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Liquid chromatography–thermospray tandem mass spectrometry for identification of a heptabarbital metabolite and sample work-up artefacts

C. E. M. HEEREMANS

Division of Analytical Chemistry, Center for Bio-Pharmaceutical Sciences, University of Leiden, P.O. Box 9502, 2300 RA Leiden (Netherlands)

A. M. STIJNEN

Division of Pharmacology, Center for Bio-Pharmaceutical Sciences, University of Leiden, P.O. Box 9503, 2300 RA Leiden (Netherlands)

R. A. M. VAN DER HOEVEN and W. M. A. NIESSEN*

Division of Analytical Chemistry, Center for Bio-Pharmaceutical Sciences, University of Leiden, P.O. Box 9502, 2300 RA Leiden (Netherlands)

M. DANHOF

Division of Pharmacology, Center for Bio-Pharmaceutical Sciences, University of Leiden, P.O. Box 9503, 2300 RA Leiden (Netherlands)

and

J. VAN DER GREEF

Division of Analytical Chemistry, Center for Bio-Pharmaceutical Sciences, University of Leiden, P.O. Box 9502, 2300 RA Leiden (Netherlands)

ABSTRACT

An unknown heptabarbital metabolite, observed in the liquid chromatogram of rat plasma and urine samples after administration of heptabarbital, was identified by liquid chromatography–thermospray tandem mass spectrometry. By applying the parent scan mode for screening and the daughter scan mode for structure elucidation, the metabolite was determined to be 5-ethyl-5-(1′-, 3′- or 6′-cycloheptadienyl)barbituric acid. It was demonstrated that artefact formation occurred when hydrochloric acid was used for conjugate hydrolysis in the sample clean-up. Identification of the artefacts was achieved by the same method. Confirmation of the structures of the metabolite and the artefacts was obtained by gas chromatography–electron impact mass spectrometry.

INTRODUCTION

Sensitivity to various drugs is dependent on the (patho)physiological status of the individual (*e.g.*, diseases, age, pregnancy). Owing to alterations in the physiological status, the pharmacokinetics or the pharmacodynamics of drugs can be influenced. In our Centre, heptabarbital [5-ethyl-5-(1′-cycloheptenyl)barbituric acid] is used as a model substrate for the investigation of these effects [1–5].

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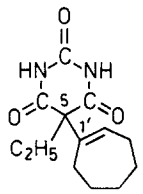


Heptabarbital (mol.wt. 250)

During pharmacological studies, a peak was observed on liquid chromatography (LC) with UV detection of a rat plasma sample after administration of heptabarbital [5], which followed the concentration–time profile of a metabolite but did not correspond to one of the known metabolites. Gilbert *et al.* [6] studied the metabolism of heptabarbital in humans and found three possible metabolites: 3′-hydroxyheptabarbital, 3′-ketoheptabarbital and 7′-hydroxyheptabarbital. Vermeulen [7] investigated the metabolic pathway of heptabarbital in male Wistar rats and identified 5-ethylbarbituric acid in the urine, which was suggested to be formed via an epoxide–diol pathway.

This paper describes the elucidation of the structure of the unknown metabolite in rat urine and plasma, employing coupled liquid chromatography (LC)–tandem mass spectrometry (MS–MS) with thermospray (TSP) ionization. The parent scan mode was used as a specific screening method for heptabarbital-related compounds, both metabolites and artefacts of hydrochloric acid hydrolysis, whereas structural information of the protonated compounds was obtained in the daughter scan mode.

The results from the LC–TSP-MS–MS experiments are compared with those obtained by gas chromatography–electron impact (EI) mass spectrometry (GC–MS) and solid-probe high-resolution (HR) measurements, which are techniques commonly used in the analysis of barbiturates.

EXPERIMENTAL

Sample clean-up and fraction collection

Urine: clean-up method 1. After addition of 1 ml of 36% hydrochloric acid to 1 ml of urine sample, the mixture was heated on a water-bath at 100°C for 30 min in order to hydrolyse possible conjugates. After cooling the mixture, 1.5 ml of saturated sodium chloride solution and 1 ml of distilled water were added. The mixture was extracted twice with 5 ml of diethyl ether. The combined ether extracts were evaporated to dryness under a stream of nitrogen.

