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Determination of malondialdehyde in human plasma by fully automated solid phase analytical derivatization☆

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ABSTRACT

Analytical derivatization (AD) increases the sensitivity of analysis by one to three orders of magnitude, stabilizes labile analytes and converts them into readily extractable products. Using a variant of this technique, we applied solid phase analytical derivatization (SPAD) to fully automate extraction, derivatization and liquid chromatography. The resulting device (AutoSPAD) determined malonyldialdehyde (MDA) from biological fluids. This biomarker of oxidative stress is highly water-soluble (500 g/L at pH 7), chemically labile and lacks any functionality that enables detection at high sensitivity. AutoSPAD utilizes column-switching technology to load DANSYL hydrazine onto the solid phase, pass the biological sample over the resulting reactor bed for derivatization on the surface to form a hydrophobic derivative suitable for increasing sensitivity of any other LC technique including LC–MS/MS. The hydrophobic solid phase retains the derivative during washing steps, following which AutoSPAD transfers the derivatized extract to the analytical column for separation and detection by fluorescence. In plasma, however, MDA exists both in free form and covalently bound to protein. Measuring MDA from plasma, therefore, required identification of appropriate protein precipitation and hydrolysis conditions. Under these conditions, the DANSYL derivative formed at only one aldehydic position but did not cyclize as reported for other reactions between hydrazine reagents and MDA. The calibration curve using ~7 μL of plasma was linear (r^2 = 0.999) in the physiological range (0.1–3 μg/mL) and the relative standard deviation of replicate determinations at 1 μg/mL was less than 5%.

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1. Introduction

A multi-laboratory study by Kadiiska et al. [1] identified malondialdehyde (MDA) biomarker of oxidative stress. Fundamental [2,3] and clinical studies of human disease [4,5] currently use this marker to assess the effects of this biological process on human disease. Such measurement, however, is difficult because this molecule is unstable, highly water-soluble and lacks a chromophore, fluorophore or electrophore. Accordingly, analytical derivatization (AD) is a requirement for its determination [1,6–12]. This problem exemplifies the utility of this analytical technique. Derivatization converts MDA into a stable, lipophilic product that, depending on the reagent, can be a chromophore, a fluorophore an electrophore or a pre-ionized molecule to improve ionization efficiency in mass spectrometry. Conventionally, however, AD is time consuming, error-prone, and has limited options for automation.

We [10,11,13–22] and others [23–29] addressed these challenges by developing solid phase analytical derivatization (SPAD) of various analytes, including MDA [10,12,14], from biological samples. Recent work shows that SPAD facilitates simultaneous extraction/derivatization by producing faster reaction kinetics for AD particularly when the sorbent is configured in a column [11]. These results provided the knowledge for development, construction and testing of a device capable of automated determination of MDA from aqueous solutions or muscle homogenate [10].

Plasma, however, presented a more difficult problem and required overcoming matrix effects not encountered in the previous study on tissue homogenate. For instance, hydrolysis used for release of MDA from its' adduct with protein in tissue homogenate was not effective for plasma analysis. Additionally, derivatization with hydrazines can produce a multiplicity of products [9] and this problem had not been completely resolved for SPAD [30]. Finally, the equipment previously employed was constructed as a research instrument [10]. This provided proof of principal but was not practical for general implementation or assembly and use by

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non-specialists who need to apply the technique and instruments to biomedical and clinical problems.

Here we report techniques that resolve these limitations, and their successful application of the AutoSPAD technology to the analysis of plasma MDA using a commercially available chromatographic system for complete automation. We also report the reaction scheme for the MDA–hydrazone formation based on mass spectrometric analysis of the product.

