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Note

Rapid analysis of hexobarbital and its main metabolites in mice by high-performance liquid chromatography

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The various derivatives of barbituric acid have been analyzed by high-performance liquid chromatography (HPLC) for a number of years [1—6]. One of these derivatives, hexobarbital [1,5-dimethyl-5-(1'-cyclohexenyl)-barbituric acid], is often used as a test compound to measure the activity of the drug-metabolizing enzymes. The duration of the narcotic action of hexobarbital is related to the concentration of the unmetabolized molecule [7—9] as well as to the half-life or clearance of hexobarbital [7, 10—12]. Analysis of these parameters can easily be done by gas chromatography. Since hexobarbital is nearly completely metabolized, the time course of the formation and elimination of 3'-hydroxyhexobarbital and 3'-keto-hexobarbital (the main metabolites) directly reflects the activity of the drug-metabolizing enzymes. Both of these polar metabolites can be analyzed by gas chromatography only after derivatization prior to chromatography [13—15].

In this paper a newly developed method will be described by which hexobarbital and its main metabolites can easily be extracted and analyzed from biological samples by HPLC.

MATERIALS AND METHODS

Chemicals

All chemicals used were of analytical grade except for acetonitrile which was of the grade "for residue analysis" (Merck, Darmstadt, G.F.R.). Hexobarbital and phenobarbital were from Bayer, Leverkusen, G.F.R.; 3'-hydroxyhexobarbital (3'-hydroxy-hb) and 3'-ketohexobarbital (3'-keto-hb) were kindly provided by Dr. N.P.E. Vermeulen, from the Gorlaeus Laboratories, Leiden, The Netherlands. Norhexobarbital and 1',2'-epoxyhexobarbital were gifts from Prof. J. Pütter from Bayer, Wuppertal, G.F.R.

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Animals

Male NMRI mice weighing 23—27 g were used for the experiments. They were housed in plastic cages on soft wood bedding at 25°C room temperature under a 12-h dark—light rhythm. The animals had free access to standard laboratory diet (Herilan®, Eggersmann, Rinteln, G.F.R.) and tap water.

Treatment of animals

Mice were injected intraperitoneally with 0.125 mg/g hexobarbital; 10, 40 and 180 min later they were killed by cervical dislocation and exsanguinated. Blood was collected and the organs rapidly resected. The organs were rinsed in ice-cold 0.9% sodium chloride and dried on a filter paper.

Extraction of hexobarbital and its metabolites

Blood or serum. A 0.2-ml sample of blood or serum was added to 1 ml of 0.01 mol/l NaH_2PO_4 (pH 2.7) and 0.02 ml of 4 mol/l sodium chloride. After the addition of 3 ml of dichloromethane, the mixture was vortex-mixed for 30 sec on a whirlmix and centrifuged for 10 min at 800 *g*; 2 ml of the organic layer were evaporated to dryness and the residue was dissolved in 1 ml or less of the eluent. A 0.05-ml portion was applied to the column for HPLC analysis.

Tissue. A 200-mg amount of tissue was homogenized in 2.4 ml of 0.01 mol/l NaH_2PO_4 (pH 2.7) using an Ultra Turrax (Janke & Kunkel, Stauffen, G.F.R.) for 15 sec at 15,000 rpm. Then 0.08 ml of 4 mol/l sodium chloride and 4 ml of dichloromethane were added. After vortex mixing for consecutive 30 sec the centrifugation and extraction steps were performed as described for serum, except that 3 ml of the organic layer were evaporated to dryness.

All the steps up to the addition of the organic solvent were done in the cold at about 0- 4°C.

Recovery

For recovery experiments hexobarbital, 3'-keto-hb, and 3'-hydroxy-hb dissolved in small amounts of 0.01 mol/l sodium hydroxide were added to serum or to organ homogenates of untreated animals to give final concentrations of 0.1 mg/ml each. After mixing and incubating at room temperature for 10 min the compounds were extracted as described above. For the determination of the concentration of hexobarbital and its metabolites in organs, the organ concentrations were corrected for the organ's blood content as described earlier [16].

