Chromatographic determination of aliphatic aldehydes in human serum after pre-column derivatization using 2,2`-furil, a novel fluorogenic reagent

Marwa Fathy Bakr Ali [a, b], Naoya Kishikawa [b], Kaname Ohyama [b], Horria Abdel-Mageed Mohamed [a], Hanaa Mohamed Abdel-Wadood [a], Ashraf Mohamed Mohamed [a], Naotaka Kuroda [b,*]

[a] Department of Pharmaceutical Analytical Chemistry, Faculty of Pharmacy, Assiut University, Assiut 71526, Egypt.

[b] Graduate School of Biomedical Sciences, Course of Pharmaceutical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan.

[*] Corresponding author. Tel.: +81 95 819 2894; fax: +81 95 819 2444.
E-mail address: n-kuro@nagasaki-u.ac.jp (N. Kuroda).

Abstract

A novel, highly sensitive and selective fluorimetric liquid chromatographic method for simultaneous determination of medium chain aliphatic aldehydes was developed. The method was based on the derivatization of aliphatic aldehydes with 1,2-di(2-furyl)-1,2-ethanedione (2,2`-furil), a novel fluorogenic reagent, to form highly fluorescent difurylimidazole derivatives. The fluorescence derivatives were separated in less than 20 min on a reversed-phase ODS column using an isocratic elution with a mixture of methanol-water (80:20, v/v%). The detection limits were from 0.19 to 0.50 nM (1-10 fmol/injection) at a signal-to-noise ratio (S/N) of 3. This method was successfully applied for monitoring of aliphatic aldehydes in healthy human sera by a simple pretreatment procedure without interferences from serum constituents.

Keywords: pre-column fluorogenic derivatization; 1,2-di(2-furyl)-1,2-ethanedione (2,2`-furil); difurylimidazole derivatives, aliphatic aldehydes (lipid peroxidation); serum analysis

Introduction

Aldehydes are carbonyl compounds widely distributed in nature and their concentrations were determined in many matrices such as food and beverages [1,2], air [3,4] and water [5-7]. These ubiquitous compounds are known to be irritant, carcinogenic, capable of binding to protein and nucleic acids and impairing their functions [8].

Medium chain length aliphatic aldehydes are produced as degradation end products of lipid peroxidation process *in vivo* [8] and their levels have been measured in different biological fluids [1,9-20]. The presence of these aldehydes has been related to various pathological conditions such as carcinogenesis, atherosclerosis, diabetes mellitus and rheumatic arthritis [10,12,18,21]. Therefore, simple, sensitive and selective method for analysis of aldehydes in biological samples is required in order to investigate their roles in the pathology of certain diseases.

However, direct analysis of aldehyde is difficult and complicated due to the lack of intrinsic chromophores or fluorophores as well as their volatility and high reactivity. Because of these limitations, derivatization reactions before their detection are required. Hydrazines are the most popular derivatization reagents for carbonyl compounds for gas and liquid chromatographic analysis. Until now, several hydrazine based reagents such as 2,4,6-trichlorophenylhydrazine (TCPH) [1], *N*-methyl-4-hydrazino-7-nitrobenzofurazan (MNBDH) [3], 2,4-dinitrophenylhydrazine (DNPH) [4,6,9-14], *O*-2,3,4,5,6-(pentafluorobenzyl)hydroxylamine (PFBHA) [5,15], dansylhydrazine (DNSH) [7], 4-(*N*,*N*-dimethylaminosulphony)-7-hydrazino-2,1,3-benzoxadiazole (DBD-H) [16] and 1,3,5,7-tetramethyl-8-aminozide-difluoroboradiaza-*s*-indacene (BODIPY-aminozide) [17] have been developed. Although various reagents have been used so far but some of

them lack selectivity and other carbonyl compounds may interfere during aldehyde determination especially in biological fluids. In addition, hydrazine reaction products exist as two isomers, *syn* and *anti*, which are subject to analytical errors [17]. In addition, hydrazine reagents are flammable, irritant to nerves, skin, and respiratory tract and unstable as they should be prepared just before analysis. Other than hydrazine reagents, 1,3-cyclohexanedione (CHD) [18,19], 4-(2-(trimethylammonio) ethoxy)benzenaminium halide (4-APC) [20], 2,3-diaminobenzene (DAB) [2] have been used for aldehyde derivatization. These reagents either performed under relatively harsh conditions [2,18], suffer from lower sensitivity [18,20] or not commercially available [20]. All these reagents are pre-column derivatization reagents, this strategy is preferred because minimal instrumentation is needed and aldehydes are more easily separated in the derivatized form.

