

[Chem. Pharm. Bull.]  
28(12)3507—3513(1980)

## Analytical Studies on Isoxazoles. II.<sup>1)</sup> A New Fluorimetric Determination of 5-Phenyl-3-isoxazolecarboxylic Acid and Its Application to the Determination of Perisoxal in Plasma

TSUNEJI UMEDA, HIROKO IMANISHI, and EIZO HIRAI

*Shionogi Research Laboratory, Shionogi and Co., Ltd.<sup>2)</sup>*

(Received June 9, 1980)

A novel fluorimetric method is described for the determination of 5-phenyl-3-isoxazolecarboxylic acid (PIA) by using 2-hydroxy-1-naphthalenecarbaldehyde (HNA); this method was utilized to provide a specific and sensitive assay procedure for perisoxal in plasma.

The developed fluorophore was 3-benzoyl-5,6-benzocoumarin (BBC), obtained by Knoevenagel condensation of benzoylacetonitrile with HNA, followed by cyclization. Linear relationships between fluorescence intensity and concentration existed over the concentration range of 35—350 ng/ml of PIA and 70—700 ng/ml of perisoxal in plasma. This method is 70 times more sensitive than the colorimetric method reported previously.

**Keywords**—5-phenyl-3-isoxazolecarboxylic acid; perisoxal; benzoylacetonitrile; fluorimetry; 3-benzoylcoumarin; 3-benzoyl-5,6-benzocoumarin; 2-hydroxy-1-naphthalenecarbaldehyde

In the previous paper,<sup>1)</sup> a new colorimetric method for the determination of 5-substituted 3-isoxazolecarboxylic acids and their derivatives by using *p*-dimethylaminobenzaldehyde (DABA) was reported. This method was based on the decomposition of 5-substituted 3-isoxazolecarboxylic acids to the corresponding  $\beta$ -ketonitriles, followed by condensation with DABA.

In the present work, we attempted to convert perisoxal into a fluorescent derivative by application of the above reaction to develop an assay method for perisoxal in human plasma. Various aromatic aldehydes were examined and it was found that salicylaldehydes reacted with benzoylacetonitrile to give fluorescence products. Because of the strong fluorescence, the condensation product with 2-hydroxyl-1-naphthalenecarbaldehyde (HNA) was suitable as a derivative for the assay of perisoxal in plasma. Perisoxal was converted into 5-phenyl-3-isoxazolecarboxylic acid (PIA) in the manner described previously.<sup>1)</sup>

### Experimental

**Apparatus**—Fluorescence spectra and intensities were measured with a Hitachi MPF-2A fluorescence spectrophotometer. Infrared (IR) spectra were measured with a JASCO DS-403G spectrometer and mass (MS) spectra were taken with a Hitachi RMU-6E spectrometer.

**Reagents**—PIA and Perisoxal: These were obtained as described in the previous paper.<sup>1)</sup>

**HNA:** Reagent-grade HNA was purchased from Tokyo Kasei Kogyo Co., Ltd. and purified by recrystallization from ethyl ether-petroleum ether, mp 82.5—84.0°.

**1.0% HNA Solution:** HNA (1.0 g) was dissolved in 100 ml of DMSO-H<sub>2</sub>O (9:1, v/v) mixture.

**2.0% K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub> Solution:** K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub> (2.0 g) was dissolved in 100 ml of 20% (v/v) H<sub>2</sub>SO<sub>4</sub>.

All other reagents used in this study were of commercial reagent grade.

**Reaction of Benzoylacetonitrile with Salicylaldehydes**—A mixture of benzoylacetonitrile (0.01 mol) and a salicylaldehyde (0.01 mol) in ethanol (20 ml) was refluxed with one drop of piperidine for 1—2 hr. The reaction mixture was cooled in an ice-water bath, then the precipitate was collected and recrystallized from ethanol. The products were identified as 3-benzoylcoumarins by comparison of their IR spectra and

1) Part I: T. Umeda, H. Imanishi, and E. Hirai, *Chem. Pharm. Bull.*, **28**, 1230 (1980).