2. Materials and methods

2.1. Chemicals and reagents

DANSYL hydrazine (DH, 98%), heptane sulfonic acid sodium salt (HSA, 98%), C1–C7 aldehydes and 1,1,3,3-tetramethoxy propane (TMP, 99%) were purchased from Sigma/Aldrich (Oakville, Canada). MDA stock was prepared by hydrolysis of TMP (10 μL) in 0.1N HCl (990 μL). The concentration of MDA in the stock was stable after 2 h (room temperature) and remained stable for 1 week when stored at 4 °C. The concentration of the stock solution was calculated based on a 60% molar conversion of TMP to MDA, according to literature [31]. Blank plasma for method development was obtained from surplus, spent stock of the Canadian Blood Services (Hamilton, ON). Acetonitrile (ACN) and methanol (MeOH, both HPLC grade) were from Caledon (Georgetown, ON) and ammonium acetate (reagent grade) was from Sigma–Aldrich (Toronto, ON). Zinc sulphate (ACS grade) was obtained from Fisher Scientific (Toronto, ON) and a 10% solution was prepared in water. A purified form of the polystyrene–divinylbenzene cross-linked co-polymer macroreticular resin, Amberlite XAD-2 was purchased from Rohm and Haas (Philadelphia, PA). It was sieved to isolate the 50–80 mesh fraction prior to use. DH reagent was prepared in methanol and was stable for up to 1 month at room temperature. This was stored in the dark inside of an outer sealed container containing activated charcoal to adsorb any volatile carbonyls present in the air. Fresh working solutions of DH were prepared daily from this stock. Human plasma samples were collected in evacuated blood collection vials containing sodium heparin, centrifuged to remove the red blood cells, and the plasma transferred to storage vials and stored at −80 °C until use. Samples were collected with informed consent under the ethics guidelines of Hamilton Health Sciences. For analysis, plasma samples were warmed to 37 °C in a water bath and treated as described below prior to analysis.

2.2. Mass spectrometric evaluation

A Waters 2695 LC system (Milford, MA) with a Phenomenex C18(2) 2 mm × 150 mm column (Oakville, ON) and a Waters\Micromass Quattro Ultima (triple quadrupole) mass spectrometer (Milford, MA) were used for the LC/MS analysis. Mass spectrometer conditions were as follows. The packed capillary voltage was 3–2.96 kV and the sampling cone voltages were from 35–54 V. The desolvation gas temperature was set to 200 °C and the desolvation gas flow was set to 107 L/h. Positive electrospray ionization was employed and the mass spectrometer was operated at low resolution. The manual derivatization of aqueous and plasma samples for MS analysis was performed as described previously [10]. Mobile phase A consisted of ACN: ammonium acetate buffer, 20 mM, pH 4.0 (10:90) and mobile phase B consisted of ACN: ammonium acetate buffer, 20 mM, pH 4.0 (90:10). Gradient elution was as follows: initial conditions: 60% A, hold 1 min. Liner ramp to 100% B over 5 min, hold at 100% B 16 min. Return to initial conditions over 0.5 min and hold 14.5 min to re-equilibrate column. An injection volume of 15 μL was used.

2.3. Apparatus

The LC system for AutoSPAD was a Dionex U3000 (Germering, Germany) equipped with two ternary gradient pumps, an on-line solvent degasser, autosampler, a thermostatted column compartment with dual 10-port switching valves, a PDA UV detector and a fluorescence detector. The left ternary pump was used for derivative elution from the SPAD cartridge and gradient elution on the analytical column. The right ternary pump delivered of DH and sample to the SPAD cartridge, as well as cartridge cleaning and re-conditioning between runs. The left 10-port valve switched the SPAD cartridge in and out of line with the separation column and the right 10-port valve was used for pre-loading the DH into its sample loop and directing it to the SPAD cartridge at the start of each run. For the right valve, in position 1–2 (ports 1 and 2 connected) the sample stream bypassed the DH loop. In the 10–1 position (ports 1 and 10 connected) the sample stream was in line with the DH loop. For the left valve, in position 1–2 the eluent stream from the SPAD cartridge bypassed the separation column. In the 10–1 position the SPAD cartridge eluent was in line with the separation column. The UV detector was set to monitor for high concentration DANSYL hydrazine peaks (λ = 350 nm) and the fluoresecence detector was set to monitor for DH–hydrazone derivatives (ex. λ = 350 nm, em. λ = 550 nm). The analytical column was a Waters NovoPak C18, 3.9 mm × 150 mm, 5 μm particle size. The syringe pump was a Sage Instruments (Cambridge, MA) model 341 fitted with a SGE (Austin, TX) gas tight syringe (1.0 mL) with a Luer tip. The syringe pump was controlled through a relay on the autosampler by means of a 12 V DC electric solenoid (part # 13FC-2C-D-12VDC, Surecon Relays, Burlingame, CA). The solenoid was powered by an external 12 V DC power supply.