Chromatographic conditions

The HPLC apparatus used consisted of a Waters 6000A solvent delivery pump (Waters Assoc., Milford, MA, U.S.A.), with the automatic sample injection system ASI 45 (Kontron Analytik, Eching, G.F.R.) with a 0.05-ml sample loop. The separation was performed on a column (25 × 0.4 cm) packed with Nucleosil® C_{18} 10 μm (Macherey & Nagel, Düren, G.F.R.) with a flow-rate of 1.9 ml/min (about 140 bar) at room temperature. The eluent was 3.5 mmol/l NaH_2PO_4 in water—acetonitrile (67:33), with the pH adjusted to 2.7 with H_3PO_4. The effluent was monitored in an LC 720 liquid chromatography

TABLE I

CONCENTRATIONS OF HEXOBARBITAL, 3'-KETOHEXOBARBITAL, AND 3'-HYDROXYHEXOBARBITAL IN SERUM, BRAIN, LIVER, AND KIDNEYS OF MALE NMRI MICE AFTER INTRAPERITONEAL INJECTION OF 0.125 mg/g HEXOBARBITAL

The data, expressed as mean ± S.D. (n = 6), are corrected for recovery; the organ concentrations are corrected for the organ's blood content.

	10 min post injections			40 min post injection			180 min post injection		
	hb*	3'-Keto-hb*	3'-Hydroxy-hb*	hb	3'-Keto-hb	3'-Hydroxy-hb	hb	3'-Keto-hb	3'-Hydroxy-hb
Serum (μg/ml)	106.1 ± 16.1	18.8 ± 6.5	69.6 ± 7.2	46.6 ± 9.5	94.2 ± 8.3	99.7 ± 8.3	0	64.2 ± 39	0
Brain (μg/g)	76.8 ± 7.0	1.8 ± 1.2	0	40.5 ± 9.8	9.5 ± 0.2	0	0	24.2 ± 12.9	0
Liver (μg/g)	142.0 ± 23.1	24.5 ± 12.0	13.5 ± 3.1	68.0 ± 8.1	66.9 ± 4.8	45.1 ± 4.6	0	37.5 ± 20.8	27.5 ± 3.8
Kidney (μg/g)	111.7 ± 13.3	7.6 ± 3.6	11.8 ± 2.4	48.1 ± 6.1	74.8 ± 11.6	61.9 ± 3.3	0	157.9 ± 56.2	64.8 ± 12.7

*For abbreviations, see text.

UV detector (Kontron). The absorption was read at 238 nm and automatically recorded on a C-1R A Chromatopac printer-plotter (Shimadzu, Kyoto, Japan).

RESULTS AND DISCUSSION

Retention times were hexobarbital 7.57 ± 0.02 min, 1′,2′-epoxy-hb 5.67 ± 0.02 min, norhexobarbital 3.95 ± 0.01 min, 2′-keto-hb 2.86 ± 0.01 min, and 3′-hydroxy-hb 2.44 ± 0.01 min. Phenobarbital (retention time 4.36 ± 0.02 min) can be used as an internal standard because it does not interfere with any of these compounds (Fig. 1).

