Communications to the Editor

[Chem. Pharm. Bull. 31(7)2523—2525(1983)]

A CONVENIENT METHOD FOR A SENSITIVE COLORIMETRIC DETERMINATION OF LIPOPEROXIDES WITH 1,3-DIPHENYL-2-THIOBARBITURIC ACID

Kenichiro Nakashima,* Taiji Ando, Kyoko Nakamura, and Shuzo Akiyama*
Faculty of Pharmaceutical Sciences, Nagasaki University,
Bunkyo-machi 1—14, Nagasaki 852, Japan

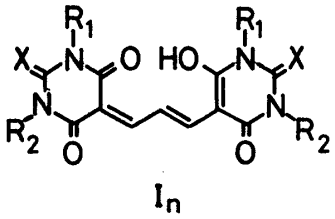
A convenient method for a sensitive colorimetric determination of malondialdehyde (MDA) with 1,3-diphenyl-2-thiobarbituric acid could be developed to apply to the determination of lipoperoxides in both rat liver and rat plasma. A linear relationship was obtained in the range of 0.12—6.25 nmol/ml of MDA. The molar absorption coefficient determined from the calibration curve was fairly large [$\varepsilon = 1.85 \times 10^5$ $mol^{-1}dm^3cm^{-1}$]. MDA was found to be 200 nmol/g wet tissue and 1.25 nmol/ml plasma of rat.

KEYWORDS——colorimetry; malondialdehyde; 1,3-diphenyl-2-thiobarbituric acid; lipoperoxide; rat liver; rat plasma

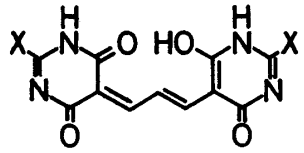
We have studied the application to colorimetry of colored compounds arising from a variety of barbituric acid (BA) with aldehydes.[1] It is well known that thiobarbituric acid (TBA) is useful as a general reagent for the determination of malondialdehyde (MDA) as an index of lipoperoxides.[2] However, the reactivity of barbituric acid with MDA has not systematically been studied. Thus, we prepared various kinds of BA derivatives, examined the reactivity of these compounds with MDA, and isolated the resulting condensation products (pigments) to check their UV(absorption) spectra. The spectral data of the pigments are shown in Table 1, from which 1,3-diphenyl-2-thiobarbituric acid (DPTBA) condensate (I_5) is found to have the largest molar absorption coefficient (ε/$mol^{-1}dm^3cm^{-1}$) in EtOH.

We wish to report that DPTBA is suitable to determine colorimetrically a trace amount of MDA and can be conveniently applied to the determination of lipoperoxides in rat liver homogenate and rat plasma.

A recommended procedure is as follows.[3] To a standard aqueous solution of MDA (1,1,3,3-tetraethoxypropane, $\leq$ 25 nmol as MDA, 1 ml) in a 10 ml glass-stoppered test tube is added a buffer solution (pH 2.00, HCl—sodium acetate, 2.5 ml)[4] and a solution of DPTBA in dimethyl sulfoxide(DMSO) (0.12 M, 0.5 ml).[5] The well-mixed solution is heated for 30 min in a boiling water bath, chilled for 5 min in a tap water, and then shaken with methylisobutyl ketone (MIBK, 4.0 ml)[6] by a shaker. After centrifugation(3000 rpm, 15 min), the absorbance of an organic layer is measured at 538 nm against MIBK. The absorption curve obtained by the method is shown in Fig. 1 with that of the reagent blank. The resulting color was very stable and the constant absorbance was kept for about 24 h. As shown in the



I_n



II_n

Table 1. Spectral Data of the Pigments (I_n and II_n)

Pigment[a]	X	R_1	R_2	$\varepsilon \times 10^{-4}$ (λ_{max}/nm)		
				EtOH	0.1 M NaOH	0.1 M HCl[b]
I_1	S	H	H	16.8 (531)	16.2 (544)	15.0 (531)
I_2	S	CH_3	H	18.5 (532)	c) (549)	15.8 (533)
I_3	S	CH_3	CH_3	17.6 (533)	c) (531)	13.7 (536)
I_4	S	C_6H_5	H	17.5 (534)	17.4 (550)	15.3 (535)
I_5	S	C_6H_5	C_6H_5	19.6 (537)	c) (538)	d)
I_6	S	C_2H_5	C_2H_5	18.1 (539)	c) (541)	15.5 (541)
I_7	O	H	H	d)	13.4 (498)	d)
I_8	O	CH_3	H	11.0 (489)	15.0 (500)	10.5 (488)
I_9	O	CH_3	CH_3	12.7 (491)	c) (491)	12.4 (490)
I_{10}	O	C_6H_5	H	12.6 (491)	13.0 (503)	12.6 (491)
I_{11}	O	C_6H_5	C_6H_5	12.6 (495)	c) (493)	13.1 (494)
II_1	NH_2			d)	12.0 (515)	d)
II_2	$NHCH_3$			d)	8.9 (521)	d)
II_3	$N(CH_3)_2$			d)	15.0 (529)	12.1 (501)

1 M = 1 mol dm^{-3}; a) I_1, I_3, and I_7 were prepared according to the literature (R. J. Shephard, J. Chem. Soc., 1964, 4410). The other compounds were isolated in this work. The molecular compositions were determined by elemental analyses and/or mass spectrometry; b) 0.1 M HCl : EtOH = 9 : 1; c) The exact value was not obtained because of the lability; d) Insoluble.

inserted figure in the upper left side of Fig. 2, the calibration curve for MDA is linear from 0.12 to 6.25 nmol.[7] The apparent molar absorption coefficient estimated from the above curve was 1.85×10^5. The procedure was readily applied to the determination of lipoperoxides in liver and plasma of Wistar rat (7 weeks old). The recovery of MDA gave fairly good results as shown in Table 2.