2.4. Preparation of SPAD cartridge

An XAD reactor bed for on-line analyte derivatization was prepared by packing ∼12 mg of XAD-2 into a 1/16″ stainless steel HPLC tube (4 cm length × 0.04″ i.d., ∼1 mm i.d.) so that sorbent was tightly packed into the entire length of the tube. Packing was retained by sealing both ends of the tube into 1/16″ i.d. stainless steel unions containing stainless steel frits (1/16″ dia., 0.2 μm pore size), both obtained from Valco Instrument Co. Inc. (Houston, TX). The unions were fitted with 0.01″ i.d. PEEK tubing (∼0.25 μm i.d.) on the opposite sides of the frits and all connections were made with standard stainless steel or PEEK HPLC nuts and ferrules. The SPAD cartridge was finally fitted onto the left 10-port switching valve as a sample loop.

2.5. Chromatographic conditions

Gradient elution was employed using the left pump, to facilitate analyte stacking at the top of the column prior to separation, sufficient separation between the DH and product peaks, and column clean up between runs. Mobile phase A consisted of ACN: ammonium acetate buffer, 20 mM, pH 4.0 (10:90) and mobile phase B consisted of ACN: ammonium acetate buffer, 20 mM, pH 4.0 (90:10). Following transfer of the MDA–hydrazone from the SPAD cartridge to the separation column (7.0 min program time, Table 1), separation of the reaction mixture required the following gradient: initial 50% A, hold 1.0 min. Change to 0% A by a linear gradient from 1.0 to 6.0 min. Hold 0% A from 6.0 to 10.5 min. Switch to 100% A from 10.5 to 11.5 min. Hold 100% A to 16 min. Switch to initial conditions (50% A, 0.3 mL/min) in 30 s. Hold to end (18 min). While in-line with the SPAD cartridge, the gradient pump flow was restricted to 0.3 mL/min. During elution, the gradient pump flow was 1.0–1.5 mL/min. All gradient pump flow and gradient composition changes were linear over the time period

Table 1
All programming steps for AutoSPAD analysis of MDA in plasma, using U3000 system. Initial conditions: auto sampler temperature 4.0 °C, column compartment at 25 °C, detectors autozero at time = 0.0 min and 8.0 min. Valve positions: Left valve, 1–2: gradient pump bypass SPAD cartridge, 10–1: SPAD cartridge in line with column; right valve, 1–2: sample prep pump bypass DH loop, 10–1: DH loop in line with SPAD cartridge; sample inject at time = 0.0 min, where blank cells are seen for a particular time point either a constant condition or a linear change occurs between previous and subsequent time points where data are provided.

Time (min)	Gradient pump, left pump			Sample prep pump, right pump				Left valve	Right valve	DH pump
	Flow (mL/min)	% A	% B	Flow (mL/min)	% A	% B	% C			
−0.60	0.00	50	50	0.06	100	0	0	1–2	1–2	
−0.50	0.00	50	50					1–2	10–1	
0.00	0.30	50	50							off
0.01								1–2	1–2	
3.00				0.06	100	0	0	10–1	1–2	
4.00				0.25	0	100	0			
6.00				0.50	0	100	0			
7.00	0.30	50	50					1–2	1–2	
8.00	1.00	50	50							
9.00										on
9.50				0.50	0	100	0			
10.00				0.50	0	0	100			
10.50										off
13.00	1.50	0	100							
13.50				0.50	0	0	100			
14.00	0.70	0	100	0.35	0	0	100	10–1	1–2	
14.10				0.35	100	0	0			
15.50				0.30	100	0	0			
17.00				0.00	100	0	0	1–2	1–2	
17.50	0.50	0	100							
18.50	0.50	100	0							
23.00	0.50	100	0							
23.50	0.30	50	50							
25.00	0.30	50	50							

indicated. Table 1 provides a complete description of all AutoSPAD steps.

