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DETERMINATION OF BARBITURATES IN MOUSE TISSUES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Procedures for determining barbiturates in mouse tissues were investigated. High-performance liquid chromatography (HPLC) with mixtures of water and methanol as the mobile phase and μBondapak C_{18} as the stationary phase is superior to gas and thin-layer chromatography with respect to ease of sample preparation, accuracy, sensitivity and time required for analysis.

The first step in the analysis, the extraction of barbiturates from tissues, was also investigated and good recoveries were achieved.

The time courses of barbiturate concentrations in mouse brain, kidneys and liver after oral administration of barbiturate–β-cyclodextrin complex to mice were determined by HPLC using UV detection at 210 nm.

INTRODUCTION

Barbiturates (BA) form stable inclusion complexes with β-cyclodextrin (β-Cyd) in aqueous solution[1–5], and the 1:1 complexes can be isolated as pure microcrystalline powders[3]. It was shown in previous papers[6,7] that β-Cyd is a useful reagent for increasing the solubility of BA, that it hardly interferes with the permeation of BA through everted rat small intestine, and that the improved solubility of BA due to inclusion complexation results in enhancement of the bioavailability of BA in rabbits and mice: the blood level of BA rises faster on oral administration of BA–β-Cyd complex than on that of an equimolar amount of the intact BA in rabbits and, further, the sleeping lag (the time from administration to loss of righting reflex) in mice is shortened and the sleeping time (the time from loss to recovery of righting reflex) is prolonged by complexation. Phenobarbital (Pheno), which forms the most stable complex and consequently shows the greatest increase in solubility, gave the greatest enhancement of the hypnotic potency on complexation with β-Cyd. On the other hand, the hypnotic potency of barbital (Bar), which has a sufficiently high solubility even in the intact form and which forms a less stable complex, was not significantly enhanced by complexation. These results may be explained by assuming that the increase in solubility by complexation accelerates the rate of absorption of BA in the

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stomach, and the complexation of BA with β-Cyd is in competition with the drug–albumin interaction[8] to increase the amount of BA reaching the brain and tends to maintain the brain level of BA for a longer time.

To prove this assumption it was proposed to determine the distribution of BA in mouse tissues on oral administration of BA–β-Cyd complex. This paper describes the determination of BA in mouse tissues by high-performance liquid chromatography (HPLC), gas chromatography (GC) and thin-layer chromatography (TLC).

EXPERIMENTAL

Apparatus

HPLC was conducted with a 6000A solvent delivery system, a U6K injector and a μBondapak C_{18} column (30 cm × 4 mm I.D.), all from Waters Assoc. (Milford, MA, U.S.A.), and a UVIDEC-100 II variable-wavelength detector (Nippon Bunko, Japan), set at 210 nm. In order to prevent contamination of the separation column, a guard column (2.3 cm × 4 mm I.D., Waters Assoc.), filled with μBondapak C_{18}-Corasil, was installed.

A Hitachi K-53 gas chromatograph with a flame-ionization detector (FID) was equipped with either a packed column (2 m × 4 mm I.D.) of 3% OV-17 on Gas-Chrom Q AW-DMCS (80–100 mesh) or a packed column (2 m × 3 mm I.D.) of 1% OV-1 on Gas-Chrom Q AW-DMCS (60–80 mesh).

Thin-layer chromatograms were scanned with a Carl Zeiss chromatogram spectrodensitometer.

