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Disposition of Heptabarbitone in the Rat: Identification of a New Metabolite by Tandem Mass Spectrometry

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Abstract—The purpose of this study was to elucidate the structure of a metabolite of heptabarbitone, the occurrence of which was reported previously in a number of pharmacokinetic and pharmacodynamic modelling studies. By application of thermospray liquid chromatography (tandem) mass spectrometry, the identity of the metabolite was proposed to be 5-ethyl-5-(1′,[3′ or 6′]-cycloheptadienyl)-barbituric acid. By measuring the ratios between the areas under the concentration time curves of the metabolite and heptabarbitone after administration of three different intravenous dosages of heptabarbitone, it was shown that the exposure to the metabolite is directly correlated with the exposure of heptabarbitone.

In-vivo pharmacodynamic studies in rats, based on the principle of pharmacokinetic and pharmacodynamic modelling represent a useful tool in investigating the influence of different physiological conditions on the pharmacodynamics of different drugs. Recently, this approach has been used for drugs acting on the central nervous system (Danhof & Levy 1984; Dingemanse et al 1988a; Mandema & Danhof 1990). In those studies, heptabarbitone was chosen as a model drug since it is not a chiral compound, thereby avoiding potential pharmacokinetic complications due to alterations in the stereoselective disposition of the enantiomers.

In all studies performed until now, an additional peak was observed in HPLC of the plasma samples after administration of heptabarbitone with a concentration vs time profile that is suggestive for a metabolite. This peak could not be accounted for by one of the known metabolites of heptabarbitone, 3′-ketoheptabarbitone, 3′-hydroxyheptabarbitone or 5-ethylbarbituric acid (Gilbert et al 1974; Vermeulen 1980).

The purpose of the present study was, therefore, to elucidate the structure of this metabolite. The dose-dependency of this metabolic route was also determined.

Materials and Methods

Chemicals

Heptabarbitone (5-ethyl-5-(1′-cycloheptenyl)-barbituric acid) was a generous gift from Ciba-Geigy (Arnhem, The Netherlands).

Animal experiments

Nine 4-month-old male BN/BiRij rats (TNO, Leiden, The Netherlands) were used for the investigations. During the period in which the experiments were performed, the rats were kept separately in Makrolon cages and in a normal 12-h light-dark cycle (light between 0700 and 1900 h). The temperature was maintained at 22–23°C. They were allowed free access to water (acidified, pH 3–4) and food (Standard diet for Rat, Mouse and Hamster, AM 1410, Hope Farms, Woerden, The Netherlands).

Each animal received all three different dosages of heptabarbitone used, 25, 45 and 85 mg kg^{-1}, with intervals of one week in a randomized order. Heptabarbitone was administered intravenously via the penile vein under a light ether anaesthesia. The freshly prepared injection solution consisted of heptabarbitone dissolved in an equimolar quantity of 1 M NaOH and made up with distilled water to a concentration of 9·4, 17·0 or 31·8 mg mL^{-1} for the three different dosages, respectively. Blood samples were taken from an incision in the tail. In order to control the body temperature of the animals, a heating-plate was used during the period the rats were sedated after administration of heptabarbitone. Urine and faeces were collected for 2 days while the rats were kept in individual metabolic cages. To minimize the influence of possible diurnal rhythm in metabolism rate (Roberts et al 1970), drug administration took place between 1000 and 1100 h, and blood sampling was carried out between 1000 and 1400 h. Urine was used for the identification of the metabolite.

Drug analysis

Heptabarbitone concentrations in plasma were determined by HPLC as described by Danhof & Levy (1985). The HPLC system consisted of a Spectroflow 400 solvent delivery system, a Promis automatic injector (both Applied Biosystems BV, Maarssen, The Netherlands), a Z-module containing a Radial-Pak C18 cartridge (Waters Associates, Milford, MA, USA) and a Spectroflow 757 UV detector (Applied Biosystems BV) set at 254 nm. Data processing was performed using a Shimadzu C-R3A reporting integrator. The detection limit was about 3 mg L^{-1} and the coefficient of variation was 8% or less in the concentration range of 25–200 mg L^{-1}.

Metabolite identification

To 5 mL urine, 7·5 mL distilled water, 12·5 mL saturated sodium chloride solution and 2·5 mL 1·6 M phosphate buffer (pH 5·5) were added and the mixture was extracted twice with 50 mL diethylether. The combined ether extracts were

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evaporated to dryness under nitrogen. The residue was dissolved in 400 μL mobile phase, of which 200 μL was injected into the HPLC. The fractions containing heptabarbitone and the metabolite were collected separately and evaporated to dryness. Blank urine samples were treated in a similar way and served as controls.