2.6. Operation of AutoSPAD

The MDA–DH derivatization reaction occurs during the first minute after injection. In the instrument method, the syringe pump loaded the DH reagent (20 μL, 10 mg/mL in methanol) into a 20 μL sample loop located on the right 10-port valve, during the previous run. While on (1.5 min), the syringe pump flow rate was 15 μL/min. Starting 30 s prior to injection the right pump transferred the DH reagent from the right valve to the SPAD cartridge in loading/reaction solution (0.5 M acetic acid, 1.7 mM heptane sulfonic acid in water) at 60 μL/min. In this way, the derivatizing reagent was loaded onto the SPAD cartridge just prior to arrival of the sample. At the time of injection, the autosampler loaded 20 μL of sample into the sample loop on the autosampler, which was then sent to the reaction cartridge in loading solution at 60 μL/min. MDA samples (standards) were prepared by diluting a MDA stock solution by the appropriate amount with water, to achieve the concentration indicated. This solution was then mixed with glacial acetic acid (100 μL sample plus 80 μL acetic acid) and placed into 200 μL glass vial inserts inside 2 mL glass sample vials with hole caps and Teflon-backed silicon septa. Plasma samples, prepared as described below, were loaded onto the autosampler directly. Samples were maintained at 4 °C in the autosampler. Other aldehydes (C1–C7) were analyzed similarly to MDA aqueous standards.

Although the reaction and trapping of product on the SPAD cartridge was complete within 1 min flow continued for a further 2 min to provide a washing of the cartridge prior to injection. Elution from the SPAD cartridge to the analytical column was by 50% ACN, 0.3 mL/min (initial mobile phase) delivered from the gradient pump in reverse flow through the SPAD cartridge, for 4 min. This yielded an elution efficiency > 90%. The left valve then switched the SPAD cartridge off-line and increased the left pump flow rate. Initiating the gradient program described above produced separation of the derivatized sample on the analytical column. During sample elution on the analytical column, the right pump cleaned the SPAD cartridge with a wash of ACN:H_2O 1:1 (5.5 min) followed by one with 100% ACN (4 min at 0.5 mL/min). At the end of the chromatographic run 100% column eluent B (90% ACN, 10% ammonium acetate buffer, 20 mM, and pH 4.0) was passed through the SPAD cartridge (0.7–0.5 mL/min, 3.5 min) to condition the cartridge for the next run. During this time, the right pump was flushed with loading/reaction solution to clear the cleaning solvent from the lines. The reduced flow rate (0.3 mL/min) for the gradient pump was employed while flow passed through the SPAD cartridge to prevent a cartridge overpressure condition (a 900 psi pressure limit was employed). The total analysis time per sample was 25 min. A more thorough cleaning of the SPAD cartridge was required for the plasma samples than was required previously for tissue homogenate, which caused the total run time to be longer than used previously. A complete list of all AutoSPAD programming steps is included in Table 1.

3. Results

3.1. Mass spectrometric analysis of MDA hydrazone product

The product(s) from the reaction between MDA and a hydrazine can have one of several structures. Derivatization can occur at one or both aldehydes. Each derivative can have a cis or trans configuration. In addition, Stalikas reported a cyclization of the mono derivative [9]. In any AD, it is important to determine the structure of the reaction product and particularly so in the case of a poly-functional molecule like MDA.

For MS structural analysis, both aqueous and plasma samples were spiked with MDA and first derivatized according to the previously described SPAD method (i.e. manual). The mass spectrometry analysis of ∼1000 μg/mL MDA sample in water revealed two peaks of substances with molecular masses 319 and 566 g/mol. This corresponded to the mono and bi forms of the acyclic MDA derivative. Both products were present in approximately equal concentrations, as evidenced by their peak areas: 1.43×10^9 for both products (data

