[Chem. Pharm. Bull. 12 (3) 370 ~ 375]

UDC 615.711.84-092.21

55. Zenzo Tamura and Toshio Imanari: Metabolism of *trans*-π-Oxocamphor.*1

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The detoxication mechanism of *trans*-π-oxocamphor (Vitacampher) (I), a rapid-acting cardiotonic, has not been clarified in spite of the relatively rapid disappearance of its activity. Kawahata[1] isolated *trans*-π-hydroxycamphor (II) and 1,7-dimethyl-2-oxo-7-norbornanecarboxylic acid (isoketopinic acid) (III) from the urine of dogs fed with a large amount of I, but the amount of either II or III was only 1~2% of that of I administered. In this paper the main metabolic pathway of I in human body when I was subcutaneously administered in a medical dose will be demonstrated.

Examination of Metabolites

First, the two expected metabolites (II and III) were examined by the procedure shown in Chart 1. Although the sublimation technique proposed by Ishikura[2] was effective for separating the metabolites from urinary impurities, some modifications were required for microanalysis to minimize technical loss. The detection limit of II or III in 50 ml. human urine was 2 and 0.2 μmole by the use of paper chromatography and gas chromatography, respectively.

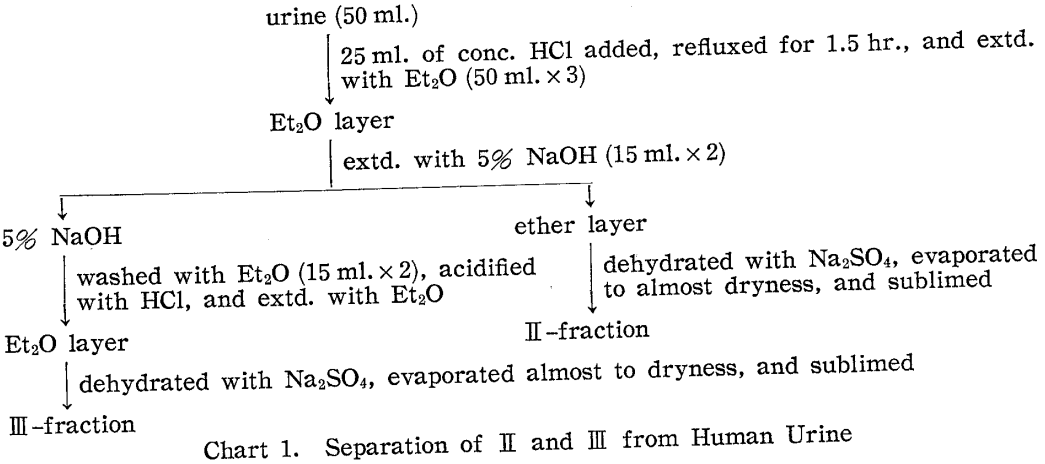

Chart 1. Separation of II and III from Human Urine

The procedure was applied to the urine of a man subcutaneously injected 20 mg. of I. From 2- and 4-hour urine, an unexpectedly large amount of III was sublimed and identified by paper chromatography, gas chromatography, and infrared spectrum. A very small quantity of II was detected in a 2-hour urine by gas chromatography shown in Fig. 1. II was not extracted without acid hydrolysis shown Chart 1. Moreover, the fact that a hydrolysis under milder acidic condition or an alkaline hydrolysis was not effective coincided with the character of *trans*-π-hydroxycamphor glucuronide (II-G) synthesized. Consequently, it is most probable that II was excreted in the form of II-G in human urine, as was pointed out by Kawahata.[1]

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*1 A part of this work was presented at the 82nd Annual Meeting of the Pharmaceutical Society of Japan, Shizuoka, November, 1962.

*2 Hongo Tokyo (田村善蔵, 今成登志男).

1) H. Kawahata: Yakugaku Zasshi, **63**, 455 (1943).

2) S. Ishikura: Private communication.

