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Fluorometric Liquid Chromatographic Determination of Aliphatic Aldehydes Arising from Lipid Peroxides[1)]

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A fluorometric method for the determination of aliphatic aldehydes using 1,3-cyclohexanedione reagent and high-performance liquid chromatography (HPLC) was developed.

Straight-chain aldehydes of C_3–C_{10} were reacted with 1,3-cyclohexanedione at 60 °C for 1 h and the fluorescent decahydroacridine derivatives[2,3)] formed were determined by HPLC on an ODS column. The calibration curves for the aldehydes were linear in the range of 0—10 ng and the relative standard deviation at the 5 ng level was about 2.3%.

This method was sensitive enough to measure the amount of aliphatic aldehydes, including 4-hydroxynonenal, generated by microsomal peroxidation in rat liver.

Keywords——lipid peroxide; aliphatic aldehyde; HPLC-fluorometric determination; 1,3-cyclohexanedione; decahydroacridine derivative

The correlation between so-called age-released disease (atherosclerosis,[4,5)] diabetes,[6,7)] cerebral apoplexy, *etc.*) and lipid peroxidation has recently been attracting considerable interest.

Lipid peroxides (LPO) arising from the peroxidation of cellular lipid and cell constituents are known to be decomposed to lower molecular weight carbonyl compounds,[8,9)] and some aliphatic aldehydes have been reported to be highly reactive[10–12)] and toxic.[13–19)] Peroxide-related diseases might thus be, at least in part, due to such aldehydes, though it is not yet clear whether these aldehydes are really physiologically significant. In order to investigate the formation and degradation of lipid peroxide-related aldehydes in tissues and the body, it is essential to develop a highly specific and sensitive assay method.

In the past, the determination of aldehydes has mostly been performed by a colorimetric method using 2,4-dinitrophenylhydrazine (2,4-DNPH) reagent. Esterbauer *et al.*[8,9)] used 2,4-DNPH reagent in the assay of peroxidized lipid of rat liver microsomes to convert aldehydes to the corresponding hydrazones, and analyzed the hydrazone mixture by a combination of thin layer chromatography (TLC)and high-performance liquid chromatography (HPLC).

In our studies for the analysis of aldehydes arising from lipid peroxides in spontaneously hypertensive rats, we used 1,3-cyclohexanedione (CHD) reagent to derivatize the aldehydes under slightly acidic conditions (Hantzsch reaction), and analyzed the fluorescent decahydroacridine derivatives thus obtained by normal or reversed-phase HPLC.[1)]

Recently, Mopper and Stahovec[20)] and Suzuki[21)] reported HPLC-fluorometric methods for the determination of various aliphatic aldehydes using 5,5-dimethyl-1,3-cyclohexanedione and CHD, respectively, and applied them to the analysis of aldehydes in environmental, industrial and food samples such as whisky.

The advantages of the use of CHD reagent are not only enhancement of the detectability of the aldehydes, but also a much milder derivatization reaction than that with 2,4-DNPH, so

that the aldehydic products produced from the peroxidized lipid might be specifically analyzable.

The present method was applied to the quantitative determination of straight-chain C_3–C_{10} aldehydes including 4-hydroxynonenal, which is one of the major aldehydic products observed in the peroxidation of rat liver microsomes by ADP–Fe^{2+}.

Experimental

Chemicals——CHD was obtained from Aldrich Chemical (Wisconsin) and was purified by recrystallization from MeOH. Propanal was obtained from Tokyo Kasei Co., Ltd., (Tokyo). Butanal, hexanal, octanal and decanal were obtained from Wako Pure Chemicals (Tokyo). 5-Hydroxypentanal was obtained from Aldrich Chemical. Other chemicals were obtained from Wako Pure Chemicals and were of reagent grade. 4-Hydroxy-2,3-*trans*-nonenal was synthesized according to the report of Esterbauer and Weger.[22] All standard samples used were straight-chain normal aldehydes.

