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Determination of malonaldehyde by coupled high-performance liquid chromatography–spectrofluorimetry after derivatization with luminarin 3

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ABSTRACT

A high-performance liquid chromatographic method with fluorescence detection is described for the specific determination of free malonaldehyde (MDA). Malonaldehyde was labelled with luminarin 3 in acetate buffer (pH 4.0) at room temperature in the dark. An aliquot of the reaction mixture was injected on to an octadecyl-bonded column using acetonitrile–imidazole buffer (30:70, v/v) as the mobile phase. The eluate was monitored with a fluorescence detector at 395 nm (excitation) and 500 nm (emission). A linear calibration graph was established between 7.2 and 90 ng/ml of MDA and the limit of quantification (LOQ) was lower than 7 ng/ml of MDA. The precision was characterized by R.S.D.s of 11% at 7.2 ng/ml and 2% at 90 ng/ml. The structure of the derivative was confirmed as the 5-hydroxy-2-pyrazoline form. UV absorbance and corrected fluorescence spectral data and quantum yields of the luminarin 3 derivative of malonaldehyde are presented.

INTRODUCTION

Several methods have been developed to determine the amount of malonaldehyde (MDA) formed by lipid peroxidation either *in vivo* or *in vitro*. The most common method MDA is based on spectrophotometric or spectrofluorimetric measurement of the condensation product formed from MDA and 2-thiobarbituric acid (4,6-dihydroxy-2-thiopyrimidine) (TBA) [1,2]. This method is not specific for MDA and often overestimates MDA levels [2–10]. Some workers have proposed more selective methods based on the chromatographic separation of MDA: in its free form [3,4]; derivatized with TBA [5,11,12] or its derivatives such as 1,3-diethyl- [6,7] or 1,3-dimethyl-, 1-methyl-, 1,3-diphenyl- and 1-phenyl-TBA [6]; derivatized with dansylhydrazine (DNSH) [8], 2-hydrazinobenzothiazole (HBT) [9], 2,4-dinitrophenylhydrazine (DNPH) [13–15], pentafluorophenylhydrazine (PFPH) [16], *p*-nitrophenylhydrazine (NPH) [17] or N-methylhydrazine (NMH) [18]; and based on the formation of a fluorescent compound derived from 1,4-dihydropyridine 3,5-dicarbaldehydes, produced by a condensation reaction of malonaldehyde, aliphatic amines and aliphatic aldehydes in neutral medium (Hantzsch reaction) [10].

Reactions of MDA with TBA, DNSH, HBT or DNPH require hot and or acidic conditions. A major drawback of these methods from a biochemical point of view is the artifactual generation of MDA from the biological materials that can be induced under the strongly acidic conditions and elevated temperatures required for the formation of the derivatives. It is therefore

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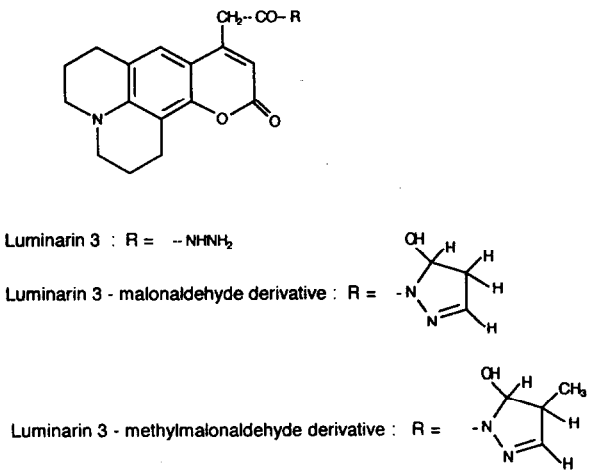

Fig. 1. Structures of luminarin 3 and its derivatives.

uncertain what proportion of the determined MDA was originally present in the sample in the free state and what proportion might have been bound or generated from precursors. Therefore, mild derivatization conditions are required for the reliable determination of free MDA.

