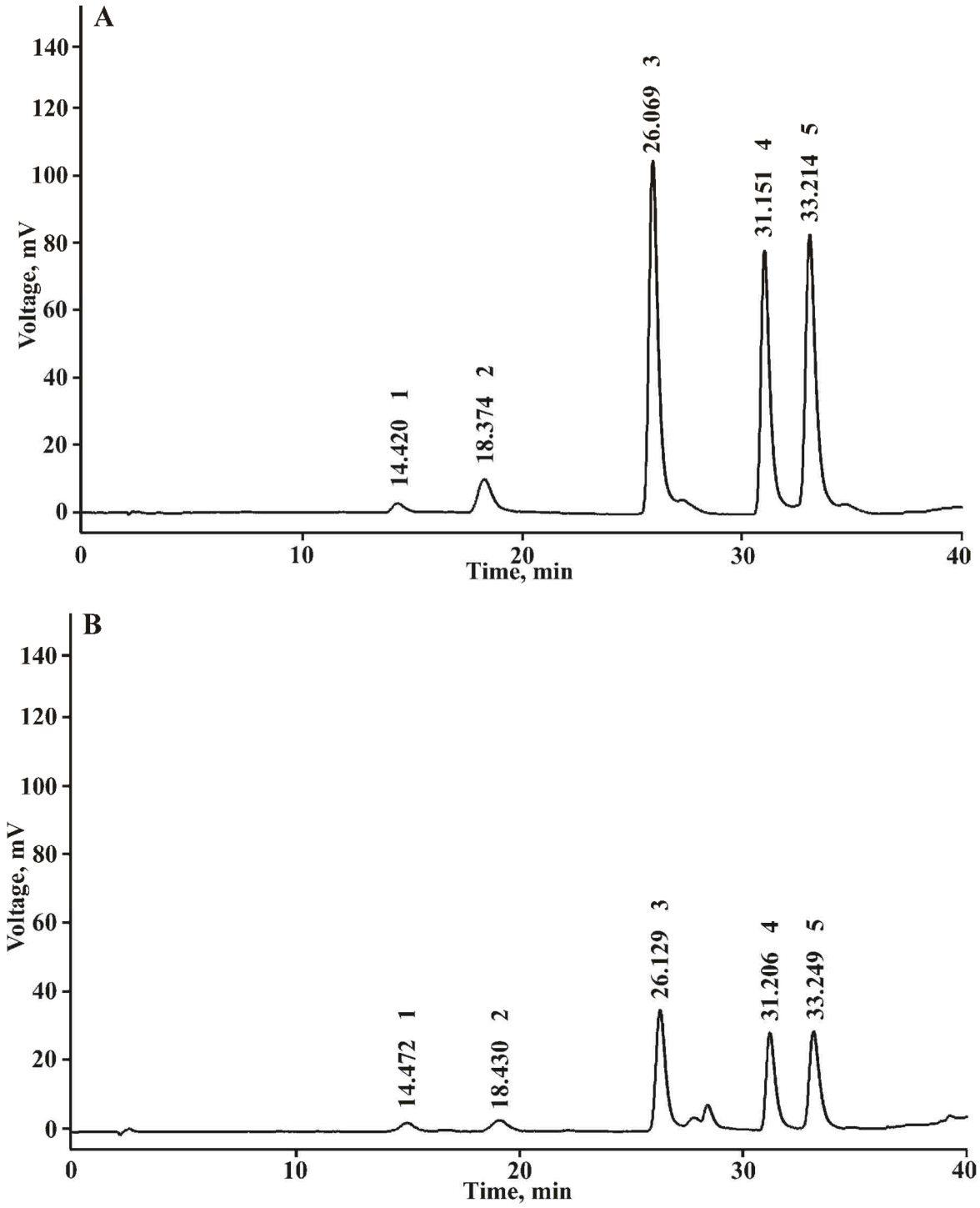
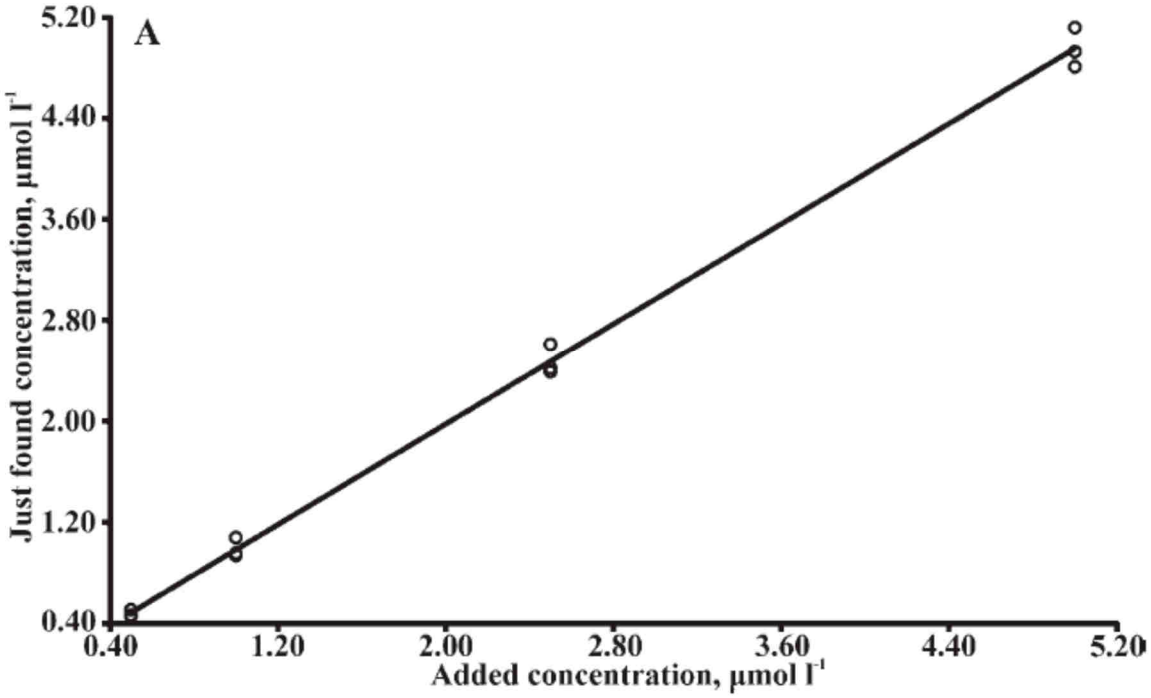