Urine: clean-up method 2. A 7.5-ml volume of distilled water, 12.5 ml of saturated sodium chloride solution and 2.5 ml of 1.6 *M* phosphate buffer (pH 5.5) were added to a 5-ml urine sample and the mixture was extracted twice with 50 ml of diethyl ether. The combined ether extracts were evaporated to dryness under a stream of nitrogen.

Plasma. To 1 ml of plasma, 5 ml of acetonitrile were added to precipitate proteins. After thorough mixing and centrifugation, the supernatant was evaporated to dryness.

Fraction collection of the peak of interest. The dried samples were dissolved in 400 μl of mobile phase and 200 μl were injected into the LC system as described by

Danhof and Levy [8]. The metabolite fraction was collected and evaporated to dryness. The collected fractions from several animals were combined in order to obtain high concentrations of the compound. The total procedure was also applied to urine samples collected before the administration of heptabarbital, providing the blanks.

Liquid chromatography–mass spectrometry and tandem mass spectrometry

The LC system used for the LC–TSP-MS–MS experiments consisted of a Model 2150 high-pressure pump (LKB, Bromma, Sweden), a Model 7125 injection valve equipped with a 20-μl loop (Rheodyne, Berkeley, CA, USA) and a 150 × 3 mm I.D. Nucleosil C_{18} (5 μm) column (Macherey-Nagel, Düren, Germany).

LC–MS and LC–MS–MS experiments were performed using a Finnigan MAT (San José, CA, USA) TSQ 70 triple quadrupole MS–MS system equipped with a Finnigan MAT TSP interface.

In the buffer ionization mode the mobile phase consisted of 50 mmol/l ammonium acetate in methanol–water (50:50, v/v), whereas in the discharge-on mode methanol–water (50:50, v/v) was used, at a flow-rate of 1.2 ml/min. The discharge potential was 1000 V. The vaporizer temperature and the repeller potential were optimized [9], while the block temperature was kept at 200°C. In the daughter and parent scan MS–MS experiments the collision energy and pressure were optimized; air was used as the collision gas.

Gas chromatography–mass spectrometry

GC–MS was performed on a Finnigan MAT Model 700 ion trap detector, combined with a Model 438A gas chromatograph (Chrompack Packard, Middelburg, Netherlands) equipped with a split injector (splitting ratio 1:40) and a 10 m × 0.25 mm I.D. CP-Sil-5 column (Chrompack). The oven temperature was kept at 50°C for 5 min and subsequently increased linearly to 250°C at 15°C/min. EI mass spectra were obtained at 1 s per scan.

High-resolution electron impact mass spectrometry

Solid-probe high-resolution (R = 15000) EI-MS was performed on a Varian MAT 711 double-focusing mass spectrometer (Varian, Bremen, Germany).

RESULTS AND DISCUSSION

Because the concentration of the compound of interest is much higher in urine, identification is performed on rat urine samples. Acidic hydrolysis using 36% hydrochloric acid is applied, because the metabolite is expected to be a hydroxyheptabarbital, which will be conjugated before excretion into urine. GC–MS with EI ionization is a commonly used technique for the analysis of barbiturates. However, because an LC peak has to be identified, LC–MS is preferred, with direct coupling of the LC system to the mass spectrometer by a TSP interface. Moreover, urine samples contain too many involatile compounds for GC–MS analysis, necessitating extensive sample pretreatment.

Optimization of the thermospray (tandem) mass spectrometry conditions

In TSP-MS three different ionization modes, namely buffer ionization, dis-

charge-on and filament-on ionization, can be used. As the sensitivity for various classes of compounds can differ dramatically between these modes, a careful choice is important. In this study, buffer and discharge-on ionization were compared. Other experimental parameters that have to be optimized are the vaporizer temperature and the repeller potential [9,10]. This optimization is performed by injecting heptabarbital (mol.wt. 250) in the column bypass mode, assuming that heptabarbital metabolites will be ionized in the same way as the parent compound owing to structural similarities. The vaporizer temperature appears not to be critical for heptabarbital because no thermal degradation is observed in the normally used temperature range of 80–120°C.