Materials

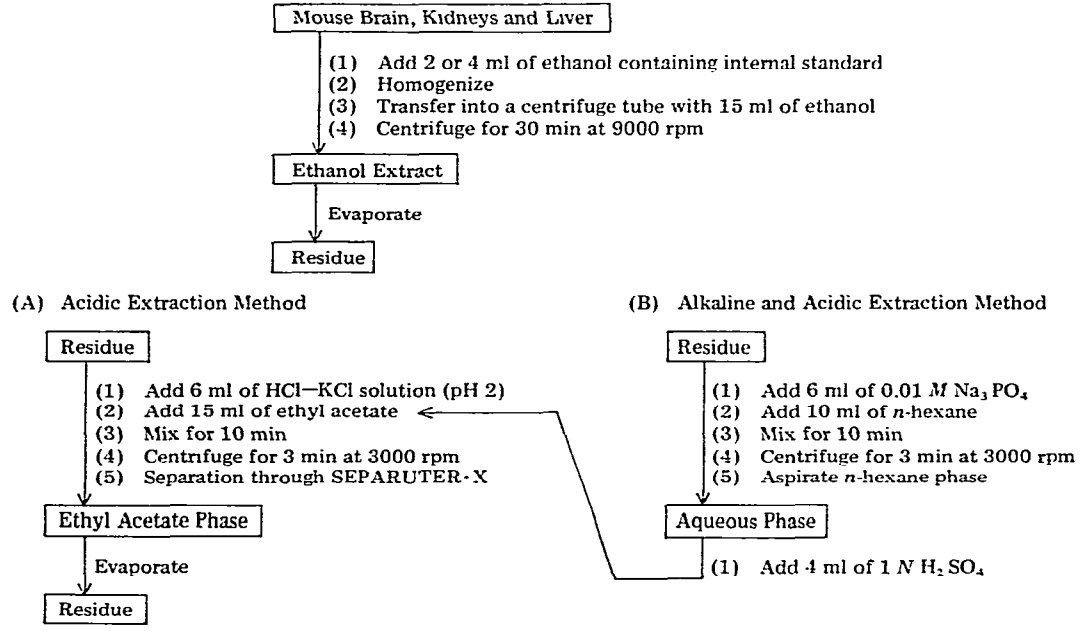
Amobarbital (Amo, m.p. 157.5–158.3°C), allobarbital (Allo, m.p. 173.2–174.1°C), barbital (Bar, m.p. 191.0–191.9°C), cyclobarbital (Cyclo, m.p. 174.1–174.5°C) and phenobarbital (Pheno, m.p. 176.3–176.8°C), of Japanese Pharmacopoeial standard, were purified by recrystallization. Pentobarbital (Pento, m.p. 132.0–133.5°C) was prepared from sodium pentobarbiturate injection solution (Somnopentyl; Pitman-Moore, Washington Crossing, MA, U.S.A.) in the usual manner and purified by recrystallization. β-Cyd was used after recrystallization from water, $[\alpha]_D^{18} = +165.5°$. In all experiments deionized, distilled water was used. The organic solvents used for extraction were of analytical-reagent grade and those used for the preparation of mobile phase systems were of reagent grade; all were freshly distilled before use. For TLC, Merck Kieselgel 60 plates (normal phase) and Merck Kieselgel Silanisiert plates (reversed phase) (both 20 × 20 cm, layer thickness 0.25 mm) were used. Reversed-phase plates were washed in methanol and were activated at 130°C for 20 min prior to use. Trimethylphenylammonium acetate (TMPA-Ac), which was used as a reagent for the methylation of BA in GC, was prepared from trimethylphenylammonium hydroxide (TMPA-H; Tokyo Kasei, Tokyo, Japan) according to the literature[9]. Other inorganic chemicals were of reagent grade.

Preparation of complexes, conditions of animals and administration of drugs

These were as described previously[7].

Extraction

The full scheme for the extraction of BA from mouse brain, kidneys and liver is shown in Scheme 1. The tissues are rapidly removed immediately after decapitation. In order to eliminate interfering tissue constituents and to enrich BA, the tissues are homogenized in ethanol, containing the internal standard (I.S.), and BA are extracted from the acidified solution with ethyl acetate, which is the most suitable solvent for the extraction of both the highly lipophilic and the less lipophilic BA and can extract BA quantitatively from BA–β-Cyd complex solutions (Table I). As liver contains a large amount of lipids, a step consisting of extraction from alkaline solution with *n*-hexane is included in order to remove most of the lipids. In order to obtain a rapid and efficient separation of the ethyl acetate phase from the aqueous phase, the extraction mixture is centrifuged and then filtered through a SEPARUTER·X (Kokubo Seiki, Tokyo, Japan) which is a new type of separator taking advantage of the difference between the surface tension of two phases.



Scheme 1. Extraction of barbiturates from mouse brain, kidneys and liver. 9000 rpm corresponds to 11,000 *g*, and 3000 rpm to 1500 *g*.

Procedure

The HPLC, GC and TLC analyses were performed according to the method of internal standardization. Calibration was effected by adding known amounts of the BA to be analysed and a definite amount of the I.S. to tissue homogenate. On the chromatogram the peak areas of the BA and of the I.S. were measured and their ratio calculated (I.S. = 1). The values obtained were plotted against concentration of the BA.