Tandem mass spectrometry (MS/MS) experiments were performed using a Finnigan MAT TSQ 70 triple quadrupole MS/MS system equipped with a Finnigan MAT thermospray (TSP) interface (Finnigan MAT, San José, CA, USA). For ionization, a discharge potential of 1000 V was used to achieve a relatively high intensity of the potential molecule, which is favourable for tandem mass spectrometry. The vaporizer temperature and the repeller potential were optimized, while the block temperature was kept at 200°C. MS/MS experiments were performed in both the parent and daughter scan modes, for screening and structure elucidation, respectively. Air was chosen as the collision gas. Heptabarbitone was used as the parent compound during optimization.

The urine samples were injected in the flow-injection-analysis (FIA) mode (Heeremans et al 1991). In daughter scan mode, one of a mixture of ions, generated in the ion source, is selected by the first mass spectrometer (MS1). In the collision chamber fragmentation is induced by collisions with the inert gas molecules. The fragments are subsequently analysed with the second mass spectrometer (MS2), providing structural information of the selected ion. In parent scan mode, MS1 is scanning over a specific mass range, one by one transmitting the ions to the collision chamber. Fragmentation is induced, but MS2 transmits only one particular fragment. All parent ions providing that particular fragment will be detected. In this way, screening for compounds with structural similarities is possible.

Data analysis

Pharmacokinetics. The areas under the heptabarbitone and the metabolite concentration-time curves (AUC) were calculated using the linear trapezoidal rule extrapolated to infinity, on the basis of the elimination rate constant, k. This constant was determined using the slope of the terminal part of the log concentration vs time profile and for each individual curve the constant derived from that curve was used. The units of measurement of the metabolite were expressed as equivalents of heptabarbitone, calculated on the basis of peak height ratios.

Statistics. The ratios between the AUC values of the metabolite and heptabarbitone after administration of the different dosages were statistically assessed using a two-way analysis of variance. The test according to Hartley showed that the variances for the different dosages were equal.

Results

In Fig. 1, HPLC of a plasma sample after administration of heptabarbitone is shown, displaying the extra peak containing the metabolite. Plasma concentration vs time profiles of heptabarbitone and the metabolite (the concentration of the metabolite is expressed as equivalents of heptabarbitone) in a representative rat following administration of the three different dosages of heptabarbitone (25, 45 and 85 mg kg^{-1}) are shown in Fig. 2. The ratios between the AUC of the metabolite and the AUC of heptabarbitone were $2{\cdot}5 \pm 0{\cdot}4$, $2{\cdot}4 \pm 0{\cdot}5$ and $2{\cdot}8 \pm 0{\cdot}3$ after administration of 25, 45 and 85 mg kg^{-1}, respectively, and appeared not to be significantly different.

Identification of the unknown metabolite was originally conducted using urine samples. Upon tandem mass spectrometry of heptabarbitone in daughter scan mode, a fragment was observed in the spectrum at $m/z = 157$, due to the loss of cycloheptadiene by collision-induced dissociation (CID) in the collision chamber. Since the metabolism of heptabarbitone was expected to occur in the cycloheptenyl group, metabolites may be expected to give a fragment at $m/z = 157$. Therefore, by performing a parent 157 scan selecting $m/z = 157$ by MS2, it is possible to screen for possible metabolites. Utilizing this procedure in urine fractions containing the unknown metabolite, a parent 157 spectrum was obtained containing fragments of $m/z = 249$, 251 and 267, respectively (Fig. 3, upper panel), which did not appear in the spectrum of blank urine (Fig. 3, lower panel). Structural information about these compounds was obtained in the daughter scan mode. The compound corresponding to the peak at $m/z = 249$ ($M = 248$) is compatible with 5-ethyl-5-cycloheptadienyl barbituric acid ($M = 248$). This structure was further confirmed by MS/MS as heptabarbitone with an additional double bond in the cycloheptenyl group, probably conjugated (at 3′ or 6′) with the other double bond (at 1′).

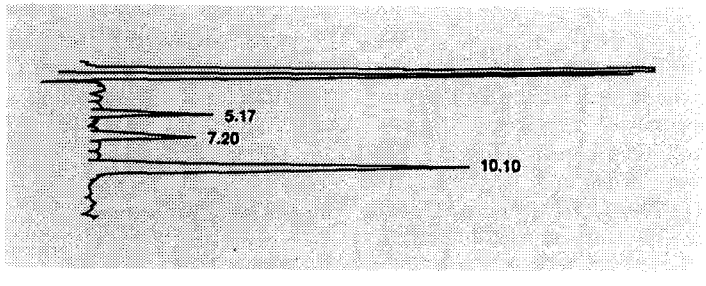

FIG. 1. HPLC of a plasma sample from a rat after administration of heptabarbitone: 5·17 min, metabolite (3′ or 7′-hydroxyheptabarbitone); 7·20 min, heptabarbitone; 10·10 min, the internal standard, secobarbitone.