2) Location: *Sagisu, Fukushima-ku, Osaka, 553, Japan.*

melting points with those of authentic samples obtained by the method of Knoevenagel and Arnot.<sup>3)</sup>

**Analytical Procedure for PIA**—In a centrifuge tube, place 1.0 ml of PIA sample solution containing 35–350 ng/ml in DMSO–H<sub>2</sub>O (1: 9, v/v). To this solution, add 1.0 ml of 1.0% HNA solution and heat the mixture at 120° for 30 min. After cooling the reaction mixture to room temperature, add 6 ml of ethyl ether and 2 ml of pH 10.6 borate buffer. Shake the mixture for 1 min and remove the aqueous layer. Wash the organic layer twice with 4 ml each of DMSO–pH 10.6 borate buffer (1: 1, v/v) and transfer it into a 10 ml volumetric flask. Evaporate the extract to dryness at 40° and dissolve the residue in DMSO–H<sub>2</sub>O (2: 8, v/v) to the mark. Measure the fluorescence intensity at 480 nm with excitation at 380 nm. Prepare a blank solution similarly but without the sample.

**Analytical Procedure for Perisoxal**—In a centrifuge tube, place 1.0 ml of perisoxal sample solution containing 70–700 ng/ml in 20% (v/v) H<sub>2</sub>SO<sub>4</sub>. To this solution, add 1.0 ml of 2.0% K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub> solution and heat the mixture in a boiling water bath for 1 hr. After cooling the reaction mixture to room temperature, add 2.0 ml of 28% (w/v) NaOH and 1.0 ml of 1 M disodium citrate and extract three times with 3 ml each of ethyl ether. Transfer the extracts into a centrifuge tube, evaporate to dryness at 40°, and dissolve the residue in 1.0 ml of DMSO–H<sub>2</sub>O (1: 9, v/v). Assay this solution as described above for PIA. Prepare a blank solution similarly but without the sample.

**Analytical Procedure for Perisoxal in Plasma**—In a centrifuge tube, place 1.0 ml of plasma sample containing 70–700 ng of perisoxal per ml and add 3 ml of 1,2-dichloroethane and 200 µl of pH 10.3 NaHCO<sub>3</sub> buffer (0.25 M). Shake the mixture for 5 min and centrifuge at 2000 rpm for 5 min. Remove the aqueous layer and add 3 ml of 0.05 N HCl to the organic layer. Shake the mixture for 5 min and centrifuge at 2000 rpm for 5 min. Remove the organic layer and add 1 ml of 1 N NaOH and 4 ml of ethyl ether to the aqueous layer. Shake the mixture for 2 min and remove the aqueous layer. Evaporate the ethereal layer to dryness at 40° and dissolve the residue in 1.0 ml of 20% (v/v) H<sub>2</sub>SO<sub>4</sub>. Assay this solution as described above for perisoxal. Prepare a blank solution similarly but without the sample.

## Results and Discussion

### Reaction of Benzoylacetonitrile with Salicylaldehydes

Benzoylacetonitrile reacted with some salicylaldehydes in the presence of piperidine to give fluorescence products. None appeared to be simple Knoevenagel condensation products, because their IR spectra did not exhibit a cyano band, but showed two carbonyl bands. The IR and MS spectra and the elemental analysis data listed in Table I indicate that 3-benzoylcoumarins are formed through Knoevenagel condensation followed by cyclization, as shown in Chart 1. These products were identified by comparison with the corresponding authentic 3-benzoylcoumarins synthesized by the condensation of ethyl benzoylacetate with salicylaldehydes.<sup>3)</sup>

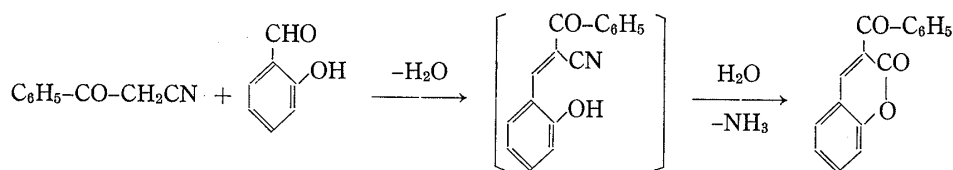


Chart 1

Few papers have appeared on the formation of 3-benzoylcoumarins by the reaction of benzoylacetonitrile with salicylaldehydes. Sakurai and Midorikawa isolated 3-benzoyl-6,8-dibromocoumarin by the condensation of benzoylacetonitrile with 3,5-dibromosalicylaldehyde in the presence of ammonium acetate.<sup>4)</sup>

