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IMPROVED DETECTABILITY OF BARBITURATES IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY BY PRE-COLUMN LABELLING AND ULTRAVIOLET DETECTION

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SUMMARY

2-Naphthacyl derivatives of barbiturates are formed in acetone at 30° within 30 min, in an essentially quantitative manner, using caesium carbonate as a catalyst. These derivatives absorb UV radiation strongly at 254 nm. Using a variable-wavelength detector set at 249 nm, 1 ng of N,N-dinaphthacylphenobarbital could be detected after chromatography using a high-performance liquid chromatograph equipped with a microparticulate reversed-phase column. This derivatization technique has the potential of allowing the determination of barbiturates, in small plasma or serum samples, in concentrations well below the therapeutic range.

INTRODUCTION

Many UV detectors used in high-performance liquid chromatography (HPLC) are single-wavelength detectors, most often operated at 254 nm. At this wavelength the barbiturates show too low a natural absorption (in their un-ionized form) to permit a sensitive determination of these compounds by HPLC. Sensitive methods are needed, for example, for monitoring plasma levels of barbiturates when limited sample volumes are available.

A considerable increase in the UV absorption of barbiturates can be achieved by post-column ionization caused by the infusion of an alkaline buffer solution[1]. This requires special equipment, which is not commonly available in routine laboratories. Another means of overcoming the problem is pre-column labelling of the barbiturates with a suitable chromophore. Alkylating agents based on phenacyl or naphthacyl groups have been utilized to improve the UV response of several classes of carboxylic acids[2–9]. The derivatization reaction is usually carried out in a non-aqueous medium (such as acetonitrile or acetone) in the presence of an alkaline catalyst (such as N,N-diisopropylethylamine[2,5,7] or a crown ether[4,6,8,9]. In the present investigation the

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suitability of α-bromo-2′-acetonaphthone (2-naphthacyl bromide) was tested for the alkylation of barbiturates.

EXPERIMENTAL

The barbiturates were obtained from different commercial sources and were used without further purification. 2-Naphthacyl bromide (NPB) from Aldrich-Europe (Beerse, Belgium) was recrystallized twice from ethanol. Caesium carbonate (reinst) was purchased from Merck (Darmstadt, G.F.R.) and was dried and finely powdered before use. Acetone, chloroform, methanol and glacial acetic acid were of p.a. grade (Merck); the acetonitrile (UV grade) was also from Merck. N,N-Dimethylacetamide (puriss.) was obtained from Fluka (Buchs, Switzerland). N,N-Diisopropylethylamine (zur Synthese, Merck) was distilled before use. Distilled water was used throughout.

Derivatization procedure

A 50-μl volume of a barbiturate solution in acetone (0.02–0.2 mg/ml was placed in a glass-stoppered centrifuge tube with the conical end drawn to a fine point, and mixed with 50 μl of NPB solution in acetone (0.4–1.6 mg/ml). Caesium carbonate (5–10 mg) was added and the tube was placed in a water-bath at 30°. After derivatization, 50 μl of glacial acetic acid were added and the contents of the tube were mixed until all of the caesium carbonate had dissolved. An aliquot of the resulting solution was injected into the liquid chromatograph. In derivatization experiments at higher temperatures, the reagent mixture was heated in a closed 8 cm × 3 mm I.D. glass capillary.

The NPB derivatives of phenobarbital [PB(NPB)$_2$] and amobarbital [AB-(NPB)$_2$] were prepared on a larger scale for the purpose of structure elucidation by mass and ^{13}C NMR spectrometry. Barbiturate (2 mmol) and NPB (4 mmol) were dissolved in 250 ml of acetone; caesium carbonate (10 mmol) was added and the mixture was stirred for 18 h at room temperature. After filtration, the acetone in the filtrate was removed under reduced pressure. The residue was taken up in 10 ml of chloroform and the resulting solution was placed in the sample reservoir of a Chromatospac Prep 100 preparative liquid chromatograph (Jobin Yvon, Longjumeau, France) equipped with a variable-wavelength UV detector. After elution with chloroform–methanol (99.5:0.5) through a column containing 100 g of silica gel (Kieselgel H nach Stahl, Merck), the appropriate fractions of the eluate containing the pure naphthacyl derivative were evaporated under reduced pressure. Upon injection into the high-performance liquid chromatograph (for conditions, see below), the thus purified derivatives of phenobarbital and amobarbital yielded a single peak in the chromatogram. A single spot appeared on a 0.25-mm silica gel F_{254} plate (5 × 10 cm, Merck), developed with chloroform–methanol (99:1) for the derivatives of phenobarbital and of amobarbital.

