Journal of Chromatography, 219 (1981) 431–435
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,244

Note

Determination of 5-ethyl-5-(1-ethylpropyl)barbituric acid in pentobarbital by high-performance liquid chromatography

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(Received July 30th, 1981)

5-Ethyl-5-(1-ethylpropyl)barbituric acid (I) can occur in pentobarbital, 5-ethyl-5-(1-methylbutyl)barbituric acid (II). Its presence is related to the use of 2-bromopentane, containing 3-bromopentane, in the synthesis of the malonate ester. 3-Bromopentane is a frequently occurring impurity of 2-bromopentane[1,2].

Several pharmacopoeias prescribe a limit test for I and II[3–6]. The test consists in the determination of the melting range of the 4-nitrobenzyl derivative. The Ph. Eur.[3] and the Ph. Helv. VI[4] prescribe a range of 136–148°C, the BP 1973[5] 136–155°C and the USP XX[6] 136–146°C. The PH. Nord. 1963[7] uses the 4-nitrobenzyl derivative for the identification and mentions a melting range of 139–157°C. The determination of melting points of 4-nitrobenzyl derivatives of barbiturates has often been applied for identification purposes[8–10]. Their use in the determination of isomer content has been discussed in the literature[11,12]. The melting point of underivatized pentobarbital cannot be used for the determination of the isomer content[13]. Jerslev *et al.* published the melting range of the 4-nitrobenzyl derivatives of a series of mixtures of I and II[12]. From these results it can be deduced that the Ph. Eur. and Ph. Helv. VI allow *ca.* 6% of I, the BP 1973 *ca.* 12% and the USP XX *ca.* 5%. The melting range in the Ph. Nord. 1963 corresponds to *ca.* 14% of I. The pharmacopoeial test for the content of I in II is not precise and is tedious to perform. We therefore examined analysis by high-performance liquid chromatography (HPLC), which is fast, easy to perform, and more precise. The same method also allows the detection of impurities other than I, and is suitable for the identification of barbiturates. HPLC has already been mentioned in recent literature as a suitable technique for the analysis of barbituric acid derivatives[14–16]. Gas–liquid chromatography (GLC), another powerful analytical technique, usually needs derivatization of barbiturates, although capillary GLC of free barbiturates has been described[17].

EXPERIMENTAL

Samples

Pure I was kindly donated by Professor B. Jerslev, Royal Danish School of Pharmacy, Copenhagen, Denmark. Samples of pentobarbital were provided by Professor J. Bosly, Institut de Pharmacie, Liège, Belgium and by Mr. Dooms, Apotheek

0021-9673/81/0000–0000/$02.50

Academisch Ziekenhuis St. Rafaël, Leuven, Belgium. For some samples the manufacturer was unknown, other samples were from Abbott (Chicago, IL, U.S.A.), Rhône-Poulenc (Paris, France) and Siegfried (Zofingen, Switzerland).

Apparatus

The HPLC apparatus consisted of a Waters pump Model 6000 A (Waters Assoc., Milford, MA, U.S.A.), a Valco injector Model CV-6-UHPa-N60 equipped with a 10-μl loop (Valco, Houston, Texas), a Pye Unicam detector model LC3UV (Pye Unicam, Cambridge, Great Britain) and a Kipp & Zonen recorder model BD40 (Kipp & Zonen, Delft, The Netherlands). Columns (25 cm × 4.6 mm I.D.) were packed with SAS-Hypersil (Shandon Southern, Cheshire, Great Britain) or with Zorbax C_8 (DuPont, Wilmington, DE, U.S.A.).

Reagents, mobile phases and operating conditions

Methanol 99+ % (Aldrich Europe, Beerse, Belgium) and distilled water were glass-distilled before use. Potassium monohydrogen phosphate and potassium dihydrogen phosphate pro analysi (E. Merck, Darmstadt, G.F.R.) were used to prepare a 0.2 *M* buffer of pH 6.0. Mobile phases used for the determination of isomer content consisted of methanol–water–0.2 *M* phosphate buffer pH 6.0 mixture in a ratio of 37:58:5 for the Hypersil column and 55:40:5 for the Zorbax column. (See figures for composition of other mobile phases.) Mobile phases were degassed by sonication. The flow-rate was set at 1.0 ml/min and the paper speed at 5 mm/min. All separations were carried out at room temperature (*ca.* 20°C). For the determination of the isomer content the detector was set at 240 nm and 0.04 a.u.f.s. (See figures for detector setting of other chromatograms.)

