ON THE METABOLIC DETOXICATION OF THYMOL IN RABBIT AND MAN

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Abstract.....The metabolic detoxication of thymol was investigated in rabbit and man. Thymol glucuronide which the aglycone is intact, was isolated from thymol medicated rabbit urine and identified as a acetyl derivative of methyl glucuronate.

The hydroxylated product of thymol, thymohydroquinone, was recognized in a small amount in thymol medicated human urine. It was presumed that thymohydroquinone is excreted as ethereal sulfuric acid conjugate in man.

Key words: thymol, parasiticide, glucuronide isolation, hydroxylation in vivo, thymohydroquinone formation.

Thymol, 1-methyl-3-hydroxy-4-isopropylbenzene, is used as an antiseptic, anthelminic, fungicide and as a reagent in the thymol turbidity test for globulin. It is also present in some mouth washes.

In the course of investigations involving the chemotherapy of alveolar echinococcosis of liver in man, thymol palmitate, palmitic acid ester of thymol, was introduced for the clinical treatment of the helminthiasis based on laboratory trials of series of several parasiticides.

It attracted our attention to investigate the detailed metabolic fate of thymol in relation to pharmacological activity of thymol palmitate. Concerning metabolic fate of thymol, it has been elucidated that thymol is readily absorbed from the intestine and excreted conjugated with glucuronic acid and sulfuric acid by man, dog and rabbit (Katsuyama and Hata, 1898, Takao 1923, Robbins 1934).

The attempts to isolate and characterize thymol glucuronide have been made, and thymol glucuronide which thymol is trichlorinated was isolated (Katsuyama and Hata 1898, Takao 1923).

In the present paper, the metabolic fate of thymol in rabbit and man was studied, especially concerning (1) the isolation and characterization of thymol glucuronide which

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the aglycone is intact, and (2) the detection of oxidized product of thymol in man which is supposed to be the main biotransformed product, using gas chromatography and thin-layer chromatography.

METHODS

Extraction and fractionation of metabolites of thymol were performed by the procedure shown in Chart. 1.

Chart. 1. Extraction and Fractionation Procedures of Metabolites

Thymol

Thymohydroquinone

Animal

The rabbits weighed 2.76–2.90 kg were used. They were fed with a standard diet (oats 50 g., carrot 100 g., and cabbage 200 g.) and were kept separately in metabolism cage designed to permit separate collection of urine and feces. Thymol filled in a capsule was administered orally in a dose of 0.5 g/kg. body weight.

Preparation of Standard Substances

Thymol: This substance was purchased from Wako Junyaku Kogyo Co..

Thymoquinone: This substance was purchased from Tokyo Kasei Kogyo Co..

Thymohydroquinone: This substance was synthesized by usual method. mp 141°–142°C.

Thymol glucuronide (Bollenback et al., 1955): To 15 g of thymol, 15 g of methyl (tri-O-acetyl- α -D-glucopyranosyl bromide) uronate and 0.1 g of p-toluene sulfonic acid were added, and heated at 100°C for 2 hours under reduced pressure. The obtained red-brown oily substance was dissolved in 200 ml of benzene and washed with 2N-KOH followed by water. The benzene layer was then dried, filtered and concentrated to a crystalline mass. Recrystallization from hot isopropyl ether gave 3.8 g of white fine needles. mp 101°–103°C.

IR: $\nu_{\text{max}}^{\text{nujol}}$ cm^{-1} : 1240, 1762 (CO).

$[\alpha]_{\text{D}}^{20} = -32.0^{\circ}\text{C}$ (C=0.4 in CHCl_3).

Anal. Calcd. for $\text{C}_{23}\text{H}_{30}\text{O}_{10}$: C, 59.22; H, 6.48; Found: C, 59.10; H, 6.32. This substance was certified to be methyl (2-isopropyl-5-methylphenyl-tri-O-acetyl- β -D-glucopyranosid) uronate.

Isolation of Thymol Glucuronide from Rabbit Urine (Aoki, 1962., Kamil et al., 1951)

Thymol (0.5 g/kg) was orally given to three rabbits (body weight 2.76–2.90 kg) and 24 hours urine specimens were collected and filtered. The filtrate was adjusted to pH 2 with 3N HCl and continuously extracted with Et_2O for 60 hours. The Et_2O extract was evaporated to dryness under reduced pressure. The resulting yellow-brown syrup was dissolved in 20 ml of H_2O and filtered. The filtrate was again evaporated to dryness under reduced pressure below 40°C. The residues were dissolved in a mixture of 90 ml of Et_2O and 10 ml of MeOH, and poured into 500 ml of petroleum ether (bp 40°–60°C) to precipitate glucuronide. The obtained red-brown precipitates were dissolved in 50 ml of MeOH and 30 ml of CH_2N_2 - Et_2O solution was added to the MeOH solution. The reaction mixture was restored in a refrigerator overnight. The solvent of the reaction mixture was removed by evaporation under reduced pressure and the residues were dissolved in 10 ml of pyridine and 10 ml of Ac_2O . After standing at room temperature for 2 days, the reaction mixture was poured into 500 ml of ice water. The obtained precipitates were dissolved in 100 ml of Et_2O and washed with 100 ml of 2N-KOH, followed by 2N- H_2SO_4 and H_2O . The solution was evaporated to dryness under reduced

pressure. The crude brown residues were dissolved in a small amount of isopropyl ether. After several recrystallizations, fine white needles were obtained. Yield 1.2 g. mp 100°–103°C

IR: $\nu_{\text{max}}^{\text{nujol}}$ cm^{-1} : 1240, 1762 (CO).