About 500 μg of the naphthacyl derivative of mephobarbital was obtained by repeated injections into the high-performance liquid chromatograph (for conditions, see below) of the derivatization mixture of 5 mg of mephobarbital, 5 mg of NPB and 5 μl of N,N-diisopropylamine in 2 ml of acetonitrile (5 h at 60°) and subsequent evaporation of the appropriate fractions of the eluate.

High-performance liquid chromatography

Analyses were conducted with a 6000 A solvent delivery system, a U6K injector, a μBondapak C_{18} column (30 cm × 4 mm I.D.), all from Waters Assoc. (Milford, Mass., U.S.A.), and a Pye Unicam LC3 variable-wavelength detector, set at 249 nm. The eluent was 80% (w/w) methanol in water, passed through a 0.2-μm filter and deaerated ultrasonically, usually at a flow-rate of 2 ml/min. Chromatography was performed at ambient temperature.

UV spectra

UV spectra were run in 80% (w/w) methanol in water in a 1-cm cell on a Cary Model 118 spectrophotometer.

Mass spectra

An AEI-MS 902 mass spectrometer was used. The ionizing current and voltage were 100 μA and 70 eV, respectively, for the recording of electron-impact spectra. All samples were introduced through the direct insertion probe and the spectra were recorded at a probe temperature of 160° for mephobarbital, 200° for amobarbital and 230° for phenobarbital.

^{13}C NMR spectrometry

A Varian CFT-20 spectrometer was used to record 20-MHz ^{13}C NMR spectra using $CDCl_3$ as the solvent.

RESULTS AND DISCUSSION

In order to verify the structures of the derivatives of phenobarbital, amobarbital and mephobarbital, their mass spectra were obtained (Table I). The molecular weights of the derivatives of phenobarbital and amobarbital (568 and 562 a.m.u., respectively) are consistent with the introduction of two naphthacyl groups. Mephobarbital, an N-methylated barbiturate, yielded a derivative with a molecular weight (414 a.m.u.) that is indicative of the introduction of one naphthacyl group. Most fragments of the three compounds could be explained as occurring by standard processes.

TABLE I

PARTIAL ELECTRON-IMPACT MASS SPECTRA OF THE NAPHTHACYL DERIVATIVES OF PHENOBARBITAL [PB(NPB)$_2$], AMOBARBITAL [AB(NPB)$_2$] AND MEPHOBARBITAL [MBNPB]

Compound	*m/e and (relative abundance, %)*
PB(NPB)$_2$	568(7); 399(5); 174(2); 168(5); 155(100); 146(7); 127(48); 117(6)
AB(NPB)$_2$	562(3); 393(3); 170(1); 168(3); 155(100); 127(22)
MBNPB	414(13); 168(2); 155(100); 127(26); 117(2)

The ^{13}C NMR spectra of the three compounds proved that the N-substituted naphthacyl derivatives of the barbiturates had been formed (the estimated shifts for the α-carbon atom of N-naphthacyl and O-naphthacyl are 49 and 70 ppm, respec-

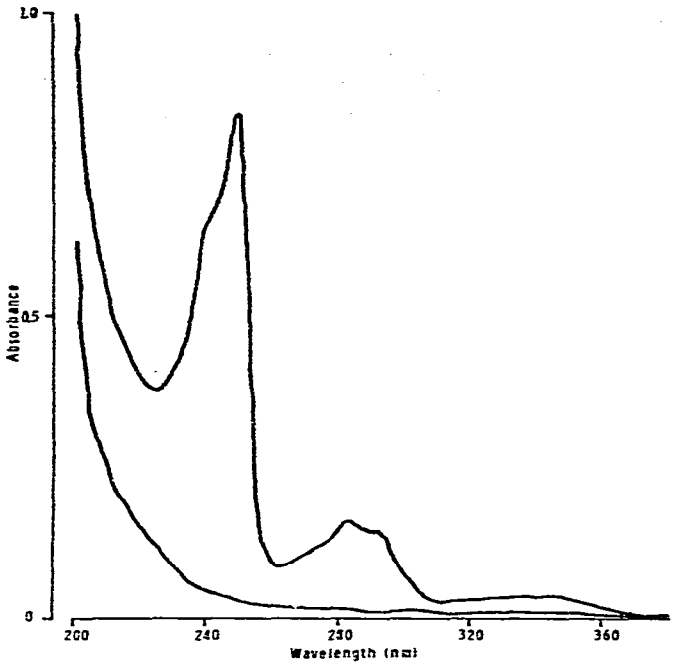

Fig. 1. UV absorption spectra of 7.0 μmol/l solutions of N,N-dinaphthacylphenobarbital and of phenobarbital in 80% (w/w) methanol in water (the lower spectrum belongs to phenobarbital).