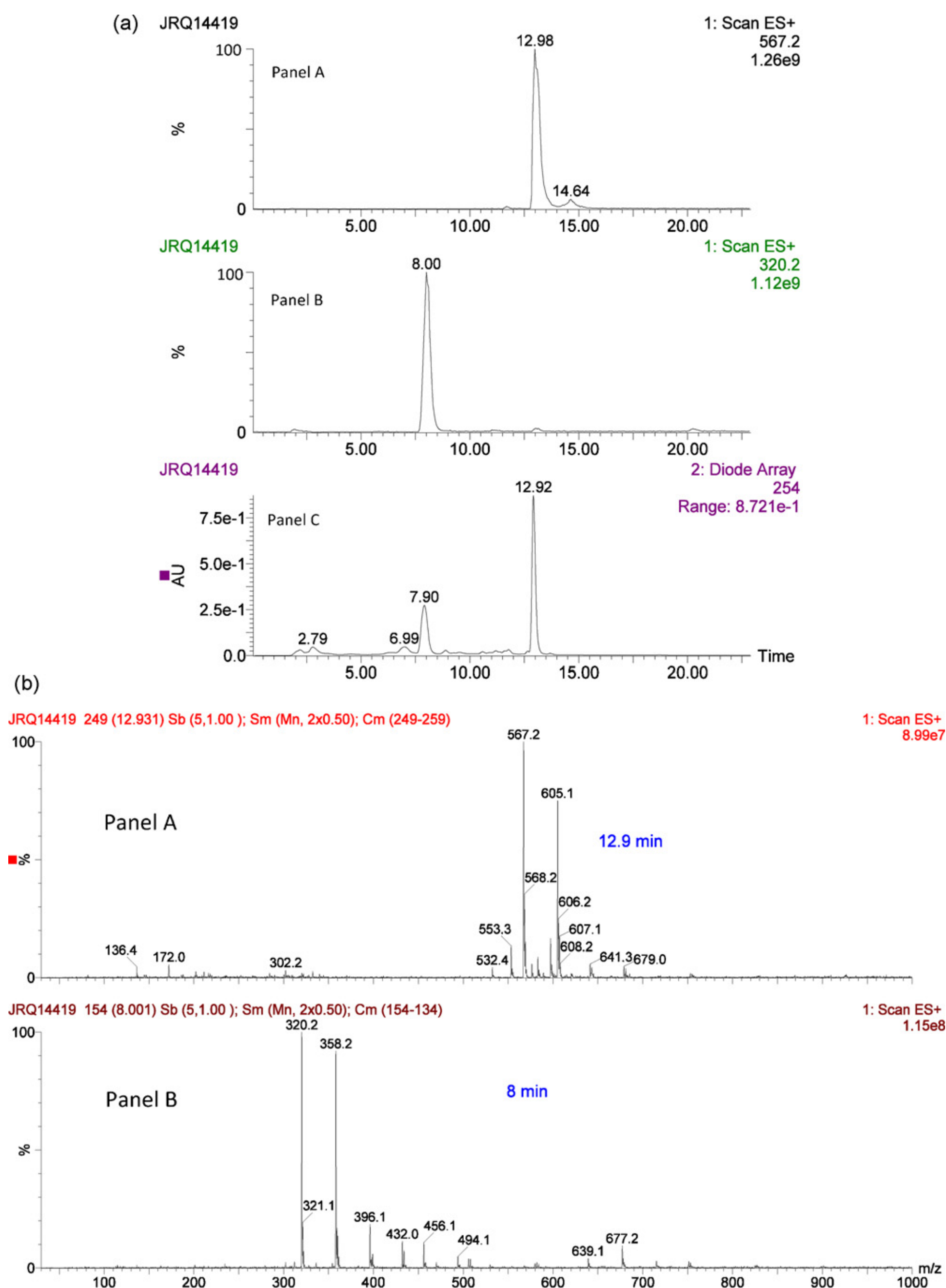

Fig. 1. Mass spectrometry analysis of MDA–DH hydrazone in blood plasma. (a) Panel A shows the extracted ion current trace at $m/z = 567.2$, panel B shows the extracted ion current trace at $m/z = 320.2$ and panel C shows the UV absorbance trace ($\lambda = 254$ nm). (b) Panel A shows the mass spectrum of the 12.9 min peak, panel B shows the mass spectrum of the 8.0 min peak. In (b) the presence of a signal at 38 m/z higher than the expected derivative mass may be due to potassium adduct ions.

not shown). Analysis of a 1000 μg/mL MDA spiked plasma sample also revealed two $[M+H]^+$ peaks at 320 and 567 m/z, which again corresponded to the protonated mono and bi forms of the acyclic MDA derivatives (319 and 566 g/mol, respectively). These were also present at approximately equal concentrations: peak areas of 1.26×10^9 for 567 m/z product and 1.11×10^9 for 320 m/z product. The chromatographic data for the plasma analysis appears in Fig. 1a and a reaction scheme based on these results appears in Fig. 2. Fig. 1b shows the mass spectra of the two products. Potassium adducts of both compounds were observed in addition to the protonated molecular ions. Because adduct ions were present in both the plasma and aqueous samples it is likely that the sources are either the mobile phase [32] or residuals from metal salts used in manufacture and shipping of XAD-2 [33]. For routine MS analysis it may be necessary to either optimize the mobile phase conditions [34], sum the two analyte peaks [35] or source a highly purified form of XAD-2.