We used the derivatizing agent luminarin 3 (Fig. 1) for the determination of low concentrations of MDA under mild conditions. Owing to its quinolizinocoumarin nucleus, luminarin 3 is a useful fluorescent and chemiluminescent label [19–21], and its hydrazine group reacts easily with carbonyl compounds, permitting the derivatization of MDA at room temperature in acetate buffer (pH 4.0).

As chemiluminescence detection requires a postcolumn reaction system with one or two additional pumps, fluorescence detection was preferred. This method is sensitive and specific for the determination of free MDA.

EXPERIMENTAL

Reagents

All reagents and solvents were of analytical-reagent grade. Luminarin 3 and 2-methyl-1,1,3,3-tetraethoxypropane (MeTEP) were obtained from Eurobio-Seratec (Les Ulis, France) and 1,1,3,3-tetraethoxypropane (TEP), imidazole and 2,4-pentanedione (acetylacetone) from Aldrich-Chemie (Steinheim, Germany). Quinine sulphate and silica gel (0.04–0.063 mm) were purchased from Merck (Darmstadt, Germany).

Standard malonaldehyde solution

A stock standard solution of MDA was prepared by acidic hydrolysis of TEP, assuming 100% conversion of TEP into MDA [15]. A 0.245-ml volume of TEP was placed into a brown 100-ml graduated flask, diluted to volume with 1% (w/v) sulphuric acid, mixed thoroughly and kept in the dark at room temperature for 2 h. This solution contains 10 m*M* MDA and can be stored at −20°C for several months. It was used to prepare a calibration graph in the range 7.2–90 ng/ml after dilution with 100 m*M* acetate buffer (pH 4.0). The calibration graph was constructed by plotting the peak-height ratio of the luminarin 3 derivative of MDA to standard luminarin 3–methylmalonaldehyde against MDA concentration.

The absorbance of 100 μM MDA solution in 1% sulphuric acid at λ_{max} (245 nm) was measured to verify the molar absorption coefficient and hence the purity of the solution.

Standard luminarin 3 solution

A 10 m*M* solution of luminarin 3 in dimethyl sulphoxide (DMSO) was prepared. This solution can be stored for several months at −20°C.

Standard luminarin 3–malonaldehyde solution

A standard solution of luminarin 3–malonaldehyde (L3–MDA) was obtained from the reaction of MDA and luminarin 3. When incubated at room temperature and stirred for 30 min, the reaction mixture was composed of 100 ml of a standard solution of MDA (72 mg, 1 mmol) and 10 ml of 100 m*M* luminarin 3 solution in DMSO (313 mg, 1 mmol). Sodium hydrogencarbonate (2.5 g) was added and the mixture was stirred gently until the acid had been neutralized (pH 7.0). The mixture was then extracted with 200 ml of dichloromethane. The extract was dried with anhydrous magnesium sulphate, filtered and evaporated to dryness under vacuum.

The residue was dissolved in 5 ml of dichloro-

methane and purified chromatographically on silica gel 60 (0.04–0.063 mm) using an elution gradient of 0 to 40% tetrahydrofuran in dichloromethane. The eluate was evaporated under vacuum and the residue was dried in a desiccator to give a yellow powder, yield 72.3%. Elemental analysis: calculated for $C_{20}H_{21}N_3O_4$, C 65.40, H 5.72, N 11.44; found, C 65.41, H 5.81, N 11.40%.

The structure of L3–MDA was also confirmed by ^1H and ^{13}C NMR spectroscopy (data not shown). The electron impact mass spectrum was characterized by ions at m/z (relative abundance, %) 367 (19.09), 349 (5.54), 282 (8.77), 281 (8.72), 256 (9.02), 255 (48.96), 254 (20.53), 227 (10.87), 226 (17.13), 68 (100.00), 67 (13.05) and 44 (34.98).

L3–MDA was prepared as a 10 m*M* solution in DMSO. This solution can be stored for several months at −20°C.