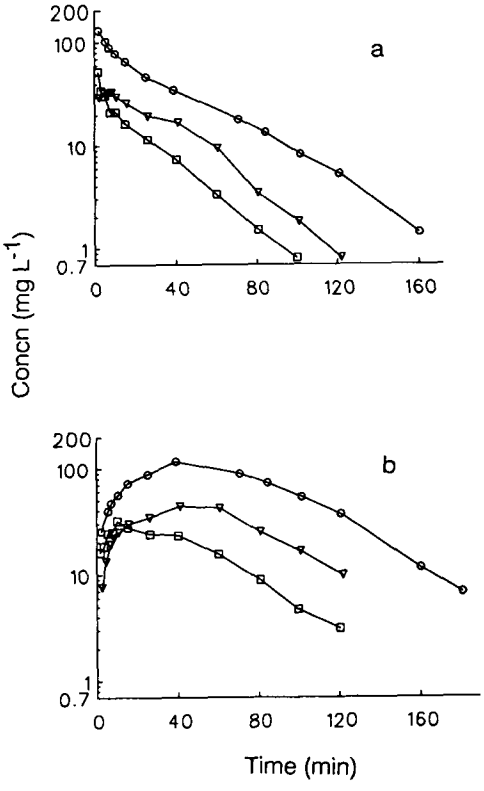

FIG. 2. Concentration vs time profiles of heptabarbitone and the metabolite (3′ or 7′-hydroxyheptabarbitone). a. Log plasma concentration vs time profiles of heptabarbitone in one representative rat. b. Log plasma concentration vs time profiles of the metabolite in the same rat. □ 25, ▽ 45, ○ 85 mg kg^{-1}.

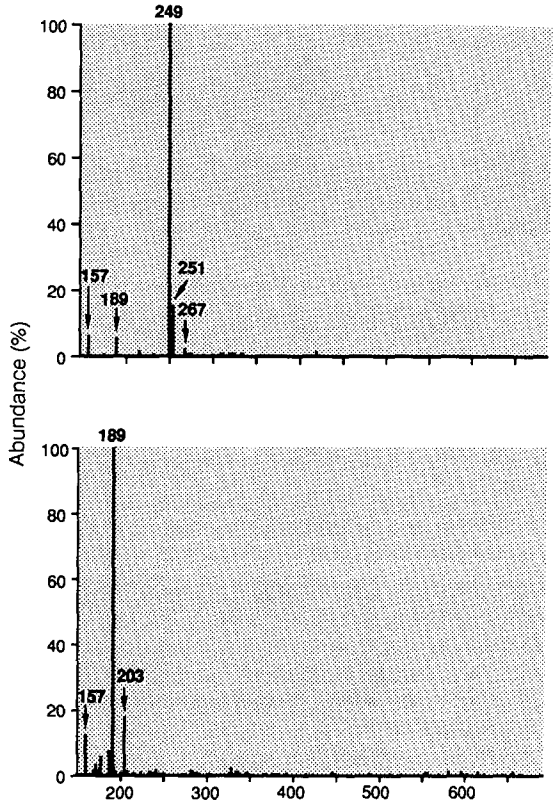

FIG. 3. Parent 157 spectra of sample and blank. Upper. Parent 157 spectrum of the collected fraction of the metabolite-containing urine sample. Lower. Parent 157 spectrum of the corresponding fraction of a blank urine sample.

The daughter spectrum (Fig. 4) showed a fragment at m/z = 93 for protonated cycloheptatriene and one at m/z = 157. The small peak in the parent 157 spectrum at m/z = 267 might be due to the addition of water to a double bond in the cycloheptadienyl group of the metabolite, probably occurring in the ion source. This was confirmed by electron-impact GC/MS analysis of the urine samples. Using this method, the fragment of 267 amu was not observed, indicating that this hydroxy-containing compound was an artefact from the thermospray ionization process (Heeremans et al 1991).

In order to confirm that the metabolite in plasma is identical to the metabolite identified in urine, a parent 157 scan, followed by daughter scans of the ions found in the parent 157 scan, was also performed on a plasma sample and a blank. The identity of the metabolite in plasma appeared to be the same as that in urine.

