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Studies on the Metabolic Fate and the Pharmacokinetics of 5-*n*-Butyl-1-cyclohexyl-2,4,6-trioxoperhydropyrimidine (BCP) in Man. III.[1] Gas-Liquid Chromatographic Determination of BCP and Its Metabolites[2]

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An investigation was carried out on the gas-liquid chromatographic determination of 5-*n*-butyl-1-cyclohexgl-2,4,6-trioxoperhydropyrimidine(BCP) and its metabolites, *i.e.*, 1-cyclohexyl-5-(3-hydroxybutyl)-2,4,6-trioxoperhydropyrimidine (II) and 5-*n*-butyl-1-(4-hydroxycyclohexyl)-2,4,6-trioxoperhydropyrimidine (IV) in human urines.

Neopentyl glycol succinate (NGS) 2% on Gas Chrom P was selected as the liquid stationary phase and a hydrogen flame ionization detector was employed.

By trimethylsilylation of the chloroform extract from a urine, BCP and IV were determined simultaneously with phenobarbital as the internal standard, for IV was subjected trimethylsilylation, while BCP was not affected by the treatment.

The metabolite II was determined selectively by heating it in an acid medium to convert into 3-cyclohexyl-7-methyl-1,2,3,4,6,7-hexahydro-5-*H*-pyrano (2,3-*d*) pyrimidine-2,4-dione (XV). Cyclobarbital or phenylbutazone was used as the internal standard in this case.

As described in the previous paper,[4] when 5-*n*-butyl-1-cyclohexyl-2,4,6-trioxoperhydropyrimidine (BCP) was administered to man, 1-cyclohexyl-5-(3-hydroxybutyl)-2,4,6-trioxoperhydropyrimidine (II) and 5-*n*-butyl-1-(4-hydroxycyclohexyl)-2,4,6-trioxoperhydropyrimidine (IV) were found as the metabolites in the urine besides unchanged BCP and two unknown substances.

The determination-procedures of the metabolites by ultraviolet (UV) absorption method were investigated to find II was determined by converting it to 3-cyclohexyl-7-methyl-1,2,3,4,6,7-hexahydro-5-*H*-pyrano (2,3-*d*) pyrimidine-2,4-dione (XV), whereas IV was not determined as the separation from the unknown substances was not successful.[1]

In this paper, an investigation was carried out on the gas–liquid chromatographic determination of B C P, II and IV.

Generally, for determination of metabolites in biological fluids or urines, selectivity and high sensitivity are required for the analytical method. Gas–liquid chromatography (GLC) fits these requirements and has been applied to the determination of barbiturates in biological fluids or urines.[5–9]

For the identification of the metabolites of BCP by GLC,[4] neopentyl glycol succinate (NGS) was selected as the liquid phase and by trimethylsilylation[10] on the chloroform

1) Part II: T. Yashiki, T. Kondo, Y. Uda, and H. Mima, *Chem. Pharm. Bull.* (Tokyo), **19**, 478 (1971).
2) This work was presented at the Meeting of Kinki Branch, Pharmaceutical Society of Japan, Kyoto, March 1969.
3) Location: *Higashiyodogawa-ku, Osaka.*
4) T. Yashiki, T. Matsuzawa, T. Kondo, Y. Uda, T. Shima, H. Mima, S. Senda, and H. Izumi, *Chem. Pharm. Bull.* (Tokyo), **19**, 468 (1971).
5) E. Brochmann-Hanssen and A.B. Svendsen, *J. Pharm. Sci.*, **51**, 318 (1962).
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9) S.K. Niyogi, V.F. Cordova, and F. Rieders, *Nature*, **206**, 716 (1965).
10) *cf.* T. Uno and H. Okuda, *Yakugaku Zasshi*, **86**, 1148 (1966); M. Yamamoto, S. Iguchi, and T. Aoyama, *Chem. Pharm. Bull.* (Tokyo), **15**, 123 (1967).