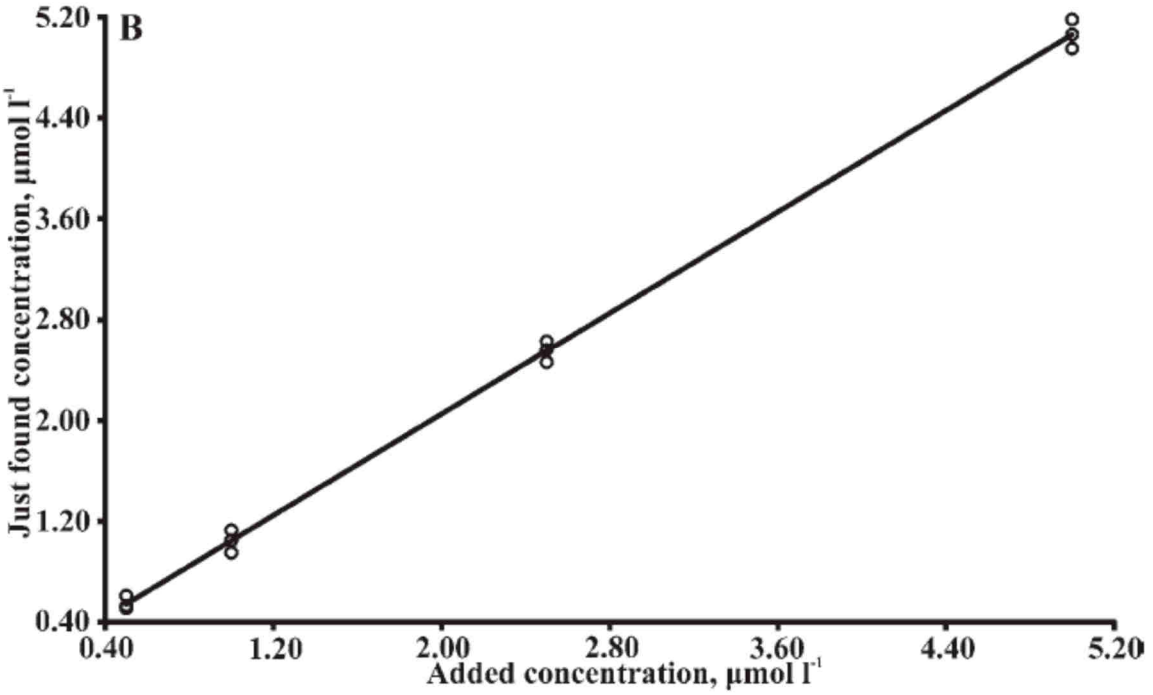