The CHD reagent was prepared by dissolving ammonium sulfate (10 g), glacial acetic acid (5 ml) and CHD (0.25 g) in about 50 ml of water and making the volume up to 100 ml with water. MeOH for preparing the standard and test solutions of aldehydes was pretreated with 2,4-DNPH and distilled before use.

Apparatus——A Spectra-Physics HPLC apparatus (type SP-8770) equipped with a Rheodyne sampling valve (model 7125) and a 100 μl sample loop was used. An ERC-ODS-1262 column (5 μm, 10 × 0.6 cm; Erma Optical Works, Ltd., Tokyo, Japan) was employed and a Hitachi F-1000 HPLC fluorescence detector was used with excitation at 380 nm and emission at 445 nm.

Procedure——Aldehyde samples in MeOH (0.5 ml) were mixed with water (0.5 ml) and CHD reagent (1.0 ml). The mixture was incubated at various pH values (adjusted with glacial acetic acid) at various temperatures for up to 3 h. The reaction mixture was poured into a Sep-pak C_{18} cartridge (Waters Co., Milford, MA) for clean-up and eluted with MeOH. This MeOH solution was subjected to HPLC under the following conditions: eluting solv., 0—18 min; MeOH : H_2O = 30 : 70, 18—32 min; THF : H_2O = 26 : 74, 32—42 min; THF : H_2O = 40 : 60, 42—50 min; THF : H_2O = 100 : 0. Flow rate: 1 ml/min.

When rat plasma or liver was used as the sample for the determination of aldehyde, 0.5 ml of 10% liver homogenate in 40 mM phosphate buffer (pH 7.4) or 0.5 ml of plasma was used. MeOH (0.5 ml) was added with mixing for 30 s followed by centrifugation at 850 × *g* for 10 min, and the supernatants (0.5 ml) were used as samples for aldehyde determination according to the procedure shown in Fig. 1.

Calculation——The amounts of aldehydes in samples were calculated from the HPLC peak areas by comparison with those of standard aldehydes.

Results and Discussion

Optimum Conditions for Derivatization

pH——As shown in Fig. 2(A), the optimum pH for the derivatization of hexanal was 5.0. When CHD reagent was added to a sample solution of rat plasma or liver homogenate, the pH of the reaction mixture was approximately 5.0 and thus the mixture was incubated without pH adjustment.

plasma or liver homogenate (0.5 ml)
- add MeOH (0.5 ml)
- mix for 30 s
- centrifuge at 850 × *g* for 10 min

supernatant (0.5 ml)
- add CHD reagent (1.0 ml)
- incubate at 60°C for 1 h

reaction mixture (1.0 ml)
- pour onto Sep-pak C_{18}
- elute with MeOH (2.0 ml)

sample for HPLC (ODS column)

Fig. 1. Procedure for the Determination of Aliphatic Aldehydes in Rat Plasma and Liver Homogenate