$[\alpha]_{\text{D}}^{20} = -36.0^{\circ}\text{C}$ (C=0.5 in CHCl_3)

Anal. Calcd. for $\text{C}_{23}\text{H}_{30}\text{O}_{10}$: C, 59.22; H, 6.48; Found: C, 59.10; H, 6.26. This substance was certified to be methyl (2-isopropyl-5-methylphenyl-tri-O-acetyl- β -D-glucopyranosid) uronate.

Analytical Method

Glucuronic acid: Total glucuronic acid was measured by the naphthoresorcinol picrate method (Ishidate and Nambara 1957).

Ethereal sulfuric acid: This was determined by Fiske's modification of benzidine method.

Gas Chromatography

The instrument used for this work was Shimadzu Model GC-4CM (PF) type gas chromatograph equipped with a hydrogen flame ionization detector. The column packing was Fluoxylate K on chromosorb. The column temperature was maintained at 150° and 200°C. The flow rate of N_2 was 50 ml/min, H_2 was 50 ml/min, and air was 1 l/min.

Thin-layer Chromatography

The samples were applied to Kieselgel HF plates (0.5 mm in thickness, activated at 110°C for 1 hour), and were developed with the solvent systems of I: $\text{PrOH-NH}_4\text{OH-H}_2\text{O}$ (6:2:1), II: $\text{CHCl}_3\text{-CH}_3\text{COOEt-HCOOH}$ (5:4:1). The plates were dried, checked by illumination from ultraviolet rays, and sprayed with the following detecting reagents: (1) Naphthoresorcinol picrate reagent: 1% naphthoresorcinol picrate in EtOH to which an equal volume of 20% H_2SO_4 was added before spraying. The sprayed plates were heated at 110°C for 1 hour. (2) Phosphomolybdic acid reagent: 5% phosphomolybdic acid in MeOH. The sprayed plates were heated at 80°C for 10 minutes. (3) Gibbs' reagent: 0.1% 2, 6-dibromo-N-chloro-P-benzoquinoneimine in EtOH. After spraying the reagent, veronal buffer was sprayed.

Enzymatic Hydrolysis of Thymol Glucuronide in Urine.

Thymol medicated human urine (0.6 g/body, orally) was treated by the procedure demonstrated in Chart. 1. The obtained [M-3] fraction was applied to enzymatic hydrolysis. Specimen from the fraction was dissolved in 20 ml of H_2O . To the solution, 15 ml of 0.1 M acetate buffer (pH 5.0) and 1 ml of β -glucuronidase-arylsulfatase solution (Boehringer, West-Germany) were added, and incubated at 37°C for 24 hours. After incubation, the reaction mixture was extracted with 20 ml of $\text{C}_2\text{H}_4\text{Cl}_2$ to obtain [M-5] fraction. The fraction was evaporated to dryness and the residues were dissolved in a small amount of MeOH and applied to thin-layer chromatography and gas chromato-

graphy.

RESULTS AND DISCUSSION

Metabolic Fate of Thymol in Rabbit

Table 1 shows a 72 hours output of glucuronic acid and ethereal sulfuric acid in the urine of three rabbits taken thymol orally. As shown in the table, excretion of glucuronic acid and ethereal sulfuric acid after the administration of thymol has markedly increased for 24 hours. Particularly, increase of glucuronic acid remarkably exceeds that of ethereal sulfuric acid in 24 hours output. From this data, it is evident that the main metabolite of thymol in rabbit may be glucuronic acid conjugate of thymol or its oxidized product.

Isolation of the glucuronic acid conjugate of thymol from rabbit urine was undertaken by the usual method. Methylation by diazomethane and successive acetylation by acetic anhydride and pyridine of the glucuronide fraction resulted in the isolation of a purified crystalline substance. Thymol glucuronide was chemically synthesized from thymol and methyl (tri-O-acetyl- α -D-glucopyranosyl bromide) uronate as triacetyl derivative of methyl glucuronate. The infrared spectrum, melting point and elemental analysis of the isolated compound were in good agreement with those of the synthesized glucuronide. From this fact, the isolated compound was confirmed to be thymol glucuronide which the aglycone is intact.

