with NaCl and extracted seven times with ether. The extract was dried over Na_2SO_4 and the solvent was evaporated. The residue was recrystallized from hexane to give white prisms of 3-hydroxyisoxazole (XXI) of m.p. 98～99°. Yield, 7.5 g. (59%). *Anal.* Calcd. for $C_3H_3O_2N$: C, 42.36; H, 3.56; N, 16.47. Found: C, 42.42; H, 3.55; N, 16.60.

Reaction of Phenylpropiolic Acid with Hydroxylamine in the presence of Sodium Hydroxide——To a mixture of hydroxylamine hydrochloride (1.39 g., 0.02 mole) and 10% NaOH (20 ml., 0.05 mole) was added a solution of phenylpropiolic acid (1.46 g., 0.01 mole) in 20 ml. of EtOH. After standing ovenight at 30°, the mixture was acidified with conc. HCl and extracted with ether. The extract was washed with H_2O, dried over Na_2SO_4 and the solvent was evaporated. The residue, crude 3-phenyl-5-isoxazolone (XXII) (1.25 g., 78%), was recrystallized from aq. EtOH to give colorless prisms of m.p. 151～152°, which showed no depression in m.p. on admixture with an authentic sample prepared from ethyl benzoylacetate and hydroxylamine.[12] *Anal.* Calcd. for $C_9H_7O_2N$: C, 67.07; H, 4.38; N, 8.69. Found: C, 66.78; H, 4.42; N, 8.77.

The measurement of IR, UV and NMR spectra were carried out by Messrs. H. Higuchi, C. Fujimura, Misses N. Sawamoto, T. Li, Y. Nakajima and M. Shimada. Microanalyses were made by Messrs. K. Ono, H. Shimada, Misses K. Saito, N. Gonda, H. Masuda and M. Yamamuro.

Summary

β-Bromocinnamonitrile and hydroxylamine afforded 3-amino-5-phenylisoxazole in the presence of alkali, unexpectedly. So the reaction of acetylenic nitriles and esters with hydroxylamine was examined under alkaline conditions. Phenylpropiolonitrile and propiolonitrile gave 3-amino-5-phenylisoxazole and 3-aminoisoxazole, respectively. Tetrolonitrile afforded a 3:1 mixture of 3-amino-5-methyl- and 3-methyl-5-amino-isoxazole in the presence of alkali. Under neutral conditions these nitriles gave the corresponding 5-aminoisoxazoles. Similarly, 3-hydroxy-, 3-hydroxy-5-methyl- and 3-hydroxy-5-phenyl-isoxazole were obtained from the corresponding acetylenic esters. In the case of methyl tetrolate, two isomers of tetrolohydroxamic acid were isolated as the intermediate. On the other hand, free phenylpropiolic acid gave only 3-phenyl-5-isoxazolone under the same alkaline conditions.

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175. Hidetoshi Yoshimura, Kazuta Oguri, and Hisao Tsukamoto : Detection of Morphine in Urine. II.*1 An Improved Method by Thin-Layer Chromatography Utilizing Potassium Platinum Iodide as the Reagent for Both Coloration and Fluorescence.

(*Faculty of Pharmaceutical Sciences, Kyushu University**2)

In the previous paper*1 of this series, the authors reported a simple and sensitive detection method for microgram quantities of morphine in urine, which consisted of three parts; a sufficient hydrolysis of conjugated morphine to free form, complete extraction of morphine with chloroform using continuous extractor, and its detection by double thin-layer chromatography. Recently Kupferberg, *et al.*[1,2] also developed a

*1 Part I. H. Yoshimura, K. Oguri, H. Tsukamoto : This Bulletin, **14**, 62 (1966).
*2 Katakasu, Fukuoka (吉村英敏, 小栗一太, 塚元久雄).
1) H. J. Kupferberg, A. Burkhalter, E. L. Way : J. Pharmacol. Exptl. Therap., **145**, 247 (1964).
2) *Idem* : J. Chromatog., **16**, 558 (1964).

new detection method for submicrogram quantities of morphine by oxidation with potassium ferricyanide to pseudomorphine, a highly fluorescent substance. This method is sensitive enough for pure morphine being able to detect as little as 0.1 μg. on thin-layer chromatogram, however its application to urinary morphine encounters certain disadvantages. The major one is a difficult distinction of the fluorescent spot due to pseudomorphine from spots due to many other fluorescent substances extracted from urine. This will disturb, more or less, a definite identification of minute quantities of morphine excreted into urine.