tively; a shift of 48 ppm was measured). The λ_{max} of $PB(NPB)_2$ was 249 nm, with a molar absorptivity of $\varepsilon = 115{,}000$, which means about a 50-fold increase in absorption compared with phenobarbital (Fig. 1). Good chromatographic properties of the naphthacyl derivatives of the barbiturates were observed using a reversed-phase column and methanol–water as the eluent. Fig. 2 shows a chromatogram of six barbiturates after derivatization with NPB. The retention volumes and the capacity factors of all derivatized compounds investigated in this study are summarized in Table II. As expected, the derivatives of the N-methylated barbiturates, mephobarbital and hexobarbital, eluted first, being less lipophilic than the compounds with two naphthacyl groups. The order of elution of the derivatives of related barbiturates — barbital, butobarbital, amobarbital, pentobarbital and secobarbital — was also clearly determined by the relative lipophilicities of the parent compounds, the derivatives of the less lipophilic compounds being eluted first.

Portions of 10 μl of solutions of $PB(NPB)_2$ in methanol, at concentrations ranging from 0.20 to 100 mg/l, were injected into the liquid chromatograph. The ratio of the mean peak height to the concentration was found to be constant (within experimental error) in the investigated concentration range (Table III), indicating that the detector response was linear up to at least 1 μg of $PB(NPB)_2$ injected. About 1 ng of $PB(NPB)_2$ could be detected under the experimental conditions (signal-to-noise ratio 2–3). Modern single-wavelength detectors, monitoring the UV absorbance at 254 nm, have an even better signal-to-noise ratio than the variable-wavelength detector used in this study. With such a single-wavelength detector it might be possible to detect sub-

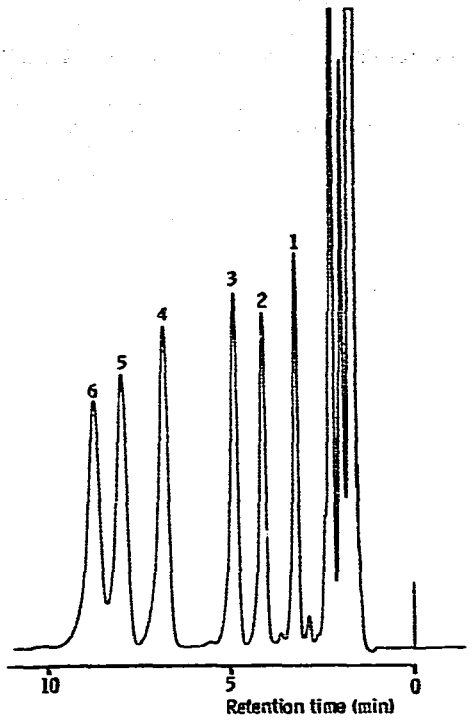

Fig. 2. High-performance liquid chromatogram on a μBondapak C_{18} column of barbiturates (each about 0.03 mg/ml) after derivatization with NPB (0.5 mg/ml in acetone) and caesium carbonate. Flow-rate, 2 ml/min; chart speed, 0.5 cm/min; sensitivity, 0.16 a.u.f.s. Peaks: 1 = hexobarbital; 2 = heptobarbital; 3 = phenobarbital; 4 = butobarbital; 5 = amobarbital; 6 = pentobarbital.

nanogram amounts of $PB(NPB)_2$, despite the lower molar absorptivity of the compound at 254 nm than at 249 nm.

The peak heights of injected $PB(NPB)_2$ were very reproducible, depending only on concentrations and not on volumes injected in the range 5–20 μl. The coefficient of variation for eight replicate 10-μl injections containing 100 ng of $PB(NPB)_2$ was 1.3%.