### Fluorescence Characteristics of 3-Benzoylcoumarins

The fluorescence characteristics of various coumarins have been studied in detail in con-

3) E. Knoevenagel and R. Arnot, *Chem. Ber.*, **37**, 4496 (1904).

4) A. Sakurai and H. Midorikawa, *J. Org. Chem.*, **34**, 3612 (1969).

TABLE I. Physical and Analytical Data for 3-Benzoylcoumarins

| Compound <sup>a)</sup> | mp (°C)<br>(lit.)              | IR $\nu_{\text{max}}^{\text{KBr}}$<br>lactone C=O<br>benzoyl C=O | MS<br>$m/e$                                     | Formula                                | Analysis (%)     |              |                |
|------------------------|--------------------------------|------------------------------------------------------------------|-------------------------------------------------|----------------------------------------|------------------|--------------|----------------|
|                        |                                |                                                                  |                                                 |                                        | Calcd<br>(Found) | C            | H O            |
| BC                     | 137—138<br>(130) <sup>b)</sup> | 1720<br>1660                                                     | 105 ( $\text{COPh}^+$ )<br>250 ( $\text{M}^+$ ) | $\text{C}_{16}\text{H}_{10}\text{O}_3$ | 76.79<br>(76.80) | 4.03<br>4.02 | 19.18<br>19.29 |
| BHC                    | 249—250<br>(237) <sup>c)</sup> | 1690<br>1651                                                     | 105 ( $\text{COPh}^+$ )<br>266 ( $\text{M}^+$ ) | $\text{C}_{16}\text{H}_{10}\text{O}_4$ | 72.18<br>(71.93) | 3.78<br>3.73 | 24.04<br>24.05 |
| BMC                    | 153—154                        | 1715<br>1660                                                     | 105 ( $\text{COPh}^+$ )<br>280 ( $\text{M}^+$ ) | $\text{C}_{17}\text{H}_{12}\text{O}_4$ | 72.85<br>(72.95) | 4.32<br>4.22 | 22.83<br>22.74 |
| BBC                    | 210—211<br>(208) <sup>d)</sup> | 1710<br>1655                                                     | 105 ( $\text{COPh}^+$ )<br>300 ( $\text{M}^+$ ) | $\text{C}_{20}\text{H}_{12}\text{O}_3$ | 79.99<br>(80.11) | 4.03<br>3.98 | 15.98<br>16.11 |

a) BC: 3-benzoylcoumarin, BHC: 3-benzoyl-7-hydroxycoumarin, BMC: 3-benzoyl-7-methoxycoumarin, BBC: 3-benzoyl-5,6-benzocoumarin.

b) See ref. 3.

c) R.K. Pandya and K.C. Pandya, *Agra Univ. J. Research*, **4**, 345 (1955) [*C.A.*, **52**, 7307 (1958)].

d) E. Knoevenagel and F. Schröter, *Chem. Ber.*, **37**, 4484 (1904).