Standard solution of luminarin 3–methylmalonaldehyde (internal standard)

Luminarin 3–methylmalonaldehyde (L3–MeMDA) was synthesized as described above from MeMDA prepared by hydrolysis of 2-methyl-1,1,3,3-tetraethoxypropane in 1% sulphuric acid for 2 h at room temperature.

L3–MeMDA was obtained in the form of yellowish brown needles, yield 53%. Elemental analysis: calculated for $C_{21}H_{23}N_3O_4$, C 66.14, H 6.04, N 11.02; found, C 66.06, H 6.18, N 10.97%.

The structure of this compound was confirmed by ^1H and ^{13}C NMR spectroscopy (data not shown) and its electron impact mass spectrum was characterized by ions at m/z (relative abundance, %) 381 (29.74), 363 (11.42), 282 (17.49), 281 (20.52), 256 (11.75), 255 (65.34), 254 (28.84), 227 (14.42), 226 (23.09), 82 (85.99), 81 (100.00), 57 (26.76), 56 (5.99), 55 (17.25), 54 (36.92), 44 (30.62) and 43 (12.02).

A 17 μM solution of L3–MeMDA was prepared: 38.1 mg of L3–MeMDA were placed in a test-tube and 4 ml of DMSO were added to obtain a 25 m*M* stock solution. This solution was diluted with the same solvent to obtain the desired concentration.

10 mM imidazole buffer solution (pH 7.0)

The pH of the solution was adjusted with concentrated nitric acid.

Acidified acetylacetone solution

A 1 *M* solution of acetylacetone in 1% sulphuric acid was prepared extemporaneously.

Apparatus

High-performance liquid chromatographic (HPLC) analysis was performed with a Chromatem 380 pump (Touzart-Matignon, Vitry sur Seine, France), equipped with a Rheodyne (Cotati, CA, USA) Model 7125 injector with a 20-μl sample loop and a Waters (Milford, MA, USA) Model 420-C fluorescence HPLC monitor. The data were processed in a Shimadzu C-R5A integrator (Touzart et Matignon). For the verification of the purity of L3–MDA and L3–MeMDA standards, a Shimadzu SPD-6A UV detector was connected to the outlet of fluorescence detector. The signal from each detector was recorded on a double-trace integrator. Corrected fluorescence and absorbance spectra were measured with a Perkin-Elmer (St. Quentin en Yvelines, France) Model LS 50 luminescence spectrometer and a Shimadzu Model UV-2100 UV–Vis recording spectrophotometer (Touzart et Matignon), respectively, in 1-cm quartz cells. Spectral band widths of 5 and 10 nm were used for the excitation and emission monochromators, respectively. Electron impact and chemical ionization mass spectra were measured on a Nermag R-1010-C mass spectrometer (Delsi-Nermag, Argenteuil, France). ^1H and ^{13}C NMR spectra were recorded on a Bruker (Bruker, Wissous, France) AC 200-MHz NMR spectrometer using [$^2H_6$]dimethyl sulphoxide as solvent and tetramethylsilane (TMS) as reference.

Chromatographic conditions

Chromatographic separation was performed using a 5-μm Nucleosil C_{18} column (150 × 4.6 mm I.D.) (Beckman, Les Ulis, France). The mobile phase was an isocratic mixture of acetonitrile and 10 m*M* imidazole buffer (pH 7.0) (30:70, v/v) at a flow-rate of 1.5 ml/min at room temperature. Filters of 395 and 500 nm were used for excitation and emission, respectively.

The absence of coumarinic contaminants in L3–MDA and L3–MeMDA standards was checked by means of liquid chromatography with fluorescence and UV detection on a 5-μm Nucleosil silica column (250 × 4.6 mm I.D.) (Interchim, Montluçon, France) using ethyl acetate–diisopropyl ether (90:10, v/v) as the mobile phase at a flow-rate of 1.5 ml/min. The UV detector wavelength was set at 360 nm; 360- and 440-nm filters were used for excitation and emission, respectively, in fluorimetric detection.