HPLC. The final residue obtained according to Scheme 1 was dissolved in 1 ml of mobile phase and centrifuged for 15 min at 3000 *g*. The supernatant solution

TABLE I

EXTRACTION EFFICIENCY OF SOLVENTS

Barbiturates (BA) were extracted from HCl–KCl solutions (pH 2) containing BA or BA–β-cyclodextrin (β-Cyd) complexes. Bar = barbital; Allo = allobarbital; Pheno = phenobarbital; Pento = pentobarbital; Amo = amobarbital; Cyclo = cyclobarbital.

Solvent	*Extraction efficiency (%)*				
	Ethyl acetate	*Diethyl ether*	*Ethylene dichloride*	*Chloroform*	*Toluene*
Bar	94.9	95.1	53.1	63.3	14.4
Bar–β-Cyd	94.0				
Allo	99.8	97.2	85.7	86.2	44.2
Allo–β-Cyd	98.5				
Pheno	100.0	99.9	90.5	93.0	51.4
Pheno–β-Cyd	98.6				
Pento	98.9	92.3	89.5	90.0	67.8
Pento–β-Cyd	100.0				
Amo	100.0	96.0	97.0	98.0	76.3
Amo–β-Cyd	99.3				
Cyclo	98.3	90.4	81.4	87.0	59.0

was filtered through a 0.45-μm membrane filter and aliquots of 5–80 μl were analysed by HPLC. The mobile phase was a mixture of water and methanol (1:1 or 3:2) passed through a 0.45-μm membrane filter and deaerated ultrasonically, usually at a flow-rate of 1 ml/min. As the I.S., Cyclo was used for the analyses of Allo, Amo, Pento and Pheno, and Allo was used for the analysis of Bar.

GC. The ethyl acetate extract was transferred into a conical test-tube and evaporated to dryness. The residue was dissolved in 20 μl of acetone, and 10 μl of methanol and 10 μl of 0.5 *M* TMPA-Ac were added to the solution. After thorough mixing, 2–3 μl of the mixture were injected into the gas chromatograph. The GC conditions were as follows: (A) the injector temperature was 200°C and the column was maintained isothermally at 150°C; (B) the injector temperature was 160°C and the column temperature was isothermal at 110°C for 8 min and then programmed from 110°C to 250°C at 3°C/min. The carrier gas was nitrogen at a flow-rate of 30 ml/min.

TLC. The sample solution was prepared by dissolving the residue obtained from the extraction procedure in 40 μl of ethanol. A series of BA standards and sample solution (2–4 μl) were applied to the plates with a 2-μl constriction pipette (Bie and Berntsen, Roedovre, Denmark). The thin layers were developed in tanks containing suitable mobile phases (see Table IV) to 16 cm from the starting line on the normal-phase plates (development time about 90 min) and to 8 cm on the reversed-phase plates (development time about 50 min). The chromatograms were scanned at a wavelength of 215 nm.

RESULTS AND DISCUSSION

High-performance liquid chromatography

The stationary and mobile phases were selected on the basis of results in the

literature[10–17]. Water–acetonitrile was also examined as the mobile phase, as this system should be favorable because of its lower absorptivity at wavelengths shorter than 220 nm. It was found, however, that this system increased the interference of brain constituents. The UV spectra of BA in the eluent (water–methanol mixture) show a maximum at 207–211 nm. In order to determine small amounts of BA, it was decided to make the measurements at 210 nm. Typical HPLC results for a standard mixture of six BA are shown in Fig. 1, with accompanying chromatograms of extracts of drug-free mouse brain, kidneys and liver. Only the blank brain extract showed the presence of substances with chromatographic retention behaviour, giving a slight overlap with Bar. In order to avoid this interference, water–methanol (3:2) was used as the eluent for the determination of Bar. Under these conditions the retention time (R.T.) of Bar was 5.90 min and that of the I.S., Allo, was 8.80 min. The retention time of Amo and Pento shown in Fig. 1 was too long (R.T. = 11.12 min) and the flow-rate of the eluent was therefore increased to 1.5 ml/min. Under these conditions the R.T. of Amo and Pento was 7.80 min and that of the I.S. (Cyclo) was 5.34 min.

