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GAS CHROMATOGRAPHIC ANALYSIS OF DIALKYLBARBITURIC ACIDS

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SUMMARY

A variety of procedures for the derivatization of barbituric acids for gas–liquid chromatography (GLC) are described and a convenient procedure for *n*-alkylation of barbiturates is reported. The gas chromatographic behaviour of derivatives (methyl, ethyl, propyl, butyl, pentyl and hexyl) is discussed on the basis of the retention index. For the identification and quantitation of 23 barbiturates in biological fluids, use of the propyl derivatives allows a clear gas chromatogram to be obtained by using only one stationary phase. Alkylation with *n*-alkylating reagents gives only the N,N′-derivative, as confirmed by nuclear magnetic resonance (NMR) and infrared spectroscopy and gas chromatography–mass spectrometry (GC–MS). The fragmentation pattern by electron-impact mass spectrometry is discussed. Alkylation with branched alkylating reagents gives up to three peaks, the major peak being the N,N′-derivative; the other peaks identified by NMR and GC, correspond to the isomeric N,O-derivatives.

INTRODUCTION

The use of derivatives for the analysis of barbituric acids by gas–liquid chromatography (GLC) is now well known. The polar nature of the acidic functions of barbiturates causes adsorption resulting in loss of material and tailing of peaks[1]. In order to obtain quantitative results at the submicrogram level, it is necessary to convert the compounds into suitable non-polar derivatives.

Among the various possibilities, the trimethylsilyl derivatives were tried but proved to be unstable because of the somewhat labile nature of the Si–N bond[2], and use of the methyl derivatives is generally preferred. Among the methods described for the preparation of these derivatives, it is observed that use is made of dimethyl sulphate in a mildly alkaline medium[3,4], diazomethane in methanol[5–7], methyl iodide and potassium carbonate[8] and "flash heater" methylations using tetramethylammonium hydroxide (TMAH)[9] or trimethylanilinium hydroxide[2]. The methylation procedure in which a quaternary ammonium base and methanol are used resulted in the formation of two chromatographic peaks, even under mild conditions, one of which was an extraneous peak caused by alkaline decomposition[2,10,11]. Recently, it was shown that alkylation with diazoalkanes gave five peaks, produced by the N,N′-,

N,O- and O,O'-dialkylbarbiturates[7], resulting from tautomerism of the barbiturate ring. The method of methylation with dimethyl sulphate is time consuming; indeed, in order to avoid the injection of materials that have a deleterious effect on gas chromatographic columns, the reagents and solvents must be removed[12].

A major disadvantage of all of these methods of methylation is that the same end product is formed from different compounds. Thus, phenobarbital (for barbiturate nomenclature, see Table I) and its 1-methyl derivative cannot be distinguished from each other. Similarly, drugs such as 1,3-dimethylxanthine (theophylline), 3,7-dimethylxanthine (theobromine) and monomethylxanthines are all converted into 1,3,7-trimethylxanthine (caffeine) and cannot therefore be distinguished from one another. On the other hand, N- or O-methylated drugs can be demethylated *in vivo*, so that the use of methylating reagents must be avoided in a study of their metabolism.

TABLE I

NOMENCLATURE OF BARBITURIC ACIDS

Name	*Substituent*		
	5	*5'*	*1*
Mephebarbital*	methyl	phenyl	
Hexobarbital	methyl	cyclohexen-1-yl	methyl
Barbital*	ethyl	ethyl	
Probarbital*	ethyl	isopropyl	
Butobarbital	ethyl	butyl	
Vinbarbital	ethyl	methyl-1-buten-1-yl	
Amobarbital	ethyl	isopentyl	
Pentobarbital	ethyl	methyl-1-butyl	
Cyclobarbital	ethyl	cyclohexen-1-yl	
Phenobarbital	ethyl	phenyl	
Mephobarbital*	ethyl	phenyl	methyl
Heptabarbital	ethyl	cyclohepten-1-yl	
Reposal	ethyl	bicyclo(3,2,1)octen-1-yl	
Vinylbital	vinyl	methyl-1-butyl	
Allobarbital*	allyl	allyl	
Brallobarbital	allyl	bromo-2-allyl	
Aprobarbital*	allyl	isopropyl	
Centralgol	allyl	β-oxypropyl	
Butalbital	allyl	isobutyl	
Nealbarbital*	allyl	neopentyl	
Secobarbital	allyl	methyl-1-butyl	
Methohexital*	allyl	methyl-1-penten-2-yl	methyl
Cyclopal*	allyl	cyclopenten-2	

* Not available in France.

We report a systematic study of the GLC behaviour of 23 barbituric acids on four stationary phases (OV-101, Dexsil 300 GC, OV-7 and SP-2250) after dialkylation (ethyl, propyl, butyl, pentyl and hexyl) according to the method of Greeley[13,14]. The retention index (RI) and variations (ΔI) are given. Propylation was successful because it greatly facilitated the separation of the 23 barbiturates by temperature-programmed GLC. The propylation procedure for both preparative and analytical

purposes gave a single, well shaped peak corresponding to the N,N'-derivative, which was confirmed by nuclear magnetic resonance (NMR) and infrared (IR) spectroscopy, and by mass spectrometry (MS). While alkylation with *n*-alkyl reagents gave only the N,N'-derivative (more than 99.5%), the use of branched alkyl reagents (isopropyl or isobutyl) gave up to three peaks, the major peak being that of the N,N'-derivative. The extraneous peaks could result from the tautomerism of the barbiturate ring and the spatial bulk of the ramified alkane.