Analysis of MDA at lower concentrations (<10 μg/mL) resulted in only one hydrazone product peak. Analytical derivatization by AutoSPAD of other short chain aldehydes (C1–C7) showed that the MDA hydrazone peak eluted well after the propionaldehyde hydrazone, and at a similar retention time as for heptanaldehyde (data not shown). Since derivatization of MDA with DH by AutoSPAD produced only one derivative, which eluted at a retention time similar to heptanal hydrazone, we expect that the product was the bis-derivative. Possibly, the bis-derivative is formed preferentially to the mono-derivative in the course of AutoSPAD because of the much higher DH:MDA or DH:XAD-2 ratios of these experiments.

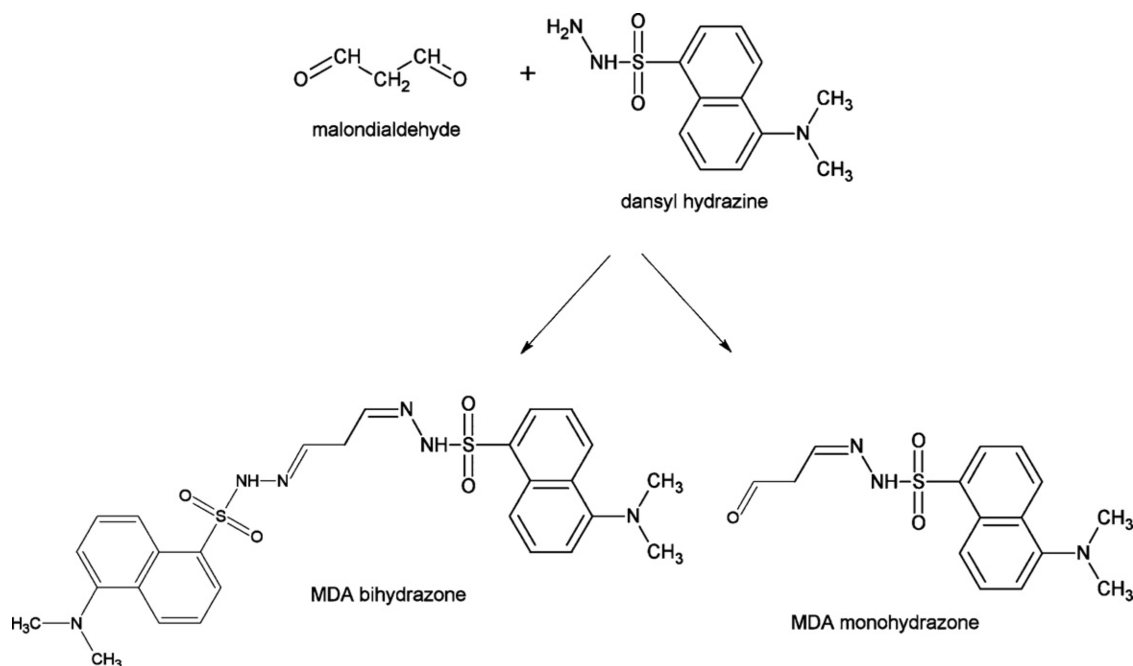

Fig. 2. Schematic of MDA–DH hydrazones formation. The calculated formula weights of the mono- and bihydrazone are 319.38 and 566.69, respectively.

Likely, the latter may be more important since the DH is always in vast excess relative to the MDA. Since the reactor bed configured in a column increases reaction rates of carbonyls, it is also possible that the second carbonyl reacts at a faster rate than the cyclization.

3.2. AutoSPAD system validation

Because the Dionex U3000 system had not previously been used for AutoSPAD analyses, its performance was first validated by injection of a series of MDA standards in water, at concentrations from 0.07 to 2 μg/mL. Each determination was performed in triplicate and the regression analysis was performed on data averages. The range of concentrations employed was determined from literature references to be clinically relevant [6,12]. Limits of detection (LOD) and limits of quantification (LOQ) were calculated from standard deviations (S.D.) of data points near the LOQ. Calculations were based on 3× S.D. for LOD and 10× S.D. for LOQ. The relative standard deviations (R.S.D.) reported in Table 2 were those observed for concentrations near the LOQ. In all cases, R.S.D. trended towards 2% at higher concentrations. The observed limits of detection are similar to those observed previously, even though the injection volume was just 60% of that used previously. The linearity and R.S.D. obtained were also similar or better than obtained previously.

