Journal of Chromatography, 114 (1975) 345–350

CHROM. 8533

ELECTRON-CAPTURE GAS CHROMATOGRAPHY OF BARBITURIC ACIDS AND DIPHENYLHYDANTOIN AFTER PENTAFLUOROBENZYLATION

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(Received April 4th, 1975)

SUMMARY

Alkylation of barbituric acids and diphenylhydantoin with pentafluorobenzyl bromide is described; the reaction is quantitative in the presence of excess of triethylamine. The electron-capture detector response of the derivatives is high, ranging from 2 to 8 $\times$ 10^{-17} moles/sec. The derivatives demonstrate excellent peak symmetry and are well suited to quantitative analysis. The structures of the derivatives have been confirmed by mass spectrometry.

INTRODUCTION

Electron-capture gas chromatography has been amply demonstrated as a most versatile technique for quantitative determinations of low concentrations of drugs and drug metabolites in biological samples. As most drugs have too low a response in electron-capture detection (ECD), electron-absorbing derivatives must be prepared. With the exception of phenols, little has been reported on the derivatization of acidic compounds for ECD. A high ECD response for carboxylic acids after alkylation with pentafluorobenzyl (PFB) bromide has, however, been reported[1]; this reagent has also been used to determine phenolic compounds[2,3].

This investigation was undertaken to evaluate PFB bromide as a reagent for the derivatization of barbituric acids and diphenylhydantoin for ECD. Conditions for derivative preparation and gas chromatographic separation were studied, as well as the response by ECD. The structures of the derivatives were confirmed by mass spectrometry.

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** Part of this work was carried out at the Department of Pharmacology, Medical University of South Carolina, Charleston, S.C., U.S.A.

EXPERIMENTAL

Reagents and chemicals

PFB bromide was supplied by Pierce (Rockford, Ill., U.S.A.). All solvents used were of pesticide grade and obtained from Fisher Scientific (Pittsburgh, Pa., U.S.A.). Triethylamine was supplied by Eastman Org. Chem. (Rochester, N.Y., U.S.A.) and was redistilled before use. The barbiturates and diphenylhydantoin were of analytical grade; their purity was better than 95%, as determined by titration and by gas chromatography on 3% OV-17 and 3% neopentyl glycol sebacate (NPGSe) columns, with flame ionization detection.

Gas chromatography

All columns were made of borosilicate glass and were washed and silanized as previously described[4]. The columns were packed with 3% of stationary phase (OV-1, OV-17 or NPGSe) supported on silanized Chromosorb W (80–100 mesh)[4].

Electron-capture detection. The instrument was a series 104, model 84, Pye gas chromatograph fitted with a 10-mC ^{63}Ni electron-capture detector operated at 250°. A pulsed voltage was used (pulse amplitude 50 V; pulse period 500 μsec; pulse width 0.75 μsec). Columns (160 cm × 4 mm I.D.) were used with a nitrogen flow-rate of 80 ml/min. The ECD response was determined by injection of known quantities of derivatives dissolved in ethyl acetate; minimum detectable quantities (in moles per sec and in picograms) were determined[4].

Flame ionization detection (FID). The instrument was a Varian Aerograph Model 600D equipped with an F & M temperature programmer, Model 240. Columns (150 cm × 2 mm I.D.) were used with a nitrogen flow-rate of 40 ml/min.

Preparation of derivatives

Macro-scale. Portions (25 mg; 10^{-4} mole) of the barbituric acids and diphenylhydantoin were separately dissolved in 0.5 ml of a 5% solution of water in methanol. Triethylamine (50 μl; $3.5 \cdot 10^{-4}$ moles) and PFB bromide (50 μl; $3 \cdot 10^{-4}$ moles) were added, and the mixtures were heated for 5 h at 50°. The reaction mixtures were left overnight at 0°, and the colorless crystals formed were collected on a filter and washed with cold 5% water solution in methanol. The recoveries were greater than 80%.

The di-PFB derivatives of phenobarbital (m.p. 112–113°) and of barbital (m.p. 165°), and the mono-PFB derivatives of mephobarbital (m.p. 125–126°), hexobarbital (oily liquid) and diphenylhydantoin (m.p. 165°) were prepared. The purity of these derivatives was better than 95% as determined by gas chromatography on OV-1 and OV-17 columns using FID.

Micro-scale. Reaction conditions were investigated using the following derivatization procedure: To 5 μg each ($\approx 3 \cdot 10^{-8}$ moles) of barbituric acids and diphenylhydantoin dissolved in 100 μl of methanol (containing 5 μg of 9-bromophenanthrene as internal standard) were added triethylamine ($1–50 \cdot 10^{-5}$ moles) and PFB bromide ($5–50 \cdot 10^{-6}$ moles), and the reaction mixture was heated for 5–120 min at 50° before gas chromatography with FID. The percentage yield of derivative was obtained from a standard curve prepared from known amounts of synthetic derivative and internal standard.