EXPERIMENTAL

Gas chromatography

GLC separations were made with a Pye Unicam Series 104 Model 84 (Cambridge, Great Britain) and a Carlo Erba 2300 (Milan, Italy) instrument, both fitted with flame ionization detectors. The columns were 2.50 m × 3 mm silanized glass tubes packed with 3.08% OV-101, 1.82% Dexsil 300 GC, 3% SP-2250 and 1.57% OV-7 on Gas-Chrom Q (Applied Science Labs., State College, Pa., U.S.A.). The retention indices were measured using *n*-alkanes in the isothermal mode. Column temperatures are given in Tables II and III.

Mass spectrometry

Mass spectra were recorded with an AEI MS-30 (Manchester, Great Britain) combined gas chromatograph–mass spectrometer (GC–MS). Sample introduction was via the gas chromatographic inlet, a Dexsil-300 GC 1.3% on Gas-Chrom Q in a 1.80 m × 4 mm glass column at 215°. The membrane temperature was 210° and the temperature source 200°. The mass spectra were recorded at 24 eV with an ionization current of 100 μA; the resolution was fixed at 1,000.

Nuclear magnetic resonance spectroscopy

The proton magnetic resonance spectra were recorded with a Jeol C 60 HC (Tokyo, Japan) spectrometer from $CDCl_3$ solutions using tetramethylsilane as internal reference.

Infrared spectrometry

The IR spectra were obtained with a Beckman IR 18 A (Fullerton, Calif., U.S.A.) spectrometer, using potassium bromide pellets.

Preparation of derivatives

For analytical purposes, alkyl derivatives were prepared by allowing 200 μg of barbiturate dissolved in 200 μl of the mixture N,N'-dimethylacetamide (E. Merck, Darmstadt, G.F.R.) – methanol – tetramethylammonium hydroxide (20% in methanol, obtained from Aldrich-Europe, Beerse, Belgium) (400:95:5, v/v/v) to react with 4 μl of iodoalkanes obtained from Merck, Riedel de Haen (Hannover, G.F.R.) and Fluka (Buchs, Switzerland). The reaction was complete within 10 min. The reagents and solvents were removed under a stream of nitrogen and the products, redissolved in hexane, were injected for GLC and combined GC–MS. The molar ratios between TMAH, alkylating reagent and barbiturate model (phenobarbital) that proved most effective for preparing the derivatives are about 21:150:5.

For preparative purposes, a total of 464 mg of phenobarbital (2 mmoles) was dissolved in a mixture of 15 ml of N,N′-dimethylacetamide, 5 ml of TMAH solution (11 mmoles) and 2 ml of methanol, the system being shaken to ensure complete dissolution. Then, to this solution were added 2 ml of iodopropane (19 mmoles). After 2 h, the product was extracted with hexane and the tetramethylammonium iodide precipate eliminated. The organic solution was washed with half-saturated sodium hydrogen carbonate solution and then with saturated silver nitrate solution. After washing it with water and drying it over sodium sulphate, the organic phase was concentrated to small volume. Crystallizations from methanol of the product obtained gave 400 mg of derivative.

For the preparation of isopropyl derivatives, a total of 5 g of phenobarbital was dissolved, 30 ml of 2-iodopropane being added to the solution maintained at 60°. The gas chromatogram of the product was examined in order to ensure the absence of phenobarbital and its monoalkylated derivatives. Crystallizations from methanol–water gave 3 g of the N,N′-derivative. The concentrated mother liquids were fractionated by thin-layer chromatography on silica gel F_{254} in the benzene–ethyl acetate (9:1) system, or by column chromatography on a silica gel 60 pre-packed column (Merck) with heptane–chloroform as the eluent.

RESULTS AND DISCUSSION

The reaction mechanism consisted of two steps: (1) the barbituric acid dissolved in the base–solvent system gave a soluble tetramethylammonium salt that was immediately formed; and (2) the anion of the soluble salt and any primary iodoalkane reacted according to a fast SN_2 mechanism to give the alkyl derivative[13].

All primary iodoalkanes (from iodomethane to iodohexane) reacted completely in less than 10 min. Formation of *n*-alkyl derivatives was satisfactory and well shaped chromatographic peaks were produced by all the barbiturates studied. The derivatives obtained were stable for several months. However, with 2-iodopropane and 1-iodo-2-methylpropane, anomalous results were obtained, which, in our opinion, may be due to the difficulty in forming the planar intermediate in the SN_2 reaction. Reactions between sterically hindered iodo compounds such as iodocyclohexane or 2-iodo-2-methylpropane and phenobarbital were negative. With 2-iodopropane, 1-iodo-2-methylpropane and 2-iodobutane about 5% of the barbiturate was unchanged or gave the monoalkyl compound.

The *n*-alkyl derivatives of 23 barbiturates were prepared in order to examine the effect on the separation of some pairs of barbiturates known to be difficult to resolve[12,15]. The results, expressed as retention indices, are given in Tables II and III. Among the derivatives and stationary phase tested, the best results were obtained using *n*-propyl derivatives on Dexsil 300 GC with programmed temperature (Fig. 1). However, under these chromatographic conditions some pairs were not resolved: allobarbital–probarbital and amobarbital–methohexital. This fact presented no inconvenience because only the amobarbital is available in France. These two pairs of barbiturates can, however, easily be resolved by using their propyl derivatives on OV-7. Reference to the retention indices (Tables II and III) could help in choosing the optimum conditions for the separation of many barbiturates.