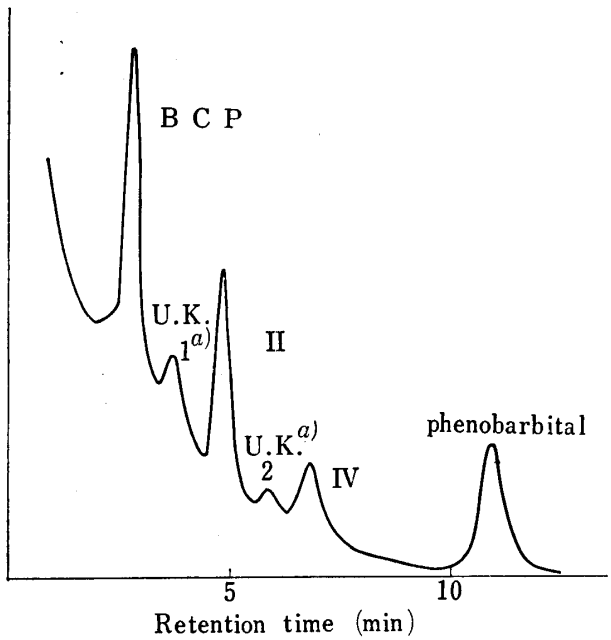

Fig. 1. Gas Chromatogram of Trimethylsilyl Derivatives of the Chloroform Extract from a Urine

NGS 2% on Gas Chrom P (60—80 mesh)
glass column : length; 50 cm, i.d.; 3 mm
temperatures: inlet 270°, column 210°, detector 250°
detection : FID
carrier gas : He
instrument : Hitachi KGL-2B.
a) The peaks of the unknown 1 and 2.
II: 1-cyclohexyl-5-(3-hydroxybutyl)-2,4,6-trioxoperhydropyrimmidine
IV: 5-*n*-butyl-1-(4-hydroxycyclohexyl)-2,4,6-trioxoperhydropyrimidine

($CHCl_3$) extract from a urine, the peaks of BCP, II and IV were separated well (Fig. 1). This method was developed for the quantitative determination of them.

Experimental

Conditions for GLC——An instrument, Hitachi KGL-2B with a hydrogen flame ionization detector (FID) was used. A glass column (75—100 cm in length, 3 mm i. d.) was packed with NGS 2% coated Gas Chrom P (60—80 mesh). Helium gas was used as the carrier (100 ml/min) and the operation-temperatures were 270° at inlet, 210° at column and 250° at detector. When the column temperature exceeded 210°, a fraction of II was converted to XV during GLC[4]. The sample volume was usually 1 μl. For fractionation of a composition, a preparative gas chromatograph, Aerograph (Varian Associates) was used.

Internal Standards——Phenobarbital (t_R 10.9 min) was selected as the internal standard[11] for the determination of BCP and IV. Though XV converted from II showed the peak at the same t_R, the formation was negligible in the conditions. Phenobarbital was free from trimethylsilylation. For the determination of II as XV, either cyclobarbital (t_R 5.8 min) or phenylbutazone (t_R 6.2 min) was used.

Trimethylsilylation of IV——To find the volume of hexamethyldisilazane (HMDS) required, 0.1—0.5 ml of HMDS were added to a $CHCl_3$ solution of IV (2.18 mg/ml) with 2 μl of trimethylchlorosilane (TMCS) and 5 μl of pyridine and the solution was kept at room temperature for 60 min. To determine the reaction period, the reaction was continued for 10—90 min after the addition of 0.2 ml of HMDS. The addition of pyridine increased the reaction rate. The trimethylsilyl (TMSi) derivative of IV was stable for at least 60 min, however, as the derivatives were generally known unstable to moisture,[11] dehydration of the $CHCl_3$ extract was carried out with Na_2SO_4 and the samples were subjected to GLC within 30 min after the treatment.

Result and Discussion

I. Investigation on the Determination of BCP, II and IV

Although BCP was extracted quantitatively with heptane from an acidified urine,[1] $CHCl_3$ was used to extract it with the metabolites simultaneously. In GLC, BCP showed the peak at the retention time (t_R) 2.8 min and the content was determined by the peak area. The peak of 5-*n*-butyl-5-hydroxy-1-cyclohexyl-2,4,6-trioxoperhydropyrimidine (I) which might be produced from BCP during the extraction or GLC, was actually negligible in the conditions.[4]

As BCP was not affected by trimethylsilylation, it was determined along with IV simultaneously after the treatment on the $CHCl_3$ extract. The lower limit of sensitivity was 0.1 μg of BCP

11) S. Sato and N. Ikegawa, "Organic Analysis," ed. by K. Tsuda, Sangyo Tosho Pub. Co. Ltd., Tokyo, 1965, p. 149.