When the procedure was applied to the urine of a horse injected 500 mg. of I, a small quantity of II was detected by gas chromatography and III was not detectable owing to the contamination of a large amount of benzoic acid which was produced by hydrolysis of hippuric acid in the urine. When extraction was carried out without hydrolysis, III was separated, but only in a small amount.

There could be a possibility that III was present in part as a conjugate in the urine and, therefore, a 5-hour urine of a man injected 20 mg. of I was reexamined by the modified separation system shown in Chart 2. About 6 mg. and 2.5 mg. of III were isolated by sublimation from f-1 and f-3 fractions, respectively, and about 0.1 mg. of II was detected in f-2 fraction by gas chromatography, but neither I nor II was detected in f-4 fraction. The result demonstrates the excretion of free and conjugated III and of II-G in the urine.

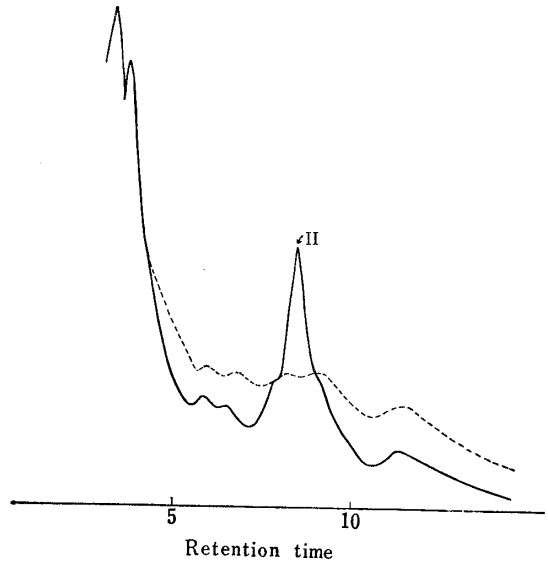

Fig. 1. Gas Chromatogram of Human Urine
column : 10%-DC 550 (on celite 545) 1.5 m. × 4 mm. i. d.
column temperature : 180°
carrier gas : N_2, 25 ml./min. at 2 kg./cm²
sens : 100.
range : 0.4 v.
—— 2-hr. urine --- 4-hr. urine

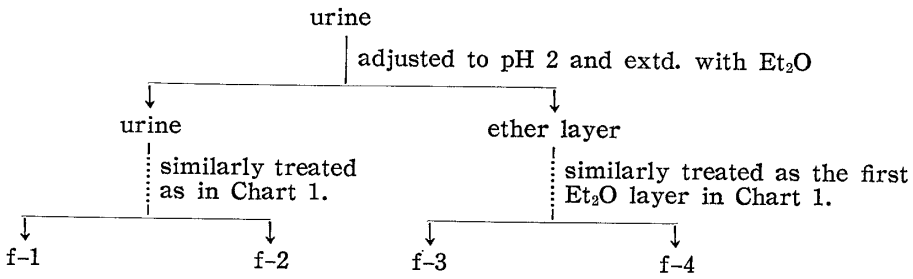

Chart 2. Separation of Conjugated and Nonconjugated Metabolites

Elucidation of Conjugate of III

After removal of free III by ether extraction, a 5-hour urine was neutralized to pH 7, lyophilized, and the residue was extracted with methanol. The extract was developed on a 40×40 cm. filter paper by an ascending method using a solvent system of butanol-acetic acid-water (4:1:5, upper layer). The paper was cut into several sections, each of which was extracted with 50% methanol. III was detected only in the extract of Rf 0.6 section by gas chromatography after acid hydrolysis, although Rf value of free III is almost 1.0. Tertiary carboxyl group of III might be combined with glucuronic acid or glycine. Actually, more than one mole of glucuronic acid was found in the extract of Rf 0.6 section for one mole of III by the naphthoresorcinol method,*3 but amino acid was not detected by ninhydrin after acid hydrolysis. Moreover, the same amount of III was recovered from the conjugate by β-glucuronidase and alkali. The result indicates that the conjugated compound of III is an ester glucuronide (III-G).