The precision of the quantitative determination of BA by HPLC was investigated by injecting a constant volume (an appropriate volume to give the optimal peak area) of BA solutions of different concentrations (at the expected concentrations in

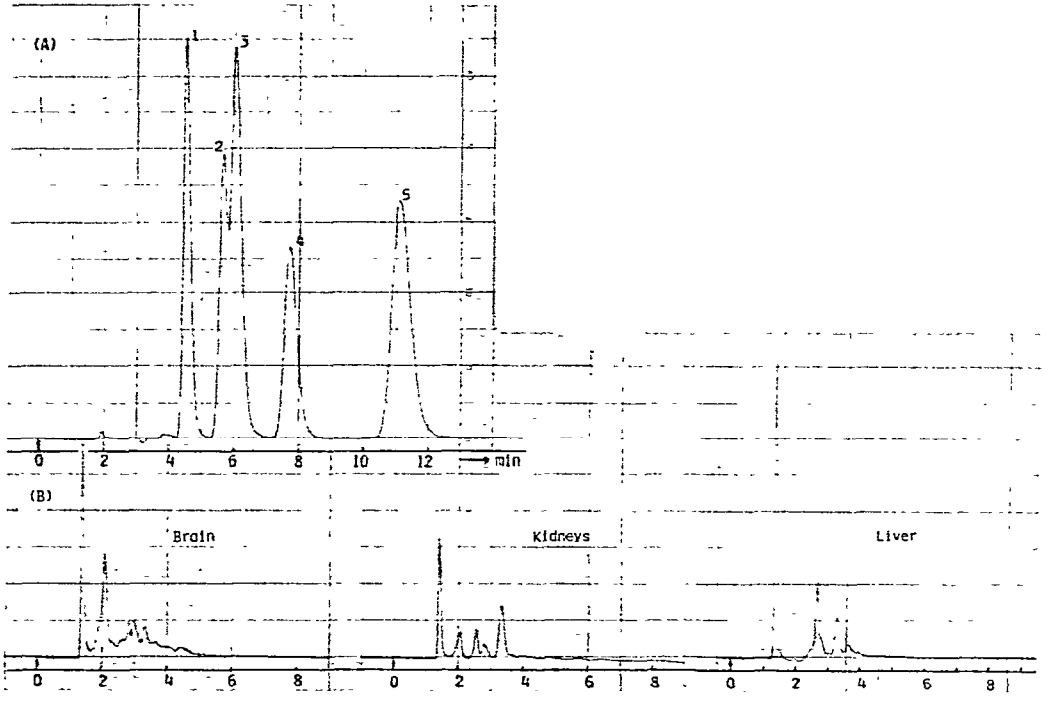


Fig. 1. (A) High-performance liquid chromatogram of a standard mixture of barbiturates (0.5 μg each): 1 = barbital; 2 = allobarbital; 3 = phenobarbital; 4 = cyclobarbital; 5 = amobarbital and pentobarbital. (B) High-performance liquid chromatograms of extracts of drug-free mouse brain, kidneys and liver. Apparatus: Waters ALC/GPC with 6000A solvent delivery system; column, μBondapak C_{18} (30 cm × 4 mm I.D.); mobile phase, methanol–water (1:1); flow-rate, 1.0 ml/min; detector, Jasco UBIDEC-100 II (210 nm, range 0.16); temperature, ambient.

tissues on administration of BA). The linearity of the calibration graphs was characterized by the correlation coefficient (Table II) and indicates that each graph has very good linearity. An advantage of HPLC is that the injection volume can be selected over a wide range. For low-concentration samples larger injection volumes result in higher precision. Standard deviations for triplicate measurements for each concentration are given in Table II.

TABLE II

LINEARITY AND PRECISION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

BA	*Injection volume (µl)*	*Concentration (µg/ml)*	*Correlation coefficient*	*Relative standard deviation (%) (n = 3)*		
				50 µg/ml	*5 µg/ml*	*0.05 µg/ml**
Allo	20	5– 50	0.99979	0.26	0.42	0.69
Amo	40	15– 75	0.99995	0.35	0.48	1.79
Bar	10	10–125	0.99986	0.14	0.35	0.74
Pento	40	5– 80	0.99984	0.26	0.52	2.20
Pheno	10	15– 90	0.99969	0.08	0.13	0.24

* For the 0.05 µg/ml concentration, the injection volume was increased steadily to 100 µl.

The detection limit of Pheno and Bar for a signal-to-noise ratio of 3 was 1.0 ng, of Allo 1.5 ng and of Amo and Pento 5.0 ng.

The recovery of the extraction procedure was tested by extraction of known amounts of BA added to blank tissue homogenate (Table III).

TABLE III

RECOVERY OF THE EXTRACTION PROCEDURE

Tested by extraction of known amounts of BA added to blank tissue homogenate.