The repeller potential not only can be used to improve sensitivity in TSP-MS, but can also induce useful fragmentation reactions in the discharge-on mode [9–12]. In the buffer ionization mode for heptabarbital an intense ammoniated molecule, $[M + NH_4]^+$, at m/z 268 and a weak protonated molecule, $[M + H]^+$, at m/z = 251 are observed at low repeller potentials. With increasing repeller potential the intensity of the ammoniated molecule (m/z 268) decreases, whereas the intensity of the protonated molecule (m/z 251) increases. However, the overall sensitivity decreases with increasing repeller potential, as is usually observed in the buffer ionization mode. Surprisingly, a fragment peak at m/z 157, due to the loss of cycloheptadiene, also appears with increasing intensity at higher repeller potentials. Fragmentation is not often observed in the buffer ionization mode [9,10]. In the discharge-on mode, fragmentation of the protonated molecule is induced at high repeller potentials without a loss of sensitivity [11,12]. Two fragment ion peaks are observed at m/z 157 and 221, explained as the result of the loss of either cycloheptadiene or ethane.

When these two ionization modes are compared, a tenfold higher signal for the protonated molecule is observed in the discharge-on mode at low repeller potentials compared with the buffer ionization mode. Because a high intensity of the protonated molecule is preferred for the detection of unknown compounds and for MS–MS analysis, further experiments were performed in the discharge-on mode at low repeller potentials. Further, the formation of ammoniated species with buffer ionization is a disadvantage, because molecular weight determinations of unknown compounds can be ambiguous when it is not clear whether only protonated species or ammoniated species also are present in the spectrum.

The possibilities of applying MS–MS in combination with LC–TSP-MS were also investigated, because of the higher selectivities that can be achieved. Further, in the daughter scan mode, structural information can be obtained from the protonated molecules observed in LC–TSP-MS.

In the daughter scan mode the protonated molecule of heptabarbital (m/z 251) is dissociated to a fragment at m/z 157, due to the loss of cycloheptadiene (Fig. 1). The fragment at m/z 95 corresponds to protonated cycloheptadiene.

LC–TSP-MS of urine samples (clean-up method 1)

LC–MS of a pretreated urine sample does not give any useful information, as no distinct differences between the urine sample and the blank sample are observed. This is probably due to the presence of non-UV-absorbing interfering compounds from the urine.

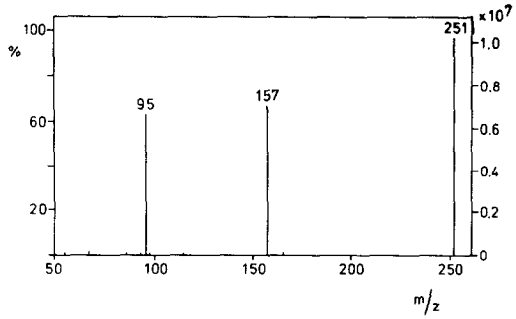


Fig. 1. Daughter-ion mass spectrum of the protonated molecule of heptabarbital (mol.wt. 250).