Fig. 2 An HPLC chromatogram of selected aldehydes (MDA, 1.00 μmol l^{-1}; FDA, 2.50 μmol l^{-1}; ADA, 5.00 μmol l^{-1}; PDA, 5.00 μmol l^{-1}) in standard solution (A) and (MDA, 0.82 μmol l^{-1}; FDA, 1.11 μmol l^{-1}; ADA, 2.09 μmol l^{-1}; PDA, 2.29 μmol l^{-1}) human plasma (B). Peaks: 1, MDA; 2, FDA; 3, ADA; 4, acetone; 5, PDA. HPLC conditions: a gradient elution; [mobile phase A: acetonitrile + deionized water + acetic acid (30:70:0.1, v/v/v) and B: acetonitrile + deionized water + acetic acid (95:5:0.1, v/v/v); Gradient: 0-1 min ... 0 % B; 1-20 min ...0-20 % B; 20-33 min ... 20-30 % B; 33-45 min ...100 % B; And 45-50 min ... 0 % B], stationary phase was an analytical column LiChroCART®, 125×4 mm i.d., PurospherSTAR® RP-18e, 5 μm fitted with a guard column LiChroCART®, 4×4 mm i.d., PurospherSTAR® RP-18e, 5 μm, the flow rate was kept constant at 0.5 ml min^{-1}, separation ran at 37 °C and aldehydes (2,4-dinitrophenylhydrazones) were monitored at 307 nm (MDA) and 356 nm (FDA, ADA, PDA)