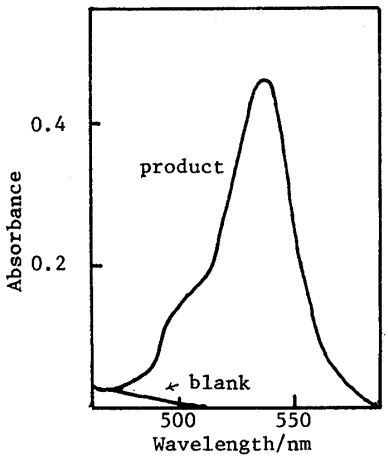


Fig. 1. Absorption Spectra of Reaction Product

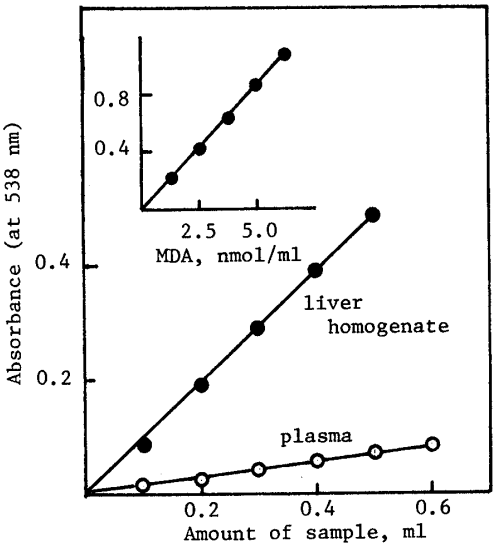


Fig. 2. Calibration Curve and Relationship between Amount of Sample and Absorbance

Table 2. Recovery and Assay of MDA in Liver and Plasma of Wistar rat (7 weeks old).

	Recovery (%)		Assay	
	Amount of MDA added (nmol/ml)		Amount of MDA	Coefficient of variation (%)
	1.25	0.5		
Liver (10% Homogenate, 0.2 ml)	95.5 (n=10)	84.6 (n=10)	200 nmol/g wet tissue	4.61 (n=10)
Plasma (0.3 ml)	82.5 (n=5)	90.9 (n=5)	1.25 nmol/ml	5.21 (n=5)

The linear relationship between the sample amount and absorbance is shown in Fig. 2. On the bases of these experiments the quantities of MDA estimated from the calibration curve are illustrated in Table 2.

In conclusion, our present method has many characteristics. 1. The preparation of the reagent (DPTBA) is easy. 2. The stock solution of DPTBA and the resulting pigment are stable. 3. The assay system is simple.[8] 4. The sensitivity is somewhat superior to the conventional TBA method.[2]

However, the method is inferior to TBA fluorometry[9] in sensitivity.

Studies on HPLC analysis of lipoperoxides by using DPTBA are now in progress.

ACKNOWLEDGMENT This work was supported in part by Grant-in-Aid for Scientific Research (No. 57570782) from the Ministry of Education, Science and Culture.

REFERENCES AND NOTES

1) a) K. Nakashima, Yakugaku Zasshi, 97, 202 (1977); b) K. Nakashima, Yakugaku Zasshi, 97, 906 (1977); c) K. Nakashima and S. Akiyama, Chem. Pharm. Bull., 28, 2518 (1980); d) K. Nakashima and S. Akiyama, Chem. Pharm. Bull., 29, 1755 (1981).
2) K. Yagi, I. Nishigaki, and H. Ohama, Vitamins (Japan), 37, 105 (1968); H. Ohkawa, N. Ohishi, and K. Yagi, Anal Biochem., 95, 351 (1979) and references cited therein.
3) After examination of the influence of the concentration on the absorbance, 1.5 x 10^{-2}M and 12.5 v/v% were estimated satisfactorily for DPTBA and DMSO, respectively.
4) Walpole's buffer was used, and the optimum pH was 1.5 – 3.0.
5) This reagent solution was very stable for about 5 months in a dark.
6) The maximum molar absorption coefficient was obtained in MIBK among MIBK, n-BuOH, iso-pentyl alcohol, and $CHCl_3$.
7) The coefficients of variation for the five replicated experiments were 0.41% (3.0 nmol/ml) and 2.96% (1.5 nmol/ml).
8) For example, good results are obtained for rat plasma, even if a deproteinization process that is generally necessary is omitted.
9) H. Tanizawa, Y. Sazuka, and Y. Takino, Chem. Pharm. Bull., 29, 2910 (1981).

(Received May 12, 1983)