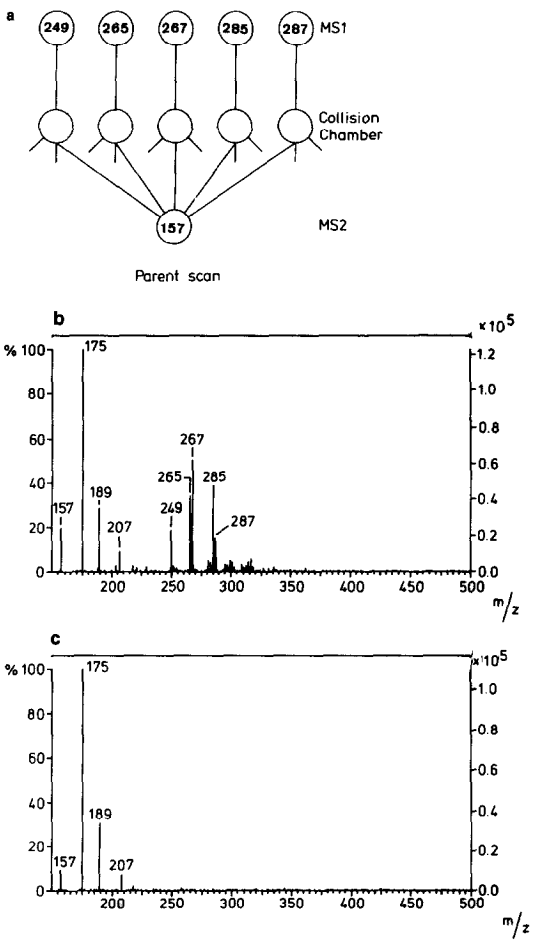


Fig. 2. (a) Parent m/z 157 scan mode; (b) parent m/z 157 mass spectrum of the collected fraction of the rat urine sample (clean-up method 1); (c) parent m/z 157 mass spectrum of a blank urine sample (clean-up method 1).

LC–TSP-MS of the collected fraction (clean-up method 1)

Off-line fraction collection of the chromatographic peak of interest, corresponding to the unknown metabolite, from the urine sample and the blank was performed using the LC system developed by Danhof and Levy [8]. The TSP mass spectrum of the collected fraction from the urine sample contains several peaks, *e.g.*, at *m/z* 249, 265, 267, 283 and 301, that are not present in the TSP mass spectrum of blank urine. It appears that the peak of interest probably consists of a number of compounds.

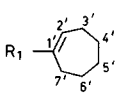
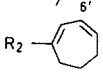
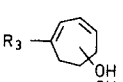
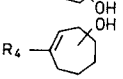
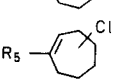
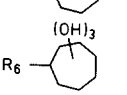
TSP-MS–MS of collected fraction of urine samples (clean-up method 1)

Because the metabolic reactions are expected to occur in the cycloheptenyl group [6,7], a daughter ion at *m/z* 157, obtained by loss of a cycloheptadiene from heptabarbital, is also expected to be formed by collision-induced dissociation (CID) in the MS–MS mode for heptabarbital metabolites. In order to screen for heptabarbital metabolites in the collected fraction of the rat urine, a parent *m/z* 157 scan (Fig. 2a) was performed in the column bypass mode. Peaks at *m/z* 249, 265, 267, 285 and 287 are observed in the parent *m/z* 157 mass spectrum of the urine sample that are not observed for the blank (Fig. 2b and c). These peaks are expected to be protonated molecules of heptabarbital-related compounds.

Subsequently, daughter-ion spectra of the above mentioned peaks were obtained and identification of the compounds was achieved (Table I). The compound with a molecular weight of 248 has an additional double bond in the cycloheptenyl group (see R_2 in Table I). The daughter-ion spectrum (Fig. 3a) shows a fragment at

TABLE I

STRUCTURES OF THE SAMPLE WORK-UP ARTEFACTS OF HEPTABARBITAL OBSERVED IN THE PARENT *m/z* 157 MASS SPECTRUM OF A RAT URINE SAMPLE AFTER CLEAN-UP METHOD 1