Excretion of III and III-G in Human Urine

According to the evidence that main metabolites of I in human urine were III and

*3 Presented by M. Ishidate, *et al.* at the 4th Symposium on Glucuronic Acid, Tokyo, June, 1958.

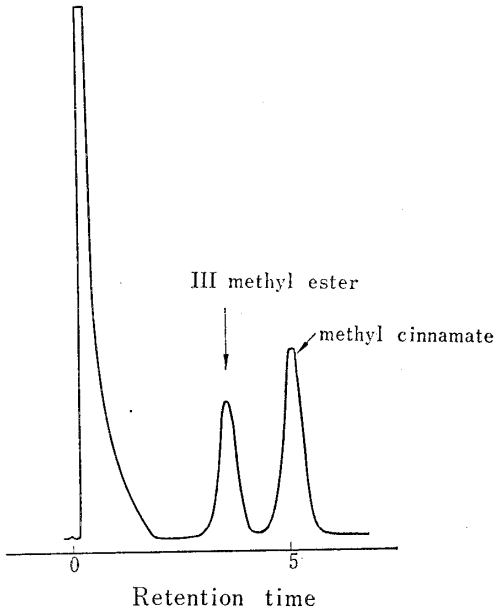

Fig. 2. Gas Chromatogram of Ⅲ Methyl Ester and Methyl Cinnamate

column: polyester succinate (Shimadzu) 1.5 m. × 4 mm. i. d.
temperature: column, 185°; sample chamber, 250°; detector, 200°
carrier gas: N_2, 25 ml./min. at 2 kg./cm²
sens: 100. range: 0.4 v.

Ⅲ-G, their excretion rate in the urine was investigated more precisely using gas chromatography. For the quantitative estimation of Ⅲ, cinnamic acid was used as an internal standard. The gas chromatogram of methyl esters of Ⅲ and cinnamic acid, and the calibration curve are shown in Figs. 2 and 3. As the sublimation was unsuitable for quantitative separation and had to be omitted, the condition of hydrolysis was reexamined. It was found that phenol and *p*-cresol were produced from normal urine by acid hydrolysis, the latter of which overlapped with the methyl ester of Ⅲ in gas chromatogram. An alkaline hydrolysis was found to produce Ⅲ from Ⅲ-G quantitatively without production of any such impurity.

Twenty mg. of I was injected subcutaneously to a man and his urine was collected at 1, 2, 3, 5 and 8 hours after the administration. Ⅲ and Ⅲ-G of each portion were determined by the procedure described in the Experimental section and the result obtained