The smallest change in retention increment (Table IV) was observed between

TABLE II

RETENTION INDICES OF DERIVATIVES OF BARBITURIC ACIDS

Column A: OV-101, 3.08%, 2.50 m × 3 mm; Column B: Dexsil 300 GC, 1.82%, 2.50 m × 3 mm.

No.	Barbituric acid	Column A						Column B					
		Methyl (168°)	*Ethyl (170°)*	*Propyl (195°)*	*Butyl (200°)*	*Pentyl (205°)*	*Hexyl (214°)*	*Methyl (170°)*	*Ethyl (170°)*	*Propyl (195°)*	*Butyl (200°)*	*Pentyl (205°)*	*Hexyl (214°)*
1	Barbital	1416	1488	1662	1820	2011	2206	1469	1532	1700	1866	2047	2230
2	Probarbital	1466	1558	1728	1898	2080	2260	1531	1597	1765	1932	2108	2300
3	Allobarbital	1498	1578	1744	1899	2058	2275	1540	1603	1767	1926	2107	2290
4	Aprobarbital	1511	1599	1770	1926	2110	2293	1567	1633	1800	1962	2137	2328
5	Butalbital	1549	1635	1798	1950	2132	2316	1593	1661	1826	1972	2173	2340
6	Butobarbital	1558	1635	1798	1950	2137	2325	1606	1662	1832	1980	2160	2343
7	Amobarbital	1597	1672	1837	1991	2171	2354	1642	1697	1860	2009	2190	2370
8	Nealbarbital	1599	1696	1860	2018	2200	2378	1641	1722	1880	2030	2213	2398
9	Vinylbital	1629	1698	1861	2018	2198	2385	1673	1723	1885	2039	2218	2395
10	Pentobarbital	1625	1707	1873	2020	2210	2394	1679	1741	1905	2059	2239	2420
11	Vinbarbital	1649	1723	1890	2040	2224	2411	1696	1757	1918	2073	2253	2432
12	Centralgol	1672	1737	1898	2050	2247	2421	1725	1784	1953	2097	2277	2455
13	Secobarbital	1665	1747	1910	2040	2243	2424	1711	1775	1935	2083	2265	2443
14	Methohexital	1714	1759	1850	1924	2026	2112	1757	1785	1861	1969	2038	2135
15	Brallobarbital	1707	1800	1970	2121	2309	2499	1769	1848	2015	2169	2335	2538
16	Hexobarbital	1799	1831	1925	2062	2107	2205	1889	1895	1990	2083	2150	2270
17	Mephebarbital	1781	1832	2000	2163	2347	2528	1876	1895	2060	2221	2403	2589
18	Phenobarbital	1828	1884	2054	2212	2395	2577	1912	1935	2105	2259	2445	2628
19	Cyclobarbital	1845	1909	2078	2236	2419	2605	1925	1960	2125	2283	2467	2648
20	Heptabarbital	1926	1994	2164	2322	2502	2690	2025	2052	2230	2375	2559	2740
21	Reposal	1991	2056	2230	2387	2568	2751	2100	2120	2290	2439	2625	2803
22	Mephobarbital	1827	1848	1946	2032	2135	2217	1911	1912	2001	2098	2195	2291
23	Cyclopal	1742	1839	1989	2143	2324	2417	1811	1864	2034	2192	2375	2557

the methyl and ethyl derivatives, so that the separation of methyl- and ethylbarbituric acids was not achieved in some instances. The plot of retention index against derivative chain length (Fig. 2) showed a slight deviation from linearity at the butyl level, and a slight increase in retention increment was observed between the butyl and pentyl substituents (see Table IV). The non-linearity of the plot of retention index against chain length for derivatized compounds has previously been described by many authors for phthalate esters[16,17], cannabinoids[18] and tetraalkylsilanes[19]. The retention increments of the monoalkyl, N-methyl compounds mephobarbital, hexobarbital and methohexital were about one half of those of the dialkyl derivatives.

The charge distribution along the alkyl chain bound to heteroatoms such as silicon[19] or nitrogen could explain the non-linearity of the plot of retention index against chain length. A positive charge located on the α-carbon atom of the alkyl chain could enhance the solubility of the dimethyl derivative in the stationary phase more than is expected for normal behaviour, especially on polar stationary phases. The variation of retention index ($\Delta I_{\text{Ethyl-Methyl}}$) decreased with the polarity of the stationary phase. Further, as judged from the ΔI values, the influence of the nitrogen atom did not extend appreciably beyond the δ-CH_2 group, so that the plot in Fig. 2 became linear from the butyl chain. This explanation was confirmed by the chromatographic behaviour of the mephobarbital derivative (N-methylphenobarbital) on sta-

TABLE III

RETENTION INDICES OF BARBITURIC ACID DERIVATIVES

Column D: SP-2250, 3%, 2.50 m × 3 mm; Column C: OV-7, 1.57%, 2.50 m × 3 mm.