During the course of the study on identification of morphine in urine, the authors found unexpectedly that potassium platinum iodide reagent which used currently for coloration of narcotics in thin-layer chromatography was also very valuable as a fluorescent reagent of morphine.

This paper describes the basis of this fluorescent reaction of morphine and its successful application to detection of morphine in urine.

Materials and Instruments

Materials——Morphine hydrochloride was purchased from commercial source and silica gel used was Silica gel G, Merck. A potassium platinum iodide reagent was prepared as follows: A mixture of 1 ml. of 10% chloroplatinic acid solution and 25 ml. of 4% potassium iodide solution was diluted with H_2O to a final volume of 50 ml. Authentic sample of pseudomorphine was synthesized by the method of Bently and Dyke.[3]

Instruments——Fluorescent excitation and emission spectra were measured by an Aminco-Bowmann Spectrophotofluorometer (wavelength uncalibrated). The fluorescent spot of thin-layer chromatography was detected by Manasulu Light (253.6 and 365 mμ; Manasulu Light Chem. Ind. Co., Ltd.).

Methods and Results

Detection of Morphine in Urine Utilizing Thin-Layer Chromatography

a) Single Thin-Layer Chromatographic Method——The MeOH solution of the urine extract*[3] was applied to the silica gel plate (0.25 mm. in thickness, activated at 105° for 30 min.) and developed with the solvent system of EtOH–dioxane–benzene–conc. NH_4OH (5:40:50:5). The plate was then dried *in vacuo* for 30 min. and sprayed with potassium platinum iodide reagent. Morphine on the chromatogram was identified as a violet-colored spot at Rf 0.25. Next, the plate was exposed to ammonia vapor until the color faded (if the color did not fade sufficiently, the plate was sprayed with the reagent and exposed to ammonia vapor once more), and left aside for 5 min. Morphine (actually converted to pseudomorphine as described below) was detected easily as a blue fluorescent spot under ultraviolet light.

b) Double Thin-Layer Chromatographic Method——If morphine could not be identified definitely by above procedure, double thin-layer chromatography*[4] would be recommended. This procedure was aimed to separate morphine from other urinary impurities which would disturb morphine detection, and to increase the sensibility of this method. Whole solution of the urine extract in a small volume of MeOH was spotted in line on the thin-layer plate (20×20 cm. in size, 0.25 mm. in thickness), authentic morphine being spotted on its end and developed with the solvent system of MeOH–*n*–BuOH–benzene–H_2O (60:15:10:15). The plate was then dried *in vacuo* for 30 min. After visualizing only the authentic morphine with potassium platinum iodide

3 See reference[1] for the extraction procedure.

4 This was essentially same as that described in the previous paper,[1] but modified a little in the thickness of silica gel plate and in the solvent system.

3) K. W. Bently, S. F. Dyke: J. Chem. Soc., **1959**, 2574.

reagent, the corresponding area on the chromatogram was scratched, collected in a 10 ml. of glass stoppered centrifuge tube, and extracted with 5 ml. of MeOH containing 2 drops of conc. NH_4OH by shaking for 3 min. After centrifugation, the solvent layer was pipetted out, the deposited silica gel being extracted similarly twice more. The residue remained after evaporation of the solvent *in vacuo* by rotary evaporator was dissolved again in a small volume of MeOH. More than a half of this extract was spotted on a thin-layer plate and morphine was identified by both color and fluorescent reaction with potassium platinum iodide reagent as described above.

Isolation, Characterization, and Identification of Fluorescent Compound——In order to isolate, characterize, and identify above fluorescent compound, a large scale reaction was performed in aqueous solution analogously with thin-layer chromatography as follows: To a stirring solution of 132 mg. of morphine hydrochloride (equivalent to 100 mg. of free morphine) in 10 ml. of H_2O was added 182 mg. of chloroplatinic acid and 1.82 g. of potassium iodide in 20 ml. of H_2O at room temperature. Concentrated ammonia was then added to this reaction mixture until the violet color faded to yellow under stirring. This solution exhibited an intense fluorescence under ultraviolet light and was proved to contain only one fluorescent compound, identical with pseudomorphine by thin-layer chromatography as described below. After allowing to stand for 30 min. the reaction mixture was evaporated to dryness. The residue was washed with a small volume of MeOH to remove unchanged morphine, dissolved in 10 ml. of conc. NH_4OH, and the solution was boiled. Slightly colored deposit obtained was again dissolved in conc. NH_4OH and boiled to yield almost colorless crystals (yield: 22 mg.).