TABLE II. Fluorescence Properties of 3-Benzoylcoumarins

| Compound | EtOH                                                                              |                   | 90% EtOH                                                                          |                   | 90% EtOH<br>(0.01 N HCl)                                                          |                   | 90% EtOH<br>(0.01 N NaOH)                                                         |                   |
|----------|-----------------------------------------------------------------------------------|-------------------|-----------------------------------------------------------------------------------|-------------------|-----------------------------------------------------------------------------------|-------------------|-----------------------------------------------------------------------------------|-------------------|
|          | $\lambda_{\text{max}}^{\text{ex}}/\lambda_{\text{max}}^{\text{em}}$ <sup>a)</sup> | RFI <sup>b)</sup> | $\lambda_{\text{max}}^{\text{ex}}/\lambda_{\text{max}}^{\text{em}}$ <sup>a)</sup> | RFI <sup>b)</sup> | $\lambda_{\text{max}}^{\text{ex}}/\lambda_{\text{max}}^{\text{em}}$ <sup>a)</sup> | RFI <sup>b)</sup> | $\lambda_{\text{max}}^{\text{ex}}/\lambda_{\text{max}}^{\text{em}}$ <sup>a)</sup> | RFI <sup>b)</sup> |
| BC       | Not detectable                                                                    |                   | Not detectable                                                                    |                   | Not detectable                                                                    |                   | Not detectable                                                                    |                   |
| BHC      | 370/432                                                                           | 83                | 370/434                                                                           | 88                | 370/434                                                                           | 95                | 370/434                                                                           | 11                |
|          | 426/462                                                                           | 58                | 426/462                                                                           | 33                | 426/462                                                                           | 1                 | 426/462                                                                           | 225               |
| BMC      | 370/428                                                                           | 27                | 370/430                                                                           | 50                | 370/430                                                                           | 55                | Not detectable                                                                    |                   |
| BBC      | 378/461                                                                           | 126               | 380/465                                                                           | 359               | 381/463                                                                           | 330               | Not detectable                                                                    |                   |

a) excitation maximum (nm)/emission maximum (nm).

b) Relative fluorescence intensity. In comparing the fluorescence intensities of 3-benzoylcoumarins ( $5.6 \times 10^{-6}$  M), that of anthracene ( $5.6 \times 10^{-8}$  M in EtOH) was taken as 100 (at 377/400 nm).

nection with natural products,<sup>5)</sup> laser dyes,<sup>6)</sup> and analytical reagents.<sup>7)</sup> The fluorescence properties of coumarins were found to depend markedly on the substituents and their positions on the coumarin molecule.<sup>8)</sup>

3-Benzoylcoumarins were stable in acidic and neutral solutions, but decomposed in strongly alkaline solution. Their fluorescence characteristics were compared in ethanol and aqueous ethanol solutions, and the results are shown in Table II. The fluorescence of 3-benzoylcoumarin (BC) was very weak and was not detectable even at a concentration of  $5.6 \times 10^{-6}$  M. 3-Benzoyl-7-methoxycoumarin (BMC) had one-seventh of the fluorescence intensity of 3-benzoyl-5,6-benzocoumarin (BBC) in 90% ethanol. The fluorescence intensity of 3-benzoyl-7-hydroxycoumarin (BHC) at the shorter wavelength band in an acidic ethanol solution was one-third of that of BBC and that of the former at the longer wavelength band in an alkaline ethanol solution was two-thirds of that of the latter. The low fluorescence intensity of BHC

5) R.H. Goodwin and F. Kavanagh, *Arch. Biochem. Biophys.*, **27**, 152 (1950); *idem, ibid.*, **36**, 442 (1952); D.G. Crosby and R.V. Berthold, *Anal. Biochem.*, **4**, 349 (1962).

6) S.C. Haydon, *Spectrosc. Lett.*, **8**, 815 (1975).

7) a) M. Machida, N. Ushijima, M.I. Machida, and Y. Kanaoka, *Chem. Pharm. Bull.*, **23**, 1385 (1975); b) M. Machida, N. Ushijima, T. Takahashi, and Y. Kanaoka, *ibid.*, **25**, 1289 (1977); c) M. Machida, M.I. Machida, T. Sekine, and Y. Kanaoka, *ibid.*, **25**, 1678 (1977); d) J.W. Woolen and P.G. Walker, *Clin. Chim. Acta*, **12**, 647 (1965); e) W. Dinges, *Anal. Chem.*, **49**, 442 (1977).

8) C.E. Wheelock, *J. Am. Chem. Soc.*, **81**, 1348 (1959).

may be caused by quenching.<sup>9)</sup> BBC gave strong fluorescence and its intensity in ethanol or DMSO was increased by the addition of water, as shown in Table III. Therefore, it is desirable that perioxal should be converted into BBC for sensitive and specific fluorimetric determination in plasma.