Metabolic Fate of Thymol in Man

It is very interesting that, any oxidized product of thymol may be excreted into thymol medicated urine as conjugated or unconjugated form. Two volunteers were

Table. 1. Glucuronic Acid and Ethereal Sulfuric Acid excreted in Rabbit
Urine before and after Administration of Thymol¹⁾

Rabbit ²⁾		Urine				
		Control	Control	24 hr	48 hr	72 hr
Glucuronic acid	A	42.0	61.5	181.6	93.3	64.5
	B	21.2	64.8	186.3	75.2	35.6
	C	61.6	39.4	183.0	45.8	29.4
	average $\pm$ S.D. ³⁾	41.6 $\pm$ 15.4	55.2 $\pm$ 11.2	183.6 $\pm$ 2.0	71.4 $\pm$ 19.5	43.2 $\pm$ 15.2
Ethereal sulfuric acid	A	8.0	22.4	40.0	26.8	12.8
	B	8.0	28.0	81.6	35.2	16.0
	C	8.0	9.6	73.6	6.4	3.2
	average $\pm$ S.D. ³⁾	8.0	20.0 $\pm$ 7.7	65.1 $\pm$ 18.0	22.8 $\pm$ 12.0	10.7 $\pm$ 5.9

1) Results are expressed mg excreted for 24 hours.

2) A (body Weight, 2.76kg), B (body weight, 2.89kg), and C (body weight 2.90kg) indicate the each rabbit which took 0.5g/body of thymol orally.

3) Standard deviation.

Fig. 1. Gas Chromatogram of the Urinary Extracts
 Chart-A : [M-1] Fraction
 Chart-B : [M-2] Fraction
 1, thymol ; 2, thymohydroquinone.

medicated thymol (0.6 g/body) and their 24 hours urine was collected. The urine was fractionated by the procedure demonstrated in Chart. 1 and the each fraction was applied to chromatographic analysis. The main results are shown in Fig. 1 and Fig. 2.

Usually, free thymol and any unconjugated oxidized product of thymol should be fractionated in [M-1] fraction, glucuronide in [M-2] and [M-3] fractions, and aglycone in [M-5] fraction. As shown in Fig. 1, [M-1] fraction which was extracted from urine at pH 5.7, contained only small amount of thymol as an unconjugated form. As glucuronic acid conjugates and ethereal sulfuric acid conjugates of phenolic compounds are usually stable at pH 5.7, the detected thymol in the [M-1] fraction was confirmed to be thymol excreted as an unconjugated form. [M-2] fraction contained large amount of thymol, and small amount of thymohydroquinone which is a hydroxylated product of thymol, as shown in Fig. 1. Ethereal sulfuric acid conjugates of phenolic compounds are usually unstable at pH 2 and liberate the free phenolic compounds, while glucuronic acid conjugates of phenolic compounds remains unchanged at pH 2.

Therefore, the origin of thymol detected in [M-2] fraction was confirmed to be

Detoxication of thymol in rabbit and man.

thymol liberated from ethereal sulfuric acid conjugate.

Concerning thymohydroquinone, it has been proved that free thymohydroquinone added to controlled urine is extracted into ether at pH 5.7. Judging from the fact that thymohydroquinone was fractionated in [M-2] fraction in this experiment, it came to the conclusion that the detected thymohydroquinone may be originated from ethereal sulfuric acid conjugate.

[M-3] fraction was applied to thin-layer chromatography in order to examine what kinds of glucuronides are formed in thymol medicated urine. The result is shown in Fig. 2. All spots shown in Fig. 2 were detected by illumination from ultraviolet rays. Spot B and C in Solvent-I, and Spot B in Solvent-II were positive in naphthoresorcinol picrate reagent for glucuronides. Spot A, B and C in Solvent-I, Spot A and B in Solvent-II were respectively scraped from the thin-layer plates and eluted. The each elute was incubated with β -glucuronidase, and the hydrolysate were extracted with $C_2H_4Cl_2$. The extracts were applied to gas chromatographic analysis. Only the extract from Spot B in the both solvent systems contained thymol and none of thymol and its oxidized product was detected from the other extracts. This result indicates that only thymol is conjugated with glucuronic acid to form thymol glucuronide.

In conclusion, in man, thymol may be metabolized to thymol glucuronide, thymol sulfate and thymohydroquinone sulfate. Formation of thymohydroquinone sulfate may

Fig. 2. Thin-Layer Chromatograms of Thymol, Its Expected Metabolite, and the Urinary Extracts with Various Solvent Systems.

(1) Solvent Systems.

I : CHCl_3 - $\text{CH}_3\text{COO Et}$ - HCOOH (5 : 4 : 1)

II : PrOH - NH_4OH - H_2O (6 : 2 : 1)

(2) Standard Substances and Urinary Extracts.

T : Thymol

TH : Thymohydroquinone

M-3 : Thymol dosed urine extract [M-3] fraction

be very small amount as compared with thymol glucuronide and thymol sulfate formation, although any quantitative determination of urinary excrements did not undertaken in the present investigation. Very small amount of unchanged thymol was excreted into urine.

The thymohydroquinone formation in man is very interesting in its correlation with the metabolic fate of 4-iodothymol, which has been reported with the detection of very

Detoxication of thymol in rabbit and man,
small amount of thymoquinone in human urine (Takada 1978).

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