Preparation of samples, reproducibility of the method

A 0.040% m/v solution of barbital in methanol–water (1:1) was used as the internal standard solution (IS). For the samples examined it was checked that no sample peak eluted together with this IS. The sample (25.0 mg) was dissolved in 5.0 ml of IS and the volume was made up to 10.0 ml with methanol–water (1:1). In order to prepare a calibration curve, 20.0 mg of I was dissolved in 20.0 ml of methanol–water (1:1). Aliquots (3.0, 2.0 and 1.0 ml) of this solution were diluted with 5.0 ml of IS and the volume was made up to 10.0 ml with methanol–water (1:1). Samples (10 μl) were repeatedly injected on the Hypersil column and peak height ratios were determined. A calibration curve for the ratio peak height/peak height IS *versus* concentration in mg/ml was obtained with a linear regression $y = 2.732x - 0.0012$ and a correlation coefficient of 0.999. When pure II was used to prepare a calibration curve, a linear regression $y = 2.014x - 0.0015$ and a correlation coefficient of 0.999 were obtained. From this it is deduced that if II were used to prepare a calibration curve to determine I, the results were to be multiplied by a conversion factor of 0.736. The reproducibility of the method was checked by injecting six times a sample of II containing I. A mean value of 4.73% of I with a standard deviation of 0.09 was obtained. The limit of detection (three times the baseline noise) was *ca.* 0.1%.

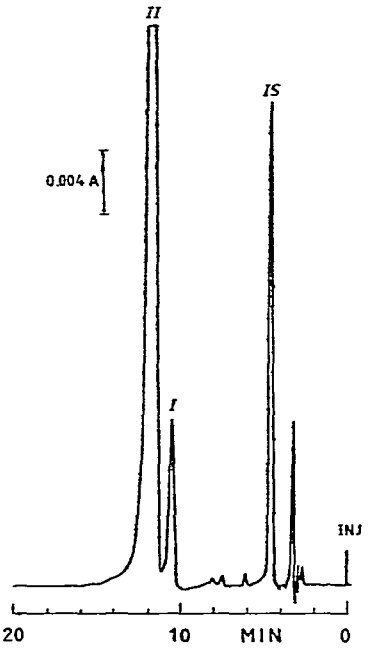

Fig. 1. Separation of 5-ethyl-5-(1-ethylpropyl)barbituric acid (I) and pentobarbital (II) in sample A3. IS (internal standard), barbital; column, SAS-Hypersil, 25 cm × 4.6 mm I.D.; mobile phase, methanol–water–0.2 *M* phosphate buffer pH 6.0 (37:48:5); flow-rate 1.0 ml/min; detection: 240 nm, 0.04 a.u.f.s.; paper speed, 5 mm/min.

RESULTS AND DISCUSSION

Fig. 1 shows a typical chromatogram obtained with the Hypersil column. I and II are well separated. The Zorbax column gives similar separations. Impurities other than I were also observed in the pentobarbital samples. The results are compiled in Table I. They were obtained with the Hypersil column. The unknown impurities are indicated by their relative retention times, the retention time of barbital (IS) being 1.0. Since the structures of these impurities were unknown, their content was expressed as isomer I. This procedure gives an estimation of the purity of the different samples.

All the samples from manufacturer C are free of isomer I. The samples from manufacturers A and B contain *ca.* 4–6% of I. Sample 3, of unknown origin, contains 5.9% of I. The melting point of the 4-nitrobenzyl derivative of this sample was 142 C, which is lower than 148°C, the value derived from literature results for an isomer content of *ca.* 6%[12]. The melting point of the nitrobenzyl derivative of sample 5, of unknown origin, was 136°C. No sample contained more than a total of 3% of unknown impurities, calculated as I.

Fig. 2 shows the separation of a series of barbiturates, obtained on the Hypersil column and on the Zorbax column. The elution on Hypersil, having a particle size of 5 μm and a shorter aliphatic chain, is faster. The aliphatic chain is probably trimethylsilyl[18]. The twelve barbiturates are not all separated. Decreasing the methanol content of the mobile phase does not improve the separation of the pairs allobarbital–

TABLE I
IMPURITY CONTENT OF PENTOBARBITAL

	Sample	Impurity content (%, m/m)*						
		Isomer I	Retention time of unknown impurities relative to barbital (IS) = 1.0					
			1.27	1.35	1.67	1.81	1.87	2.33	4.07
Manufacturer A	1	5.2	0.3	0.4					
	2	4.9	0.3	0.4		0.5			
	3	5.0		0.3	0.3	0.2			
	4	4.8		0.4	0.3	1.8	1.1		
Manufacturer B	1	4.7	0.3	0.3					
	2	6.1		0.4		0.5			
Manufacturer C	1	ND**		0.5			1.1		
	2	ND		0.3					
	3	ND		0.9		0.6			
	4	ND		0.3		0.3			
	5	ND		0.4	0.4	0.4			0.7
	6	ND		0.4	0.5		0.5		0.6
Manufacturer unknown	1	ND		0.3					
	2	ND		0.5			0.7		
	3	5.9		0.3			0.4		
	4	ND		0.3			0.4	0.4	
	5	ND		0.2	0.4		0.4		0.5

* Impurities other than isomer I are calculated as I.
** ND = Not detectable (less than 0.1 %).

