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Note

Stereospecific analysis of racemic hexobarbital in rat blood by enantioselective capillary gas chromatography

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Racemic hexobarbital (HB) is a widely used model substrate, for which sleeping times, half-lives or clearances have been used as parameters to estimate changes in the activity of the hepatic cytochrome P-450 enzyme system [1, 2] A complication in the interpretation of these parameters in terms of drug-metabolizing enzyme activity is the fact that the enantiomers are metabolized at different rates To simplify interpretation, one should make sure that the enantiomers of HB do not reciprocally influence each other's metabolism or pharmacological effect, and that the ratio of their rates of metabolism is independent of the relative abundance of the different subspecies of hepatic cytochrome P-450 participating in HB metabolism

In principle, there are several possibilities for determining enantiomers in biological fluids, such as derivatization with suitable chiral reagents followed by liquid or gas chromatography [3], labelling with chiral immunoglobulin antibodies [4], or direct separation on chiral high-performance liquid chromatographic (HPLC) [5] or gas—liquid chromatographic (GLC) [6, 7] stationary phases.

Recently, a procedure for simultaneous analysis of pseudoracemic HB in blood was described [8] For this purpose, N_1-C^2H_3-*R*(−)HB was synthesized and combined with N_1-CH_3-*S*(+)HB in equimolar amounts to afford a pseudoracemic mixture of HB This mixture was administered orally to rats and the pharmacokinetics in blood and the metabolic profile in the urine of pseudo-

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racemic HB were determined using gas chromtography combined with mass fragmentography (GC—MF) Recently, a stereoselective radioimmunoassay (RIA) for the enantiomers of HB was published [4]. Although cross-reactivity of the *d*- and *l*-antisera with the respective HB enantiomers was negligible, significant cross-reactivity towards some HB metabolites was reported

Both GC—MF and RIA, however, require sophisticated synthetic or biochemical techniques in conjunction with advanced analytical equipment. Therefore, in the present study an attempt was made to evaluate possibilities and limitations of enantioselective GLC in studying the pharmacokinetics of racemic HB in the rat. Enantioselective GLC has, in principle, been shown to be suitable for resolution of pure reference HB enantiomers [7, 9].

EXPERIMENTAL

Materials

The sodium salt of hexobarbital [1,5-dimethyl-5-(1′-cyclohexenyl)barbituric acid, Evipan®] was purchased from Bayer (Leverkusen, F R G) Sodium methohexital [1-methyl-5-(1′-methyl-2-pentynyl)-5-(2-propenyl)barbituric acid] was obtained from Eli Lilly (Lahn-Giessen, F.R G) XE-60-L-Valine-(*S*)-α-phenylethylamide (XE-60-S) was a generous gift from Dr. W A. König (Hamburg, F R.G) *S*(+)HB and *R*(−)HB were generous gifts from Professor J Knabe (Universität des Saarlandes, Saarbrücken, F R G.). Dichloromethane (DCM) and light petroleum (PE; b.p. 40–60°C) were purchased from J T Baker (Deventer, The Netherlands) and were freshly redistilled before use

Animal treatment

Male Wistar rats were used, with a mean body weight of 206 g Their housing, maintenance and cannulation of the carotid artery have been described previously [10] Per 100 g body weight, 0 25 ml of isotonic saline containing 10 0 mg/ml racemic (±)HB was injected via the carotid artery cannula. This corresponded to a dose of 12 5 mg/kg of each enantiomer The dose was flushed in with 0 25 ml of isotonic saline At regular intervals for up to 50 min, 100-μl blood samples were taken from the carotid artery The blood samples were stored at − 20°C until analysis

