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11. Hidetoshi Yoshimura, Kazuta Oguri, and Hisao Tsukamoto : Detection of Morphine in Urine.

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There have been considerable informations concerning the detection of morphine in urine, but the methods currently used are still lacking in adequate sensibility.

Thus, several papers[1,2] have described that it is difficult to detect morphine in the urine excreted by amimal or human when more than 24 hours had passed after the drug administration. This seems to be quite reasonable from the data of Elliott, *et al.*[3] using N-$^{14}CH_3$ labeled morphine that the major portion of radioactivity was excreted within 24 hours in both nonaddict and addict human.

On the other hand, Gross and Thompson[4] have shown that the dog continued to excrete a small amount of morphine for at least 6 days, and Milthers[5] has obtained the similar result on rats. Experiment[3] on human using N-$^{14}CH_3$ labeled morphine has also indicated that excretion of radioactivity was not complete in 24 hours, but continued over a few days after the medication.

These results suggest that detection of morphine will be possible even for urine excreted when it has passed a day or more after the adminstration, if the extraction of morphine from the urine is complete and the detection procedure is sensitive enough.

This paper will describe the simple procedure affording virtually complete extraction of morphine including sufficient hydrolysis of conjugated morphine to free form and the sensitive detection method by doubly performed thin-layer chromatography. It will provide a definite evidence on judgement whether morphine is administered or not.

Materials and Methods

Materials——Free morphine (monohydrate) was prepared from a commercial sample of the morphine hydrochloride and recrystallized repeatedly from MeOH.

Administration of Morphine——Animals used were male albino rabbits weighing about 3.0 kg. The desired amount of morphine was injected subcutaneously in a volume of 1.0 ml./kg. of body weight.

Extraction of Morphine from Urine——To 50 ml. of urine was added 28 ml. of conc. HCl and 1 ml. of 40% $NaHSO_3$ solution. The mixture was heated on a boiling water bath for 30 min. To this hydrolysate was again added 1 ml. of 40% $NaHSO_3$ solution. The mixture was adjusted to pH 9.0 with 30% NaOH solution, diluted with 50 ml. of 3.0*M* phosphate buffer, pH 9.0, and extracted with $CHCl_3$ for 3 hr. in continuous extractor shown in Fig. 1. For the estimation of morphine, the $CHCl_3$ layer was dried over anhyd. Na_2SO_4 and evaporated to dryness. The residue obtained was further dried under reduced pressure and dissolved in 1 ml. of MeOH.

Estimation of Morphine——The following two methods were used in this study.

a) Colorimetric method : The method was essentially the same as described by Woods and Muehlenbeck.[6] An aliquot of 10 to 50 μl. of MeOH solution of the urine extract described above, containing about 50 μg. of morphine, was transferred into 50 ml. centrifuge tube with a Hamilton microsyringe and evaporated the solvent on a water bath. The residue obtained was dissolved in 5 ml. of 0.5*N* HCl solution.

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1) H. Asahina, M.Ôno : Kôseikagaku Kenkyu Hôkoku, **1962**, 21.
2) E. Hosoya : *Ibid.*, **1962**, 11.
3) H. W. Elliott, *et al.* : Proc. Soc. Exptl. Biol. Med., **85**, 77 (1954).
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6) L. A. Woods, H. E. Muehlenbeck : J. Pharmacol. Exptl. Therap., **111**, 64 (1954).