TABLE III. Fluorescence Properties of 3-Benzoyl-5,6-benzocoumarin in Various Solvents

| Solvent            | Excitation maximum (nm) | Emission maximum (nm) | RFI <sup>a)</sup> |
|--------------------|-------------------------|-----------------------|-------------------|
| Ethyl acetate      | 375                     | 444                   | 16                |
| Benzene            | 380                     | 450                   | 17                |
| Acetone            | 374                     | 454                   | 27                |
| 1,2-Dichloroethane | 384                     | 460                   | 44                |
| Chloroform         | 382                     | 452                   | 63                |
| MeOH               | 378                     | 470                   | 307               |
| EtOH               | 378                     | 461                   | 126               |
| 90% EtOH           | 380                     | 465                   | 359               |
| 70% EtOH           | 380                     | 470                   | 712               |
| 50% EtOH           | 380                     | 472                   | 894               |
| 30% EtOH           | 382                     | 478                   | 1690              |
| 10% EtOH           | 382                     | 483                   | 2590              |
| DMSO               | 378                     | 462                   | 126               |
| 90% DMSO           | 378                     | 464                   | 203               |
| 70% DMSO           | 380                     | 471                   | 521               |
| 50% DMSO           | 381                     | 476                   | 1300              |
| 30% DMSO           | 382                     | 482                   | 3500              |
| 20% DMSO           | 382                     | 482                   | 3960              |
| 10% DMSO           | 384                     | 484                   | 4540              |
| 5% DMSO            | 384                     | 484                   | 5110              |
| 1% DMSO            | 384                     | 484                   | 5930              |

a) Relative fluorescence intensity.

Table III shows the fluorescence properties of BBC in various solvents. With increasing solvent polarity, the emission maximum of BBC shifted to longer wavelength in the same way as with other coumarins<sup>6,7c)</sup> and its fluorescence intensity was increased. As the percentage of water in aqueous ethanol or DMSO was increased, both the absorption and emission bands were red-shifted and the fluorescence intensity increased markedly. DMSO-H<sub>2</sub>O (2:8, v/v) mixture was selected as a suitable solvent to measure the fluorescence of BBC, taking into account the above results and the solubility of BBC. An adequate linearity was observed between fluorescence intensity and concentration in the range of 3—1300 ng/ml of BBC in the mixed solvent.

### Determination of PIA

The fluorescence excitation and emission spectra of the final reaction mixture of PIA (excitation maximum, 380 nm; emission maximum, 480 nm) were identical with those of BBC dissolved in DMSO-H<sub>2</sub>O (2:8, v/v) mixture, as shown in Fig. 1. The developed fluorophore was stable for at least 2 hr.

DMSO was useful in that this fluorescence reaction could be performed at relatively high temperature, and in that it could dissolve a large amount of HNA, while the reaction was accelerated by the addition of water as in the case of the color reaction of PIA.<sup>1)</sup> The effect of water in aqueous DMSO on the fluorescence development was investigated. As shown in

9) W.R. Sherman and E. Robins, *Anal. Chem.*, **40**, 803 (1968).

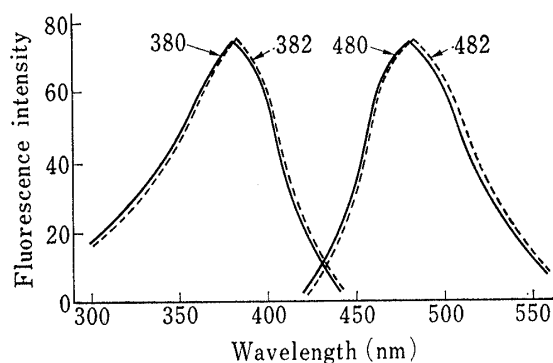


Fig. 1. Excitation and Emission Spectra of BBC and a Reaction Mixture of PIA with HNA

BBC: BBC was dissolved in DMSO-H<sub>2</sub>O (2:8, v/v) at the concentration of  $1.5 \times 10^{-7}$  M.

---: excitation and emission spectra.

Reaction mixture: An aliquot (1.0 ml) of  $1.5 \times 10^{-6}$  M PIA solution was treated according to the standard procedure.

—: excitation and emission spectra.