Fluorescence and absorbance measurements

Fluorescence quantum yields were determined with reference to quinine hydrogen sulphate in 0.05 *M* sulphuric acid, according to Parker and Rees [22]. Quantum yields were measured at room temperature without deoxygenation. The intrinsic fluorescence sensitivity was expressed by the equation $IFS = \phi\varepsilon/H$, where ϕ is the quantum yield, H is the half band width (cm^{-1}) and ε is the molar absorptivity ($l\ mol^{-1}\ cm^{-1}$) [23].

Derivatization procedure

A 200-μl volume of 100 m*M* acetate buffer solution (pH 4.0) containing malonaldehyde was mixed with 10 μl of 10 m*M* luminarin 3 solution and 10 μl of 17 μ*M* L3–MeMDA (internal standard) in a glass test-tube. The mixture was protected from light and kept at room temperature for 30 min for the optimum formation of the luminarin 3–MDA derivative. Then 10 μl of acidified 1 *M* acetylacetone solution were added to the mixture to react with excess reagent. After 5 min, an aliquot of the reaction mixture was injected on to the HPLC column.

RESULTS AND DISCUSSION

Condensation of luminarin 3 with malonaldehyde and methylmalonaldehyde. Characterization of the derivatives

The condensation reaction of luminarin 3 with dicarbonyl compounds may involve one or two luminarin 3 molecules. When one molecule is involved, a ring formation reaction may take place, producing a pyrazole or 5-hydroxy-2-pyrazoline derivative.

MDA and MeMDA react with luminarin 3, a nucleophilic reagent, in acidic medium to form the derivatives shown in Fig. 1. The identity and purity of the luminarin 3 derivatives of malonaldehyde and methylmalonaldehyde were confirmed by elemental analysis, mass spectrometry (MS), ^{1}H and ^{13}C NMR spectroscopy and HPLC analysis. The results demonstrated that the condensation reaction between luminarin 3, MDA and MeMDA is equimolar and that 5-hydroxy-2-pyrazoline derivatives are formed.

The purity of the L3–MDA and L3–MeMDA standards was determined to be >99% by HPLC on Nucleosil silica (normal-phase) and Nucleosil ODS (reversed-phase) columns with both UV and fluorescence detection. The luminarin 3 derivative of MDA showed a single peak in both chromatographic systems. The luminarin 3 derivative of MeMDA showed two peaks in normal-phase chromatography. However, when a reversed-phase system with acetonitrile was used, only a single peak was obtained. These two peaks were confirmed to be the *threo* (70%) and *erythro* (30%) isomers by NMR spectroscopy.

The electronic impact mass spectra of L3–MDA and L3–MeMDA show fragmentation patterns consistent with the proposed structures. The evidence for the 5-hydroxy-2-pyrazoline form was that L3–MDA and L3–MeMDA showed a parent ion at m/z 367 and 381, respectively, with additional ions at m/z 349 and 363, respectively, corresponding to $[M-18]^{+\bullet}$. These ions result from the loss of hydroxyl and hydrogen from the 5- and 4-positions of the pyrazoline ring, respectively. The most abundant ions were at m/z 68 and 81, respectively. These ions correspond to the loss of a pyrazole ring, which appears, with the gain of a hydrogen atom and itself, respectively, from ions at m/z $[M-18]^{+\bullet}$. The remainder of the molecule appears in the form of carboxyl fragments (at m/z 282 or 281) which lose CO to give the fragment at m/z 255. This latter species fragments further, by the ejection of CO from the lactone ring and a hydrogen atom, to give the fragment at m/z 226.