In this study, simple, sensitive and selective method based on conversion of aliphatic aldehydes to fluorescent derivatives was developed according to the synthesis reaction of lophine derivatives [23-25]. First, aliphatic aldehydes were derivatized with 1,2-di(2-furyl)-1,2-ethanedione (2,2`-furil), α-dicarbonyl compound, in the presence of ammonium acetate to form highly fluorescent difurylimidazole derivatives and then these derivatives were separated by HPLC. Additionally, the developed method was successfully applied for pre-column fluorogenic derivatization of the studied aldehydes in human serum samples with simple pretreatment method and without any interference from biological components. To our knowledge, using 2,2`-furil for monitoring aliphatic aldehydes in human serum was not reported, which indicates the novelty of our method.

2. Experimental

2.1. Chemicals and Reagents

Pentanal, hexanal, heptanal, octanal, decanal, undecanal (used as an internal standard, I.S.), 2,2`-furil and ammonium acetate were purchased from Wako Pure Chemical Industries (Osaka, Japan). Nonanal was purchased from Sigma–Aldrich (Milwaukee, WI, USA). Methanol was obtained from Kanto Chemical Company (Tokyo, Japan). Acetic acid was purchased from Nacalai Tesque (Kyoto, Japan). The water used was purified by a Simpli Lab UV (Millipore, Bedford, MA, USA). All reagents were used as received. The stock solution of aldehydes and an internal standard (5 mM) were prepared in methanol. To obtain reproducible results, daily working solutions were prepared by diluting the stock solutions with methanol to obtain the required concentrations. All prepared solutions were stored at 4 °C. Reagent solutions were prepared by weighing an appropriate amount of 2,2`-furil and ammonium acetate and dissolving in methanol and acetic acid, respectively.

2.2. Apparatus

The HPLC system consisted of a Shimadzu LC-20AT pump (Kyoto), a Shimadzu RF-20AXS fluorescence detector, a Rheodyne (Cotati, CA, USA) 7125 injector with a 20 μL sample loop, and an EZChrom Elite chromatography data acquisition system (Scientific software, Pleasanton, CA, USA). Chromatographic separation was performed on a Cosmosil 5C18MS-II (250 mm × 4.6 mm, I.D., 5 μm) from Nacalai Tesque by an isocratic elution with a mixture of methanol-water (80:20, v/v%) at ambient temperature and a flow rate of 1.0 mL min^{-1}. The fluorescence detection wavelengths were set at 355 nm for emission and at 250 nm for excitation. The Fluorescence spectra were recorded on a Shimadzu RF-

1500 spectrofluorophotometer. Yamato HF-41 heating block (Tokyo) was used for aldehyde derivatization.

2.3. Fluorescence derivatization procedure for aldehyde

In a screw-capped vial, a 50 μL of aldehyde solution, 50 μL of 4.0 mM of 2,2`-furil and 50 μL of 3.0 M ammonium acetate were added. After vortex-mixing, the reaction mixture was heated at 100 °C for 30 min using a heating block, and then cooled. An aliquot of 20 μL was injected into the HPLC system after filtration through a 0.45-μm cellulose acetate membrane filter.

2.4. Assay procedure for aliphatic aldehydes in human serum

To a 50 μL of serum sample, 10 μL of I.S. (0.85 ng mL^{-1} undecanal) was added and then 200 μL of methanol was added to denature proteins. The mixture was vortexed for 30 sec and centrifuged at 2200 *g* for 20 min at 4 °C. A 50 μL of supernatant was taken and then introduced into the derivatization reaction.

The present experiments were approved by the Ethics Committee of the School of Pharmaceutical Sciences, Nagasaki University, and performed in accordance with established guidelines.

3. Results and discussion

3.1. Fluorescence characteristics of the derivatives

Figure 1 shows the fluorescence spectra obtained from decanal (200 nM) after derivatization, the fluorescence was detected at excitation and emission maxima of 250 and 355 nm, respectively (Fig. 1a). Although both 2,2`-furil and ammonium acetate are non-fluorescent compounds,

fluorescence was observed from reagent blank without addition of any aldehydes (Fig. 1b). The fluorescence of the reagent blank might be attributed to the presence of short-chain aliphatic aldehydes as impurities either in solvents or reagents or incorporated from the air during the derivatization procedure [20]. Under the same reaction conditions, acetone and 2-hydroxyacetophenone were introduced into the reaction but no fluorescence was observed. This confirms the selectivity of the method to aldehyde.

In order to elucidate the structure of the fluorescent derivative, the derivatization mixtures of heptanal and octanal were analyzed by EI–MS (JMSDX 303 electron impact mass spectrometer, JEOL, Tokyo). The most abundant ion peaks were found at *m/z* 284 and 298 and these peaks could be attributed to the fluorescent heptanal and octanal derivatives, respectively as shown in Fig. 2.

3.2. Optimization of derivatization conditions

A typical chromatogram of a standard mixture of aliphatic aldehydes and reagent blank are shown in Fig. 3. All of the peaks of aldehyde derivatives including I.S. were detected on the chromatogram within 30 min. Blank peaks were observed in each chromatogram before 5 min, which might be due to the presence of short-chain aliphatic aldehydes as described in section 3.1.