Barbituric acid	*Column C*						*Column D*					
	Methyl (166°)	*Ethyl (167°)*	*Propyl (180°)*	*Butyl (188°)*	*Pentyl (226°)*	*Hexyl (244°)*	*Methyl (160°)*	*Ethyl (163°)*	*Propyl (190°)*	*Butyl (191°)*	*Pentyl (228°)*	*Hexyl (246°)*
Barbital	1508	1584	1750	1918	2113	2307	1609	1659	1833	1978	2190	2383
Probarbital	1600	1650	1809	1981	2175	2308	1675	1724	1896	2055	2294	2483
Allobarbital	1603	1676	1833	1995	2190	2381	1704	1753	1920	2069	2271	2461
Aprobarbital	1622	1695	1855	2017	2213	2408	1721	1773	1940	2092	2255	2455
Butalbital	1652	1726	1878	2022	2220	2414	1742	1793	1953	2097	2294	2482
Butobarbital	1662	1726	1881	2043	2230	2418	1753	1796	1957	2106	2302	2490
Amobarbital	1698	1760	1913	2070	2262	2447	1786	1825	1984	2134	2328	2511
Nealbarbital	1698	1784	1935	2093	2286	2475	1791	1850	2012	2159	2357	2542
Vinylbital	1742	1791	1946	2110	2297	2484	1841	1868	2028	2174	2375	2562
Pentobarbital	1729	1799	1953	2114	2304	2493	1823	1868	2031	2180	2379	2566
Vinbarbital	1767	1828	1980	2143	2330	2517	1870	1909	2068	2217	2413	2600
Centralgol	1817	1876	2028	2187	2376	2565	1945	1965	2140	2279	2482	2671
Secobarbital	1770	1839	1990	2148	2338	2525	1865	1909	2070	2214	2413	2599
Methohexital	1845	1887	1963	2046	2151	2253	1957	1983	2070	2149	2255	2362
Brallobarbital	1849	1937	2083	2242	2447	2643	1980	2031	2200	2336	2556	2752
Hexobarbital	1957	1979	2063	2145	2265	2374	2093	2092	2189	2254	2390	2499
Mephebarbital	1953	1984	2136	2300	2493	2698	2103	2099	2266	2421	2630	2823
Phenobarbital	1988	2026	2182	2342	2538	2739	2134	2140	2309	2450	2667	2862
Cyclobarbital	1988	2036	2193	2358	2553	2740	2123	2140	2310	2447	2667	2860
Heptabarbital	2067	2121	2278	2441	2642	2839	2206	2225	2400	2530	2759	2953
Reposal	2132	2186	2345	2507	2712	2911	2278	2296	2471	2607	2834	3030
Mephobarbital	1988	2011	2097	2179	2297	2405	2134	2129	2228	2303	2434	2542
Cyclopal	1876	1941	2097	2259	2461	2655	2000	2034	2206	2355	2565	2758

tionary phases C and D (Fig. 2). Indeed, the curves C and D showed a very clear break around the butyl chain, due to the influence of the methyl radical on the chromatographic behaviour. A similar observation was made by Yalkowsky *et al.*[20] on the basis of physicochemical results.

The structure of *n*-alkyl derivatives was ascertained by IR and NMR spectroscopy and GC–MS. IR spectra showed the disappearance of the N–H stretching band at 3100–3300 cm^{-1}, the presence of a broad C=0 stretching band in the 1670–1700 cm^{-1} range and strong bands in the 1350–1400 cm^{-1} range, due to the N–R group. The NMR spectra permitted unequivocally the assignment of the N,N′-alkyl structure (Table V). The NMR spectrum of dipropylphenobarbital showed a triplet of four protons at 3.95 ppm (N–CH_2–), a sextuplet of four protons at 1.65 ppm (β-CH_2) and a triplet of six protons at 0.90 ppm (ω-CH_3). The NMR spectrum of monopropylmephobarbital confirmed this assignment: a triplet of two protons at 3.92 ppm (N–CH_2), a sextuplet of two protons at 1.65 ppm (β-CH_2), a triplet of three protons at 0.89 ppm (ω-CH_3) and a singlet at 3.39 ppm of three protons, corresponding to the N–CH_3 group. The NMR spectrum of *n*-alkyl derivatives exhibited no resonances corresponding to an O–R group in the 5.50 ppm region.

Table VI lists the mass spectra of di-*n*-alkyl derivatives of phenobarbital. The mass spectra of phenobarbital, N,N′-dimethyl and diethyl derivatives have been re-

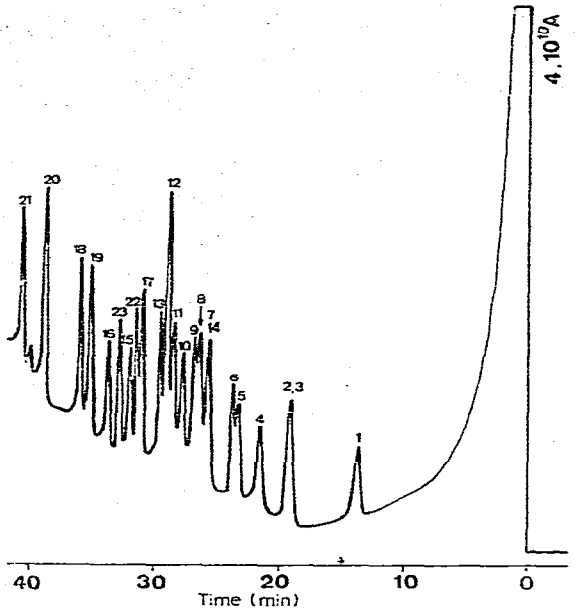


Fig. 1. Gas chromatogram of 23 di-*n*-propylbarbiturates. Gas chromatographic conditions: 2.50 m × 4 mm glass column coated with Dexsil 300 GC 1.82% on Gas-Chrom Q; temperature conditions: 15 min at 140°, then programmed at 4°/min. Peak numbers correspond to the numbers in Table II.

ported previously[7,21,22]. Elimination of ethylene fixed on the C-5 atom by a McLafferty rearrangement produced the base peak; this fragmentation mechanism decreased while the chain length increased. Elimination of the alkenyl radical (C_nH_{2n-1}) from N-alkylbarbiturates upon electron ionization follows the concepts described for N-alkylsuccinimides[23] and N-alkyluracils[24]. In this mechanism, two hydrogen atoms from the β- and α- carbon atoms were transferred to vicinal carbonyl oxygen atoms. Table VI shows that an N-alkyl chain length of at least three carbon atoms is necessary to induce this mode of fragmentation.