Extraction procedure

To 100 μl of blood in a conical tube, 5 ml of the extraction solvent DCM–PE (4 6) were added followed by 100 μl of a 10 μg/ml solution of methohexital in the same solvent After extraction for 30 s on a whirlmixer and centrifugation for 5 min at 600 *g*, the upper organic layer was transferred to another conical evaporation tube and evaporated under a gentle stream of nitrogen to a volume of approx. 100 μl Subsequently, 500 μl of a 0.1 *M* solution of sodium hydroxide were added and the organic layer was extracted for 30 s on a whirlmixer and centrifuged. The washing procedure of the alkaline phase was kept short in order to prevent the possible decomposition of HB under alkaline conditions The upper organic layer was discarded and the aqueous phase was acidified with 50 μl of 1 *M* hydrochloric acid. Finally, the aqueous layer was extracted again with DCM—PE (4·6) and the organic layer

evaporated to approx. 10 μl and not to complete dryness, in order to prevent possible sublimation of HB Of this solution, 1 μl was injected into the gas chromatograph.

Gas chromatography

All measurements were carried out on a Carlo Erba 2300 gas chromatograph equipped with a flame ionization detector Column temperature was 150° C, and a 1 5 split ratio was used. Inlet pressure was 1 7 kP/cm. Carrier gas flow-rate was 1 5 ml/min; injection temperature was 200° C and detector temperature was 250° C.

A glass capillary, support-coated open tubular (SCOT) column was used (25 m × 0.35 mm I.D.). The deposition of a Silanox 101 support layer and polysiloxane deactivation of the capillary wall were carried out as described previously [10] The column was statically coated with 0 1% (w/v) XE-60-S in dichloromethane

Data analysis

Peak-height ratios of the internal standard and HB enantiomers were used to calculate blood concentrations with the aid of a calibration curve In each rat, blood elimination half-lives of the enantiomers were determined by linear regression of the log blood concentration versus time curves Area under the curve (AUC) values were calculated with the aid of the logarithmic trapezoidal rule method The systemic clearance, Cl_s, was calculated according to the equation $Cl_s = D_{1a}/\mathrm{AUC}$, where D_{1a} is the intraarterial dose

RESULTS AND DISCUSSION

Analysis of HB enantiomers in blood

In Fig 1A, a representative gas chromatogram is shown, obtained following extraction from blood spiked with 10 μg/ml internal standard methohexital (peak 1), 2 5 μg/ml *R*(−)HB (peak 2) and 2 5 μg/ml *S*(+)HB (peak 3) The retention times were 23 5, 33 and 34 min, respectively The resolution factor, R_s, was 0 65 and remained constant after multiple injections The identity of the peaks representing the two HB enantiomers was established with the aid of the pure reference compounds These were also used to exclude inversion of the enantiomers at any stage of the analytical procedure, including extraction and GLC analysis In Fig 1B, a chromatogram of a blank extract is shown From these chromatograms it can be concluded that for this type of analysis flame ionization detection is sufficiently sensitive and selective The calibration curves were linear in the range 1- 25 μg/ml for each enantiomer The lowest measurable concentration of each enantiomer was 0 5 μg/ml The overall recovery of the extraction procedure was 64%. This is lower than the recovery expected of a single-step extraction procedure, which is in the order of 90% [1]. A back-extraction, however, was necessary to eliminate, as much as possible, interfering endogenous material from the extract. This would otherwise accumulate on the column, thus contaminating the stationary phase and resulting in a visible decrease in resolution after each consecutive injection At a concentration of 2 5 μg/ml of racemic HB, the intra-assay coefficient of