To this solution 10 ml. of 2% *p*-nitrobenzoyl chloride in $CHCl_3$, 1 ml. of 40% $NaHSO_3$ solution and 1 ml. of 40% NaOH solution were added. The mixture was shaken mechanically for 40 min. and centrifuged for 2 min. The aqueous layer (upper) was removed carefully. The $CHCl_3$ layer was washed by shaking for one min., first with 5 ml. of borate buffer, pH 9.0 and then with 5 ml. of phosphate buffer, pH 5.0. Each washing was followed by centrifugation and removal of the aqueous layer. An aliquot of 8 ml. of the buffer-washed $CHCl_3$ was pipetted into 50 ml. centrifuge tube and shaken for 10 min. with 0.5 ml. of methylorange reagent.[6] The mixture was centrifuged, and a portion of 5 ml. of the $CHCl_3$ solution was transferred to 50 ml. centrifuge tube by introducing the pipette directly through the center of the $CHCl_3$ surface, care being taken not to touch any of the methylorange reagent. This was shaken with 4 ml. of *N* HCl solution for 5 min. and centrifuged. The amount of morphine was calculated from the optical density determined with a Hitachi Model EPU-2A Spectrophotometer at 515 mμ.

b) Gas chromatographic method: The instrument used for this work was a Shimadzu Model GC-1B Gas Chromatograph equipped with a hydrogen flame ionization detector (dual column and differential flame type). The columm was a stainless steel U-tube 2.25 m. long and 4 mm. in inside diameter. The column packing was 1.5% SE-30 on Chromosorb W (60~80 mesh), which was treated with hexamethyldisilazane. The column temperature was maintained at 210°, the sample chamber temperature was 300°, and the detector cell temperature was 230°. Nitrogen was used for the carrier gas with a flow rate of 35 ml./min.

Methanol solution of the urine extract described above was injected with a Hamilton microsyringe. The usual sample size was about 2 μl. corresponding to about 5 to 10 μg. of morphine. The amount of morphine in the sample was calculated from the standard curve by measuring the peak areas. The standard curve, which was made by running through the same procedure using purified free morphine, was desirable to be checked with new standard solutions every day that unknown samples were gas chromatographed.

Thin-layer Chromatography——It was carried out by use of silica gel plate (silica gel G, Merck), 20×20 cm. in size, 1 mm. or 0.25 mm. in thickness, activated at 105° for 30 min. The thiker plate (1 mm.) was allowed to stand overnight before activation.

All of the viscous solution of the extract in a small volume of MeOH was spotted in line on the thicker plate (1 mm.), authentic morphine being spotted on its end and developed with the solvent system of EtOH, dioxane, benzene, and conc. NH_4OH (5:40:50:5). After visualizing only the authentic morphine with potassium platinum iodide reagent, the corresponding area on the chromatogram was scratched, collected in a flask, and extracted with 30 ml. of MeOH containing a few drops of conc. NH_4OH.

A part of the extract was then spotted on the thinner plate (0.25 mm.), developed with the solvent system of dioxane and conc. NH_4OH (60:5) and sprayed with the same reagent as above.

Results and Discussion

Extraction of Morpnine

Morphine is a polar compound which possesses the phenolic hydroxyl and tertiary amino groups in the molecule and therefore difficult to be extracted from the aqueous solution with organic solvent by shaking. One of the most widely used solvent for the extraction of morphine is the mixture of chloroform and isopropyl alcohol (3:1). Appropriate recovery has been obtained in the extraction of morphine from the urine with this solvent by shaking when the urine has been saturated with sodium bicarbonate and adjusted to pH 9.0 with conc. ammonium hydroxide.[7,8] However, formation of troublesome emulsion occurs at every extraction and so additional time-wasting procedure must be necessary to remove the emulsion.

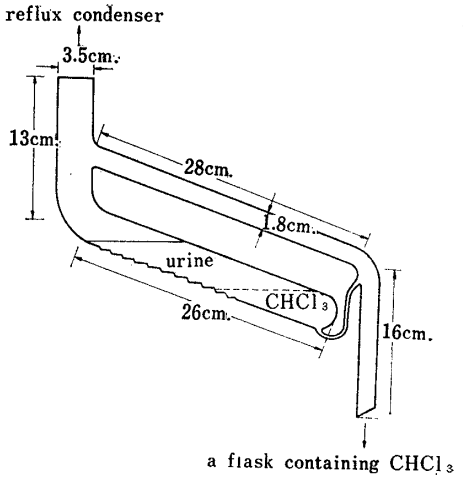

Fig. 1. Continuous Extractor with Chloroform

7) K. Milthers: Acta pharmacol. et toxicol., **18**, 199 (1961).
8) S. Okui: Kôseikagaku Kenkyu Hôkoku, **1962**, 33.