In all the mass spectra, the ion arising from the elimination of R–N=C=O from the molecular ion provided structural information. The ions at *m/e* 189 and 174

TABLE IV

VARIATION OF RETENTION INDEX AS A FUNCTION OF CHAIN LENGTH FOR THE N-*n*-ALKYLBARBITURATES

$\Delta I = RI_{Ethyl} - RI_{Methyl}$. a: N,N′-derivatives; and b: N-methyl, N-alkyl derivatives.

ΔI	*OV-101*		*Dexsil 300 GC*		*OV-7*		*SP-2250*	
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*
Ethyl—methyl	77	33	58	12	62	29	42	11
Propyl—ethyl	167	94	166	87	155	81	166	98
Butyl—propyl	155	80	154	97	162	83	145	70
Pentyl—butyl	185	102	182	80	194	106	205	134
Hexyl—pentyl	186	88	181	104	193	114	190	110

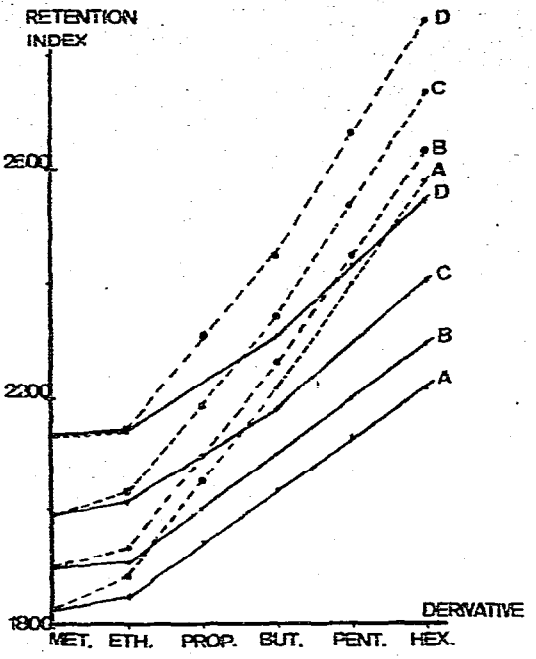


Fig. 2. Plot of retention index against derivative chain length for phenobarbital (dotted line) and mephobarbital (full line) on four stationary phases A, B, C and D (see Tables II and III).

TABLE V

NUCLEAR MAGNETIC RESONANCE FREQUENCIES FOR ISOPROPYL ISOMERS OF PHENOBARBITAL AND MEPHOBARBITAL (ppm DOWNFIELD FROM TMS IN $CDCl_3$) NMR frequencies of the propyl derivatives are given for comparison.

Barbiturate	*Substituent*					
	5'	*5*		*1*	*3*	*4*
	C_6H_5	CH_3	CH_2			
Phenobarbital						
Free	7.34	0.94	2.46	10 (NH)	—	—
N,N'-Propyl	7.34	0.94	2.50	3.95 (N–CH_2); 0.90 (ω-CH_3)	1.65 (–CH_2–)	—
Isopropyl						
Isomer 1	7.32	0.95	2.43	5.08 (N–CH<) 1.42 (CH_3 Isopropyl)	1.52 (CH_3 Isopropyl)	—
Isomer 3	7.34	0.95	2.40	5.08 (N–CH<) 1.36 (CH_3 Isopropyl)		5.54 (O–CH<) 1.42 (CH_3 Isopropyl) 1.30 (CH_3 Isopropyl)
Mephobarbital						
Free	7.34	0.94	2.49	9.38 (NH)	3.39 (N–CH_3)	—
N-Propyl	7.32	0.94	2.48	3.92 (N–CH_2) 1.65 (–CH_2–) 0.89 (ω-CH_3)	3.39 (N–CH_3)	
N-Isopropyl						
Isomer 1	7.34	0.94	2.49	5.09 (N–CH) 1.50 (CH_3 Isopropyl) 1.42	3.39 (N–CH_3)	

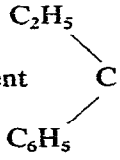
TABLE VI

RELATIVE INTENSITIES (R) OF PRINCIPAL FRAGMENTS IN MASS SPECTRA OF N,N'-n-ALKYLPHENOBARBITAL

The fragmentation scheme is discussed in the text.

Ion	*R*						
	H	CH_3	C_2H_5	C_3H_7	C_4H_9	C_5H_{11}	C_6H_{13}
M	20	5	3	9	9	12	10
$M-CH_3$	7	4	3	—	—	—	—
$M-C_2H_5$	—	—	—	7	14	18	10
$M-C_2H_4$	100	100	100	100	54	45	42
$M-C_nH_{2n-1}$	—	—	—	20	70	83	100
$M-CH_3-C_nH_{2n-1}$	—	—	—	3	7	5	4
$M-C_2H_4-C_nH_{2n}$	—	—	8	27	12	12	10
M−RNCO	6	4	5	10	7	5	5
$M-C_nH_{2n}-C_nH_{2n-1}$	—	—	—	3	7	7	7
$M-C_2H_4-2\,C_nH_{2n}$	—	—	—	20	7	7	5
$M-C_{n-1}H_{2n-2}$	—	—	—	—	8	7	8
189	7	—	7	20	13	12	12
174	—	—	—	13	10	12	10
146	15	27	65	97	100	100	92
118	9	19	17	20	13	16	15
117	12	20	22	20	15	16	15
91	6	4	6	7	7	10	10

were produced by the successive eliminations of C_nH_{2n} and RCON, and C_nH_{2n}, RCON and CH_3, respectively, from the molecular ion.