TABLE II

RETENTION VOLUMES (V_R) AND CAPACITY FACTORS (k') OF DERIVATIZED BARBITURATES

Compound	*No. of naphthacyl groups*	V_R *(ml)*	k'
Mephobarbital	1	6.3	1.17
Hexobarbital	1	6.5	1.24
Heptobarbital	2	8.4	1.90
Barbital	2	9.8	2.38
Phenobarbital	2	9.9	2.41
Butobarbital	2	13.7	3.72
Amobarbital	2	16.0	4.52
Pentobarbital	2	17.5	5.03
Secobarbital	2	19.0	5.55

TABLE III

PEAK HEIGHTS OF N,N-DINAPTHACYL PHENOBARBITAL [PB(NPB)$_2$] AFTER HPLC OF SOLUTIONS OF VARYING CONCENTRATION

Concentration (mg/l)	*Sensitivity of detector (a.u.f.s.)*	*Peak height* (cm)*	*Peak height at 0.02 a.u.f.s.*	*Peak height (0.02 a.u.f.s.)/ concentration*
0.20	0.02	0.68	0.68	3.4
1.00	0.02	3.45	3.45	3.45
10.0	0.08	8.38	33.5	3.35
100	0.64	10.44	334	3.34

* Each value is the mean of three determinations.

The derivatization of phenobarbital with NPB was investigated at temperatures ranging from room temperature to 80°, with N,N-diisopropylamine and caesium carbonate as catalysts, and acetone, N,N-dimethylacetamide and acetonitrile as the solvents. Elevated temperatures were necessary for the complete derivatization of the compounds with diisopropylamine. Acetone, in conjunction with caesium carbonate, was found to be an excellent medium for the derivatization of the barbiturates at temperatures no higher than 30°.

After derivatization, the reaction was stopped by the addition of glacial acetic acid in order to prevent the degradation of NPB into potentially interfering compounds. The percentage of phenobarbital converted into its N,N-dinaphthacyl derivative was investigated under varying conditions, using the previously prepared PB(NPB)$_2$ as the external standard. The results are summarized in Table IV. Phenobarbital, at a concentration of 0.05 mg/ml, was almost completely derivatized at 30° within 30 min with a 0.2 mg/ml NPB solution. The conversion of the other barbiturates into their naphthacyl derivatives was also investigated. All compounds, at concentrations up to 0.1 mg/ml, were completely derivatized within 30 min at 30° when using a 0.8 mg/ml NPB solution in acetone with caesium carbonate as the catalyst.

Recently, a sensitive HPLC method for the determination of some barbiturates (and related compounds) in serum has been reported[10]. After extraction of the serum with chloroform, the compounds were chromatographed on a reversed-phase column,

TABLE IV

DERIVATISATION OF PHENOBARBITAL WITH 2-NAPHTHACYL BROMIDE (NPB)

NPB concentration (mg/ml)	*Phenobarbital concentration (mg/ml)*	*Time (min)*	*Conversion (%)*	
			30°	*80°*
0.2	0.05	10	72	100
0.2	0.05	20	89	
0.8	0.10	20	92	
0.2	0.05	30	97	
0.2	0.01	30	100	
0.8	0.10	30	100	100
0.2	0.05	40	100	
0.8	0.10	40	100	
0.2	0.05	50	100	

without derivatization, with 15% acetonitrile in water, and were detected by UV absorption at 195 nm. In our experience considerable interference in the chromatograms by endogenous compounds in the serum or plasma can occur after a single extraction step, particularly when detecting at low wavelengths and when using low methanol or acetonitrile concentrations in the mobile phase. With the described derivatization procedure the barbiturates are converted into products that can be detected at 254 nm. At this wavelength, contrary to detection at 195 nm, methanol can be used as the co-solvent in the eluent instead of the very expensive UV-grade acetonitrile. A high methanol concentration (80%, w/w) must be used in order to elute the NPB derivatives.

Interferences in the chromatograms caused by underivatized compounds in the serum or plasma are therefore unlikely. Acidic compounds, particularly higher fatty acids, which are extracted from the plasma by organic solvents and are derivatized by NPB, might be a source of interference. However, the naphthacyl derivative of palmitic acid did not elute even after 3 h with 80% (w/w) methanol in water at a flow-rate of 2 ml/min; using pure methanol as the eluent the main peak of the naphthacyl derivative was eluted after 6 min (flow-rate 1 ml/min).

The derivatization of barbiturates as described above might form the basis of a very sensitive method for the determination of these compounds in minute plasma or serum samples. For instance, the therapeutic range of phenobarbital in plasma is 15–40 μg/ml[11]. If a minimal amount of 5 ng per injection (signal-to noise-ratio 10) can be determined with acceptable precision, and 50% of the entire sample extract after derivatization is injected, then a plasma phenobarbital level of 0.4 μg/ml could be determined in plasma samples of 10 μl.

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