This difficulty was overcome by the simple and effective extraction with chloroform using continuous extractor which was devised in this laboratory (Fig. 1).

Table I shows a typical experimental result when a morphine solution (1.869 mg. in 100 ml. of water) was saturated with sodium bicarbonate, adjusted to pH 9.0 with a few drops of conc. ammonium hydroxide, and extracted with chloroform over a period of 2 to 20 hours. Maximum recovery (about 85%) was obtained in 3 and 5 hours-extractions. Prolongation of extraction time resulted in a decrease of recovery which was probably due to lability of morphine in alkaline medium.

Table I. Recovery of Morphine by Continuous Extraction

Extraction time (hr.)	Recovery (%)[a]	Extraction time (hr.)	Recovery (%)[a]
2	77.5	10	72.2
3	84.5	20	51.2
5	85.7		

a) Methylorange method was used for estimation of morphine extracted.

To avoid such decomposition of morphine during the extraction, a small amount of sodium hydrogen sulfite was added to the urine as the antioxidant. The resulting mixture was then diluted with the same volume of 3.0*M* phosphate buffer, pH 9.0, which was considered to be better agent in maintaining the pH constantly and preventing the formation of emulsion. Nearly quantitative recovery was obtained under this condition.

Hydrolysis of Conjugated Morphine in Urine

Although a small part of morphine administered is metabolized to normorphine[5,9] and codeine,[10] a large part is excreted as the conjugated forms together with unchanged free morphine in the urine.[4,11,12] It is therefore very reasonable to hydrolyze the conjugates before the extraction procedure and detect them as the free morphine on judgement whether morphine has been administered or not by use of the urine.

The simple hydrolysis procedure which has been employed so far for the detection of morphine in urine is to make the urine to 5% hydrochloric acid concentration and heat it for 30 minutes on water or oil bath. This is quite convenient, but seems not to be sufficient to liberate most of morphine involved, considering the finding[13] that part of bound morphine is freed only by autoclaving (5% HCl).

The present experiment has been undertaken in order to find out the hydrolysis condition which liberates most of morphine from the conjugates in the urine. A typical experiment was carried out as follows: Portions (50 ml.) of 24 hours urine of male albino rabbit injected with morphine (10 mg./kg.) subcutaneously was adjusted to the hydrochloric acid concentration of 5, 10, and 15 per cent respectively, and hydrolyzed for 30 minutes or 60 minutes on a boiling water bath. Free morphine which was excreted unchanged in the urine and liberated after the hydrolysis was extracted continuously and estimated by both colorimetric and gas chromatographic methods as described under Methods.

9) A. L. Misra, S. J. Mulé, L. A. Woods: J. Pharmacol. Exptl. Therap., **132**, 317 (1961).
10) C. Elison, H. W. Elliott: *Ibid.*, **144**, 265 (1964).
11) L. A. Woods: *Ibid.*, **112**, 158 (1954).
12) J. M. Fujimoto, E. L. Way: *Ibid.*, **121**, 340 (1957).
13) A. Stolman, C. P. Stewart: "Toxicology," Vol. 1, 123 (1960), Academic Press, New York and London.

As shown in Table Ⅱ, the hydrolysis in 5% hydrochloric acid concentration was found to afford insufficient liberation of morphine even after one hour heating. The recovery was increased along with the hydrochloric acid concentration and the maximum obtained after 30 minutes hydrolysis in that of 15%. Taking more strong condition (60 min. in 15% HCl conc. or 30 min. in 20% HCl conc.), the recovery remained in approximately same value as that of 30 minutes hydrolysis in 15% hydrochloric acid concentration.