$[M+H]^+$ *m/z*	Structure
251 heptabarbital	
249	
265	
267	
285	
301	

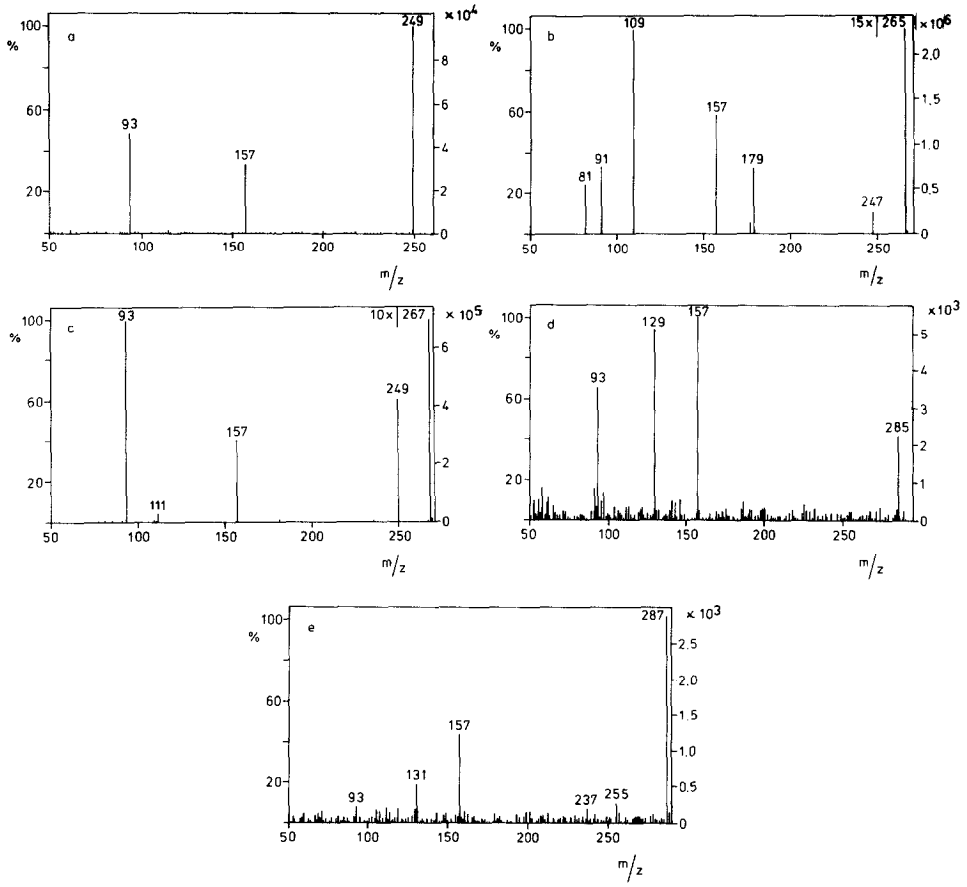


Fig. 3. Daughter-ion mass spectrum of the various protonated molecules observed in the parent *m/z* 157 mass spectrum of the urine sample. (a) *m/z* 249; (b) *m/z* 265; (c) *m/z* 267; (d) *m/z* 285; (e) *m/z* 287.

m/z 93 for protonated cycloheptatriene, while the daughter-ion spectrum of heptabarbital (mol.wt. 250) shows a fragment at *m/z* 95 for protonated cycloheptadiene (R_1).

The daugther-ion spectra of *m/z* = 265 and 267 (Fig. 3b and c, respectively) show similarities.

The compound corresponding to a protonated molecule at *m/z* 267 has a hydroxy group in the cycloheptenyl moiety (R_4). Loss of water from the protonated molecule and the abstracted protonated ring at *m/z* 111 results in fragments at *m/z* 249 and 93, respectively.

The compound corresponding to a protonated molecule at *m/z* 265 has an additional double bond and a hydroxy group in the cycloheptenyl group (R_3). Loss of water from the protonated molecule and the abstracted protonated ring (*m/z* 109) results in similar fragments at *m/z* 247 and 91. The daughter at *m/z* 179 is probably formed by loss of 2 HNCO.

The daughter-ion spectrum of *m/z* 285 (Fig. 3d) is not easily interpreted. Fragments are observed at *m/z* 129 and 93. The fragment at *m/z* 93 is likely to be protonat-

ed cycloheptatriene, as observed for the compounds corresponding with the protonated molecules at *m/z* 249 and 265. The ions at *m/z* 129 and 93 show a mass difference of 36 u, which might correspond to the loss of HCl from *m/z* 129. In the parent *m/z* 157 spectrum an isotope peak of *m/z* 285 is observed at *m/z* 287 with a 3:1 ratio, respectively, suggesting the presence of chlorine.

The daughter-ion spectrum of *m/z* 287 (Fig. 3e) proves the presence of a chlorine in the cycloheptenyl group. Fragments are observed at *m/z* 131 and 93 with a mass difference of 38 u, corresponding to the loss of $H^{37}Cl$.