Mass spectrometry

An LKB 9000 gas chromatograph–mass spectrometer was used with a glass column (180 cm × 4 mm I.D.) packed with 1% of OV-17. The ion-source temperature was 270°, the trap current was 60 μA, the ionizing voltage was 70 eV and the scan time was 10 sec. Molecular ions and main-fragment ions characteristic of some of the barbituric acid derivatives are listed below.

Di-PFB barbital: *m/e* 181 ($C_6F_5CH_2$, 100% relative intensity); *m/e* 516 (M − 28, 56%, with *m** 489.4); *m/e* 292 (24%); *m/e* 278 (20%); *m/e* 544 (M^+, 17%); and *m/e* 501 (M − 43, 17%, with *m** 486.4).

Di-PFB phenobarbital: *m/e* 181 ($C_6F_5CH_2$, 100%); *m/e* 564 (M − 28, 76%, with *m** 537.3); and *m/e* 592 (M^+, 25%).

Mono-PFB mephobarbital: *m/e* 398 (M − 28, 100%, with *m** 371.8); *m/e* 181 ($C_6F_5CH_2$, 58%); and *m/e* 426 (M^+, 20%).

Other main fragments of the barbituric acid derivatives agreed well with the main fragments of the underivatized compounds[5].

The base peak of mono-PFB diphenylhydantoin (*m/e* 180) was the same as for the underivatized compound[6]; other main fragments of this derivative were at *m/e* 181 (41%), *m/e* 432 (M^+, 33%), *m/e* 403 (M − 29, 18%), *m/e* 355 (M − 77, 11%) and *m/e* 251 (M − 181, 8%).

RESULTS AND DISCUSSION

When examined by gas chromatography–mass spectrometry, all derivatives demonstrated molecular and fragment ions consistent with the incorporation of one or two PFB groups (see Experimental).

Methanol was selected as medium for the alkylation reaction by virtue of its being a good solvent both for the samples and for the derivatizing reagent; triethylamine was used as the base catalyst for the alkylation reaction. Potassium carbonate, which has been used to catalyze the alkylation of simple phenols and carboxylic acids[1,2], caused hydrolysis of the barbituric acids and diphenylhydantoin.

The triethylamine concentration needed for quantitative alkylation is, however, critical, as shown in Fig. 1 for barbital. No alkylation takes place in the absence

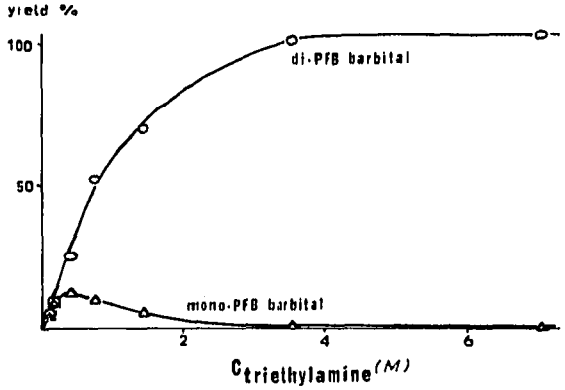

Fig. 1. Effect of triethylamine concentration on the PFB alkylation of barbital. Barbital (5 μg) was dissolved in 100 μl of methanol; PFB bromide (1 μl) was used. Internal standard: 5 μg of 9-bromophenanthrene; temperature, 50°; reaction time, 30 min.

of triethylamine, and at low triethylamine concentrations a mixture of the mono- and di-PFB derivatives is obtained. A triethylamine concentration of more than 0.3 moles/l is necessary for complete and reproducible alkylation. The amount of PFB bromide, the reaction temperature and the reaction time are relatively less important. At a triethylamine concentration of 0.3 moles/l complete alkylation of the eight barbiturates listed in Table I was accomplished after 60 min at 50° using only a moderate excess of PFB bromide (1 μl; $5 \cdot 10^{-6}$ moles).

TABLE I

EFFECT OF PFB ALKYLATION ON RELATIVE RETENTION OF BARBITURIC ACIDS

Column: 3% OV-17 operated at 210°. Reference compound: 9-bromophenanthrene (retention time 4.5 min).