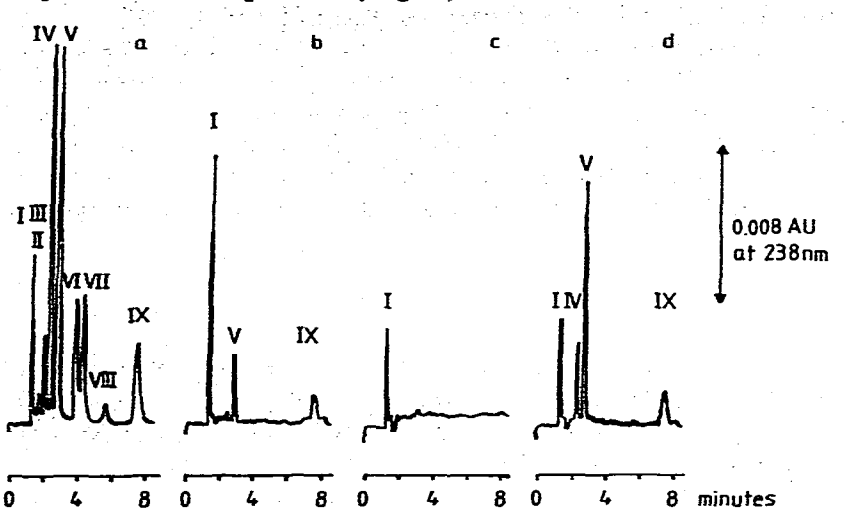

Fig. 1. Chromatographic analysis of hexobarbital (hb) and its metabolites on RP C_{18}, 10 μm. *Chromatographic conditions: column*, 25 × 0.4 *cm*; *eluent*, 3.5 *mmol/l* NaH_2PO_4 *in water*—acetonitrile (67:33), pH 2.7; detection at 238 mm. (a) Artificial mixture of hexobarbital, some of its possible metabolites, and phenobarbital. I = Solvent front; II, III = unidentified degradation products of 1′,2′-epoxy-hb; IV = 3′-hydroxy-hb (1.29 μg); V = keto-hb (0.37 μg); VI = nor-hb (1.11 μg); VII = phenobarbital (0.78 μg); VIII = 1′,2′-epoxy-hb (0.65 μg); IX = hexobarbital (0.88 μg). (b) Brain extract 40 min after the intraperitoneal administration of 0.125 mg/g hexobarbital. I = Solvent front; V = keto-hb; IX = hexobarbital. (c) Kidney extract of an untreated animal. I = solvent front. (d) Kidney extract 40 min after the intraperitoneal administration of 0.125 mg/g hexobarbital. I = Solvent front; IV = hydroxy-hb; V = keto-hb; IX = hexobarbital.

The recovery was 95.6 ± 3.2% for hexobarbital, 80.8 ± 2.5% for 3′-keto-hb, and 41 ± 1.3% for 3′-hydroxy-hb (calculated from 10 independent determinations). The detection limit was 0.15 μg/ml serum and 0.1 μg/g organ for hexobarbital and 3′-hydroxy-hb, respectively, and 0.05 μg/ml serum and 0.05 μg/g organ for 3′-keto-hb.

No interfering material was extracted from the organs (Fig. 1c). The acid-buffered extraction solution and eluent were used to improve efficiency by ionisation suppression and to improve stability of hexobarbital and its metabolites during extraction and chromatography.

In a pilot study the concentrations of hexobarbital and its main metabolites in blood, brain, liver, and kidneys of male NMRI mice at different time in-

tervals after application of hexobarbital were investigated, and the results are given in Table I. Within the first 10 min after intraperitoneal injection hexobarbital penetrates the brain and accumulates in the liver where it is metabolized. It is rapidly converted and has disappeared from the blood and organs within 3 h. The most hydrophilic metabolite, 3′-hydroxy-hb, does not penetrate the brain but reaches high concentrations in serum, liver, and kidneys. 3′-Keto-hb is found in high concentrations in serum, liver, and kidneys and to a lesser degree within the brain, where it can be analyzed even 3 h after the intraperitoneal injection of hexobarbital. The time course and the recovery for hexobarbital and the metabolites 3′-keto-hb and 3′-hydroxy-hb were nearly the same as those described by Vermeulen et al. [17] for the rat.

In conclusion, the method described allows the rapid simultaneous determination of hexobarbital and its metabolites from biological samples in a simple analytical system. Thus the activity of the drug-metabolizing enzymes can easily be determined. In NMRI mice hexobarbital is mainly metabolized to 3′-hydroxyhexobarbital and 3′-ketohexobarbital. The ratio of the concentrations of hexobarbital and its metabolites differs for the various organs and the blood.

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