In order to obtain higher fluorescence intensities, derivatization conditions including reagent concentrations, reaction temperature and time were optimized by using a standard mixture of the studied aliphatic aldehydes (5 nM each). In case of 2,2`-furil and ammonium acetate concentrations, the maximum and constant fluorescence intensities were obtained in the presence of more than 3.0 mM of 2,2`-furil (Fig. 4a) and 2.5 M of ammonium acetate (Fig. 4b), respectively. Therefore, 4.0 mM of

2,2`-furil and 3.0 M of ammonium acetate were chosen as optimal concentrations. The reaction temperature and time have a great influence on the derivatization process and the fluorescence intensity. The fluorescence intensities increased with an increase in the reaction temperature and reached maximum at more than 90 °C. At 100 °C, the maximum fluorescence intensities were achieved by heating for more than 25 min. As a result, 100 °C and 30 min were chosen as reaction temperature and time, respectively.

3.3. Validation study

A standard mixture of the studied aliphatic aldehydes with different concentrations was analyzed under the optimized derivatization conditions. The linear calibration ranges, regression equations, correlation coefficients and detection limits of aldehydes were calculated and summarized in Table 1. The calibration curves showed good linearity between concentration and peak area ratio with correlation coefficients from 0.998 to 0.999 and the detection limits were from 0.19 to 0.50 nM (1-10 fmol/injection) at a signal-to-noise ratio (S/N) of 3. The sensitivity of the proposed method was 23-66 times more sensitive compared with GC-MS using TCPH as a derivatization reagent [1], 10-24 times compared with HPLC-UV using DNPH [11,14], 2.5 times compared with GC-MS using PFBHA [15], 3-5 times compared with LC-MS using DNPH [9] and 1.5-3 times compared with HPLC-FL using BODIPY-aminozide [17]. The proposed method showed high sensitivity without requiring expensive instrumentation which may be not available in all laboratories. Also, the derivatization reagent was stable and relatively safe compared with hydrazine reagents.

The reproducibility of the proposed method was examined using three different concentrations of the studied aldehyde (5, 10 and 15 nM).

The precision was expressed by relative standard deviation (RSD). It was found that RSD values for intra-day (n=5) and inter-day (n=5) precision were 0.3-2.9% and 0.6-3.9%, respectively. It was confirmed that the proposed method have sufficient reproducibility.

3.4. Determination of aliphatic aldehydes in human serum

The validation studies of biological fluid were carried out using pooled serum sample pre-analyzed for aldehyde contents before spiking with standard aliphatic aldehyde solutions. Calibration curves of pooled serum samples spiked with a mixture of C_6-C_{10} over concentration range from 0.8 to 15 nM were performed to assess linearity of the proposed method in biological fluids and the results were summarized in Table 2. Among studied aldehydes, pentanal (C_5) was difficult to be determined in serum samples due to its small amount and presence of interfering peaks derived from serum components.

To evaluate intra and inter-day precision, five replicates of three sets of serum samples spiked with three different concentration levels (5, 10 and 15 nM) of standard aldehyde solutions were performed. It was found that RSD values for intra-day (n=5) and inter-day (n=5) precision were 1.0-6.2% and 1.2-8.2%, respectively. The recovery of the proposed method was determined by using serum samples spiked with standard mixture of aldehydes at the three concentrations (5, 10 and 15 nM) before protein precipitation. Recoveries were expressed as [(found amount/ spiked amount) x100] and found to be more than 88%, which is higher than previous methods [13,14] and the results are listed in Table 3.

3.5. Real sample analysis

To evaluate the feasibility of the proposed method, it was applied for the determination of free aliphatic aldehydes in the sera from healthy

subjects. Figure 5 shows typical chromatograms of spiked serum sample and healthy human serum determined by the proposed method. The peaks of hexanal, heptanal, nonanal and decanal could be detected clearly without any interference from biological components. The chromatogram indicates the high selectivity of the proposed method. On the other hand, the peak of pentanal was difficult to be detected as explained before in section 3.4., while the peak of octanal was not detected in the human serum. The concentrations of aliphatic aldehyde in healthy human sera are summarized in Table 4 and the values were in good agreement with other previous reports [9,10].

Conclusion

For the first time, we have demonstrated the feasibility of pre-column fluorogenic derivatization of aliphatic aldehydes in serum using 2,2`-furil. Aliphatic aldehydes were converted to fluorescent difurylimidazole derivatives by the reaction with 2,2`-furil in the presence of ammonium acetate, and the derivatives were determined sensitively by HPLC-FL system. The proposed method was successfully applied to determine aliphatic aldehydes in human serum without any interference from the biological components after simple pretreatment method. Since the proposed method is simple, rapid, sensitive and reproducible, the method should be suitable for routine analysis of large numbers of clinical samples for the evaluation of lipid peroxidation process.