Choice of optimum conditions for derivatization

The reaction of MDA ($pK = 4.46$) with luminarin 3 proceeded at room temperature under weakly acidic conditions (pH 4.0). In

order to confirm that the present procedure is specific for free MDA, 100 μM of TEP, a precursor of MDA, was treated at pH 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 and at temperatures of 25 and 50°C. The amount of MDA formed was determined from the absorbance at 245 nm. This study showed (Fig. 2) that the best yields for conversion of TEP to MDA were obtained by incubation at 50°C. At pH 1.0, whatever the temperature, we observed 100% transformation of TEP into MDA. At pH 3.0, 60% of MDA was formed at 50°C in 60 min, whereas at 25°C and pH 3.0 or 4.0 no MDA was formed after 120 min. This phenomenon has been observed previously by Kawai *et al.* [17]. These results demonstrate that the present procedure is specific for free (genuine) MDA.

The procedure reported in a previous paper [19] required strongly acidic conditions (0.1 M H_2SO_4) for the formation of luminarin 3 derivatives. Under these conditions MDA precursors are hydrolysed to give MDA. Accordingly, the determination of free MDA and that generated from its precursors could be obtained from the difference between MDA levels prior to and after acid hydrolysis.

Concerning the derivatization of MDA by luminarin 3, the influence of the reaction time and luminarin 3 concentration on the peak-height ratio of L3–MDA to L3–MeMDA was also studied. The peak-height ratios were almost constant after about 30 min and over the range of luminarin 3 concentrations investigated (0.05–0.5 μmol). The other important factor for analytical application is the thermal stability of the luminarin 3–MDA derivative. To prevent degradation of the product, the reaction temperature should not exceed room temperature (25°C) [19]. Under these conditions the luminarin 3–MDA derivative was stable for at least 24 h. These experiments indicated that the optimum conditions were those used in the derivatization procedure described above.

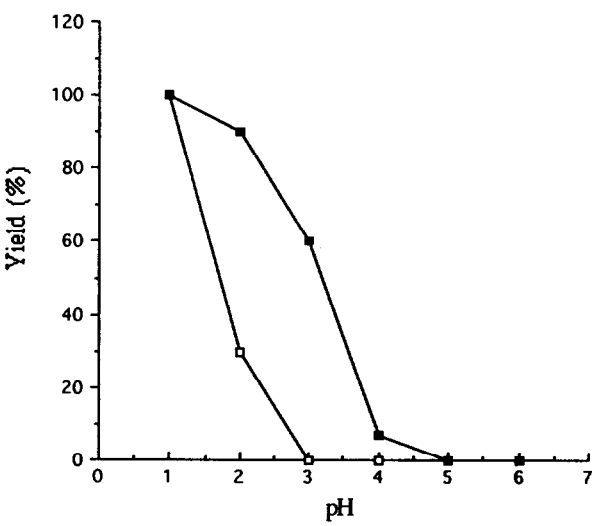

Fig. 2. Effect of pH on hydrolysis of TEP (0.1 μmol/ml), (□) at 25°C for 120 min and (■) at 50°C for 60 min. Formation of MDA was determined by UV spectrophotometry at 245 nm.

Liquid chromatography

The selected chromatographic conditions were used to separate luminarin 3 from its derivatives with MDA, MeMDA and acetylacetone. The mobile phase components, acetonitrile and imidazole buffer, were chosen for their ability to

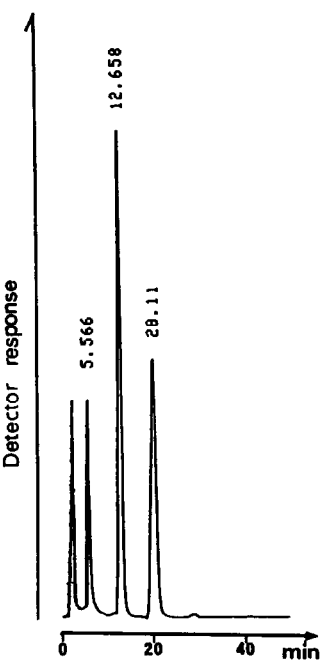

Fig. 3. Elution profile of a mixture of standard solutions of luminarin 3 (at 5.6 min) and luminarin 3 derivatives of MDA (at 12.7 min) and MeMDA (at 20.1 min) in reaction mixture (8 mV full-scale). A 15-pmol amount of each derivative was injected on to the column. For other details, see text.