As seen in Table Ⅲ, this procedure was further shown to be even favorable in comparison with the sealed tube method, although the procedure itself was much simpler than the latter.

Table Ⅱ. Effect of the Hydrochloric Acid Concentration and Time of Heating on Hydrolysis of the Conjugates in Urine

HCl Concentration (%)	Time (min.)	Morphine (mg./50 ml. Urine)	
		M.O. method	G.C. method
0	0	0.85	0.71
5	60	4.25	3.8
10	30	6.9	6.2
10	60	7.75	7.8
15	30	9.0	8.95

Table Ⅲ. Comparison of Hydrolysis in Open Vessel with That in Sealed Tube

	HCl Concentration (%)	Time (min.)	Temp. (°C)	Morphine (mg./50 ml. Urine)[a)]
Sealed tube	5	30	140	9.3
Open vessel	15	30	100	9.5

a) Gas chromatographic method was used. Amount of unchanged free morphine in 50 ml. of this urine sample was determined to be of 0.88 mg.

It should be noticed that no loss of morphine was observed during the hydrolysis and extraction procedure when normal urine, to which 5 mg. of morphine was added, was heated for 30 minutes in 15% hydrochloric acid concentration and extracted.

Detection of Morphine in the Extract by Double Thin-layer Chromatography

As far as the first 24 hours urine after the drug administration is concerned, detection of morphine can be performed without difficulty since it contains enough morphine to be extracted by the present method and detected by ordinary thin-layer chromatography.

On the contrary, the urine taken after more than a day may contain only a very small amount of morphine. Although the continuous extraction with chloroform will result in virtually complete recovery of morphine, it will bring together a large amount of other substances being contained in the normal urine. These substances will certainly disturb the detection of microgram quantities of morphine by thin-layer chromatography.

In fact, the extract from 50 ml. of the rabbit urine excreted between 24 and 48 hours after subcutaneous injection of morphine (10 mg./kg.) was proved to be difficult to detect morphine by its direct application to ordinary thin-layer chromatography.

In such a case, separation of interfering materials from morphine should be necessarily performed before the detection procedure, and this was successfully carried out by thin-layer chromatographic separation using rather thicker sillica gel plate

(1 mm. in thickness) as described under Methods. All the extract from one urine sample could be applied at once to this particular plate, by which morphine was separated fairly well from most of the impurities. The area corresponding to morphine on the chromatogram was extracted with methanol containing a few drops of conc. ammonium hydroxide, and followed by ordinary thin-layer chromatography for detection of morphine.

This procedure is not complicated taking only a short time, and at the same time shows much higher sensibility compared with that of previously known method. The smallest amount of morphine in the urine which could be detected without fail was found to be of 10 μg. by the experiment in which a certain amount of morphine was added to the normal rabbit urine (50 ml.) and followed by the present method (see Table Ⅳ).

Table Ⅳ. Amount of Morphine Detectable in Urine

Morphine						
μg./50 ml. Urine	100	50	20	10	5	2
	++	++	+	+	±	−

++: immediately colored +: colored after a few hours −: not colored at all

A portion of 50 ml. of every 24 hours urine after the subcutaneous administration of various doses was further examined by this method (see Table Ⅴ). It was proved that detectable amount of morphine was still excreted after a week into the urine of rabbit to which 10 mg./kg. of morphine was injected. In the urine of rabbit administered in a dose of 3 mg./kg., morphine could be detected certainly after 3 days.

Table Ⅴ. Detection of Morphine in Every Day Urine after Administration of Morphine

Dose (mg./kg.)	Day							
	1[a]	2	3	4	5	6	7	8
10	++	++	++	+	+	+	+	±
5	++	++	++	+	+	±	−	−
3	++	++	+	±	−	−	−	−

a) Single thin-layer chromatography was adopted in this case.