In addition, the compounds with molecular weights of 282 and 300, observed in LC–MS, can be explained by the addition of one and two water molecules, respectively, at the double bonds in the cycloheptadienyl group of the compound with a molecular weight of 264 u. However, these compounds are not intensely observed in the parent *m/z* 157 scan, although a daughter-ion spectrum of *m/z* 301 shows a fragment at *m/z* 157.

It is not likely that the identified compounds given in Table I are eluted in the same region of the liquid chromatogram. Probably the hydroxy-containing components are formed in the ion source by addition of water to the double bonds (see below).

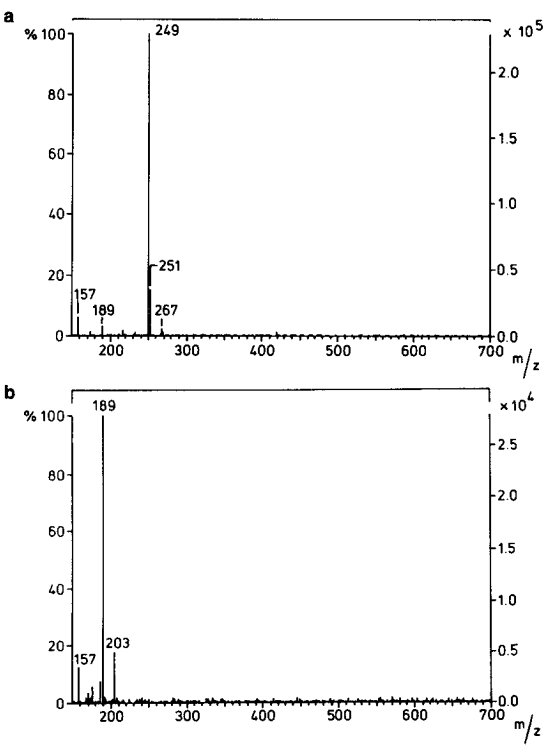
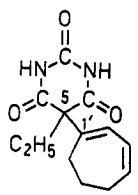


Fig. 4. (a) Parent *m/z* 157 mass spectrum of the collected fraction of the rat urine sample (clean-up method 2); (b) parent *m/z* 157 mass spectrum of a blank urine sample (clean-up method 2).

TSP-MS–MS of collected fraction (clean-up method 2)

Chlorinated compounds are not expected to be formed in metabolic reactions. Therefore, the sample clean-up (method 1), using hydrochloric acid for the hydrolysis of conjugated metabolites, was changed. Boiling with hydrochloric acid apparently introduces artefacts. When sample clean-up method 2 was used, the unknown peak was still observed in the liquid chromatogram obtained with UV detection. After fraction collection of the peak of interest, a parent *m/z* 157 scan was performed on the urine sample and also on a blank in the column bypass mode (Fig. 4). The peak at *m/z* 249 is the base peak of the spectrum of the urine sample. The structure of this protonated molecule is confirmed in the daughter scan mode to be heptabarbital with an additional double bond, probably conjugated (at the 3′- or 6′-position) with the other double bond at the 1′-position [5-ethyl-5-(1′-, 3′- or 6′-cycloheptadienyl)barbturic acid].



5-ethyl-5-(1′-, 3′- or 6′-cycloheptadienyl)barbituric acid (mol.wt. 248)

The daughter-ion spectrum of *m/z* 249 is in agreement with the former experiments, showing fragments at *m/z* 93 and 157. The small peak in the parent *m/z* 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. The daughter-ion spectrum of *m/z* 267 also corresponds with the previously obtained spectrum, showing daughter-ions at *m/z* 111 and 93.

TSP-MS–MS of collected fraction of plasma samples (clean-up method 2)

A parent *m/z* 157 scan was also performed on the collected fraction of a plasma sample in which the concentration of the unknown compound was much lower, and on a blank. The plasma sample contained the metabolite (mol.wt. 248) with the additional double bond in the cycloheptenyl group [5-ethyl-5-[1′-, 3′- or 6′-cycloheptadienyl)barbituric acid].