This compound was proved to be a sole product with fluorescent character produced in above reaction and to have the same Rf value with that of authentic sample of pseudomorphine by thin-layer chromatography (see Table I).

Table I. Rf Value of the Reaction Product of Morphine with K_2PtI_6

Solvent system				Pseudomorphine[a]	Reaction product[a]
60 benzyl alc.,	30 EtOH,	10 conc. NH_4OH		0.63	0.65
30 benzyl alc.,	30 $CHCl_3$,	30 EtOH,	10 conc. NH_4OH	0.54	0.54

a) dissolved in 0.05*N* NaOH

As shown in Fig. 1, it was further demonstrated that the excitation and emission spectra of this compound and of pseudomorphine were quite identical each other. The complete identity of both compounds was finally established by the comparison of their infrared absorption spectra (Fig. 2).

This oxidation of morphine to pseuodmorphine could not be observed by either potassium iodide or chloroplatinic acid only.

Sensibility of Method——The smallest amount of morphine that could be identified by above thin-layer chromatographic method was dependent upon what wavelength of excitation light was used, being 0.1~0.2 μg. by the short wave lamp (253.6 mμ) and 0.025~0.05 μg. by the long wave lamp (365 mμ).

This result seemed quite strange, because it was evident from the excitation spectrum shown in Fig. 1 that the intensity of fluorescence was stronger at 253.6 mμ than at 365 mμ. The lower sensibility by use of the short wave lamp on thin-layer chromatogram could be explained by the absorption of the excitation light by excess of the potassium platinum iodide reagent*[5] and silica gel, both of which absorbed

*[5] UV $\lambda_{max}^{H_2O}$ 227 mμ, $\lambda_{min}^{H_2O}$ 314 mμ.

deeply the light in this region of wavelength. In fact, while the emission spectrum of pseudomorphine was not affected in the presence of potassium platinum iodide reagent, the excitation spectrum was quite influenced by this reagent, disappearing the peak of 247 mμ (the first peak in the excitation spectrum, Fig. 1).

When this method was applied to urinary extract, however, higher sensibility was obtained by the short wave lamp than the long one. This was attributable to the fact that by use of the long wave lamp, there exhibited a continued fluorescence all over the chromatogram probably owing to urinary components, and it was actually very difficult to distinguish the fluorescent spot of pseudomorphine from others. Therefore, only the short wave lamp can be used for detection of urinary morphine.

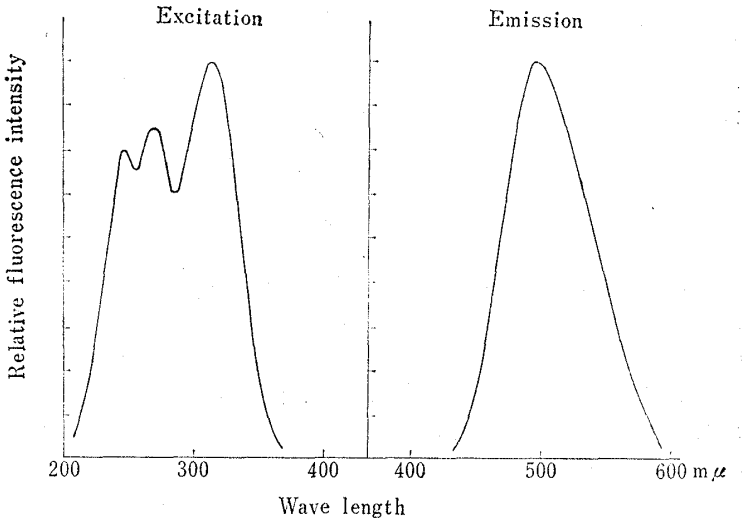

Fig. 1. Excitation and Emission Spectra of Pseudomorphine

The excitation spectrum was obtained by setting the emission monochromator at 495 mμ. The emission spectrum was obtained by setting the excitation monochromator at 247 mμ (wavelength uncalibrated).