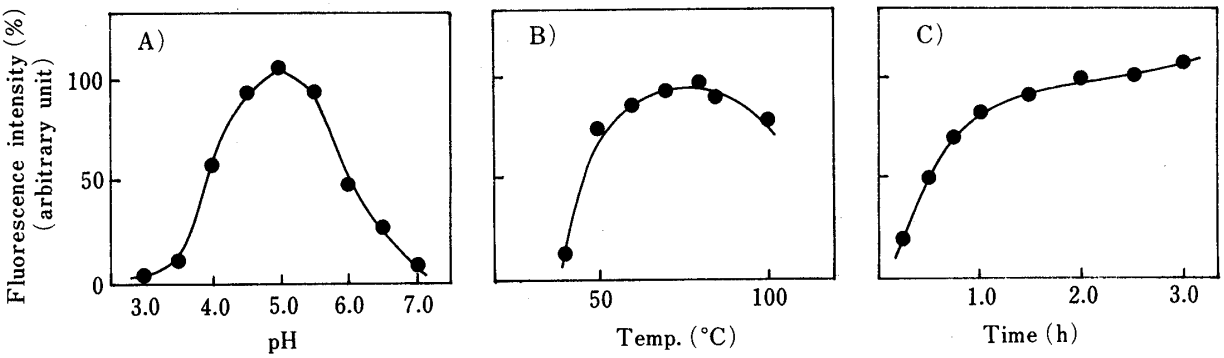

Fig. 2. Effects of pH, Temperature and Time on the Reaction of Hexanal with CHD

A) Effect of pH. Hexanal was incubated with CHD at 60 °C for 1 h. B) Effect of reaction temperature. The reaction mixture was incubated at pH 5.0 for 1 h. C) Effect of reaction time. The reaction mixture was incubated at pH 5.0 and 60 °C.

TABLE I. Detection Limits and Response Factors of Various Aldehydes Relative to Hexanal

Aldehyde	Average peak area[a] (mm^2)	Standard deviation (mm^2)	Response factor relative to hexanal[b]	Lowest quantifiable limit (pg)
Propanal	358.2	13.2 (3.7%)	1.08 (0.63)[c]	4.02
Butanal	205.7	7.2 (3.5)	0.62 (0.45)	7.00
Pentanal	95.6	1.0 (1.0)	0.29 (0.25)	15.06
Hexanal	333.2	8.2 (2.5)	1.00 (1.00)	4.32
Heptanal	113.7	1.3 (1.2)	0.34 (0.39)	12.66
Octanal	286.5	11.2 (3.9)	0.86 (1.10)	5.03
Nonanal	282.0	3.0 (1.1)	0.85 (1.21)	5.11
Decanal	367.1	8.7 (2.7)	1.10 (1.72)	3.92
5-Hydroxypentanal	393.4	11.3 (2.9)	1.18 (1.20)	3.66
4-Hydroxynonenal	81.7	0.4 (0.5)	0.25 (0.39)	17.63

a) Peak area was calculated from the chart run at a detector sensitivity of $\times 0.5$. *b*) Relative response factor was obtained by dividing the fluorescence intensity of each aldehyde–CHD by that of hexanal–CHD. *c*) Numbers in parentheses represent response factor relative to hexanal on the basis of nmol aldehydes. Each standard sample contained 5 ng aldehyde/10 μl injected into the HPLC column.

Temperature——As shown in Fig. 2(B), the optimum temperature for the derivatization was 80 °C. In order to minimize artifact formation and side reactions, we employed 60 °C as the standard reaction temperature.

Time——As shown in Fig. 2(C), the development of the fluorescence depends strongly on reaction time. We selected 1 h as the standard reaction time.

Fluorescence Spectrum

Saturated and straight chain aldehydes of carbon numbers 3—10, 4-hydroxynonenal and 5-hydroxypentanal showed similar fluorescence spectra with Ex. max. 380 nm and Em. max. 445 nm.

Detection Limit

When precautions were taken to minimize aldehyde contamination arising from reagents and solvents, the detection limit was about 7.8 pg (78 fmol as provisional $M_r = 100$) per injected ten aldehydes with a signal-to-noise ratio of 3.0. This corresponds to 0.78 nM levels of aldehydes in an injection volume of 100 μl (Table I).