Compound	$t_{rel.}$	
	Underivatized	*Derivatized*
Barbital	0.10	0.33 (mono) 1.12 (di)
Hexobarbital	0.45	1.26 (mono)
Mephobarbital	0.55	1.39 (mono)
Allobarbital	0.16	1.36 (di)
Aprobarbital	0.16	1.45 (di)
Amobarbital	0.21	1.66 (di)
Pentobarbital	0.23	1.87 (di)
Phenobarbital	0.83	4.63 (di)
9-Bromophenanthrene	1.00	1.00

Diphenylhydantoin under these conditions formed only the mono-PFB derivative, formation of which was, however, quantitative and reproducible.

All the derivatives were stable in solution for several months.

The influence of PFB alkylation on the retention times of barbituric acids is demonstrated in Table I for a 3% OV-17 column, which gives acceptable peak symmetry for the underivatized compounds. In spite of the large increase in molecular weight for each PFB group incorporated, the retention-time increase of the derivatives is less than expected, there being a 3–4 fold increase in retention time for each PFB group.

The ECD response of some of the derivatives is shown in Table II. The response is very high and about the same regardless of whether one or two PFB groups are incorporated. High ECD response has also been reported for other derivatives containing a pentafluorophenyl group, emphasizing that the pentafluorophenyl group is the major electrophore in all these derivatives[7–9]. The ECD response is only slightly temperature-dependent[10], the response to di-PFB barbital being decreased by a factor of about 2 by increasing the detector-cell temperature from 220 to 290°.

The gas chromatographic separation of some of the derivatives is shown in Fig. 2. The peak symmetry is remarkable in spite of the small amounts (1–4 pg) injected. The minimum detectable amount of di-PFB barbital on this column was 0.05 pg.

The results of repeated injections of a few picograms of the di-PFB derivatives of barbital and phenobarbital are given in Table III. The excellent reproducibility

TABLE II

ECD RESPONSE

Compound	*Minimum detectable amount*	
	*10^{-17} Moles/sec**	*Picograms***
Di-PFB barbital	2.2	0.05
Di-PFB phenobarbital	2.6	0.07
Mono-PFB mephobarbital	3.6	0.11
Mono-PFB hexobarbital	3.4	0.10
Mono-PFB diphenylhydantoin	8.2	0.25

* A signal three times the background noise level[4].
** At t_R = 3 min for a column with 3600 theoretical plates.

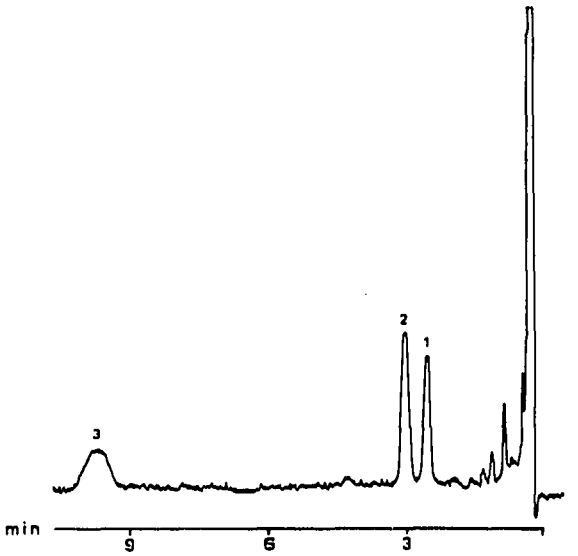

Fig. 2. GC–ECD of picogram amounts of PFB alkylated barbituric acids. Column: 3% OV-17 operated at 230°. 1 = 0.9 pg of di-PFB barbital; 2 = 3.8 pg of mono-PFB mephobarbital; 3 = 2.1 pg of di-PFB phenobarbital.

TABLE III

REPRODUCIBILITY OF REPEATED INJECTIONS OF PICOGRAM QUANTITIES OF DI-PFB DERIVATIVES OF BARBITAL AND PHENOBARBITAL

Conditions as for Fig. 2; 8 injections of each sample.

Compound	*Amount injected (pg)*	*Peak area (cm^2)*
Di-PFB barbital	0.9	0.94 ± 3.1%
Di-PFB phenobarbital	2.1	1.70 ± 2.9%

clearly shows that it is possible quantitatively to measure the PFB derivatives of barbituric acids in the 1–2 pg range. It was also established that the linearity of the pulsed-mode ^{63}Ni detector used in this work ranged from the minimum detectable amounts up to several hundred picograms, about a 1000-fold range of linearity.

In conclusion, these results demonstrate that PFB bromide as an alkylating agent forms with barbituric acids and diphenylhydantoin stable derivatives that are highly sensitive to ECD and well suited to quantitative determination. Applications of this technique to microlitre samples of biological origin are currently in progress.

ACKNOWLEDGEMENTS

I thank Miss Barbro Näslund for excellent technical assistance and Mrs. Kristina Walle for assistance in preparing the manuscript.

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