Notes

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Hisao Tsukamoto, Hidetoshi Yoshimura, and Satoshi Toki : Metabolism of Drugs. XVIII.¹⁾ The Metabolic Fate of Methylhexabital (5-Cyclohexenyl-3,5-dimethylbarbituric Acid). (8).¹⁾ The Quantitative Determination of Main Biotransformation Products of Methylhexabital in the Urine of Rabbits by Ultraviolet Spectrophotometry.

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The latest work¹⁾ in this series has shown that ureide [1-(2-cyclohexenylpropionyl-3-methylurea)], 3-OH-MHB** (5-(3-hydroxy-1-cyclohexenyl)-3,5-dimethylbarbituric acid), 3-keto-MHB [5-(3-oxo-1-cyclohexenyl)-3,5-dimethylbarbituric acid] and 3-keto-nor-MHB, [5-(3-oxo-1-cyclohexenyl)-5-methylbarbituric acid] were separated and identified by buffered paper chromatography from the urine of rabbits administered methylhexabital (MHB, 5-cyclohexenyl-3,5-dimethylbarbituric acid).

A method of estimating a small amount of urinary metabolites of MHB was required during the course of an investigation on the metabolism of MHB.

This paper describes a method for the simultaneous determination of three main metabolites (3-OH-MHB, 3-keto-MHB, and 3-keto-nor-MHB) in rabbit urine by the use of buffered paper chromatography in conjunction with ultraviolet spectrophotometry, and presents a relative concentration of each metabolite in the 24-hour urine.

This procedure was essentially the same as those described in the previous paper²⁾ about the excretion rate of metabolites of ethylhexabital (5-cyclohexenyl-5-ethylbarbituric acid). The estimation of the concentration of ureide and MHB-M (VI) was not made, because the former had no absorption peak and structure of the latter was not clarified as yet.

Materials and Methods***

MHB (m.p. 142~143°) was supplied by Dainippon Seiyaku Co. Ltd., 3-keto-MHB (m.p. 160~161°) and 3-keto-nor-MHB (m.p. 215~216°(decomp.)) were prepared by the oxidation³⁾ of MHB and nor-MHB, respectively. 3-OH-MHB was obtained^{4,5)} from the urine of rabbits. The extinction measurements were made with a Hitachi photoelectric spectrophotometer with standard 10-mm² quartz absorption cell.

In borate-NaOH buffer (pH 11) solution, the extinction at 238, 240, and 244 mμ was directly proportional to the concentration of 3-keto-nor-MHB, 3-keto-MHB, or 3-OH-MHB, in accordance with the Lambert-Beer law (Fig. 1).

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** As shown in previous study 3-OH-MHB is composed from two diastereoisomeric α- and β-3-OH-MHB, and both exhibit the same R_f value and identical ultraviolet absorption spectra.

*** All melting points are uncorrected.

1) Part XVII : H. Tsukamoto, H. Yoshimura, S. Toki : This Bulletin, 6, 15(1958).

2) H. Tsukamoto, E. Takabatake, T. Ariyoshi : *Ibid.*, 3, 459(1955).

3) H. Tsukamoto, H. Yoshimura, S. Toki : *Ibid.*, 4, 363(1956)

4) *Idem.* : *Ibid.*, 4, 368(1956).

5) H. Yoshimura : *Ibid.*, 5, 561(1957)

Fig 1. Relation between Extinction at Max Absorption and Concentration of 3-Keto-nor-MHB, 3-Keto-MHB, or 3-OH-MHB

Fig. 2. Chromatogram of MHB Metabolites in Urine

Therefore, on the basis of spectrophotometric data, the concentration of the metabolites can easily be determined.

MHB was given orally by stomach tube to male rabbits after fasting for 24 hrs. as freshly prepared aq. solution containing 1.1 equiv. NaOH in a dose of 100, 200, and 300 mg./kg. body weight. These doses were given to three rabbits alternately, every 7 days. The urine was collected by catheter 24 hrs. later, and the urine excreted already in the cage was collected into a bottle. These were combined and filtered through a cotton.

Determination of metabolite in urine—In a glass-stoppered tube were placed 1.0 cc. of urine, 0.5 cc. of H_2SO_4 (0.85~6.0%; adjusting pH of medium to 3.0), and 12.5 cc. of AcOEt. After shaking the mixture for 10 mins., 10 cc. of the AcOEt phase was pipetted out and evaporated to dryness. The residue was dissolved in 0.5~1.0 cc. of MeOH and a portion of this solution was applied on a filter paper. A quantity of 20~50 γ of barbiturates in a volume of 0.05~0.1 cc. was suitable for chromatography. Samples were subjected to ascending chromatography for 16~18 hrs. on Toyo Roshi No. 50 filter paper (2×40 cm.) which was previously treated with borate-NaOH buffer (pH 11), using BuOH saturated with borate-NaOH (pH 11) as the solvent. After development the strips were removed and air-dried. One of them was sprayed with 1% aq. solution of $NaIO_4$ and 1% $KMnO_4$ solution, then the other strips were cut into sections at approximate R_f values corresponding to each spot (Fig. 2).