Reference:

[1] Y.C. Fiamegos, C.D. Stalikas, Anal. Chim. Acta 609 (2008) 175.

[2] G. de Revel, L. Pripis-Nicolau, J.-C. Barbe, A. Bertrand, J. Sci. Food Agric. 80 (2000) 102.

[3] A. Büldet, U. Karst, Anal. Chem. 71(1999) 1893.

[4] M.C. Prieto-Blanco, M.P. Iglesias, P. Lopez-Mahia, S.M. Lorenzo, D. P. Rodriguez, Talanta 80 (2010) 2083.

[5] S.-W. Tsai, C.-M. Chang, J. Chromatogr. A 1015 (2003) 143.

[6] Y.-L. Lin, P.-Y. Wang, L.-L. Hsieh, K.-H. Ku, Y.-T. Yeh, C.-H. Wu, J. Chromatogr. A 1216 (2009) 6377.

[7] A. Mainka, K. Bächmann, J. Chromatogr. A 767 (1997) 241.

[8] S.-S. Kim, D.D. Gallaher, A.S. Csallany, Lipids 34 (1999) 489.

[9] R. Andreoli, P. Manini, M. Corradi, A. Mutti, W.M.A. Niessen, Rapid Commun. Mass Spectrom. 17 (2003) 637.

[10] H. Xu, D. Song, Y. Cui, S. Hu, Q.-W. Yu, Y.-Q. Feng, Chromatographia 70 (2009) 775.

[11] C.-E. Banos, M. Silva, Talanta 77 (2009)1597.

[12] H. Xu, L. Lv, S. Hu, D. Song, J. Chromatogr. A 1217 (2010) 2371.

[13] L. Lili, H. Xu, D. Song, Y. Cui, S. Hu, G. Zhang, J. Chromatogr. A 1217 (2010) 2365.

[14] H.-J. Zhang, J.-F. Huang, B. Lin, Y.-Q. Feng, J. Chromatogr. A 1160 (2007) 114.

[15] N. Li, C. Deng, N. Yao, X. Shen, X. Zhang, Anal. Chim. Acta 540 (2005) 317.

[16] K. Nakashima, Y. Hidaka, T. Yoshida, N. Kuroda, S. Akiyama, J. Chromatogr. B 661 (1994) 205.

[17] X.-J. Xiong, H. Wang, W.-B. Rao, X.-F. Guo, H.-S. Zhang, J. Chromatogr. A 1217 (2010) 49.

[18] A.E. Holley, M.K. Walker, K.H. Cheeseman, T.F. Slater, **Free Radic. Biol. Med.** 15 (1993) 281.

[19] M. Matsuoka, N. Imado, T. Maki, K. Banno, T. Sato, Chromatographia 43 (1996) 501.

[20] M. Eggink, M. Wijtmans, R. Ekkebus, H. Lingeman, I. J.P. de Esch, J. Kool, W.M.A. Niessen, H. Irth, Anal. Chem. 80 (2008) 9042.

[21] J.R. Requena, M.-X. Fu, M.U. Ahmed, A.J. Jenkins, T.J. Lyons, S.R. Thorpe, Nephrol. Dial. Transpl. 11 (1996) 48. .

[22] M. Vogel, A. Büldet, U. Karst, Fresenius J. Anal. Chem. 366 (2000) 781.

[23] K. Nakashima, H. Yamasaki, N. Kuroda, S. Akiyama, Anal. Chim. Acta 303 (1995) 103.

[24] N. Kishikawa, M. Wada, Y. Ohba, K. Nakashima, N. Kuroda, J. Chromatogr. A 1057 (2004) 83.

[25] N. Kishikawa, M. Nakao, M.S. Elgawish, K. Ohyama, K. Nakashima, N. Kuroda, Talanta 85 (2011) 809.

Figure captions:

Fig. 1. Excitation and emission spectra of (a) derivatized decanal (200 nM) and (b) reagent blank at excitation and emission maxima at 250 nm and 355 nm, respectively.

Fig. 2. The reaction scheme for fluorogenic derivatization of saturated aliphatic aldehydes with 2,2`-furil.

Fig. 3. Chromatograms obtained from (a) reagent blank and (b) standard solution mixture of aldehydes (5 nM each) after derivatization. Peaks: 1= pentanal, 2= hexanal, 3= heptanal, 4= octanal, 5= nonanal and 6= decanal.

Fig. 4. Optimization conditions of derivatization reaction of standard mixture of studied aldehydes (5 nM each): effects of (a) 2,2`-furil concentration, and (b) ammonium acetate concentration on fluorescence intensity. The data represent error bars of three determinations.

Fig. 5. Representative chromatograms of (a) spiked serum sample with aldehydes (5 nM each) and (b) serum of healthy human, peaks 2-6 as in Fig. 3.