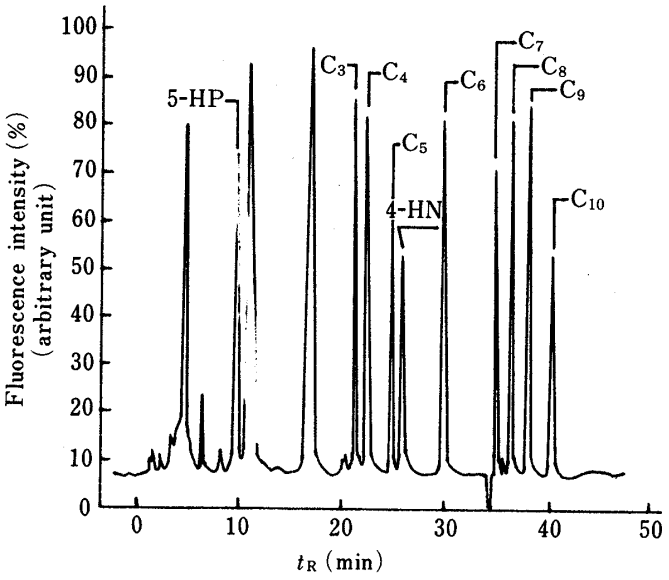

Fig. 3. HPLC Chromatogram of Aldehyde–CHD

C_3, propanal; C_4, butanal; C_5, pentanal; C_6, hexanal; C_7, heptanal; C_8, octanal; C_9, nonanal; C_{10}, decanal; 4-HN, 4-hydroxynonenal; 5-HP, 5-hydroxypentanal. A mixture of aldehydes (1.0 ppm) was treated with CHD as described under Experimental and 10 μl of the sample solution was injected into an ODS column. The detector sensitivity was 0.5.

TABLE II. Elution Characteristics of Aldehyde–CHDs on an ERC-ODS-1262 Column

Aldehyde	t_R (min)	Standard deviation (min)	Capacity factor (K')	Separation relative to hexanal (R_s)
5-Hydroxypentanal	9.88	0.28 (2.83%)	4.6	23.5
Propanal	21.45	0.24 (1.12)	10.8	11.6
Butanal	22.65	0.13 (0.57)	11.6	9.3
Pentanal	25.60	0.34 (1.33)	12.9	6.5
4-Hydroxynonenal	26.20	0.12 (0.46)	13.5	5.2
Hexanal	29.65	0.26 (0.88)	15.7	—
Heptanal	35.05	0.13 (0.37)	18.6	8.0
Octanal	36.38	0.17 (0.47)	19.3	8.8
Nonanal	38.00	0.18 (0.47)	20.2	10.9
Decanal	40.23	0.17 (0.42)	21.4	13.0

Eluting solvents were MeOH–H_2O (30:70) and THF–H_2O (26:74, 40:60, 100:0). Running conditions for HPLC were as described in Experimental.

Chromatographic Conditions

The reversed-phase column was capable of resolving all aldehyde–CHDs tested. A typical chromatogram is shown in Fig. 3. An excellent gradient elution profile was achieved with MeOH–H_2O and THF–H_2O mixtures in the ratios described under Experimental.

Calibration Curves and Fluorescence Responses of Aldehydes

Calibration curves for ten standard aldehydes in the range of 0—10 ng were linear.

A fluorescence peak area of 10 ng of hexanal produced 665.0 mm^2, and the fluorescence responses of the fluorophores from nine aldehydes, relative to that from hexanal, are listed in Table I. Response factors relative to hexanal varied from 0.25 to 1.18. The fluorescence intensity of decanal–CHD was highest (11471.9 mm^2/nmol) whereas that of pentanal–CHD was lower (1648.3 mm^2/nmol) than those of other aldehydes on a nanomolar basis.

Precision——Ten standard aldehydes were analyzed 5 times by reversed-phase HPLC. The average relative standard deviation was 2.3% at the 5 ng levels as shown in Table I.

Elution Characteristics of Aldehydes——Separation of aldehyde–CHDs by HPLC is summarized in Table II.

TABLE III. Recoveries of Aldehyde–CHDs from Sep-pak C_{18} Treatment

Aldehyde	Recovery (%)
Propanal	91.8
Butanal	111.1
Pentanal	85.4
Hexanal	97.5
Heptanal	91.2
Octanal	104.6
Nonanal	110.2
Decanal	118.4
5-Hydroxypentanal	83.2
4-Hydroxy-2,3-*trans*-nonenal	99.1

Reacted solutions of 500 ng of each aldehyde with CHD were subjected to Sep-pak C_{18} treatment.