BA	*Recovery (%)*		
	Brain	*Kidneys*	*Liver*
Allo	100.5–103.5	99.5–105.3	97.2–103.2
Amo	99.6–103.7	98.2–102.5	96.5–103.0
Bar	95.5– 97.1	94.9– 97.4	93.7– 94.8
Pento	95.6– 99.0	93.9–102.2	97.4– 99.9
Pheno	99.6–102.9	100.7–103.1	99.0–101.1

Gas chromatography

Numerous methods for the determination of BA by GC have been reported, and flash-heating methylation[18–27] appears to be the best. As reagents for this methylation, tetramethylammonium hydroxide (TMA-H)[18,19], TMPA-H[20–27] and TMPA-Ac[9] have been used. On the basis of results obtained in our laboratory we chose TMPA-Ac as the methylation reagent; it caused neither cleavage of the BA molecule nor the formation of lower methylation products. A single peak appeared on the chromatogram, corresponding to the respective 1,3-dimethyl derivative. Fig. 2A shows a typical gas chromatogram for a standard mixture of BA on a column packed with 3%

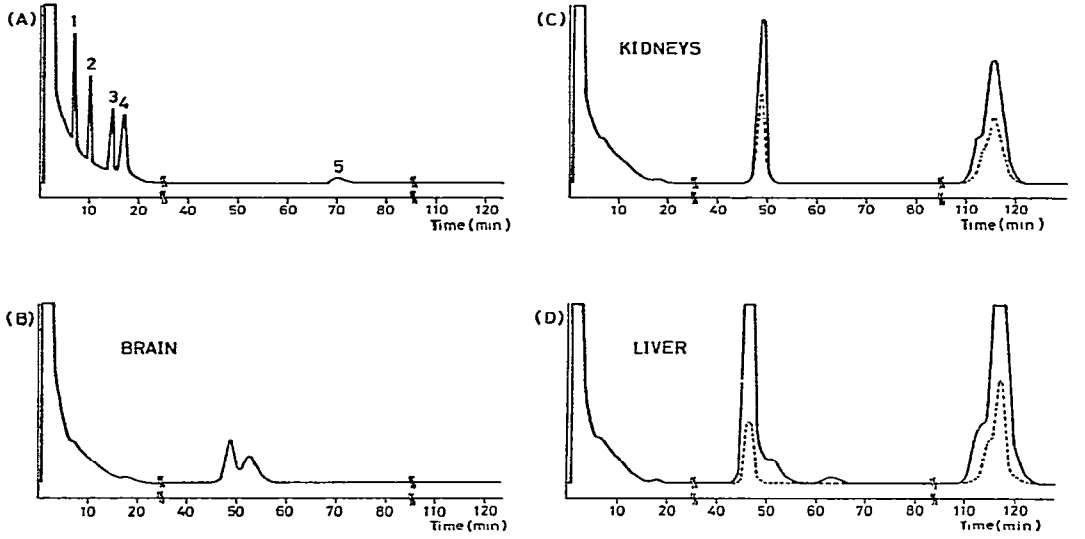


Fig. 2. (A) Gas chromatogram of a standard mixture of barbiturates (1 μg each) in 0.5 *M* trimethylphenylammonium acetate: 1 = barbital; 2 = allobarbital; 3 = amobarbital; 4 = pentobarbital; 5 = phenobarbital. (B)–(D) Gas chromatograms of extracts of drug-free mouse brain, kidneys and liver in 0.5 *M* trimethylphenylammonium acetate. Apparatus: Hitachi Model K53; column, 3% OV-17 on Gas-Chrom Q (2 m × 4 mm I.D.); detector, FID; column temperature, 150°C; carrier gas, nitrogen; flow-rate, 30 ml/min. ----, Results obtained employing an extraction method with barium hydroxide solution.

OV-17, obtained using GC conditions (A). Fig. 2B–D show gas chromatograms of extracts of blank mouse brain, kidney and liver under the same conditions. The substances present in the tissue extracts do not interfere in the analyses of BA except Pheno. Under these conditions Allo can be used as the I.S. for the determination of Bar, Amo and Pento. For the determination of Allo, Amo was chosen as the I.S. The ratio of the peak area of the BA under analysis to the peak area of the I.S. was linearly related to the amount of each BA over the range studied (3–20 μg), even after extraction from tissue homogenate.