In this experiment, single thin-layer chromatography (ordinary method) could be applied sufficiently to the first 24 hours urine of rabbit. Detection on unknown sample is therefore desirable to perform first with single method, and only in the case to be unable to detect morphine double method should be applied.

All of these results was obtained by the investigation on rabbit, however it would be extended similarly to the urine or blood of human who had received illegally morphine or other narcotics mixture containing morphine.

In fact, the urine samples of several persons who might use narcotics illegally at the adequate date, but could not be proved it by the currently using method, was confirmed certainly to contain morphine by the present method although the cases were not so many.

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Summary

A simple and sensitive method for extraction and detection of morphine in urine was described.

The continuous extraction method showed a virtually complete recovery of morphine and prevented the formation of emulsion unavoidable in the extraction by shaking.

The hydrolysis condition which led to liberation of most of morphine from the conjugates in urine was found to be that of heating for 30 minutes, in the 15% hydrochloric acid concentration. This procedure was simple, but afforded the comparable result to that of sealed tube hydrolysis in the 5% hydrochloric acid concentration.

Double thin-layer chromatographic method was successfully applied first to the separation of morphine from a large amount of impurtities in the urine extract using thicker plate (1 mm.) and then to the final detection of morphine using ordinary one (0.25 mm.).

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12. Tetsuji Kametani, Seiichi Takano, Kazuko Masuko, and Fujinori Sasaki: Bisbenzylisoquinoline Alkaloids and Related Compounds. V.*1 A Total Synthesis of Diastereoisomeric Mixture of Liensinine.*2,*3

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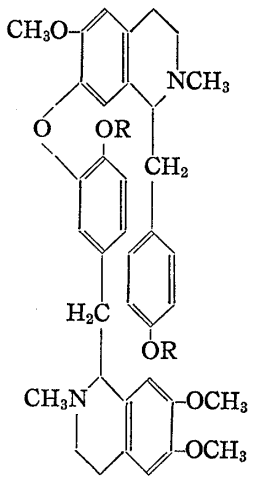
The isolation of a new alkaloid, liensinine, with the composition of $C_{37}H_{42}O_6N_2$, m.p. 95~99°, from the embryo loti of *Nelumbo nucifera* GAERTN. was described by Chao-yuan, *et al.*[1] and it was then shown that liensinine possessed the structure (I), mainly based on the results of Hoffmann degradation and potassium permanganate oxidation.[2]

The purpose of the present investigation was to study the Ullmann reaction between both tetrahydroisoquinoline derivatives, X and XVII, in order to obtain diastereoisomeric mixture of O,O-dibenzylliensinine (II) as a possible intermediate for the synthesis of I′, possessing the same planar structure as natural liensinine (I). Benzylation of 3-bromo-4-hydroxyphenylacetic acid[3] (III) with benzyl chloride in the presence of sodium ethoxide in ethanol afforded 3-bromo-4-benzyloxyphenylacetic acid (IV), which was converted into the acid chloride (V).



I : R = H
I′ : R = H
II : R = $CH_2C_6H_5$

*1 Part IV. T. Kametani, K. Fukumoto: J. Chem. Soc., **1964**, 6141.
*2 This forms Part CXXXII of "Studies on the Syntheses of Heterocyclic Compounds" by T. Kametani.
*3 This work was reported at the Tohoku Branch Meeting of Pharmaceutical Society of Japan, February 20, 1965.
*4 Kita-4-bancho, Sendai (亀谷哲治, 高野誠一, 増子和子, 佐々木藤紀).
1) Chao Tse-yuan, Chou Yun-lee, Young Tao-tsin, Chou Tsan-quo: Scientia Sinica, **11**, 215 (1962).
2) Pan Pei-chuan, Chou Yun-lee, Sun Tsun-tsi, Kao Yee-sheng: *Ibid.*, **11**, 321 (1962).
3) H. Kondo, S. Uyeo: Yakugaku Zasshi, **53**, 557 (1933).