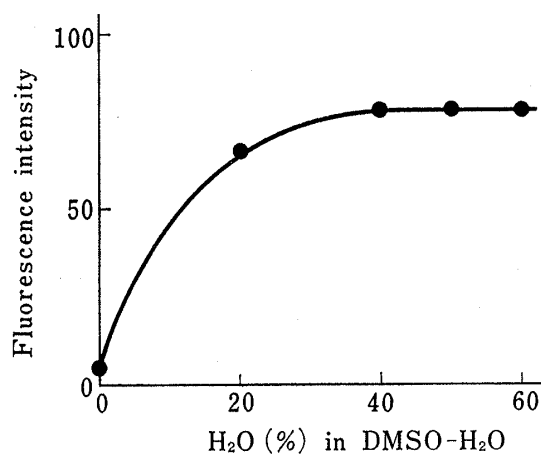


Fig. 2. Effect of H<sub>2</sub>O% (v/v) in DMSO-H<sub>2</sub>O on Fluorescence Development

Aliquots (1.0 ml) of PIA solution containing 210 ng/ml were treated according to the standard procedure but with various levels of H<sub>2</sub>O% (v/v) in DMSO-H<sub>2</sub>O.

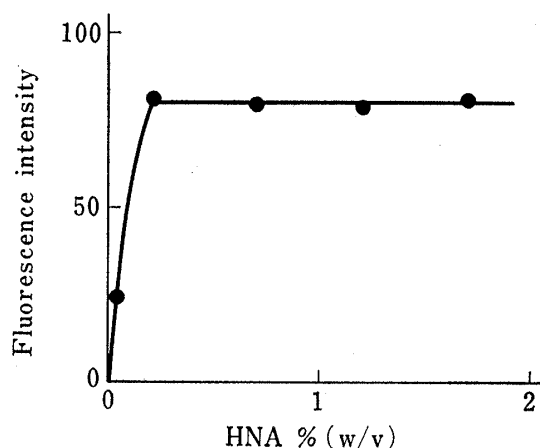


Fig. 3. Effect of HNA Concentration on Fluorescence Development

Aliquots (1.0 ml) of PIA solution containing 210 ng/ml were treated according to the standard procedure but with various concentrations of HNA.

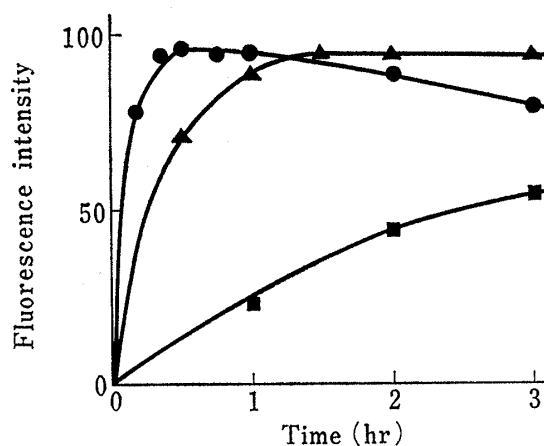


Fig. 4. Effects of Reaction Temperature and Time on Fluorescence Development

Aliquots (1.0 ml) of PIA solution containing 210 ng/ml were treated according to the standard procedure except that various reaction times were used at the following temperatures.

—●—: 120°, —▲—: 100°, —■—: 80°.

Fig. 2, maximum and constant fluorescence intensity was obtained in the range of 40–60% (v/v) H<sub>2</sub>O.

As shown in Fig. 3, maximum and constant fluorescence intensity was obtained in the range of 0.2–1.7% (w/v) HNA.

The rate of the fluorescence reaction at various temperatures was examined. As shown in Fig. 4, maximum and constant fluorescence intensity was obtained in the range of 90–180 min at 100° and 20–60 min at 120°.