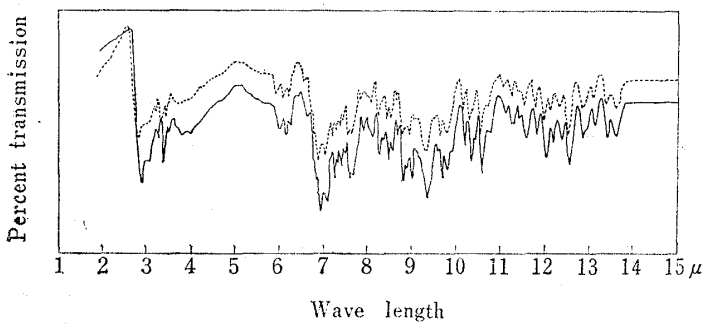

Fig. 2. Infrared Absorption Spectra of Pseudomorphine (mono hydrate) and Reaction Product (KBr)

——— pseudomorphine ····· reaction product

The sensibility of this method for urinary morphine was examined as follows: To 50 ml. of normal rabbit or human urine was added morphine hydrochloride solution containing 200, 100, 50, and 30 μg. of free morphine. The mixture was extracted continuously with $CHCl_3$ after acid treatment as reported previously*[1] and the extract was applied to single thin-layer chromatographic method. The smallest amount of morphine in this urine sample that could be identified easily was 50 μg./50 ml. by either coloration or fluorescence with potassium platinum iodide reagent. If double thin-layer chromatographic method was performed, the sensibility increased up to 10 μg./50 ml. by both reactions.

Table II. Rf Values of the Narcotics and Their Reactivities to Coloration or Fluorescence with Potassium Platinum Iodide Reagent

Solvent system / Sample	5 EtOH, 40 Dioxane, 50 Benzene, 5 conc. NH_4OH			60 MeOH, 15 *n*-BuOH, 10 Benzene, 15 H_2O		
	Rf value	Coloration	Fluorescence	Rf value	Coloration	Fluorescence
Morphine	0.25	+	+	0.19	+	+
N-Allylnormorphine	0.27	+	+	0.56	+	+
Codeine	0.50	+	−	0.28	+	−
Oxycodone	0.75	+	−	0.29	+	−

The extension of this method to other closely related narcotics was also studied and the same result as of Kupferberg, *et al.*[1,2], was obtained; the phenanthrene nucleus of morphine with a phenolic hydroxyl group seemed to be necessary for the formation of the fluorophore. The result was summarized together with their Rf values in Table II.

Discussion

Characteristics of the method described here are condensed in higher reliability and simplicity. The potassium platinum iodide reagent which was used widely for detection of morphine as a color reagent in thin-layer chromatography was now evaluated also as a fluorescent reagent. Morphine in urine could be thus identified more definitely than other methods by successive performance of color and fluorescent reactions on the same thin-layer chromatogram without any complexity.

During the course of this study, it was experienced that even human who had never received morphine or other narcotics excreted quite often morphine-like substances which gave violet color with potassium platinum iodide reagent around the spot of morphine in thin-layer chromatography. This might mislead judgement of identification. It seemed that such confusing cases were encountered more on smokers rather than on non-smokers. Identification of morphine, therefore, should be performed not only by coloration on thin-layer chromatogram, but also by other methods.

Fortunately none of these substances appeared as fluorescent spots according to the present method, and so above misjudgement by coloration only could be avoided.

This fluorescent reaction was clarified to be based on oxidation of morphine to pseudomorphine, the same principal as in unique ferricyanide method of Kupferberg, *et al.*[1,2] and both methods showed about the same level of sensibility for authentic morphine. However, when these were applied to detection of urinary morphine, the present method afforded better result than that of ferricyanide method, because in the latter method background-fluorescence of chromatogram interfered to confirm the presence of minute quantities of morphine in urinary extract.

On the other hand the present method was limited to the detection only and could not be applied to quantitative determination of morphine because the oxidation reagent of this method strongly absorbed the excitation light and disturbed the linearity of the assay method.

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Summary

A simple and reliable identification method of morphine was developed by successive performance of color and fluorescent reactions with potassium platinum iodide reagent utilizing the same thin-layer chromatogram; after separation by thin-layer chromatography morphine could be identified as a violet-colored spot with above reagent and then reconfirmed as a blue fluorescent spot under ultraviolet light after the chromatogram was exposed to ammonia vapor.

The fluorometric identification is based on the essentially same principal to that of Kupferberg, *et al.*[1,2], however the present method has certain advantages to identify morphine, especially excreted in urine, in respect of simplicity and reliability.

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