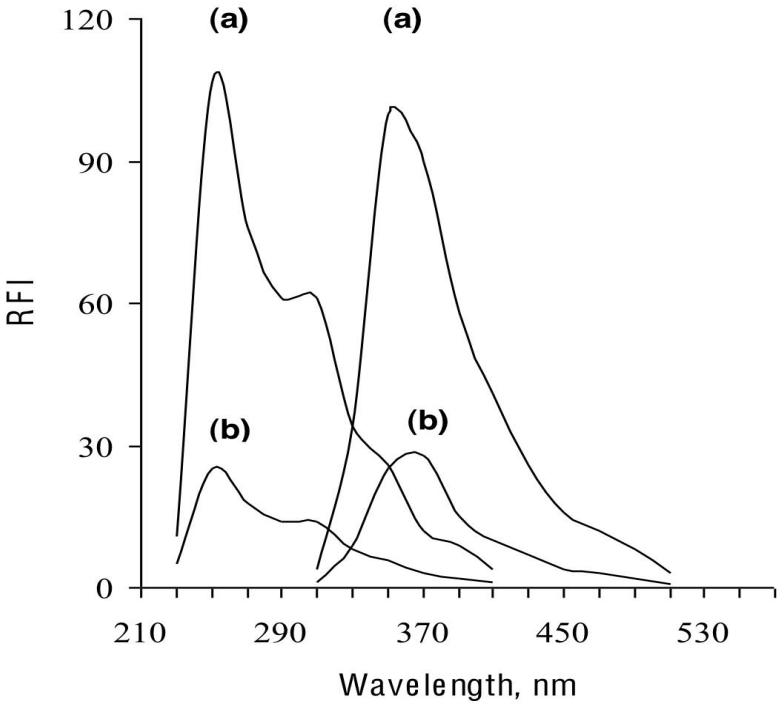

Fig.1. Excitation and emission spectra of (a) derivatized decanal (200 nM) and (b) reagent blank at excitation and emission maxima at 250 nm and 355 nm, respectively.

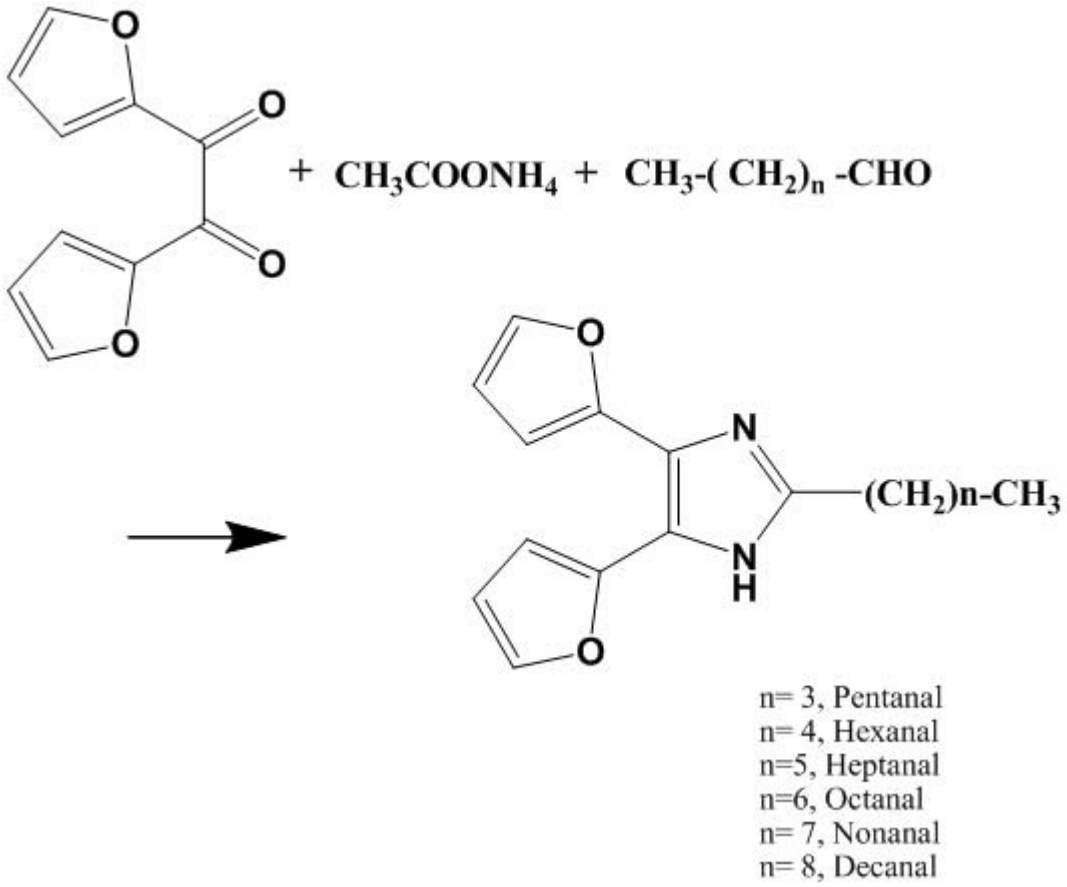

Fig.2. The reaction scheme for fluorescence derivatization of saturated aliphatic aldehydes with 2, 2′-furil