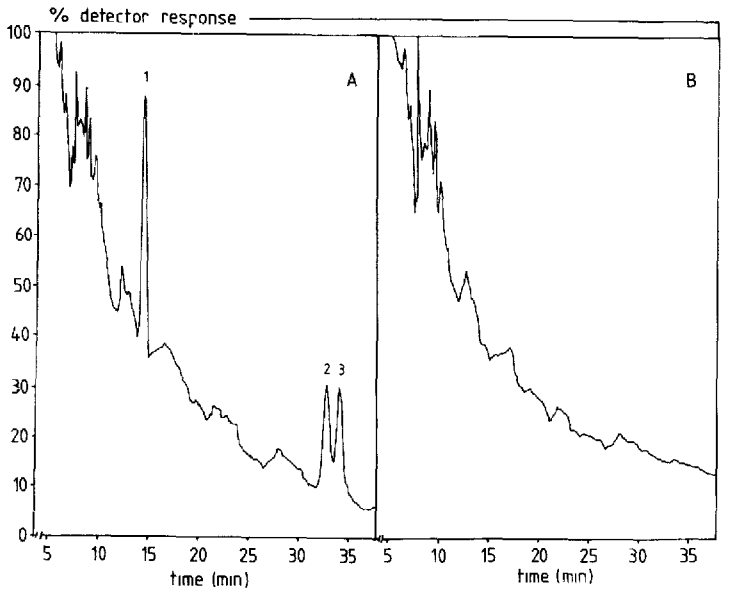

Fig 1 Gas chromatograms of rat blood extracts (A) Extract of rat blood spiked with 10 μg/ml methohexital (1), 2 5 μg/ml *R*(−)HB (2) and 2 5 μg/ml *S*(+)HB (3) (B) Blank blood extract

variation (C V) was 6 2%, (n = 5) and the inter-assay C V was 9 4% (n = 5)

The analytical method described here has the advantage that the pharmacokinetics of the enantiomers of HB in each other's presence can be studied without prior, expensive and time-consuming labelling of the enantiomers, enabling detection by highly specialized GC—MF techniques However, the procedure also has some disadvantages Firstly, metabolites of HB cannot be measured by this technique and secondly, the extraction procedure is laborious and time-consuming.

In addition, the XE-60-S SCOT column had a low temperature stability At oven temperatures of 170—180° C, separation of HB enantiomers was still efficient at much lower retention times, but at this temperature some bleeding of the column began to manifest itself, making quantitative determinations and long-term use impracticable As a result of the relatively low volatility of HB, therefore, very long retention times observed at 150° C had to be accepted.

Pharmacokinetics in blood

In Fig 2, a typical blood concentration versus time profile of *R*(−)HB and *S*(+)HB following intraarterial administration of 25 mg/kg racemic HB is presented In five rats, the $t_{½}$ of *S*(+)HB was 15 9 ± 2.7 min (mean ± standard error of the mean). The $t_{½}$ for *R*(−)HB was slightly, but not significantly shorter, 13 4 ± 1 4 min The Cl_s of *S*(+)HB was 130 8 ± 32 8 ml/min kg and the Cl_s of *R*(−)HB was 93 2 ± 21.9 ml/min kg

In conclusion, it can be stated that at the present stage of development of optically active stationary phases, the requirement of eliminating as completely as possible the endogenous substances interfering with analysis will preclude routine application of this type of column to the separation of enantiomers

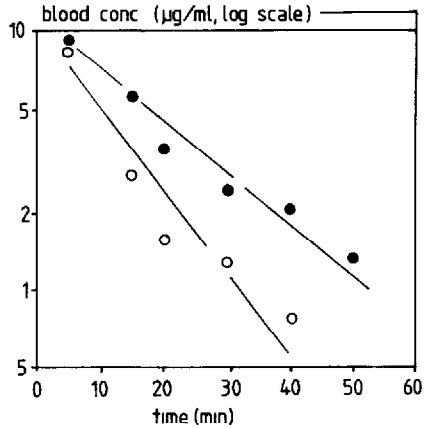

Fig 2 Representative blood concentration versus time curve of intraarterially administered racemic HB in a dose of 25 mg/kg (•) 12 5 mg/kg *R*(−)HB, (○) 12 5 mg/kg *S*(+)HB

extracted from a complex biological matrix However, with the advent of increasingly efficient and thermostable chiral stationary phases, enantioselective GLC may develop into a practical alternative for the current enantioselective labelling or derivatization techniques

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