The determination of Pheno was performed by using a column packed with 1% OV-1 under GC conditions (B). The R.T. of Pheno was 21.6 min and Pento, with an R.T. of 13.8 min, could be used as the I.S. Many peaks arising from substances present in the tissue extracts appeared after 25 min. These peaks do not affect directly the analyses of BA like the strong peaks appearing in Fig. 2B–D, but it takes a long time to remove them from the separation column. To eliminate the interfering substances, which were assumed to be mainly higher fatty acids, an extraction method with barium hydroxide solution[28] in which fatty acids are converted into poorly soluble barium salts and then filtered off was attempted. However, this treatment resulted only in a slight decrease of the peak heights concerned.

Another limitation of GC is the very small injection volume of the sample solution, so that a sample solution with a much higher concentration than that used for HPLC must be prepared by using a very small volume of the solvent. Because of this difficulty, GC cannot compete with HPLC in terms of accuracy.

Thin-layer chromatography

Many TLC techniques have been used for the separation and identification of BA, and the determination of BA by TLC has also been investigated[29–32]. In most of the investigations normal-phase plates were used. TLC techniques using reversed-phase plates have been developed recently, and in this work the two types of plate were compared for the determination of BA. The mobile phases were selected on the basis of results in the literature or obtained in our laboratory.

Two detection methods, one of which measures the absorbance of non-ionized BA molecules at a wavelength near the UV absorption maximum (215 nm) and the other the absorbance of ionized molecules at 240 nm (for the first ionized form) or 255 nm (for the second ionized form) after spraying alkaline reagents (ammonia, sodium hydroxide or a suitable buffer solution), were compared. The former method was simpler and had better reproducibility.

The R_F values of the BA studied are given in Table IV. On the normal-phase plate, blank tissue extractions showed the presence of substances having chromatographic retention behaviour within the R_F range of the BA. Moreover, the mutual resolution of the five BA was not very good even on longer development, and hence a suitable I.S. was not available. On the other hand, the reversed-phase plate gave better resolution with a shorter development. In the latter instance the constituents of tissue were concentrated at the starting point and the solvent front, and the background in the region between R_F 0.2 and 0.8 was completely flat. The following conditions were consequently employed for TLC: the stationary phase was Merck Kieselgel Silanisiert (reversed-phase), the mobile phase was methanol–water (1:1) and the chromatograms were measured at 215 nm without spraying with a reagent. The I.S. for Bar, Allo and Pheno was Pento, and that for Amo and Pento was Bar. Under these conditions the tissue constituents did not affect the determination of the BA and the calibration graphs were linear over the range 1–8 μg.

The main disadvantage of TLC is that quantitative analysis is possible in only very limited concentration ranges. Therefore, in order to conduct exact analyses it is

TABLE IV

R_F VALUES OF THE BARBITURATES

The thin layers were developed to 16 cm from the starting line on the normal-phase plates and to 8 cm on the reversed-phase plates. Chromatograms were measured at 215 nm without spraying with a reagent.

Stationary phase	*Mobile phase*	*R_F Value*				
		Bar	*Allo*	*Pheno*	*Amo*	*Pento*
Kieselgel 60 (normal)	$CHCl_3$–$(CH_3)_2CO$ (8:1)	0.23	0.32	0.24	0.34	0.37
	C_6H_6–$CH_3COOC_2H_5$ (3:1)	0.34	0.47	0.42	0.49	0.48
Kieselgel Silanisiert (reversed)	CH_3OH–H_2O (1:1)	0.71	0.62	0.60	0.40	0.38
	CH_3OH–H_2O (2:3)	0.58	0.44	0.42	0.21	0.22

necessary to know previously the approximate concentration of each BA in the tissue being analysed. However, TLC may be useful for preliminary assays of BA in tissues because of its simplicity.