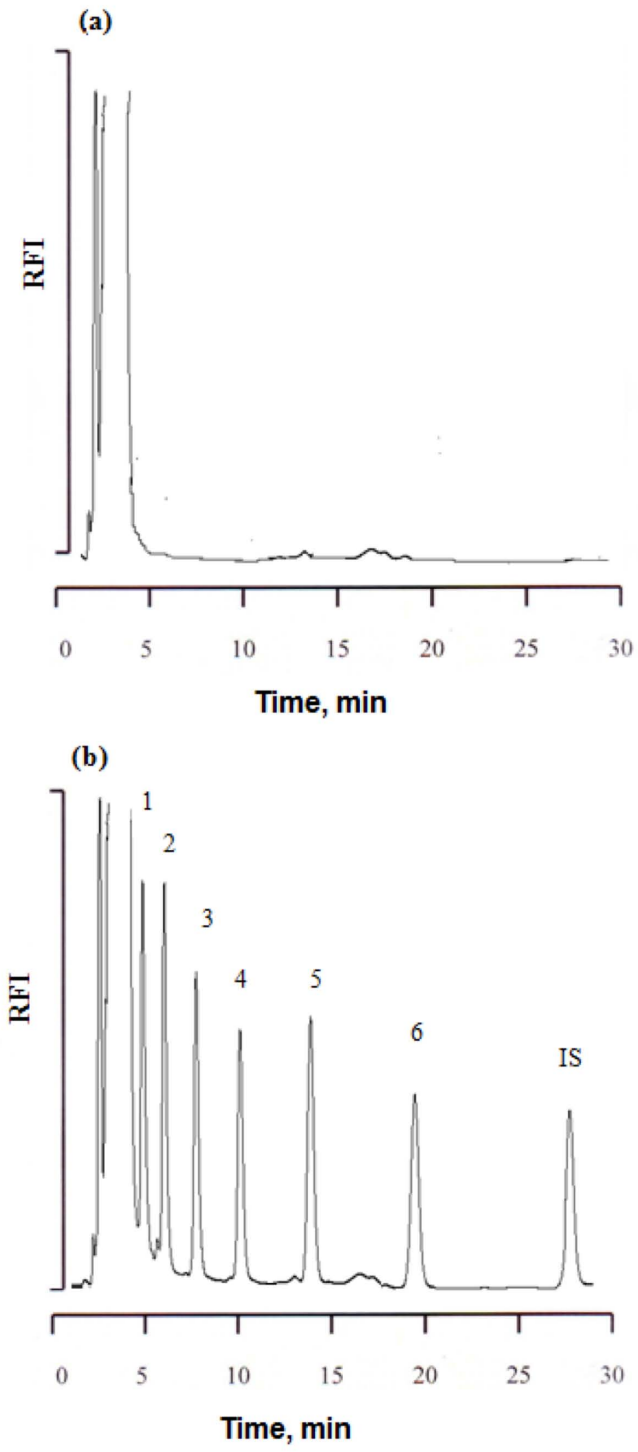

Fig.3. Chromatograms obtained from (a) reagent blank and (b) standard solution mixture of aldehydes (5 nM each) after derivatization. Peaks: 1 = pentanal, 2 = hexanal, 3 = heptanal, 4 = octanal, 5 = nonanal and 6 = decanal..