In all, three sections each with R_f 0.05~0.20, 0.30~0.375, and 0.375~0.525 were cut out for 3-keto-nor-MHB, 3-keto-MHB, and 3-OH-MHB, respectively. These sections were eluted with 5.0 cc. of borate-NaOH buffer (pH 11) and the extinction was measured at each wave length of maximum optical density; 3-keto-nor-MHB at 238 $m\mu$, 3-keto-MHB at 240 $m\mu$, and 3-OH-MHB at 244 $m\mu$. For the blank, 24-hr. urine collected just before the administration of MHB was examined in the same manner as the sample.

Results and Discussion

When known amounts of a mixture of authentic 3-keto-nor-MHB, 3-keto-MHB, and 3-OH-MHB were added to the urine of rabbits, extracted, and estimated according to the method described, recovery rate was 87% for 3-keto-nor-MHB and 3-OH-MHB and 85% for 3-keto-MHB.

Bush, *et al.*⁶⁾ reported that after administration of MHB to dogs, only a trace of unchanged material and of nor-MHB, a small amount of keto-MHB I and II, and 5~6% of 3-keto-nor-MHB were isolated in the urine.

Previously,^{4,5)} we isolated six metabolites from the urine of rabbits receiving MHB. The yield of these products as pure crystals was 0.2~0.5% (3-keto-nor-MHB), 1.0~2.0% (3-keto-MHB), 4.0~5.0% (α -3-OH-MHB), 1.9~2.5% (β -3-OH-MHB), 0.2~0.3% (ureide), and 0.3~0.5% (MHB-M(VI))(total 8.9~9.5%).

Now, as shown in Table I, the present method gave a more precise results concerning the yield of the three main metabolites. From these results it was clear that the major product is 3-OH-MHB, and that 3-keto-nor-MHB and 3-keto-MHB are excreted almost equally. There was scarcely any marked difference between respective yield and dosage, and as for the total quantity of excreted metabolites, the difference depending on dosage was not significant.

A small quantities of ureide and MHB-M(VI) would be added to the total yield described above but the fate of remainder was undetectable.

A number of ultraviolet spectrophotometric procedures⁷⁾ have been developed for the determination of barbiturates in the biological fluids and organs, but those were difficult to simultaneously determine the excreted compounds which contained barbituric acid ring.

TABLE I. Yield of 3-Keto-nor-MHB, 3-Keto-MHB, and 3-OH-MHB in the 24-hr. Urine of Rabbits Administered MHB

Dose (mg./kg.)		100				200				300			
Rabbit No.		A	B	C	Average	A	B	C	Average	A	B	C	Average
Wt. (g.)		2280	2050	2310		2258	2352	2504		2325	2340	2390	
Exptl. date(1957)		July	May	June		June	July	July		July	June	July	
		2	28	25		25	2	9		9	25	2	
Urine Vol. (cc.)		125	124	175		87	158	194		142	81	203	
Metabolite													
3-Keto-nor-MHB	Concn. (γ /cc.)	87.5	76.6	37.5		143.8	137.5	137.5		224.0	350.0	162.5	
	Amt. excreted (mg.)	12.5	10.8	7.5		14.4	24.9	30.7		36.6	33.3	37.9	
	Yield(%)	5.4	5.3	3.3	4.7	3.2	5.3	6.1	4.9	5.2	4.8	5.3	5.1
3-Keto-MHB	Concn. (γ /cc.)	90.6	67.2	68.8		234.4	62.5	93.8		168.8	225.0	131.3	
	Amt. excreted (mg.)	13.3	9.8	14.2		24.0	11.6	21.4		28.2	21.4	31.4	
	Yield(%)	5.8	4.8	6.2	5.6	5.3	2.5	4.3	4.0	4.0	3.2	4.4	3.9
3-OH-MHB	Concn. (γ /cc.)	278.1	236.0	190.6		900.0	406.3	321.9		681.3	1331.3	387.5	
	Amt. excreted (mg.)	40.0	33.9	38.2		90.0	73.8	71.7		111.2	123.0	90.4	
	Yield(%)	17.4	16.7	16.6	16.9	20.0	15.7	14.3	16.7	15.8	17.6	12.6	15.3
Total yield(%)		28.6	26.8	26.1	27.2	28.5	23.5	24.7	25.6	25.0	25.6	22.3	24.3

6) M. T. Bush, T. C. Butler, H. L. Dickinson : J. Pharmacol. Exptl. Therap., **108**, 104(1953).

7) e.g. L. R. Goldbaum : Anal. Chem., **24**, 1604(1952); S. Goldschmidt, W. Lamprecht, E. Helmreich : Z. physiol. Chem., **292**, 125(1953).

The present procedure based on separation of urinary products by paper chromatography, elution of each section, and estimation by ultraviolet spectrophotometry is able to simultaneously determine the three metabolites in the urine of rabbits receiving MHB.

Bush, *et al*⁽⁶⁾, suggested that keto-nor-MHB is produced by the demethylation of previously oxidized MHB. Although from our experiment it was clear that the amount of the hydroxylation product of MHB is predominant, the pathway and the mechanism of degradation of MHB will be clarified by further studies on the interconversion of these metabolites, and for these purpose, our quantitative method will be expedient.

Summary

Method of simultaneous determination of three main metabolites of MHB in urine by paper chromatography in conjunction with ultraviolet spectrophotometry was described.

Approximately 25.7% of MHB administered was converted to 3-keto-nor-MHB (4.9%), 3-keto-MHB (4.5%), and 3-OH-MHB (16.3%), and excreted in amounts independent of the dosage administered.

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