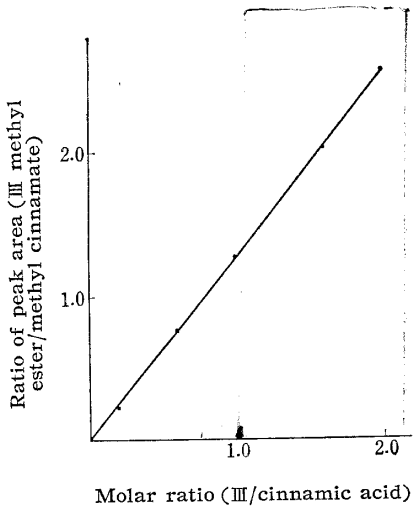

Fig. 3. Calibration Curve for Ⅲ

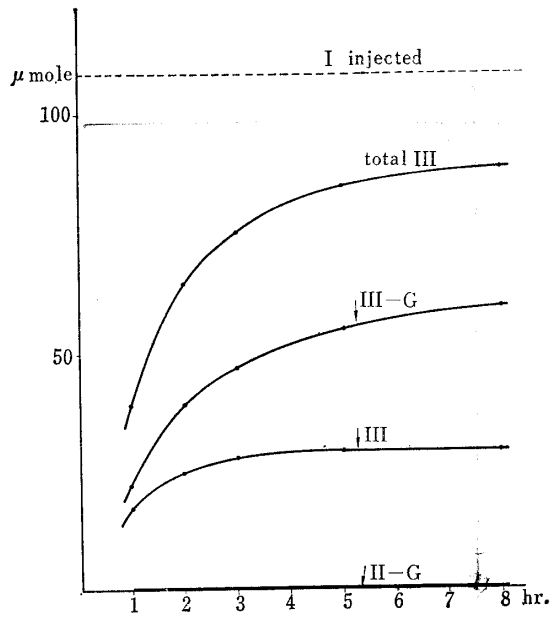

Fig 4. Cumulative Urinary Excretion of *trans*-π-Oxocamphor Metabolites

is shown in Fig. 4, which demonstrates that more than 80% of the administered I was excreted as Ⅲ and Ⅲ-G in 8 hours. Consequently the main metabolic pathway of I in human body can be summarized as shown in Chart 3.

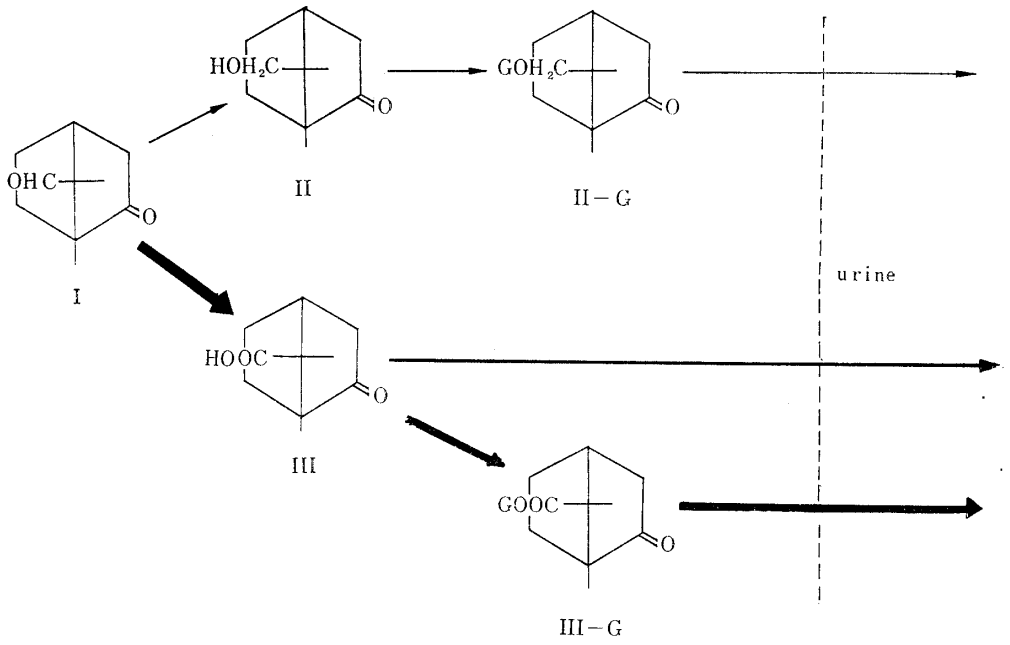

Chart 3. Metabolic Pathway of I in HumanBody

G : glucuronic acid moiety

Experimental

Materials——*trans*-π-Oxocamphor: Commercially available Vitacampher (1% aq. solution) was used.

trans-π-Hydroxycamphor and isoketopinic acid : Gifts from Yoshitomi Pharm. Ind., Ltd.

trans-π-Hydroxycamphor glucuronide : To a solution of 4.7 g. of methylbromotriacetylglucuronate and 4.3 g. of *trans*-π-hydroxycamphor dissolved in 37 ml. of $CHCl_3$ 3.7 g. of Ag_2CO_3 was added and the mixture was stirred for 6 hr. at room temperature. After filtration, $CHCl_3$ was evaporated in a reduced pressure, the yellowish viscous residue was dissolved in MeOH, and the same volume of H_2O was added to produce a white precipitate, which was collected and recrystallized from MeOH to white needles. The yield of methyl triacetylhydroxycamphorglucuronate (IV) was 50%.