A
5.20
4.40
3.60
2.80
2.00
1.20
0.40
Just found concentration, µmol l-1
0.40
1.20
2.00
2.80
3.60
4.40
5.20
Added concentration, µmol l-1


B
5.20
4.40
3.60
2.80
2.00
1.20
0.40
Just found concentration, µmol l-1
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1.20
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5.20
Added concentration, µmol l-1

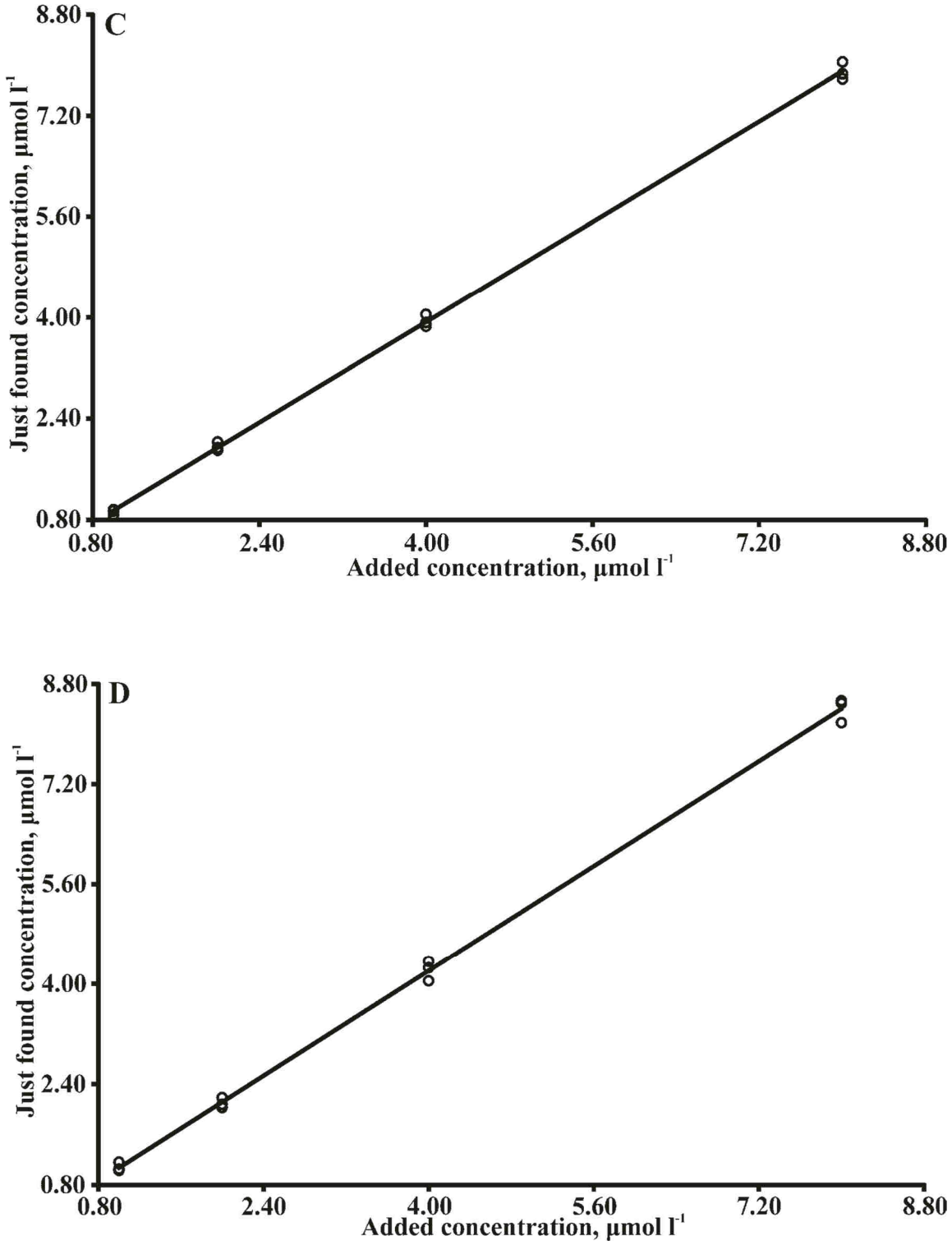

Fig. 3 Typical recovery experiment: malondialdehyde (A), formaldehyde (B), acetaldehyde (C), and propionaldehyde (D). Values of triplicate assays are recorded. The slopes correspond to the mean recovery of 98.3 % (*S.D.* = 1.6 %) for malondialdehyde, 104.4 (*S.D.* = 4.0 %) for formaldehyde, 96.9 % (*S.D.* = 3.0 %) for acetaldehyde, and 105.9 % (*S.D.* = 1.7 %) for propionaldehyde

the cases, the coefficients of variation were below ±12 %. Regarding the analytical performance in quantitative analysis, the spike recoveries for selected aldehydes are gathered in Fig. 3. Calibration curves were linear over the whole range tested; i.e., 0.10-10.00 μmol l^{-1}), when the regression lines obtained by combining five standard curves are summarised in Table II. The lowest concentration that could be detected and, at the same, quantified with acceptable accuracy and precision was 0.10 μmol l^{-1} for all aldehydes (corresponding to 1.7 pmol $inject^{-1}$). Finally, the limit of detection for MDA, FDA, ADA and PDA, defined as a signal-to-noise (*S/N*) ratio of 3:1, was 0.03 μmol l^{-1} (for 0.5 pmol $inject^{-1}$).

Table II Average parameters of five calibration curves for the developed HPLC method

Standard	Regression equation	Mean slope, 95% confidence interval
MDA[1]	$y = 4239.5x + 201.5$	4239.5 (4032.6-4475.2)
FDA[1]	$y = 4990.2x + 433.1$	4990.2 (5272.4-4721.5)
ADA[1]	$y = 4236.1x + 285.6$	4236.1 (4455.8-4044.5)
PDA[1]	$y = 3568.2x + 275.6$	3568.2 (3751.0-3412.9)
Standard	Intercept (μmol l^{-1})[2] 95% confidence interval	Correlation coefficient
MDA[1]	–0.05 (from – 0.08 to – 0.01)	0.9981
FDA[1]	–0.09 (from – 0.13 to – 0.05)	0.9986
ADA[1]	–0.07 (from – 0.11 to – 0.02)	0.9989
PDA[1]	0.08 (from 0.12 to 0.03)	0.9983

[1]Eight-point for the determination of analytical parameters; [2]The *x*-intercept (μmol l^{-1}) is the point at which the line crosses the *x*-axis; MDA – malondialdehyde; FDA – formaldehyde; ADA – acetaldehyde; PDA – propionaldehyde

Conclusion

A new HPLC method with UV detection for the determination of selected aldehydes as markers of oxidative stress in human plasma was developed, offering sufficiently high sensitivity for measuring aldehydes in plasma at the physiological level. All aldehydes of interest were also detectable in real samples — in whole blood of the volunteers / donors.

Acknowledgments

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