The ion at m/e 146, corresponding to the fragment  , arises from

the fragmentation of the barbiturate ring containing an unsaturated C-5 substituent[7,25]. This ion was the base peak or nearly the base peak when increasing the chain length. The fragment $M-C_{n-1}H_{2n-2}$ was attributed to the α-cleavage mechanism of the alkyl chain with transfer of one hydrogen atom.

This fragmentation pattern was confirmed by the mass spectra of monoalkyl derivatives of mephobarbital (Table VII). As expected, the loss of C_2H_4 and C_nH_{2n-1} from the molecular ion occurred to a significant extent. The loss of HNCO or isocyanate RNCO ($R = CH_3$ or C_nH_{2n+1}) was observed in all mass spectra[26]. Similarly, the mass spectra showed fragments corresponding to the loss of CH_3 plus C_nH_{2n-1} and C_2H_4 plus C_nH_{2n}. The first step consisting in the loss of C_2H_4 by a McLafferty rearrangement was followed in all examples by a classical McLafferty rearrangement with elimination of C_nH_{2n} and not by the elimination of the alkenyl radical C_nH_{2n-1}.

The retention indices of branched dialkylbarbiturates were lower than those of n-dialkyl homologues (Table VIII). The retention times of diisopropyl- and diisobutylphenobarbital were close to those of diethyl- and dipropylphenobarbital, respectively.

The gas chromatograms of the products of the reaction between phenobarbital and 2-iodopropane exhibited three peaks (Fig. 3), peak 1 accounting for 90% of the

TABLE VII

RELATIVE INTENSITIES (*R*) OF PRINCIPAL FRAGMENTS IN MASS SPECTRA OF N-*n*-ALKYLMEPHOBARBITAL

The fragmentation pattern is discussed in the text.

Ion	*R*						
	H	CH_3	C_2H_5	C_3H_7	C_4H_9	C_5H_{11}	C_6H_{13}
M	15	6	3	9	15	16	13
M−CH_3	3	4	4	4	4	—	—
M−C_2H_4	100	100	100	100	90	85	63
M−C_2H_5	—	—	—	6	15	17	13
M−CH_3NCO	10	4	7	6	8	15	5
M−C_nH_{2n-1}	—	—	—	18	80	100	100
M−C_2H_4−C_nH_{2n}	—	—	5	35	30	23	17
M−CH_3−C_nH_{2n-1}	—	—	—	—	15	14	10
M−RNCO	3	4	3	6	6	6	5
M−CH_3−RNCO	—	8	7	—	—	—	—
M−C_2H_4−RNCO	—	17	12	14	13	10	8
189	—	—	—	13	13	13	10
174	—	—	—	9	10	11	10
146	15	21	58	96	100	81	60
118	18	16	16	24	30	22	13
117	15	15	20	47	40	26	21
91	8	8	9	18	15	14	8

TABLE VIII

RETENTION INDICES OF BRANCHED ALKYL DERIVATIVES OF PHENOBARBITAL AND MEPHOBARBITAL

Barbiturate	*Column*			
	OV-101	*Dexsil 300 GC*	*OV-7*	*SP-2250*
Phenobarbital				
Isopropyl:				
Isomer 1	1928	1963	2092	2168
Isomer 2	1993	2034	2163	2243
Isomer 3	2056	2138	2261	2365
Methyl-2 propyl	2143	2103	2265	2371
Methyl-1 propyl	2093	2145	2210	2335
Mephobarbital				
Isopropyl:				
Isomer 1	1889	1957	2027	2167
Isomer 2	1946	2023	2090	2237
Isomer 3	2020	2135	2189	2355

total products. As the molecular weights with these peaks were identical, the compounds were presumably the result of tautomerism of the barbiturate ring; they were separated by column chromatography on silical gel.

The major product was identified by spectrometric methods as the N,N'-diisopropyl derivative. The IR spectrum showed the absence of N–H stretching bands, and the presence of a broad C=O stretching band in the 1670–1700 cm^{-1} range and a

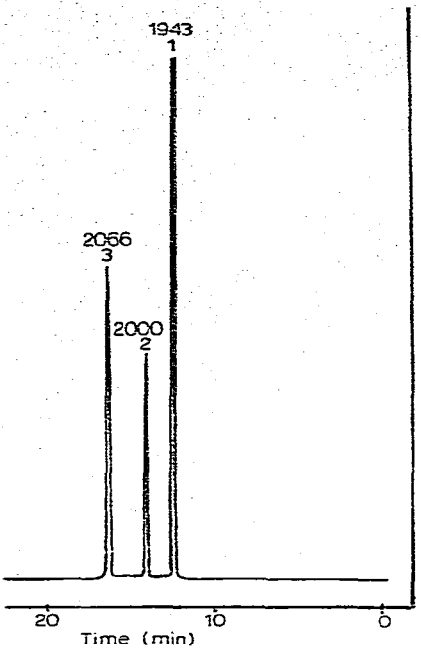
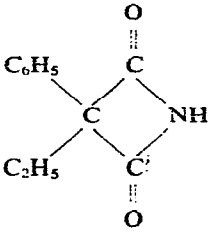
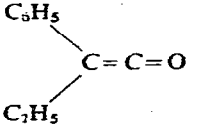