promote peroxyoxalate chemiluminescence detection as reported by Tod and co-workers [20,21] for other luminarin derivatives. Fig. 3 shows typical chromatograms of a mixture of standard solutions of luminarin 3, luminarin 3–MDA and MeMDA derivatives. Their retention times were 5.6, 12.5 and 20.1 min, respectively. The luminarin 3–MDA derivative was well separated from the reagent and internal standard. For this separation of pure standards, the limit of detection was evaluated as the concentration that gave a signal-to-noise ratio of 3. A limit of detection of 0.45 ng/ml (0.125 pmol per injection) was found for L3–MDA, which is lower than values given in the literature for other HPLC methods [3–6,10,13,14]. It should be noted that derivatization with DNSH [8] and NMH [18] can give similar detection limits. Beljean-Leymarie and Bruna [9] used a gas chromatographic (GC) method after derivatization of MDA with HBT and reported a detection limit of 0.04 pmol per injection. Although the method is highly sensitive for MDA, it requires hot and acidic conditions for more efficient formation of the derivative. To our knowledge, only the GC method developed by Tomita *et al.* [16] gives a higher sensitivity than the present method.

In practice, when the derivatization was performed on smaller amounts of MDA, the unreacted reagent seriously interfered with the detection of the luminarin 3–MDA derivative. To reduce this interference we added an excess of acetylacetone which, under these conditions, reacts with most of the excess of luminarin 3. This reaction gives two (additional) products,