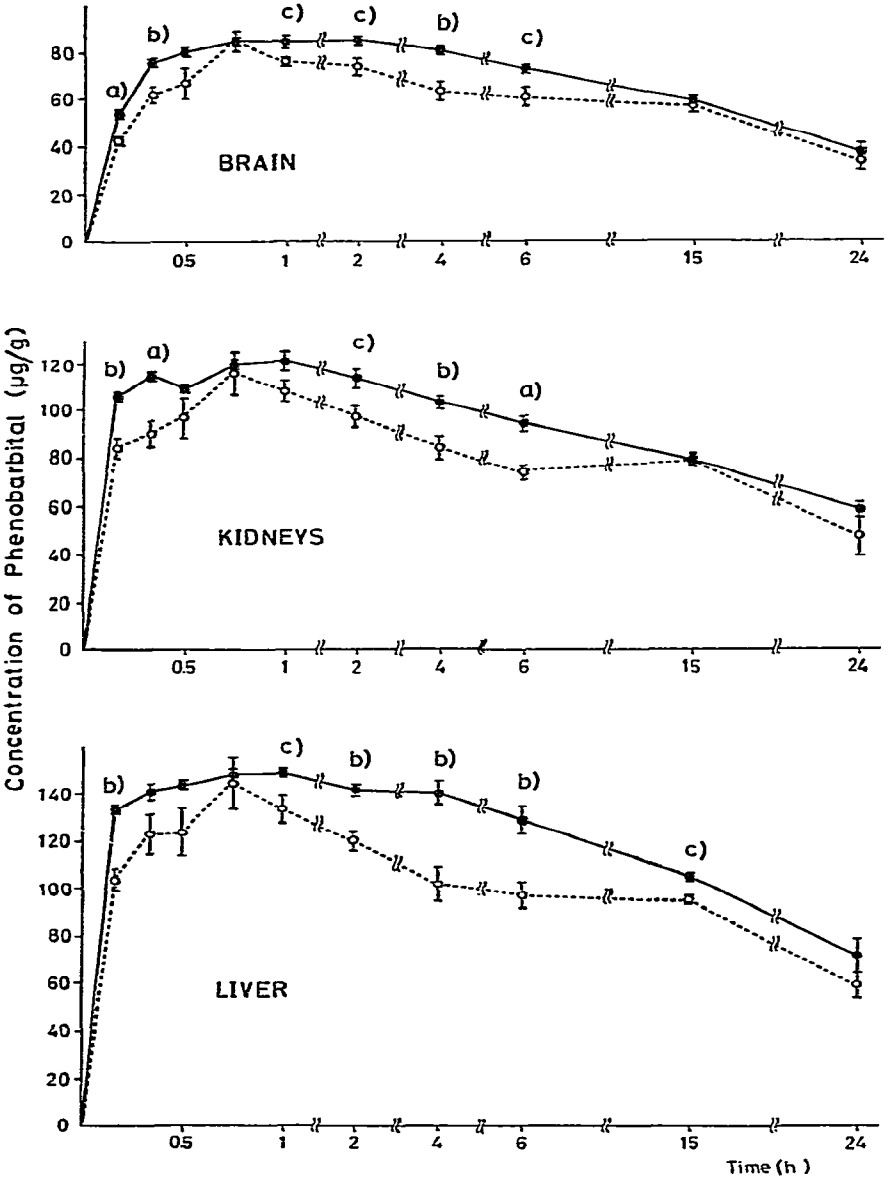


Fig. 3. Time course of the concentrations of phenobarbital in mouse brain, kidneys and liver after oral administration of phenobarbital–β-cyclodextrin (●) and phenobarbital (○). Dose: 4.5 µmol (1045 µg as phenobarbital) per 10 g. Each point and vertical bar indicates the mean ± standard error for six mice. (a), (b), (c): Significantly different from intact phenobarbital by two-tailed Student's *t*-test; (a) $p < 0.001$; (b) $p < 0.01$; (c) $p < 0.05$.

Time courses of the concentrations of phenobarbital and barbital in mouse brain, kidneys and liver after oral administration of phenobarbital–β-cyclodextrin and barbital–β-cyclodextrin, respectively

As the results described above suggested that the HPLC is the most useful method for the determination of BA in mouse tissues, the time courses of the con-