Metabolic pathway

A possible mechanism for the formation of the metabolite is a hydroxylation to 3′-, 4′-, 6′- or 7′-hydroxyheptabarbital, followed by a dehydration step. The pharmacological behaviour of this metabolite is discussed elsewhere [13].

GC–MS of collected fractions combined with high-resolution MS

GC–EI-MS is generally used in the analysis of barbiturates. The EI fragmentation is relatively well understood [14]. After fraction collection the samples are much cleaner and the amount of non-volatile materials is greatly reduced. For these reasons, GC–MS analysis of the collected fractions was performed.

The GC–MS data confirmed the results from the LC–TSP-MS and -MS–MS studies. However, the identification was hampered by the absence of molecular ions in barbiturate EI mass spectra. After clean-up method 1 the compounds with R_2 and R_5 were observed. These data confirm that the hydroxy-containing compounds (R_3, R_4 and R_6 in Table I) are artefacts from the TSP ionization process. The presence of the chlorine atom in R_5 was confirmed by solid-probe HR-MS. After clean-up method 2, the compound with R_2 was observed, which is in agreement with the TSP-MS–MS data. The clean-up method with hydrochloric acid hydrolysis is responsible for the presence of the compound with the chlorinated cycloheptenyl group (R_5).

CONCLUSIONS

Using a combination of TSP-MS–MS in the parent and daughter scan modes, the unknown metabolite observed in rat plasma and urine samples after administration of heptabarbital was determined to be 5-ethyl-5-(1′-, 3′- or 6′-cycloheptadienyl)barbituric acid. It has been demonstrated that acidic hydrolysis with 36% hydrochloric acid can introduce artefacts. The identity of the metabolite and the sample work-up artefacts was confirmed by GC–EI-MS, but not as straightforwardly as with TSP-MS–MS.

REFERENCES

1 J. Dingemanse, M. Polhuijs and M. Danhof, *J. Pharmacol. Exp. Ther.*, 246 (1988) 371.
2 J. W. Mandema and M. Danhof, *J. Pharmacokinet. Biopharm.*, 18 (1990) 459.
3 A. M. Stijnen, S. H. van der Voort, C. F. A. van Bezooijen and M. Danhof, in K. W. Woodhouse, C. Yelland and O. F. W. James (Editors), *The Liver Metabolism and Ageing*, Eurage, Rijswijk, 1989, p. 57.
4 A. M. Stijnen, A. M. Bergveld, J. W. Mandema, C. F. A. van Bezooijen and M. Danhof, in preparation.
5 J. Dingemanse, D. Thomassen, B. H. Mentink and M. Danhof, *J. Pharm. Pharmacol.*, 40 (1988) 522.
6 J. N. T. Gilbert, B. J. Millard, J. W. Powell and W. B. Whalley, *J. Pharm. Pharmacol.*, 26 (1974) 123.
7 N. P. E. Vermeulen, *Ph.D. Thesis*, University of Leiden, Leiden, 1980, pp. 103–109.
8 M. Danhof and G. Levy, *J. Pharmacol. Exp. Ther.*, 232 (1985) 430.
9 C. E. M. Heeremans, R. A. M. van der Hoeven, W. M. A. Niessen, U. R. Tjaden and J. van der Greef, *J. Chromatogr.*, 474 (1989) 149.
10 C. Lindberg and J. Paulson, *J. Chromatogr.*, 349 (1987) 117.
11 W. H. McFadden and S. A. Lammert, *J. Chromatogr.*, 385 (1987) 201.
12 W. M. A. Niessen, R. A. M. van der Hoeven, M. A. G. de Kraa, C. E. M. Heeremans, U. R. Tjaden and J. van der Greef, *J. Chromatogr.*, 478 (1989) 325.
13 A. M. Stijnen, C. E. M. Heeremans, C. F. A. van Bezooijen, W. M. A. Niessen and M. Danhof, in preparation.
14 B. J. Gudzinowicz and M. J. Gudzinowicz, *Analysis of Drugs and Metabolites by GC/MS*, Vol. 2, Marcel Dekker, New York, 1977, pp. 1–184.