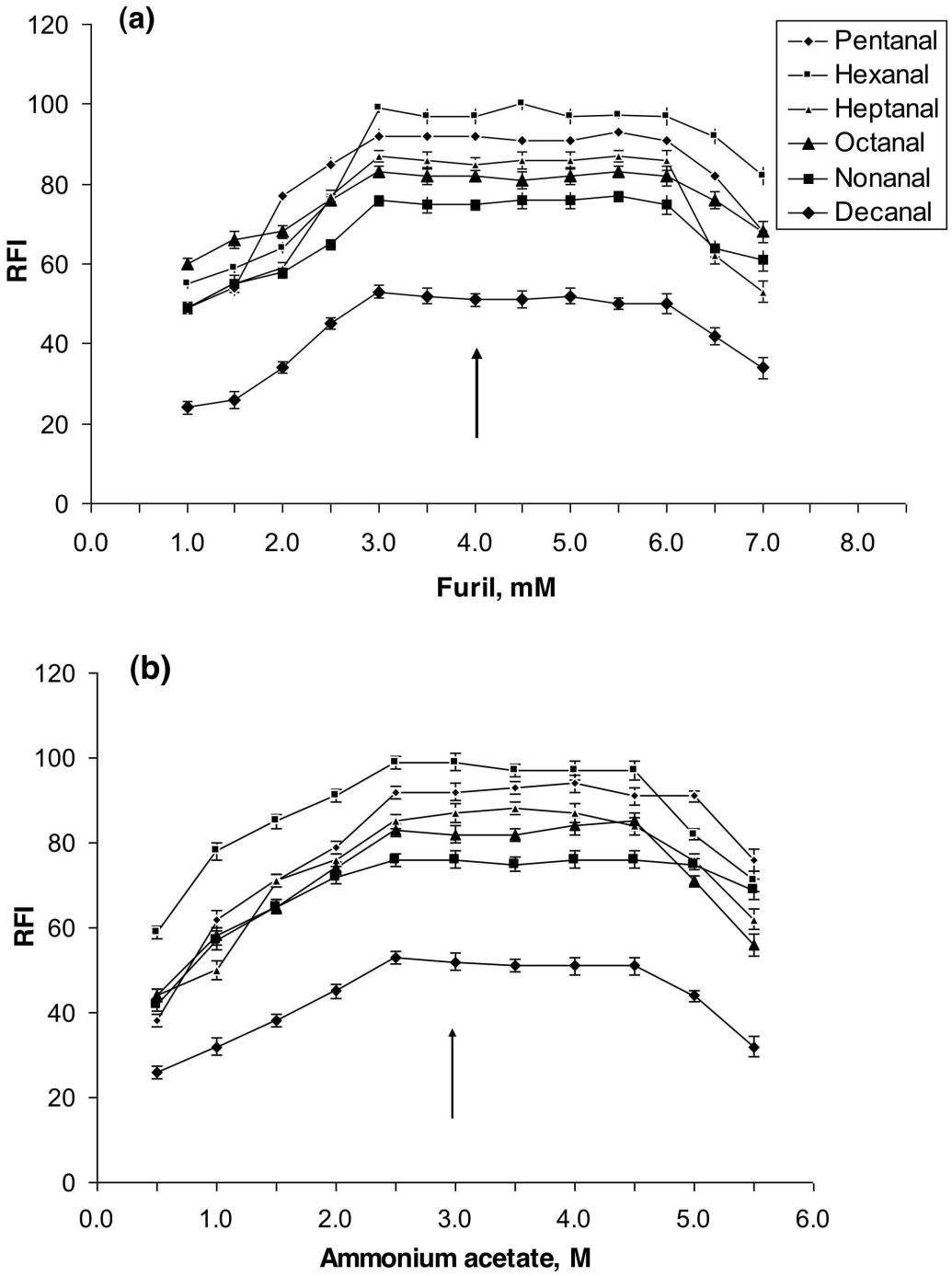

Fig.4. Optimization conditions of derivatization reaction of standard mixture of studied aldehydes (5 nM each): effects of (a) 2,2′ -furil concentration, and (b) ammonium acetate concentration on fluorescence intensity. The data represent error bars of three determinations..