The metabolite IV showed the peak at t_R 6.9 min after trimethylsilylation on the $CHCl_3$ extract and was determined by this peak with phenobarbital as the internal standard. Investigations on the conditions for trimethylsilylation and on the stability of the TMSi derivative of IV showed that the addition of 0.2 ml of hexamethyldisilazane (HMDS), 2 μl of trimethylchlorosilane (TMCS), 5 μl of pyridine and keeping the solution at room temperature for 30 min were enough for the reaction and the product was stable at least for 60 min. The lower limit of sensitivity of IV was 0.3 μg.

The TMSi derivatives of the $CHCl_3$ extract showed a peak at t_R 4.8 min which was coincident with the peak of the same derivative of the authentic standard of II. To confirm the assignment, the composition of the peak was fractionated to find that the infrared (IR) spectrum was coincident with that of XV[4] (Fig. 2). Similar fractionation of the TMSi derivative of the authentic standard of II also gave the IR spectrum of XV.

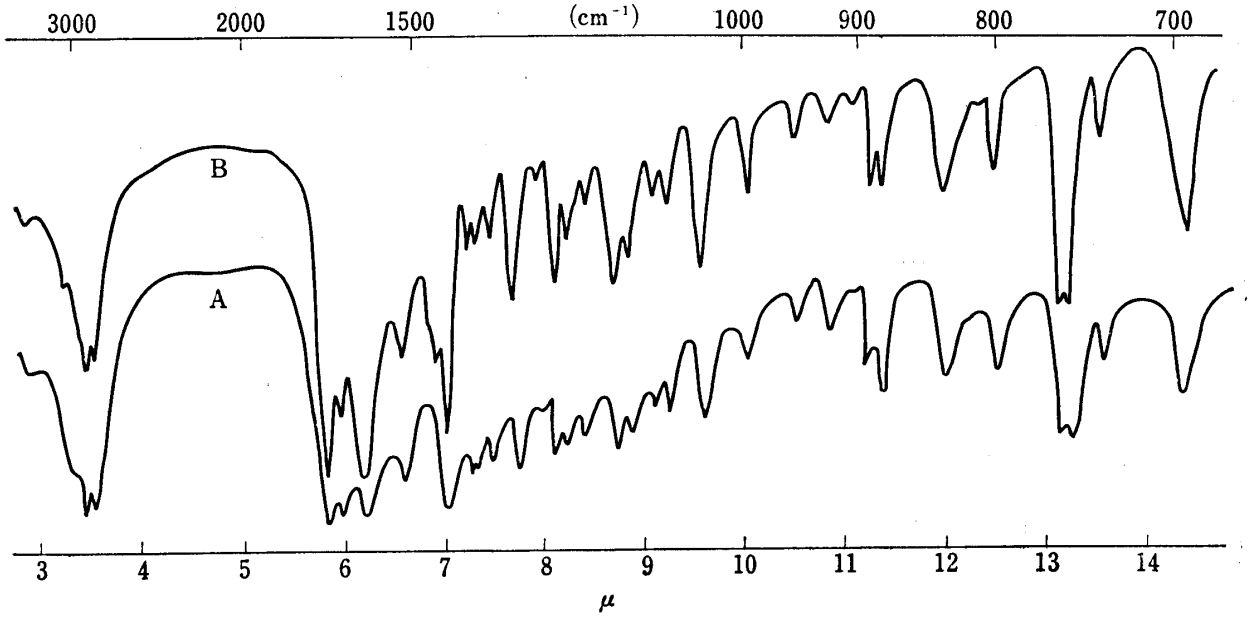

Fig. 2. IR Absorption Spectra of XV and the Substance Fractionated from the Peak of Trimethylsilyl Derivative of II in the Chloroform Extract (KBr Disk)

A: fractionated substance B: authentic standard of XV
II: 1-cyclohexyl-5-(3-hydroxybutyl)-2,4,6-trioxoperhydropyrimidine
XV: 3-cyclohexyl-7-methyl-1,2,3,4,6,7-hexahydro-5-*H*-pyrano (2,3-*d*) pyrimidine-2,4-dione

On the other hand, XV (mp 287°) showed a peak at t_R 10.9 min and the peak was not affected by trimethylsilylation. Moreover, in the search for the urinary metabolites of BCP, an intact $CHCl_3$ extract was subjected to GLC and the composition of a peak whose t_R corresponded to that of XV was fractionated. The substance was recognized as XV by the IR spectrum, though XV was not the metabolite but an artifact converted from II.[4]

These facts supported the assignment of the peak at t_R 4.8 min to the metabolite II and the conversion might occur by the heat to prevent the condensation of a gas for fractionation after the vapor of II passed the detector.