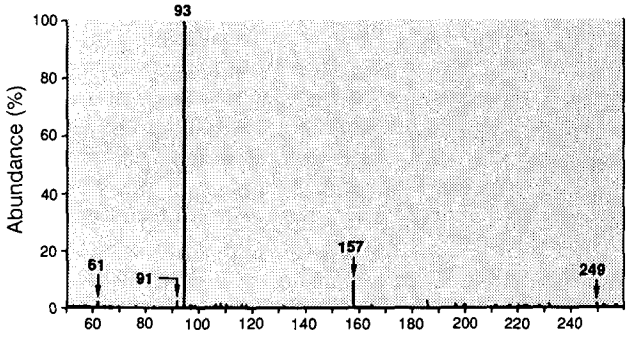

FIG. 4. Daughter spectrum of the protonated molecule at m/z = 249, observed in the parent 157 spectrum of the metabolite-containing urine sample.

Discussion

In this study, a structure of the metabolite of heptabarbitone (Dingemanse et al 1988b) measured in plasma was further examined. Because of the high amount of metabolite needed for the analytical procedures, the HPLC-fraction of the urine (collected after administration of heptabarbitone) containing a peak with the same retention time as that of the metabolite peak in plasma was used.

The strategy to identify the structure of the metabolite on the basis of tandem mass spectrometry was started by generating a daughter spectrum of heptabarbitone. This resulted in peaks at m/z = 95, 157 and 251 (results not shown). The peak at m/z = 157 is that of the barbiturate ring obtained by loss of a cycloheptadiene and the peak at m/z = 95 corresponds with protonated cycloheptadiene. Because the metabolic reactions were not expected to take place in the part of the molecule yielding this peak at m/z = 157, this fragment was also expected in the daughter

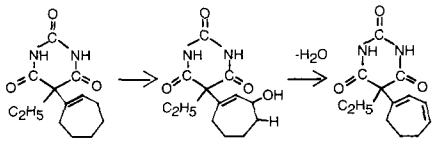

FIG. 5. Possible mechanism by which the metabolite, 5-ethyl-5-(1′,[3′ or 6′]-cycloheptadienyl)-barbituric acid, can be formed. First heptabarbital is hydroxylated to 3′ (or 7′)-hydroxyheptabarbitone and then a dehydration step takes place generating the metabolite.

spectrum of the unknown metabolite. Therefore, the parent 157 scan was performed, which resulted in peaks at m/z = 249 (highest intensity), 251 and 267 (Fig. 3 upper panel). The peak at m/z = 267 was shown to be an artefact from the thermospray MS/MS ionization process (Heeremans et al 1991). When the metabolite peak in plasma was analysed, only the peak at m/z = 249 was found in the parent 157 scan. The daughter spectrum of this peak yielded a relatively high peak at m/z = 93 (Fig. 4). Comparing this finding with the daughter spectrum of heptabarbitone, which yields a peak at m/z = 95 arising from cycloheptadiene, the m/z = 93 in the metabolite daughter spectrum can be attributed to cycloheptatriene. This indicates that the metabolite contains an extra double bond in the cycloheptenyl ring of heptabarbitone. From these results, it is proposed that the metabolite is 5-ethyl-5-(1′,[3′ or 6′]-cycloheptadienyl)-barbituric acid (M = 248). The second double bond is expected to be at the 3′ or 6′ position, since conjugated double bonds are more stable than non-conjugated.

A possible mechanism via which the metabolite can be formed is a hydroxylation to 3′ or 7′-hydroxyheptabarbitone followed by a dehydration step (Fig. 5). The formation of 3′-hydroxyheptabarbitone has already been reported (Gilbert et al 1974) in man, but its formation is likely to occur also in rats, since the formation of the 3′-hydroxy-metabolite of hexobarbitone, a structurally very similar barbiturate, is the predominant metabolic route in dog, rabbit, rat, mouse and man (Yoshimura 1957; Bush & Weller 1972).

After administration of three different intravenous dosages of heptabarbitone (25, 45 and 85 mg kg^{-1}), the ratio between the AUC of the metabolite and the AUC of heptabarbitone appeared to be similar, indicating that the exposure to the metabolite is directly correlated with the exposure to the mother compound. An important question is whether the newly identified metabolite possesses pharmacological activity. For a number of barbiturates, in-vivo metabolites have indeed been shown to be pharmacologically active (Irrgang 1965; Waddell 1965; Yamamoto et al 1978). Dingemanse et al (1988a) however, by using an indirect technique, showed that no active metabolites of heptabarbitone were formed. This might be explained by a lower pharmacological activity of the metabolite compared with heptabarbitone or a higher specific extinction of the metabolite compared with heptabarbitone (with the concentrations of the metabolite being lower than suggested by the observed peak heights).

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