Although the intensity of HNA under the conditions of fluorescence measurement was very weak, a large excess of HNA interfered with the assay of PIA. The fluorescence product, BBC, was successfully extracted with ethyl ether from the alkaline reaction mixture containing excess HNA, whereas HNA, because of its phenolic hydroxy group, remained in the aqueous layer. The extraction was quantitative in the range of pH 9.0–11.0, while the amount of HNA in the extract was markedly decreased with increase of pH and became constant in the

range of pH 10.3–11.3. Therefore, the reaction mixture was adjusted to pH 10.6. The amount of HNA in the extract was further decreased by washing twice with pH 10.6 borate buffer–DMSO (1:1, v/v) mixture. In this procedure, DMSO in the washings was effective for the removal of HNA from the ethereal extract, while the amount of BBC in the extract did not decrease upon washing with the borate buffer containing 20–50% (v/v) DMSO.

A linear relationship was obtained between fluorescence intensity and concentration in the range of 35–350 ng/ml of PIA.

The precision of the assay procedure was examined on replicate runs for ten sample solutions containing 180 ng/ml of PIA. The coefficient of variation was 1.0%.

### Determination of Perisoxal

On heating in a boiling water bath, perisoxal was easily oxidized by  $K_2Cr_2O_7$  to yield PIA, as described previously,<sup>1)</sup> followed by reaction with HNA. Maximum and constant fluorescence intensity was obtained in the concentration range of 1.0–2.0% (w/v)  $K_2Cr_2O_7$ .

The reaction mixture after the oxidation of perisoxal should be partly neutralized to extract PIA efficiently. As shown in Fig. 5, maximum and constant fluorescence intensity was obtained in the pH range of 3.0–4.0. Therefore, 2.0 ml of 28% (w/v) NaOH and 1.0 ml of 1 M disodium citrate were added to the mixture to hold the pH between 3.0 and 4.0. Three extractions with 3 ml each of ethyl ether were enough to extract PIA completely.

A linear relationship was obtained between fluorescence intensity and concentration in the range of 70–700 ng/ml of perisoxal.

The precision of the assay procedure was examined on replicate runs for ten sample solutions containing 350 ng/ml of perisoxal. The coefficient of variation was 2.2%.

### Determination of Perisoxal in Plasma

Perisoxal in plasma was efficiently extracted with 1,2-dichloroethane in the range of pH 10.0–13.0. It was desirable to extract perisoxal between pH 10.0 and 10.5, in order to avoid emulsion formation in a more strongly alkaline solution.

Back-extraction was required to clean up the extract from plasma. A quantitative transfer of perisoxal from the extract into the aqueous phase was performed by acidification with 3 ml of  $5 \times 10^{-3}$ – $1 \times 10^{-1}$  N HCl. After the aqueous extract had been made alkaline with 1 ml of 0.2–1.5 N NaOH, perisoxal was completely extracted with 4 ml of ethyl ether again.

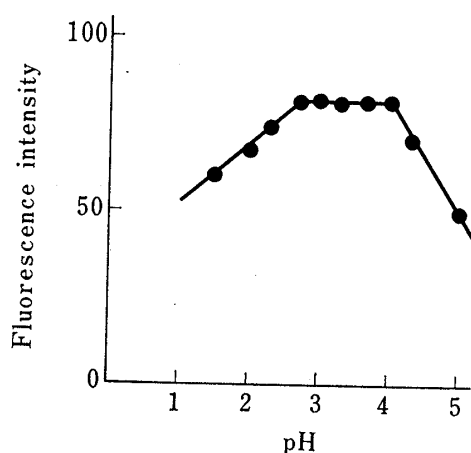


Fig. 5. Effect of pH of PIA Extraction on the Fluorescence Development

Aliquots (1.0 ml) of perisoxal solution containing 390 ng/ml were treated according to the standard procedure but at various pH values.

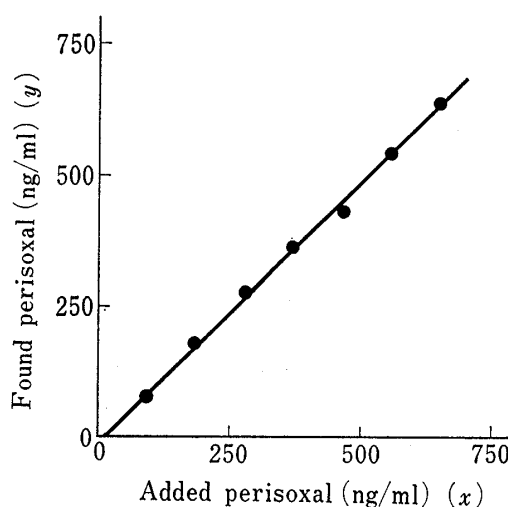


Fig. 6. Recovery of Perisoxal from Plasma

Aliquots (1.0 ml) of plasma containing 90–650 ng of perisoxal per ml were treated according to the standard procedure.

regression equation:  $y = 0.9957x - 12.3$ ,  $s = 13.7$ .