Preliminary determination of II by the peak at t_R 4.8 min revealed that fairly consistent determination-values were obtained with those obtained by UV absorption method when a blank urine was fortified with the authentic samples of II or a urine collected at small dose such as 300 mg of BCP was used as the sample. However, when a urine collected at large dose such as 900 mg, especially collected in 4—10 hours after the administration was used as the sample, larger values were obtained than those by UV absorption method. This might be due to the tailing of the peaks of BCP and unknown 1 or partially to the increase of the base line near the peak of II when the concentration of the drug was high in a body.

Therefore, a reaction that II was converted to XV by heating it in an acid medium[4,12] was also applied to determine it more selectively, *i.e.*, the $CHCl_3$ extract from a urine was transferred to 0.1N sodium hydroxide (NaOH) solution, which was acidified and heated. After cooling, XV was extracted quantitatively with $CHCl_3$ and the solution was subjected to GLC without pre-treatment. From the peak of XV, the content of the original II was determined (Fig. 3).

Although the conditions for the conversion of II to XV were already investigated in the UV absorption method,[1] re-examination revealed that heating the extract at 100° for 30 min in 3N hydrochloric acid (HCl) was enough for the reaction. Over the acidity of 4N HCl, side reactions occurred and another peak appeared to interfere the determination of XV. The compound XV was stable at least for 60 min and the lower limit of sensitivity was 0.5 μg of XV.

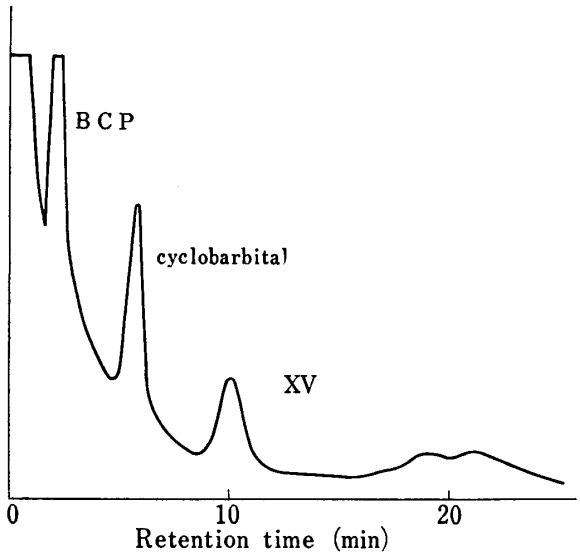

Fig. 3. Gas Chromatogram for the Determination of the Metabolite II as XV.

internal standard: cyclobarbital
II: 1-cyclohexyl-5-(3-hydroxybutyl)-2,4,6-trioxoperhydropyrimidine
XV: 3-cyclohexyl-7-methyl-1,2,3,4,6,7-hexahydro-5-*H*-pyrano (2,3-*d*)pyrimidine-2,4-dione

II. Determination-Procedures for Total BCP, II and IV

The determination-procedures by GLC are as follows.

Determination of Total BCP and IV——To 15—30 ml of a urine, is added equal volume of 6N HCl and they are extracted with 150—300 ml of $CHCl_3$ by shaking it for 25 min. If necessary, the $CHCl_3$ extract is washed with 30—60 ml of aqueous solution of 50% zinc chloride.[1] After dehydrated with sodium sulfate, the extract is evaporated *in vacuo* below 40°. The residue is dissolved in 0.5—1.0 ml of $CHCl_3$ and trimethylsilylation is made with 0.5 ml of HMDS, 2 μl of TMCS, 5 μl of pyridine and by keeping the solution at room temperature for 30 min. Then the solvent is evaporated with nitrogen gas and the $CHCl_3$ solution of phenobarbital is added. After GLC, the contents of BCP and IV are determined by the relative peak areas. To correct the conditions of GLC, the authentic samples are added to a blank urine and the recoveries should be measured similarly.

Determination of II——The $CHCl_3$ extract is evaporated *in vacuo* below 40° and the residue is dissolved in 20 ml of 0.1N NaOH to which is added equal volume of 6N HCl and heated at 100° for 30 min. After cooling, the solution is extracted twice with 300 ml of $CHCl_3$ and the solvent is evaporated again *in vacuo* below 40°. Addition of 0.5—1.0 ml of $CHCl_3$ solution of cyclobarbital or phenylbutazone makes a sample. After GLC, the content of II is determined as XV by the relative peak area.

III. Calibration Lines

Authentic samples of BCP, II and IV were dissolved in a blank urine at various concentrations and determined according to the standard procedures.