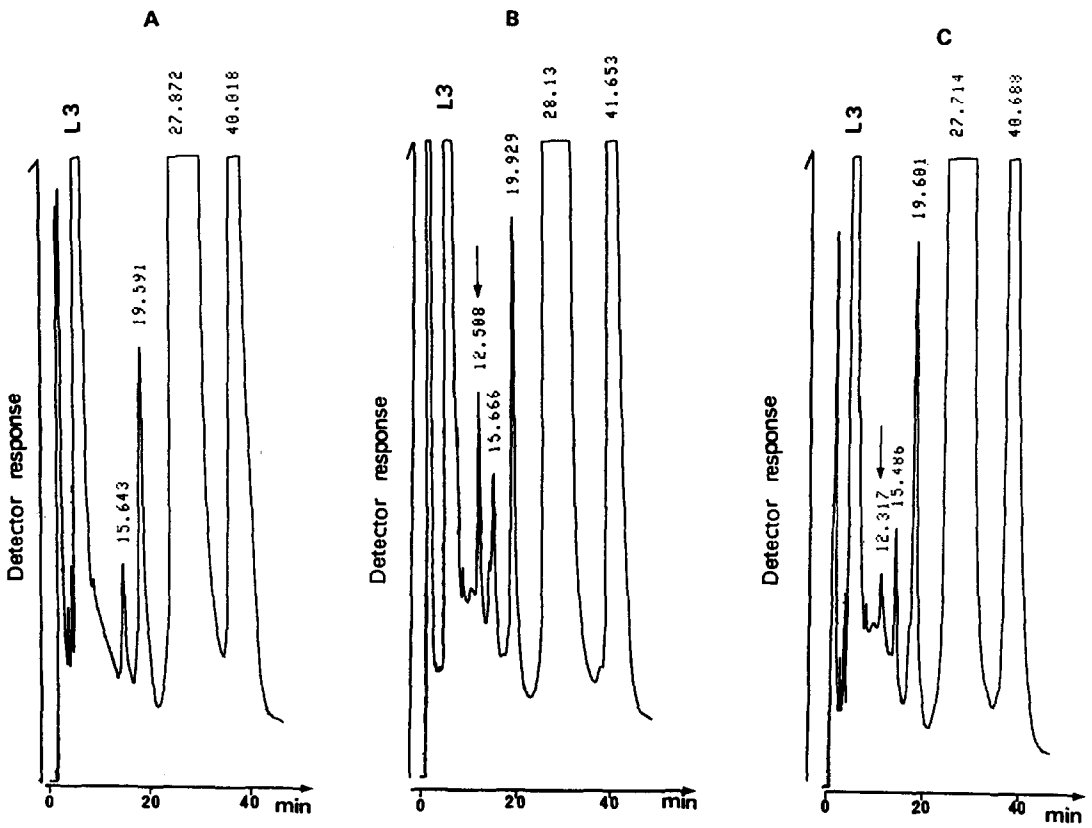

Fig. 4. Separation of luminarin 3–MDA derivative with fluorescence detection. (A) Blank; (B) chromatogram of the reaction mixture when 36 ng/ml of MDA were derivatized (4 mV full-scale); (C) chromatogram of the reaction mixture when 7.2 ng/ml of MDA were derivatized (4 mV full-scale). Numbers on peaks indicate retention times in minutes. For other details, see text.

TABLE I

UV ABSORPTION AND FLUORESCENCE DATA FOR LUMINARIN 3-MDA IN ACETONITRILE AND ACETONITRILE–10 m*M* IMIDAZOLE BUFFER (pH 7.0)

Acetonitrile concentration (%)	λ_{ex} (nm)	λ_{em} (nm)	ε^a (l mol^{-1} cm^{-1})	ϕ^b	IFS^c
0	401	502	22 952	0.39	3.85
30	402	495	28 072	0.39	4.34
50	399	491	28 682	0.51	5.63
70	399	485	28 976	0.51	5.60
100	394	464	32 139	0.71	8.55

[a] Molar absorptivity.
[b] Quantum yield.
[c] Intrinsic fluorescence sensitivity.

with peaks at 28.4 and 40.7 min, which were proved to be the pyrazole and monohydrazone forms of the luminarin 3–acetylacetone derivative, respectively (by liquid chromatography–mass spectrometry; data not shown). Fig. 4 shows the chromatograms of the reaction mixture obtained with (A) a blank acetate buffer solution and (B and C) standard solutions of MDA [(B) 36 and (C) 7.2 ng/ml]. Although these chromatograms are complex, experiments with spiked plasma samples displayed no additional peaks (different reaction conditions; data not shown). Therefore, owing to the good selectivity of the reagent, further inferences are not be expected.

Calibration graphs constructed for MDA with L3–MeMDA as internal standard ($n = 6$) were linear in the range 7.2–90 ng/ml, corresponding to 1.74–21.74 pmol of injected MDA derivative. The regression line was described by the following equation:

$$\text{ratio of peak heights} = 0.0129 \cdot \text{concentration (ng/ml)} + 0.0270 \quad (r^2 = 0.999)$$

The intra-run relative standard deviations were 10.6% for 7.2 ng/ml, 7.3% for 18 ng/ml, 5.6% for 36 ng/ml, 2.4% for 72 ng/ml and 1.8% for 90 ng/ml of MDA ($n = 6$). Hence the limit of quantification, *i.e.*, the lowest concentration that can be determined with an R.S.D. of less than 15%, was lower than 7 ng/ml (Fig. 4C).

Spectral properties

The influence of mobile phase composition on the absorbance and fluorescence properties of the luminarin 3–MDA derivative was studied.

As shown in Table I, whereas the position of the excitation maxima varied little with the composition of the solvent, the emission maxima showed a marked bathochromic effect with increased polarity of the acetonitrile–imidazole buffer mixture. This is due to the effect of the increased dipole–dipole interaction between the slightly polarized state of the molecule and the solvent characteristic of a $\pi \rightarrow \pi^*$ fluorescence emission.

Variations in the intensity of fluorescence of the luminarin 3–MDA derivative with the acetonitrile content of the mobile phase were studied by determining the relative quantum yields and intrinsic fluorescence sensitivity. These parameters doubled when the acetonitrile content in the mobile phase increased from 0 to 100%. Hence the whole composition range of mobile phases is compatible with highly sensitive detection.