TABLE IV. Recovery of Aldehydes from Rat Plasma or Liver Homogenate

	Hexanal (μg)			5-Hydroxypentanal (μg)		
	Liver homog.	Liver homog. heated	Plasma	Liver homog.	Liver homog. heated	Plasma
1	6.64	9.59	9.59	8.60	11.21	10.16
2	6.41	9.30	9.81	8.37	10.98	9.54
3	5.71	9.69	10.18	8.75	10.50	9.29
4	5.38	9.09	9.09	8.45	11.64	9.47
5	5.45	9.04	10.19	8.83	10.95	9.74
Mean±S.D.	5.92±0.57 (59.2%)	9.34±0.29 (93.4%)	9.77±0.46 (97.7%)	8.60±0.19 (86.0%)	11.06±0.42 (110.6%)	9.64±0.33 (96.4%)

Hexanal or 5-hydroxypentanal (10 μg) was added to rat plasma or liver homogenate, which was then centrifuged and treated as described under Experimental. Figures in parentheses indicate the recoveries (%).

Clean-Up of the Fluorescent Products

The recoveries of aldehyde–CHD from Sep-pak C_{18} are shown in Table III. The average recovery of ten standard aldehydes was 99.3%.

Aldehyde Recoveries from Rat Plasma and Liver

Table IV shows the recoveries of hexanal and 5-hydroxypentanal from rat plasma and liver to which 10 μg of each aldehyde was added. The recoveries of the aldehydes from the plasma were 97.7% and 96.4%, respectively. The recoveries of the aldehydes from liver, however, were time-dependent; they decreased for a short period of time after the addition of the aldehydes to liver homogenate. In the case of hexanal, the recovery was 59.2% when it was analyzed just after its addition. The recoveries were 22% and 18.4% after 2 and 60 min of incubation, respectively.

When liver homogenate heated at 100 °C for 1 min was used, the recoveries of hexanal and 5-hydroxypentanal were 93.4% and 110.6% respectively. The addition of MeOH to plasma or liver homogenate was very effective for obtaining a good recovery.

Aldehydes in Rat Plasma and Liver

Typical chromatographic patterns of aldehydes in normal rat plasma and liver treated with CHD according to the procedures of Fig. 1 are shown in Fig. 4. Each peak was characterized on the basis of the retention times of the standard aldehyde–CHDs under

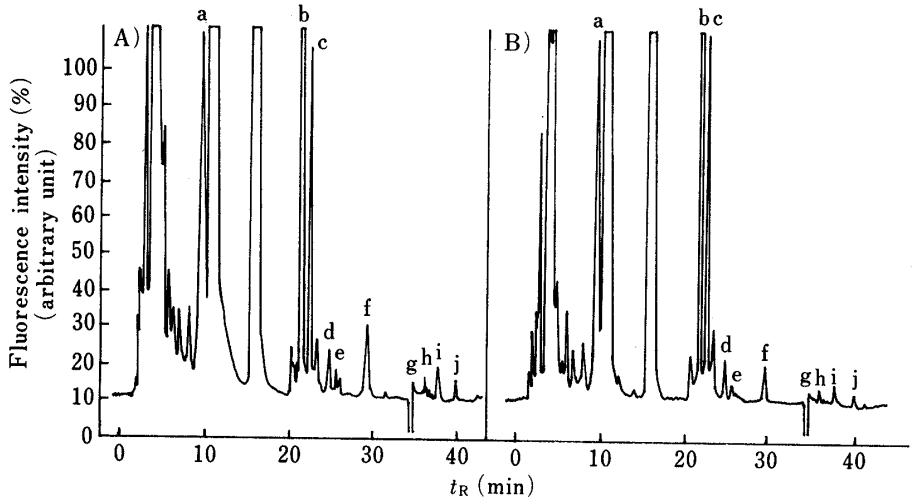

Fig. 4. HPLC Chromatograms of the Rat Liver (A) and Plasma (B)

a—j represent the following aldehydes: a, 5-HP (reference aldehyde); b, C_3; c, C_4; d, C_5; e, 4-HN; f, C_6; g, C_7; h, C_8; i, C_9; j, C_{10}.