Fig. 3. Gas chromatogram of the isopropylated derivatives of phenobarbital. Gas chromatographic conditions: 60 m × 0.29 mm glass capillary column coated with OV-101; oven temperature, 220°; helium flow-rate, 1.5 ml/min. Retention indices of isomers 1, 2 and 3 are indicated.

triplet band in the 1330–1370 cm^{-1} range due to the N–CH–$(CH_3)_2$ group. The NMR spectrum permitted unequivocally the assignment of the structure of this compound. The data obtained in the NMR study are shown in Table V. The spectrum exhibited resonances at 5.08 ppm corresponding to the two protons of N–CH. Between the vicinal protons of the N-propyl and the vicinal proton of the N-isopropyl group there was a significant difference, from 3.95 to 5.08 ppm. An additional feature merits further comment. The N,N′-isopropyl derivative showed non-equivalence of its methyl protons of the isopropyl chain, which is most likely due to the unequal anisotropic environment. This finding was confirmed by the NMR spectrum of isopropylated mephobarbital in which the ω-methyl protons of the isopropyl chain were non-equivalent. Further evidence for the N,N′-isopropyl derivative was obtained from the mass spectrum (Table IX). The base peak at *m/e* 146 was present in all of the isopropyl isomers and was found by other workers[7,25] to correspond to the fragment $\begin{matrix} C_2H_5 \\ \diagdown \\ \quad C=C=O^+ \\ \diagup \\ C_6H_5 \end{matrix}$. Other fragments providing structural information were: *m/e* 288, produced by elimination of ethylene by a McLafferty rearrangement; *m/e* 275 (M−41), due to the elimination of the olefin C_3H_5, the fragmentation characteristic for the N-alkylbarbiturate with which the presence of two carbonyl group seems to be necessary[24,25]. Fragments *m/e* 246 and 204 corresponded to the loss of one

TABLE IX

RELATIVE INTENSITIES OF FRAGMENTS IN MASS SPECTRA OF ISOPROPYL ISOMERS OF PHENOBARBITAL AND METHOBARBITAL

Fragment	*Phenobarbital*				*Mephobarbital*			
	m/e	*Isomer*			*m/e*	*Isomer*		
		1	*2*	*3*		*1*	*2*	*3*
M	316	4	6	7	288	16	4	4
M−15	301	2	<1	<1	273	2	2	3
M−28	288	23	51	3	260	58	100	8
M−41	275	2	6	—	247	8	16	8
M−42	274	—	—	9	246	—	—	19
M−28−42	246	5	15	38	218	27	34	100
M−RNCO	231	3	5	44	231*	4	2	19
M−C_2H_4−R−R′−2H	204**	3	8	15	203***	6	6	4
	189	5	14	87	189	7	13	53
	146	100	100	100	146	100	95	93
——	118	7	13	37	118	19	27	28
Φ−C=C=O	117	15	23	37	117	27	36	44
Tropylium ion	91	4	6	15	91	8	15	20

* R = CH_3.
** R = R′ = C_3H_7.
*** R = C_3H_7; R′ = CH_3.

and two C_3H_6 groups (42 a.m.u.), respectively, from the ion at *m/e* 288. The fragment *m/e* 189 was produced by the successive elimination of C_3H_6 and C_3H_7NCO (85 a.m.u.)

Isomer 2 was identified as a N,O-diisopropylphenobarbital from the mass spectrum (Table IX) and the retention index (Table VIII). Although the NMR spectrum was not obtainable owing to the very small amount produced, evidence of the N,O-diisopropyl structure was provided by the gas chromatogram of isopropylated mephobarbital. This compound, N-methylphenobarbital, gave only three isomers on isopropylation. As one methyl group is fixed in this compound, the products of isopropylation must be the N,N′- and N,O-diisopropyl isomers (compounds 2 and 3). Taking isomer 1 as a reference, fairly constant retention increments were obtained for the isomers 2 and 3 for phenobarbital and mephobarbital, indicating similar structures. The isomers 1 and 2 showed a similar fragmentation pattern in MS. Many metastable ions provided further evidence of this fragmentation. The transition 316→288 gave a metastable ion at *m/e* 262.5: the transition 288→246 was confirmed

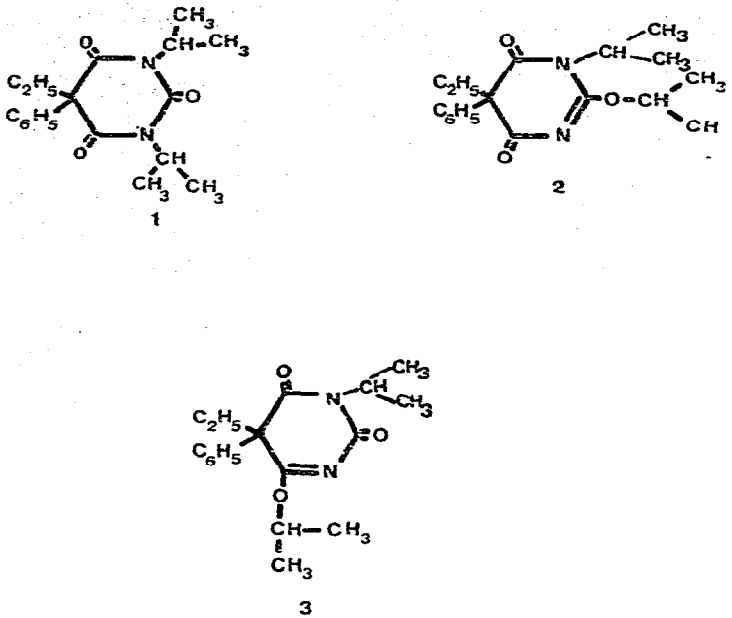


Fig. 4. Structure of diisopropylphenobarbital isomers 1, 2 and 3.

by a metastable ion at *m/e* 210. The loss of the fragment C_3H_7NCO from the molecular ion was confirmed by a metastable ion at *m/e* 169. From these results, the unequivocal assignment of isomer structure 2 was not feasible. By assignment of the structure of isomer 3, this ambiguity was removed.