In the regression analysis of data on perisoxal in plasma, the agreement between the added and the found amounts of perisoxal was reasonable, as shown in Fig. 6, indicating that perisoxal was completely recovered from the plasma.

The precision of the assay procedure was examined on replicate runs for ten plasma samples containing 350 ng of perisoxal per ml. The coefficient of variation was 3.1%.

Seven compounds,  $M_1$ — $M_7$  in Table IV, have been isolated and identified as the main metabolites of perisoxal.<sup>10)</sup> The plasma samples containing perisoxal and these metabolites were prepared as directed in Table IV and assayed by the proposed procedure. Table IV shows that there was reasonable agreement between the added and the found amounts of perisoxal. Thus, it is concluded that these metabolites do not interfere in the assay of perisoxal in plasma. It is likely that the metabolites are removed in the extraction procedure.

TABLE IV. Determination of Perisoxal in Plasma in the Presence of Its Main Metabolites ( $M_1$ — $M_7$ )

| No. | Added (ng) |            |            |            |            |            |            | Found (ng)        |                   |
|-----|------------|------------|------------|------------|------------|------------|------------|-------------------|-------------------|
|     | $M_1^{a)}$ | $M_2^{b)}$ | $M_3^{c)}$ | $M_4^{d)}$ | $M_5^{e)}$ | $M_6^{f)}$ | $M_7^{g)}$ | Perisoxal ( $x$ ) | Perisoxal ( $y$ ) |
| 1   | 15         | 15         | 397        | 15         | 5          | 5          | 184        | 91                | 99                |
| 2   | 15         | 15         | 397        | 15         | 5          | 5          | 184        | 182               | 169               |
| 3   | 15         | 15         | 397        | 15         | 5          | 5          | 184        | 272               | 289               |
| 4   | 10         | 10         | 397        | 5          | 0          | 0          | 92         | 363               | 345               |
| 5   | 10         | 10         | 265        | 5          | 0          | 0          | 92         | 454               | 418               |
| 6   | 5          | 5          | 132        | 15         | 5          | 5          | 46         | 544               | 535               |
| 7   | 0          | 0          | 66         | 0          | 0          | 0          | 46         | 636               | 621               |

Regression equation:  $y=0.9545x+7.2$ ,  $s=16.2$ .

a) 5-(2-Hydroxyphenyl)-3-(1-hydroxy-2-piperidinoethyl)isoxazole.

b) 5-(3-Hydroxyphenyl)-3-(1-hydroxy-2-piperidinoethyl)isoxazole.

c) 5-(4-Hydroxyphenyl)-3-(1-hydroxy-2-piperidinoethyl)isoxazole.

d) 3-[1-Hydroxy-2-(3-hydroxypiperidino)ethyl]-5-phenylisoxazole.

e) 3-[1-Hydroxy-2-(4-hydroxypiperidino)ethyl]-5-phenylisoxazole.

f) 3-[1-Hydroxy-2-(2-oxopiperidino)ethyl]-5-phenylisoxazole.

g) 3-[1-Hydroxy-2-(N-oxidopiperidino)ethyl]-5-phenylisoxazole.

In conclusion, the developed fluorimetric methods is more specific for PIA and perisoxal and 70 times more sensitive than the colorimetric method reported previously.<sup>1)</sup> Therefore, this method should be suitable for drug level determination of perisoxal in human plasma with relative freedom from interference by the metabolites of perisoxal.

10) S. Hashimoto, M. Shizu, and S. Takahashi, *Chem. Pharm. Bull.*, **24**, 1757 (1976).