3.3. Development of plasma preparation regime

The hydrolysis regime previously optimized for tissue homogenate samples produced no MDA derivatives when used in plasma, likely due to the significantly higher protein load in plasma. Albumin is the major plasma protein and its concentration typically varies between 38 and 52 mg/mL [36]. By comparison the tissue homogenate samples analyzed previously had total protein concentrations in the range of 2–3 mg/mL. Other authors have reported alternative hydrolysis strategies for MDA analysis from blood. These involved treatment with NaOH [6], phosphoric acid [37], sulfuric acid [7], and trichloroacetic acid [38]. It was necessary to evaluate all four of these strategies for successful development of one suited to determination of MDA from plasma by AutoSPAD. In all cases, following hydrolysis, when required, the pH was adjusted to neutral, and acetic acid was added to achieve the sample concentration previously employed. Of these alternative hydrolysis conditions, only the method of Grotto et al. [6] (NaOH hydrolysis) produced a MDA peak in the analysis, with an observed 40% recovery of the MDA hydrazone from the plasma samples but the technique of Grotto et al. [6] required higher sample dilution which degraded limits of detection.

These data indicated that hydrolysis alone was not sufficient. The final technique required separate hydrolysis and protein precipitation steps and with the hydrolysis preceding protein precipitation. Protein precipitation by solvent is unsuitable since organic solvent would dilute the sample and would interfere with the adsorption of the derivatization product to the SPAD reactor bed. Accordingly alternative strategies were evaluated specifically: zinc sulphate (10%), trichloroacetic acid (10%), and ammonium sulphate (sat.) performed as described by Polson et al. [39]. None of the methods

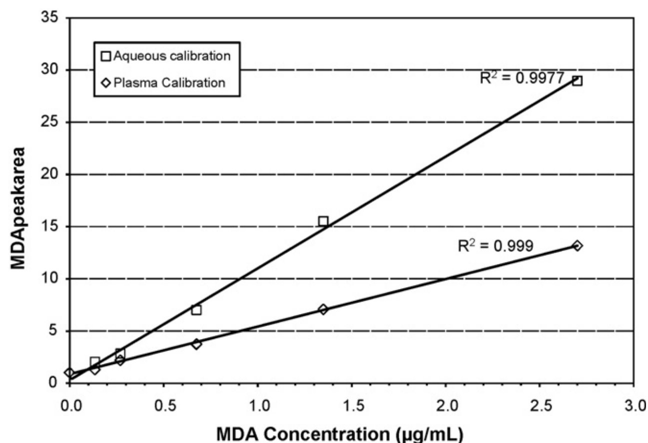

Fig. 3. Comparison of aqueous and plasma MDA calibration obtained by AutoSPAD on Dionex U3000. All samples were subjected to hydrolysis conditions and protein precipitation prior to analysis.

Table 2
Calibration figures of merit for aqueous sample analysis with and without hydrolysis/protein precipitation steps, and plasma analysis with sample hydrolysis and protein precipitation.

	Standards	Standards hydrolysed	Plasma hydrolysed
Range (μg/mL) regression	0.0675–2.0 $y = 31.50x + 0.2289$	0.135–2.7 $y = 10.71x + 0.3002$	0.135–2.7 $y = 4.55x + 0.8699$
r^2	0.9992	0.9977	0.9990
LOD (μg/mL)	0.016	0.022	0.041
LOQ (μg/mL)	0.054	0.074	0.14
%R.S.D.	8.0	25	6.5

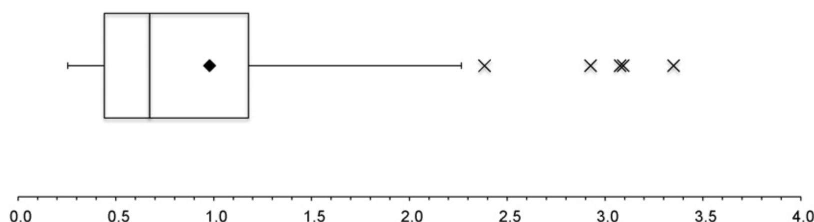

Fig. 4. Box and Whiskers plot of patient plasma MDA concentration data. The box encompasses the 50th percentile of data points. The vertical line in the box represents the median data point. The solid diamond represents the average data point. The horizontal error bars extend to the lowest and highest valid data points. The data points indicated by 'x' are high outliers. There were no low outliers.