The relations between relative sample weight (X) and the relative peak area (Y) are shown in Table I, II, III for BCP, the metabolite IV as the TMSi derivative and the metabolite II as XV respectively. Fig. 4, 5 also show the same relations.

12) S. Senda and H. Izumi, *Yakugaku Zasshi*, **89**, 266 (1969).

The regression equations obtained are $Y=1.229X-0.030$ for B.C.P., $Y=0.790X-0.063$ for IV with phenobarbital as the internal standard and $Y=0.385X-0.067$(A), $Y=0.804X-0.146$(B) for II as XV with cyclobarbital and phenylbutazone as the internal standard respectively.

Table I. Relation between Relative Sample Weight and Relative Peak Area of BCP to Phenobarbital in the Calibration by GLC

Concn. in blank urine (μg/ml)	5.61	8.10	15.00	18.75
Relative sample weight (X)	0.187	0.270	0.500	0.625
Relative peak area (Y)	0.201	0.295	0.580	0.715
	0.187	0.270	0.620	0.725
	0.231	0.306	0.594	0.755
Mean	0.206	0.290	0.598	0.732
Standard deviation	0.022	0.018	0.020	0.021

$syx=0.021$, relative standard deviation (at $Y=0.5$)$=4.2\%$
syx=stanmdard deviation from the estimated regression line
internal standard: phenobarbital 1.5 mg, blank urine: 50 ml

Table II. Relation between Relative Sample Weight and Relative Peak Area of the Metabolite IV as the Trimethylsilyl Derivative to Phenobarbital in the Calibration by GLC

Concn. in blank urine (μg/ml)	9.72	13.50	18.00	22.50
Relative sample weight (X)	0.324	0.450	0.600	0.750
Relative peak area (Y)	0.165	0.300	0.390	0.510
	0.184	0.313	0.425	0.530
	0.210	0.290	0.415	0.540
Mean	0.186	0.301	0.410	0.527
Standard deviation	0.023	0.011	0.018	0.015

$syx=0.017$, relative standard deviation (at $Y=0.5$)$=3.4\%$
internal standard: phenobarbital 1.5 mg, blank urine: 50 ml
IV: 5-*n*-butyl-1-(4-hydroxycyclohexyl)-2,4,6-trioxoperhydropyrimidine

Table III. Relation between Relative Sample Weight and Relative Peak Area of the Metabolite II as XV to Cyclobarbital in the Calibration by GLC

Concn. in blank urine (μg/ml)	10.6	21.2	31.8	42.4	53.0
Relative sample weight (X)	0.799	1.599	2.398	3.198	3.999
Relative peak area (Y)	0.210	0.606	0.902	1.160	1.486
	0.242	0.530	0.858	1.210	1.420
	0.236	0.544	0.821	1.130	1.500
	0.250	0.566	0.898	1.192	1.400
	0.240	0.526	0.820	1.098	1.562
Mean	0.236	0.554	0.860	1.158	1.474
Standard deviation	0.015	0.033	0.040	0.045	0.065

$syx=0.040$, relative standard deviation (at $Y=1.0$)$=4.0\%$
internal standard: cyclobarbital 0.663 mg
blank urine: 50 ml
II: 1-cyclohexyl-5-(3-hydroxybutyl)-2,4,6-trioxoperhydropyrimidine
XV: 3-cyclohexyl-7-methyl-1,2,3,4,6,7-hexahydro-5-*H*-pyrano(2,3-*d*) pyrimidine-2,4-dione

The relative standard deviations are 4.2% (at $Y=0.5$) for BCP, 3.4% (at $Y=0.5$) for IV and 4.0% (at $Y=1.0$) for II(A).

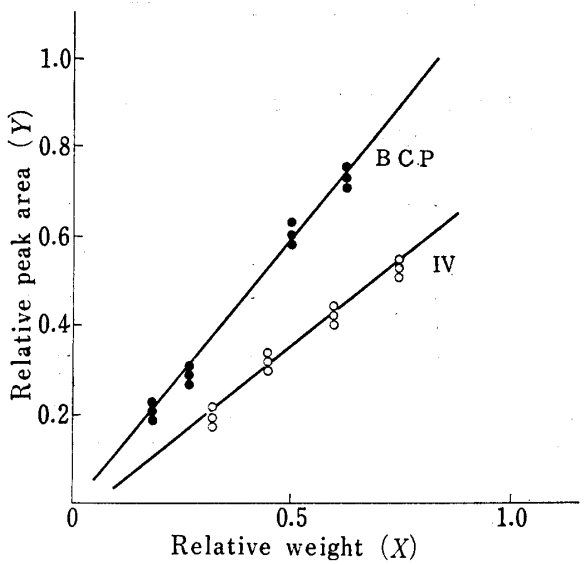

Fig. 4. Calibration Lines of BCP and Trimethylsilyl Derivative of IV with Phenobarbital as the Internal Standard