To 2.3 g. of IV, 40 ml. of 5% KOH-MeOH was added and the mixture was kept at 30° for 10 min. The clear hydrolyzed solution was shaken with 12 g. of Amberlite IR-120 resin (H form). After removal of the resin by filtration, the solution was evaporated and yellowish viscous residue (II-G) was obtained. II-G was recrystallized from EtOH, m.p. 198.5~200°. *Anal.* Calcd. for $C_{16}H_{24}O_8$: C, 55.80; H, 7.40. Found : C, 55.65; H, 7.08.

β-Glucuronidase : β-Glucuronidase of the step-4 by Fishman, *et al.*[3] was prepared from calf liver.

Instrument——Shimadzu GC-IB gas chromatograph, equipped with H_2 flame ionization detector, was used.

Detection of II and III by Paper Chromatography——2,4-Dinitrophenylhydrazones of II and III, which were prepared by the same method as that of camphor[4] were developed by the descending method using Toyo Roshi No. 51 A filter paper and with solvent system of benzene-ligroine (2:3) saturated with 20% HCl. Rf values of 2,4-dinitrophenylhydrazones of II and III were 0.85 and 0.3, respectively.

Hydrolysis of II-G——Conditions of hydrolysis of chemically synthesized II-G were examined. One ml. of β-glucuronidase solution (12000 units/ml.) was added to 10 ml. of acetate buffer (pH 5.0) containing 1μmole of II-G and incubated at 38° for 3.5 hr. It was found from the determination of glucuronic acid by naphthoresorcinol method*[3] that more than 90% of II-G was hydrolyzed in this condition. In urine, however, hydrolysis of II-G was not successful under similar condition and II was not detected by paper chromatography. This fact indicates the presence of hydrolysis inhibitor in urine. Accordingly, usual hydrolysis with HCl was applied to the urine and the recovery of II was almost quantitative.

Solvent Extraction——Distribution coefficient of II between H_2O and Et_2O was determined by gas chromatography as $C_{H_2O}/C_{Et_2O} \simeq 2/3$, and accordingly the recovery of II by Et_2O extraction in Chart 1

3) W. H. Fishman, P. Bernfeld : "Methods in Enzymology," **1**, 262 (1955).
4) Y. Fujita, K. Nakahara : Nippon Kagaku Zasshi, **61**, 77 (1940).

was not quantitative. pKa of III was measured by pH titration as 4.45 (at 25°) and III was extracted completely with Et_2O from urine at pH 2.

Purification by Sublimation——Further purification of II and III was achieved by sublimation. When the sample contained less than 200 μg. of the compounds, glass tube (20 cm. × 5 mm. internal diam.) was sealed in a reduced pressure. After heating in a metal furnace at 180° for 2 hr., II or III deposited on the tube near the outlet of furnace.

Separatory Determination of III and III-G in Urine——Three ml. of urine containing more than 10 μg. of III was adjusted to pH 2 with HCl and Et_2O solution of a certain amount of cinnamic acid was added. The tube was filled up to 7 ml. with Et_2O, stoppered, and shaken well. When emulsion occurred, Et_2O*4 and urine layer were separated by centrifugation. Et_2O layer was transfered to another tube and evaporated to a small volume, to which Et_2O solution of CH_2N_2 was added until the solution became yellow. The solvent was evaporated off immediately at as lower a temperature as possible*5. The residue was dissolved in Me_2CO and submitted to gas chromatography. From the ratio of the two peak areas measured by the half-width method (III methyl ester peak area/methyl cinnamate peak area), the amount of III was estimated using a calibration curve (Fig.3).

In order to determine total III (free III + III-G), alkaline hydrolysis was carried out. To 3 ml. of urine. 1 ml. of 5*N* NaOH was added and heated at 70° for 10 min. After this hydrolysis, estimation was made according to the procedure described above. The amount of III-G was estimated by substracting the amount of III from that of total III.