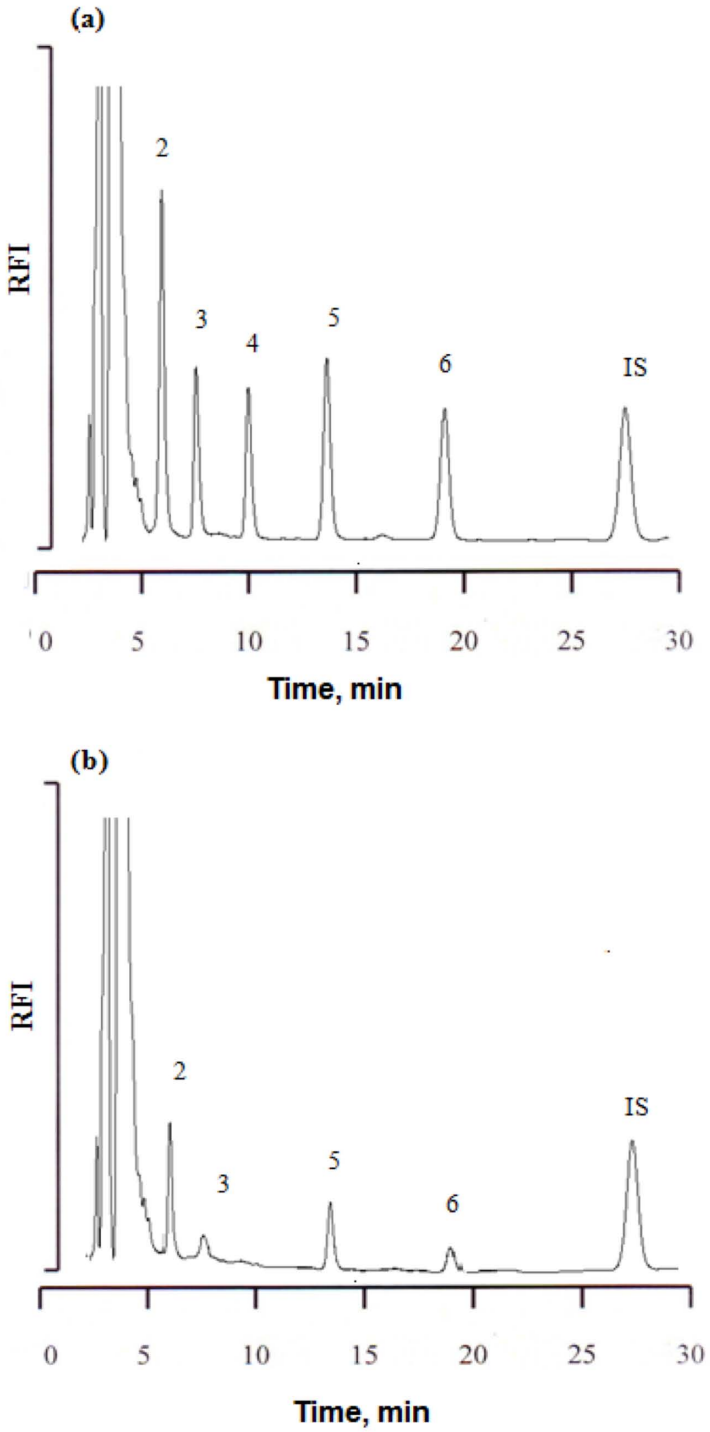

Fig.5. Representative chromatograms of (a) spiked serum sample with aldehydes (5 nM each) and (b) serum of healthy human, peaks 2–6 as in Fig. 3.

Table 1: Calibration curves, correlation coefficient and detection limits for standard aldehyde solutions

Compound	Range, nM	Calibration curve [a], n=3	r	LOD [b], nM (fmol/injection)
Pentanal	1.5-15	Y= 0.09 + 0.49 X	0.9980	0.5 (10)
Hexanal	1.0-15	Y= - 0.01 + 0.47 X	0.9994	0.29 (6)
Heptanal	0.8 -15	Y= - 0.04 + 0.51 X	0.9994	0.25 (5)
Octanal	1.1 -15	Y= - 0.08 + 0.55 X	0.9990	0.29 (6)
Nonanal	0.6 - 15	Y= 0.06 + 0.53 X	0.9994	0.19 (1)
Decanal	0.8-15	Y= 0.07 + 0.46 X	0.9994	0.21 (4)

[a] Y= peak area ratio; X= sample concentration (nM).
[b] S/N= 3.

Table 2: Calibration curves, correlation coefficient and detection limits for spiked serum samples

Compound	Range, nM	Calibration curve [a], n=3	r	LOD [b], nM (fmol/injection)
Hexanal	1.3 -15	Y= - 0.03 + 0.27 X	0.9992	0.4 (8)
Heptanal	0.9 -15	Y= 0.05 + 0.29 X	0.9997	0.3 (6)
Octanal	1.7 -15	Y= 0.03 + 0.35 X	0.9989	0.5 (10)
Nonanal	1.0 - 15	Y=- 0.02 + 0.34 X	0.9996	0.3 (2)
Decanal	0.8 -15	Y= - 0.03 + 0.33 X	0.9998	0.25 (5)

[a] Y= peak area ratio; X= sample concentration (nM).
[b] S/N= 3.

Table 3: Recovery and precision of the proposed method for aldehydes in serum samples

Aldehyde	Spiked amount (nM)	Intra-day (n=5)			Inter-day (n=5)		
		Found	RSD %	Recovery %	Found	RSD %	Recovery %
Hexanal	5	4.7	2.8	94.0	4.6	4.5	93.3
	10	9.4	1.5	94.8	9.38	1.9	93.8
	15	15.3	2.3	102.3	14.1	2.7	94.1
Heptanal	5	5.1	5.8	102.1	5.06	8.2	101
	10	10.3	1.7	103.0	10.2	2.5	102
	15	14.4	2.8	96.0	14.5	1.8	97
Octanal	5	4.55	3.0	91.0	4.4	3.9	88
	10	10.3	1.4	103	10.5	4.3	105
	15	14.9	1.1	99.4	14.9	1.8	99.4
Nonanal	5	4.9	2.5	99.8	4.76	7.1	95.0
	10	10.1	1.7	101	10.2	2.8	102
	15	15.1	1.4	101	15.5	1.7	103
Decanal	5	5	6.2	100	4.8	6.4	97
	10	10.1	1.0	101	9.9	1.2	99
	15	15.3	2.9	102	15.4	2.9	103

Table 4: Serum concentration levels of aldehyde in healthy subjects (n=5)

Subject	Hexanal Conc. (nM)	Heptanal Conc. (nM)	Nonanal Conc. (nM)	Decanal Conc. (nM)
Range	1.8 -3.0	1.1-1.4	0.9-1.5	0.7-1.5
Mean ± SD	2.36 ± 0.49	1.21 ± 0.13	1.20 ± 0.23	1.24 ± 0.48