released endogenous MDA from the protein during precipitation, but the zinc sulphate method was superior in terms of minimal interference with the derivatization/hydrazone trapping efficiency. After evaluation of various regimes for plasma hydrolysis and protein precipitation, the following proved to be the optimized technique: plasma samples (50–100 μL) were treated with an equal volume of glacial acetic acid, followed by vortex mixing for 20 s and heating at 45 °C for 60 min for MDA hydrolysis. Next of a third volume of zinc sulphate solution (10%) was added for protein precipitation. The samples were again vortex mixed, allowed to sit for 20 min, and then centrifuged for 10 min. The clear supernatant was injected directly (20 μL). Although recovery of endogenous MDA released from protein was only 43%, treatment with higher quantities of acetic acid, higher temperatures or times of hydrolysis resulted in inferior performance in terms of amount of hydrazone product obtained. If the protein precipitation step occurred prior to the hydrolysis step, there was no formation of the MDA hydrazone.

Fig. 3 shows the results of a MDA plasma calibration using MDA spiked into plasma. Incubation of samples at room temperature (1 h) permitted MDA binding to plasma proteins. Following protein adduct formation the plasma underwent the treatment previously described. An aqueous calibration with samples treated similarly is shown for comparison. The slope of the plasma calibration here is lower than that obtained for aqueous samples prepared without the hydrolysis steps, due to the greater dilution required by the hydrolysis and protein precipitation procedures. Despite the dilution factor and the analysis from human plasma, the coefficient of correlation, is nevertheless, 0.999.

The y-intercept for the aqueous standards is representative of the background levels of MDA in the analysis, which is determined to be 0.03 μg/mL. Because this is below the limit of detection for plasma analysis, it was safe to disregard it for the biological samples. As expected, the blood bank plasma contained an amount of endogenous MDA. From the negative x-intercept this level is determined to be 0.2 μg/mL. For external standard calibration, the curve was adjusted to account for this endogenous MDA level.

Table 2 cites the regression analysis data and other figures of merit for this technique. The limit of detection for plasma samples was somewhat higher than for similarly treated aqueous samples due to incomplete hydrolysis. The level of sensitivity observed was, however, still appropriate for the expected levels of MDA expected in human plasma samples.

3.4. Analysis of plasma samples from humans

To assess applicability of the technique we determined MDA in a variety of human plasma samples. This would demonstrate feasibility and determine if the general inter-individual variability in human plasma samples could compromise the analysis. Possible sources of error included swamping the reagent by very high concentrations of MDA or other carbonyls, or inter-individual variation in matrix effects that could inhibit the reaction. Samples from 60 patients were collected either before or after routine surgical procedures. As such, the expected MDA concentrations would range from normal to elevated, due to surgical stress. Although the number of samples analyzed was not sufficient to determine specific correlations with surgery, a range of MDA concentrations were found from expected normal to elevated levels. Fig. 4 presents the results of the analysis of all patient samples in a box and whisker plot. The literature suggests that normal MDA levels are in the range of 0.2–0.3 μg/mL [6,12]. We observed a range of MDA concentrations from 0.26 to 3.35 μg/mL with a median value of 0.67 μg/mL. Of the 60 samples, 22 had concentrations below 0.5 μg/mL, 25 were in the range 0.5–1.2 μg/mL and 13 samples had high MDA concentrations (>1.4 μg/mL). These 60 samples were a subset of more than 1000 plasma samples used in method development, quality control and replicate analysis on the same AutoSPAD cartridge and without loss of response. These data demonstrated the robustness of the method and confirmed that human plasma, despite its high inter-individual variability in lipids and other normal constituents did not compromise derivatization of MDA by AutoSPAD.

4. Conclusions

We have reported a completely automated analysis of malondialdehyde from plasma samples, using commercially available instrumentation, implemented without significant modification of the instrument. A revised structure of the MDA hydrazone is proposed based on MS analysis of the product m/z. Total analysis time, after plasma hydrolysis and protein precipitation but which included analytical derivatization, clean-up and chromatographic separation was just 25 min. The method is accurate, sensitive and applicable to limited sample volumes. Analysis of a large number of samples from patients did not interfere with the analytical method. The flow rates and chromatographic conditions employed

are appropriate for mass spectrometric analysis for additional selectivity and sensitivity.

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