Isomer 3 was identified as having the structure 3 (Fig. 4), the retention index confirming a N,O-structure (Table VIII). The NMR spectrum exhibited resonances attributable to a N-isopropyl group at 5.08 ppm and to an O-isopropyl group at 5.54 ppm. In the mass spectrum (Table IX), considerable differences from the results for the MS of isomers 1 and 2 were observed. The ion at *m/e* 288, especially, being insignificant; elimination of the ethylene fragment by a McLafferty rearrangement was difficult because of the presence of an isopropyl group on carbon atom 4. The ion at *m/e* 274, owing to the loss of C_3H_6 by the McLafferty reaction, was absent for the compounds 1 and 2. Moreover, considerable differences in relative ion abundances were observed for certain ions, especially the ions at *m/e* 246, 231 and 189 due to the loss of C_3H_6 plus C_2H_4 by McLafferty reactions, to the elimination of C_3H_7NCO, which can be lost easily by a retro-Diels–Alder mechanism, and to the elimination of C_3H_6 plus C_3H_7NCO, respectively. The abundance of these ions was consistent with the compound having structure 3. Similarly, the mass spectrum of isomer 3 obtained by isopropylation of mephobarbital showed the same fragmentation pattern and the same relative ion abundance. The assignment of the structure of isomer 3 being unequivocal, isomer 2 accordingly has the structure 2 (Fig. 4).

Identification and quantitation of barbiturates in biological fluids

Many GLC methods for the identification and measurement of barbiturates

in biological fluids have been proposed and summarized by several authors[12,15]. In most of these methods the methyl derivatives were formed according to different procedures. Identification was based on the retention times relative to an internal standard on two different stationary phases: an apolar type, OV-101 or SE-30, and a polar type, OV-225 or NPGA, using temperature programmed GLC. Quantification was carried out by using an internal standard added to the biological fluid. Several of these methods were unsatisfactory for use as (i) the methylation of different compounds gave the same compound as phenobarbital and mephobarbital; (ii) some interferences could be resolved only by the simultaneous use of two stationary phases; and (iii) identification by means of relative retention times was not accurate.

In our procedure, we used the propyl derivatives analysed on Dexsil 300-GC with programmed temperature as described in Fig. 1. Under these conditions only allobarbital–probarbital and amobarbital–methohexital were not resolved. To bring about an enhancement in the identification of barbiturates, two internal standards, aprobarbital and mephebarbital, were added to the biological fluid, and a retention index relative to the two standards was defined; this barbiturate retention index compared with the methylene unit index was more accurate than relative retention times, the coefficient of variation of the barbiturate retention index being about 0.5% in five GLC analyses of phenobarbital. Under the same conditions, the coefficient of variation of relative retention times increased to about 2%.

We used as extraction and purification procedures, the procedures previously described by Brachet-Liermain *et al.*[15]. After extraction, the dried residue was dissolved in 400 μl of the mixture N,N'-dimethylacetamide, methanol and TMAH (20% in methanol) (400:95:5). The propyl derivatives were prepared by adding 8 μl of iodopropane. After 10 minutes of reaction, the mixture was dried in a stream of nitrogen and finally dissolved in 200 μl of hexane–ethanol (90:10); 2 or 4 μl of this solution were taken for analysis by GLC.

After being identified by their barbiturate retention index, the barbiturates were quantitated by using alternatively two internal standards. This procedure allowed

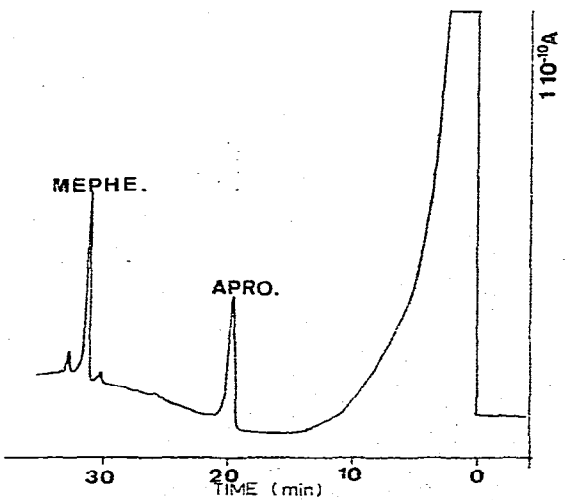


Fig. 5. Blank obtained on 3 ml of plasma from a normal subject.

us to show that no interference peaks occurred with retention times similar to that of the standards. The blood extract blank obtained under our chromatographic condition swith the two internal standards is given in Fig. 5.

CONCLUSION

The procedure described here has been demonstrated to be sensitive enough to permit measurement of barbiturates in plasma obtained from patients receiving barbiturate therapy. The method has several advantages over previously used GLC methods.

The formation of propyl derivatives does not require the use of hazardous material such as diazomethane or material that has a deleterious effect on GLC columns. The derivatization reaction gives suitable chromatographic peaks and allows any *n*-alkyl derivative to be easily prepared without formation of isomers.

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