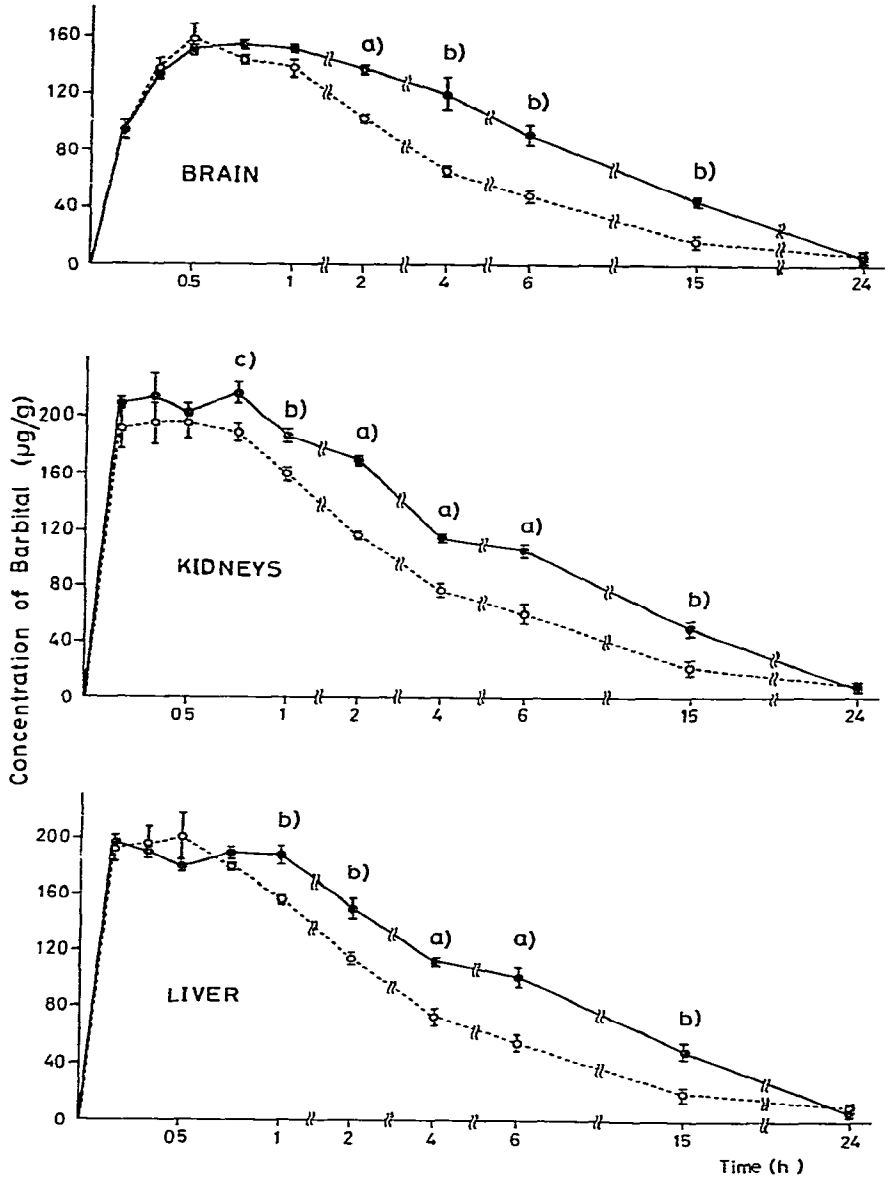


Fig. 4. Time course of the concentrations of barbital in mouse brain, kidneys and liver after oral administration of barbital–β-cyclodextrin (●) and barbital (○). Dose: 10.9 μmol (2008 μg as barbital) per 10 g. Each point and vertical bar indicates the mean ± standard error for six mice. (a), (b), (c): Significantly different from intact barbital by two-tailed Student's *t*-test; (a) $p < 0.001$; (b) $p < 0.01$; (c) $p < 0.05$.

centrations of Pheno (Fig. 3) and Bar (Fig. 4) in mouse brain, kidneys and liver after oral administration of Pheno–β-Cyd and Bar–β-Cyd, respectively, were measured by HPLC and were compared with those obtained after oral administration of an equimolar amount of the corresponding intact drug. The doses administered were the minimal amounts producing sufficient hypnotic activity (1.2 times the ED_{50} of intact BA in the case of long-acting types, Pheno and Bar)[7].

With Pheno, the concentrations in brain at the initial stage, to 20 min from administration of Pheno–β-Cyd and during 1–6 h after administration were significantly higher than those obtained following administration of intact Pheno. This result indicates that the complexation of Pheno with β-Cyd accelerates the rate of absorption of Pheno and maintains the brain level of Pheno for a longer time. The time courses of the concentrations of Pheno in mouse kidneys and liver showed a similar tendency to that in brain and therefore it seemed that the complexation did not cause partial distribution.

With Bar, there was no difference in the brain concentrations following administration of the complex and the intact drug for up to 1 h, indicating that the complexation of Bar with β-Cyd did not affect the rate of absorption of Bar. In spite of the result for the Bar-induced sleeping time, on which the complexation did not have any significant effect, after 2–15 h significant differences in the brain concentrations following administration of Bar–β-Cyd and Bar were observed. This may be explained if the brain level of Bar after 2 h had already decreased below the hypnotic threshold, even on the administration of the complex. In any event, the complexation of BA with β-Cyd maintains the BA concentrations in tissues for a longer time, irrespective of the stability of the complex.

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