The regression equations are
$Y=1.229\ X-0.030$ for BCP,
$Y=0.790\ X-0.063$ for IV
IV: 5-*n*-butyl-1-(4-hydroxycyclohexyl)-2,4,6-trioxoperhydropyrimidine

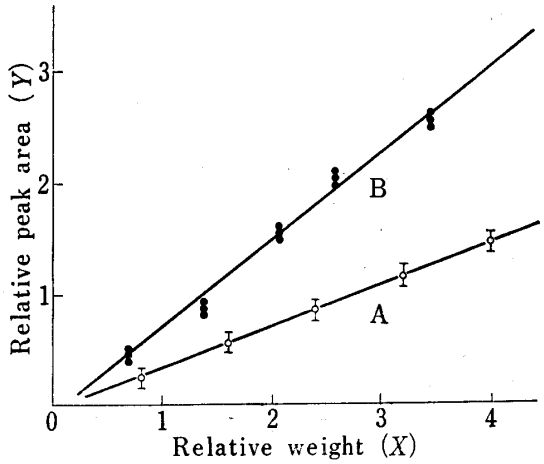

Fig. 5. Calibration Lines of the Metabolite II as XV with Cyclobarbital (A) and Phenylbutazone (B) as the Internal Standards

The regression equations are $Y=0.385\ X$-0.067 for A (○: average, ⊢⊣: standard deviation ×2) and $Y=0.804\ X-0.146$ for B.
II: 1-cyclohexyl-5-(3-hydroxybutyl)-2,4,6-trioxoperhydropyrimidine
XV: 3-cyclohexyl-7-methyl-1,2,3,4,6,7-hexahydro-5-*H*-pyrano(2,3-*d*)pyrimidine-2,4-dione

IV. Determination Values Measured by GLC and UV Absorption Method

To a healthy male, 300 mg of BCP crystals were administered and the urine was collected on which total BCP and its metabolites, II and IV were determined periodically by GLC and also by UV absorption method. The determined values of total BCP and II were compared between the two methods. As shown in Table IV, fairly good coincidences were observed.

Table IV. Amount of BCP, Its Metabolites, II and IV determined by UV Absorption Method and/or Gas-Liquid Chromatography in the Urine after Oral Administration of 300 mg of BCP to a Healthy Male

Time (hr)	BCP (mg) UV[a]	BCP (mg) GLC[a]	II (mg) UV[a]	II (mg) GLC[b]	IV(mg) GLC[a]
2	1.26±0	1.0±0.1	0.96±0.08	—	—
4	4.16±0.01	3.9±0.1	2.57±0.02	2.4±0.3	0.9±0.1
6	3.52±0.02	3.4±0.3	2.20±0	2.2±0.1	1.3±0.2
8.5	3.66±0.04	3.4±0.3	1.84±0.03	2.2±0.3	0.8±0.1
24	16.40±0	16.6±0.4	7.17±0.05	7.1±0.7	3.0±0.1
36	11.60±0	13.8±0.6	7.07±0.05	6.3±0.1	1.6±0.6
48	8.43±0.09	6.1±0.3	4.93±0.26	5.5±0.2	1.1±0
60	5.83±0.04	4.7±0.3	4.56±0.08	5.5±0.3	0.8±0.1
72	5.77±0.07	5.4±0.2	4.33±0.03	4.3±0.2	0.5±0.1
84	3.68±0.02	3.1±0	3.84±0	3.5[c]	0.5±0.1
96	3.13±0.05	2.8±0.1	2.66±0.07	2.2[c]	—

a) average and deviation ($n=2$) Phenobarbital was the internal standard for GLC.
b) average and standard deviation ($n=3$) Phenylbutazone was the internal standard for GLC.
c) Measured once.
—: trace
II: 1-cyclohexyl-5-(3-hydroxybutyl)-2,4,6-trioxoperhydropyrimidine
IV: 5-*n*-butyl-1-(4-hydroxycyclohexyl)-2,4,6-trioxoperhydropyrimidine