Identification of Phenol and *p*-Cresol in Acid Hydrolysate of Normal Urine——This experiment was undertaken to see if there was any compound in urine that interfere with the determination of III and III-G. (a) and (b) in Fig. 5 show the gas chromatogram of blank test using normal urine. Arrows indicate the position of peaks of III methyl ester and methyl cinnamate. Any substance that interfered

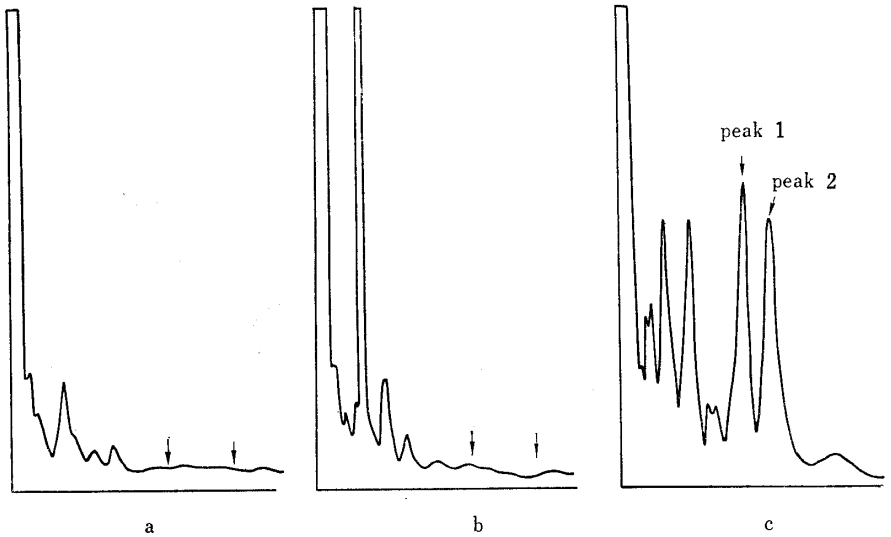

Fig. 5. Gas Chromatogram of Normal Urine

a : blank test for determination of free III
b : blank test for determination of total III
c : see text.

with quantitative analysis of III was not present in normal urine or its alkaline hydrolysate. When urine was not fresh or hydrolyzed with HCl, the gas chromatogram of (c) in Fig. 5 was obtained. In this case, determination of III was impossible because peak 2 overlapped with that of III methyl ester. The compounds of peaks 1 and 2 were investigated. It was assumed that they were phenols since they were not extracted with aq. $NaHCO_3$ but were extracted with aq. NaOH from Et_2O layer. The retention time of peak 1 corresponded to that of phenol and peak 2 to *p*- or *m*-cresol.

The compounds of peaks 1 and 2 were collected after gas chromatographic separation. These were led to azo compounds with *p*-diazobenzenesulfonic acid and submitted to paper chromatography using

*4 When the urine was not fresh and contained free phenols, it was necessary to remove them by extracting III and cinnamic acid with 5% Na_2CO_3. The extract was acidified with HCl and reextracted with Et_2O.

*5 Double bond of cinnamic acid reacts slowly with excess of CH_2N_2.

the solvent system of BuOH-2% Na_2CO_3 (1:1, upper layer) with the paper treated with 4% Na_2CO_3. Rf values of two substances agreed with those of phenol and *p*-cresol.

The authors are grateful to Professor Emeritus M. Ishidate for his continuous encouragement throughout this work. They are also indebted to Miss M. Hatakeyama for technical assistance and to Yoshitomi Pharm. Ind., Ltd. the supply of the samples. This study was supported in part by a grant from the Iatrochemical Foundation to which the authors thanks are due.

Summary

The fate of medical dose of subcutaneously administered *trans*-π-oxocamphor (I) in human body was investigated. *trans*-π-Apocamphorcarboxylic acid (III) and its ester glucuronide (III-G) were detected as the main urinary metabolites of I, while *trans*-π-hydroxycamphor glucuronide was detected as a minor one. Determination by gas chromatography demonstrated that more than 80% of administered I was excreted as III and III-G during 8 hours.

(Received October 9, 1963)