CONCLUSIONS

Owing to its nucleophilic and fluorescence properties and the stability of its derivatives, luminarin 3 is a suitable derivatization reagent for the sensitive and selective HPLC with fluorescence detection of MDA. The method allows

the determination of small amounts of MDA with mild conditions of derivatization, without the formation of interfering by-products. In addition, the derivatization reagent and chromatographic conditions permit chemiluminescence detection.

The limiting factor with respect to the sensitivity of the present method was not detection but derivatization. In future work, acetylacetone could be replaced with a more efficient scavenger to remove all remaining luminarin 3. Even so, the limit of detection was well below that of other HPLC methods and its limit of quantification should allow the precise determination of free MDA in biological fluids. One example given in the literature is the concentration of MDA in normal human urine, which is in the range 10–60 ng/ml [16]. In several instances, the MDA concentration in urine has been shown to be correlated with lipid peroxidation processes following exposure to certain xenobiotics [14,15].

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REFERENCES

1 T. Hirayama, S. Miura, M. Araki, Y. Takeo and T. Watanabe, *J. Assoc. Off. Anal. Chem.*, 73 (1990) 590.
2 M.J. Richard, B. Portal, J. Meo, C. Coudray, A. Hadjian and A. Favier, *Clin. Chem.*, 38 (1992) 704.
3 W.A. Behrens and R. Madère, *Lipids*, 26 (1991) 232.
4 C. Largillière and S.B. Mélancon, *Anal. Biochem.*, 170 (1988) 123.
5 M.J. Richard, P. Guiraud, J. Meo and A. Favier, *J. Chromatogr.*, 577 (1992) 9.
6 K. Nakashima, T. Ando, T. Nakamizo and S. Akiyama, *Chem. Pharm. Bull.*, 33 (1985) 5380.
7 J. Therasse and F. Lemonnier, *J. Chromatogr.*, 413 (1987) 237.
8 T. Hirayama, N. Yamada, M. Noharaa and S. Fukui, *J. Assoc. Off. Anal. Chem.*, 66 (1983) 304.
9 M. Beljean-Leymarie and E. Bruna, *Anal. Biochem.*, 173 (1988) 174.
10 K. Kikugawa, T. Kato and A. Iwata, *Anal. Biochem.*, 174 (1988) 512.
11 G. Lepage, G. Munoz, J. Champagne and C.C. Roy, *Anal. Biochem.*, 197 (1991) 277.
12 M.A. Carbonneau, E. Peuchant, D. Sess, P. Canioni and M. Clerc, *Clin. Chem.*, 37 (1991) 1423.
13 S. Kawai, M. Tomita and K. Kasashima, *J. Chromatogr.*, 495 (1989) 235.
14 M.A. Shara, P.H. Dickson, D. Bagchi and S.J. Stohs, *J. Chromatogr.*, 576 (1992) 221.
15 T. Ekström, P. Garberg, B. Egestad and J. Högberg, *Chem.-Biol. Interact.*, 66 (1988) 177.
16 M. Tomita, T. Okuyama, Y. Hatta and S. Kawai, *J. Chromatogr.*, 526 (1990) 174.
17 S. Kawai, T. Fuchiwaki, M. Tomita and T. Higashi, *J. Chromatogr.*, 514 (1990) 29.
18 H. Tamura and T. Shibamoto, *Lipids*, 26 (1991) 170.
19 F. Traoré, M. Tod, J. Chalom, R. Farinotti and G. Mahuzier, *Anal. Chim. Acta*, 269 (1992) 211.
20 M. Tod, J.-Y. Legendre, J. Chalom, H. Kouwatli, M. Poulou, R. Farinotti and G. Mahuzier, *J. Chromatogr.*, 594 (1992) 386.
21 M. Tod, M. Prevot, J. Chalom, R. Farinotti and G. Mahuzier, *J. Chromatogr.*, 542 (1991) 295.
22 C.A. Parker and W.T. Rees, *Analyst*, 85 (1960) 587.
23 J.B.F. Lloyd, *J. Chromatogr.*, 178 (1979) 249.