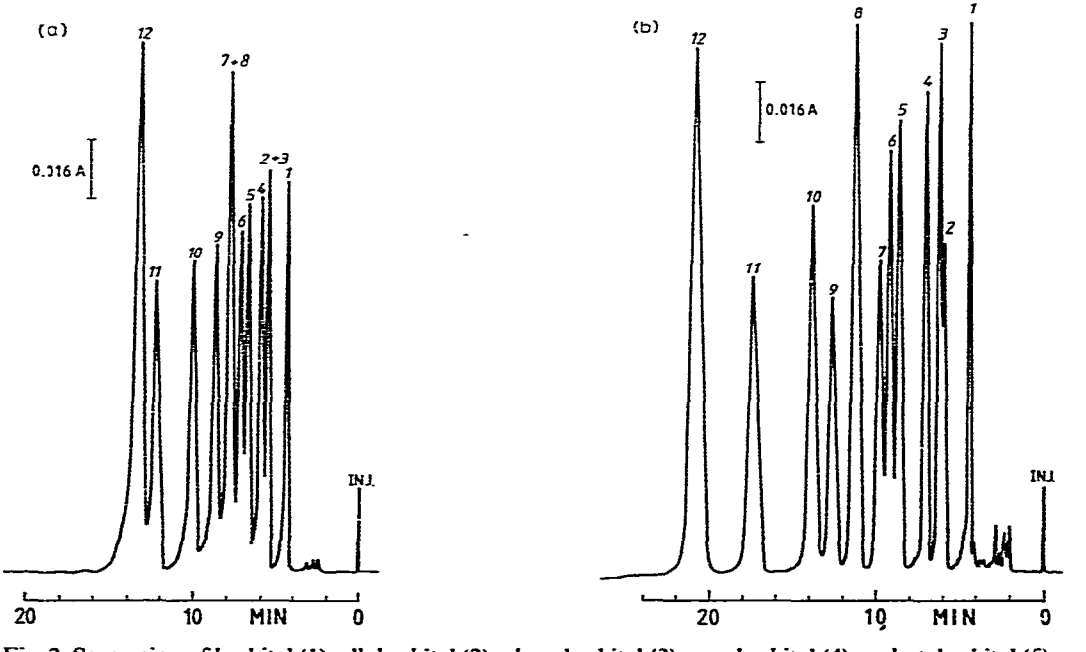

Fig. 2. Separation of barbital (1), allobarbital (2), phenobarbital (3), aprobarbital (4), secbutabarbital (5), butobarbital (6), butalbital (7), methylphenobarbital (8), 5-*sec.*-butyl-5-ethyl-2-thiobarbituric acid (9), pentobarbital (10), secobarbital (11) and thiopental (12). Column, 25 cm × 4.6 mm I.D.; flow-rate, 1.0 ml/min; detection, 254 nm, 0.16 a.u.f.s.; paper speed, 5 mm/min. (a) SAS-Hypersil. Mobile phase, methanol–water–0.2 *M* phosphate buffer pH 6.0 (40:55:5). (b) Zorbax C_8. Mobile phase, methanol–water–0.2 *M* phosphate buffer pH 6.0 (55:40:5).

phenobarbital and butalbarbital–methylphenobarbital. The elution on the Zorbax column, with 7-μm particles and a octylsilyl chain, is slower but the separation is more complete.

The use of a pH 6.0 buffer for the preparation of the mobile phase has several advantages. The column packing material will have a longer lifetime than with more alkaline mobile phases, containing for example ammonium carbonate, which has been proposed for use in HPLC of barbiturates[16]. Furthermore, since it is well known that the absorbance of barbiturates is influenced by the pH of the solution, the peak height can change when different qualities of distilled water and methanol are used for the preparation of the mobile phase. This was observed in the routine use of HPLC of barbiturates during practical exercises by students.

The results show that the isomer content of pentobarbital, as well as the content of other impurities, can easily be determined by HPLC on reversed-phase materials of different chain lengths, using buffered methanol–water mixtures as the mobile phase. With some adjustment of the methanol content in the mobile phase, the same system has also been used for the analysis of other barbiturates.

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