various elution conditions (*e.g.* different ratios of MeOH : H_2O and THF : H_2O). Higher contents of C_3 and C_4 aliphatic aldehydes were found as compared to other aldehydes. When this analytical method was applied to rats given CCl_4 (3 ml/kg body weight) orally, higher levels of hexanal (1.3 μg/g liver, 0.32 μg/ml plasma) and 4-hydroxynonenal (26.86 μg/g liver) were observed (control rats: hexanal, 0.74 μg/g liver, 0.28 μg/ml plasma; 4-hydroxynonenal, 13.08 μg/g liver). This method was able to measure sensitively the amounts of aliphatic aldehydes generated in rat liver mitochondria and in microsomal peroxidation by ADP-Fe^{2+} The details of the analytical results for aldehyde formed in the above *in vivo* and *in vitro* peroxidation reactions will be reported in a subsequent paper.

References and Notes

1) A summary of this work was presented at the 7th Meeting of the Japanese Society of Lipid Peroxide Research, Nagoya, 1983 (Abstract p. 14), at the 19th Meeting of the Council for the Spontaneously Hypertensive Rat, Kamakura, 1983 (Abstract p. 66) and at the 104th Annual Meeting of the Pharmaceutical Society of Japan, Sendai, 1984 (Abstract p. 403).
2) M. Pesez and J. Bartos, "Colorimetric and Fluorimetric Analysis of Organic Compounds and Drugs," ed. by M. K. Schwartz, Marcel Dekker Inc., New York, 1974, pp. 283—284.
3) M. Pesez and J. Bartos, *Talanta*, **14**, 1097 (1967).
4) A. L. Tappel, "Lipid Peroxidation and Fluorescent Molecular Damage to Membranes in Pathobiology of Cell Membranes 1," ed. by B. F. Trump and A. D. Astila, Academic Press, New York, 1975, pp. 145—170.
5) S. H. Moncada, E. A. Higgs and J. R. Vane, *Lancet*, **1**, 18 (1977).
6) M. Tsujii, M. Tamura, M. Morio, K. Kimura, M. Higuchi, Y. Matsuoka, I. Suwa, K. Umeita, C. Kubota and Y. Okuno, *Nara Igaku Zasshi*, **26**, 477 (1975).
7) S. Yamazaki, *Rinshobyori*, **19**, 128 (1975).
8) A. Benedetti, M. Comporti and H. Esterbauer, *Biochim. Biophys. Acta*, **620**, 281 (1980).
9) A. Benedetti, R. Fulceri, M. Ferrali, L. Ciccoli, H. Esterbauer and M. Comporti, *Biochim. Biophys. Acta*, **712**, 628 (1982).
10) W. Yoshioka and N. Kaneda, *Yukagaku*, **23**, 321 (1974).
11) N. Kaneda, *Eiyo To Shokuryo*, **30**, 71 (1977).
12) E. Schauenstein, *J. Lipid Res.*, **8**, 417 (1977).
13) N. R. DiLuzio, *Fed. Proc.*, **32**, 1875 (1973).
14) E. D. Wilkinson, *Biochem. J.*, **99**, 657 (1966).
15) A. C. Allison, *Nature* (London), **205**, 141 (1965).
16) C. E. Mengel, *Am. J. Med. Sci.*, **255**, 341 (1968).
17) G. L. Plaa and H. Witschi, *Ann. Rev. Pharmacol. Toxicol.*, **16**, 125 (1976).

18) R. O. Recknagel and E. A. Glende, Jr., *Handbook of Physiology*, **9**, 591 (1977).
19) A. L. Tappel, *Fed. Proc.*, **32**, 1870 (1973).
20) K. Mopper and W. L. Stahovec, *J. Chromatogr.*, **256**, 243 (1983).
21) Y. Suzuki, *Bunseki Kagaku*, **34**, 314 (1985).
22) H. Esterbauer and W. Weger, *Monatsh. Chem.*, **98**, 1884 (1967).