Journal of Chromatography, 154 (1978) 349–352
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 10,849

Note

Determination of phenobarbital and *p*-hydroxyphenobarbital by densitometry of thin layers

S. S. LEVIN, M. F. SCHWARTZ, D. Y. COOPER and J. C. TOUCHSTONE
School of Medicine, University of Pennsylvania, Philadelphia, Pa. 19104 (U.S.A.)
(First received August 22nd, 1977; revised manuscript received January 3rd, 1978)

During studies of cytochrome P450 activity in rats by induction with phenobarbital (PB), it became of significance to determine methods of detection and quantitation of the drug and its metabolite, *p*-hydroxyphenobarbital (PPB). Previous attempts at separation have been carried out by gas chromatography which necessitated derivatization of the compounds[1]. In addition, gas chromatography has proved insensitive for detection of PPB under the conditions investigated.

The present report describes densitometry *in situ* of thin-layer chromatograms for quantitation of both PB and PPB without derivatization. The method proved sensitive for detection in various rat tissues and excretions, indicating that with proper usage, quantitation of the compounds by thin-layer densitometry can be both reproducible and reliable.

EXPERIMENTAL

Apparatus

The chromatograms were scanned with the Schoeffel 3000 spectrodensitometer, which was equipped with an SDC 3000 density computer. The densitometer was provided with photomultiplier tubes allowing for both transmission and reflectance. Thin layers were scored into 20 lanes of 10 mm width by a Schoeffel scoring device. Samples were applied with a Hamilton 10-μl syringe and dried upon application by a Model 6000 hair drier (Master Appliance, Racine, Wisc., U.S.A.).

Materials

Analtech silica gel GF precoated glass plates of dimensions 20 × 20 cm and 250 μm thickness were used for separation of PB and PPB and their subsequent quantitation. All plates were washed in methanol prior to use. Solvents used for preparation of mobile phase systems were reagent grade ethyl acetate, methanol and Baker analyzed concentrated ammonium hydroxide. Glass tanks of dimensions 7 × 22 × 22 cm, containing 100 ml of the mobile phase were used for development of chromatograms.

Reference standards

Phenobarbital was obtained from Merck (Darmstadt, G.F.R.). *p*-Hydroxy-

phenobarbital was obtained from Aldrich (Milwaukee, Wisc., U.S.A.) and through the courtesy of Dr. T. C. Butler, University of North Carolina[2]. The standards were dissolved in *tert.*-butanol at a concentration of 1 μg/μl.

Extraction method

Samples were frozen until extracted. The method followed was a modification of that reported by Levy and Schwartz[3]. To 5 ml of urine in a 50-ml glass stoppered conical centrifuge tube were added 2 ml of saturated NaH_2PO_4 solution. This gave a final pH of 4.5. This was extracted twice with 5 volumes of methylene chloride, and aliquots from this applied to the thin-layer chromatography (TLC) plates.

Procedure

Plates were activated at 115° for ½ h prior to application of standards. A series of PB and PPB aliquots (1–8.0 μg) were applied to the same area on each lane (two standards per spot), 2 cm from the bottom. Alternating lanes were spotted to allow for double beam scanning by the densitometer. The thin layers were developed in saturated paper lined tanks containing ethyl acetate–methanol–ammonium hydroxide (82:15:4) as the mobile phase to 2.5 cm from the top of the plate. Chromatograms were observed under short-wave UV light and marked for scanning by the densitometer. The spots were distinguishable upon excitation of the chromatogram with the UV light due to the fluorescence "quenching" effect[4]. The spots absorb the incident light, thereby decreasing the excitation of the fluorescent medium in the area of the spot. Hence, we observe the gradient between the well-defined areas of non-fluorescence and the rest of the fluorescent medium. The same principle enables the densitometer to detect a signal of decreased transmitted or reflected light. (In this case transmitted, since the transmission mode proved more sensitive.) These findings were also reported by Touchstone *et al.*[5]. The chromatograms were scanned at a wavelength of 255 nm.

RESULTS AND DISCUSSION

R_F values for the method described were 0.68 and 0.79 for PB and PPB, respectively. These resulted in linear calibration curves after densitometry. Fig. 1

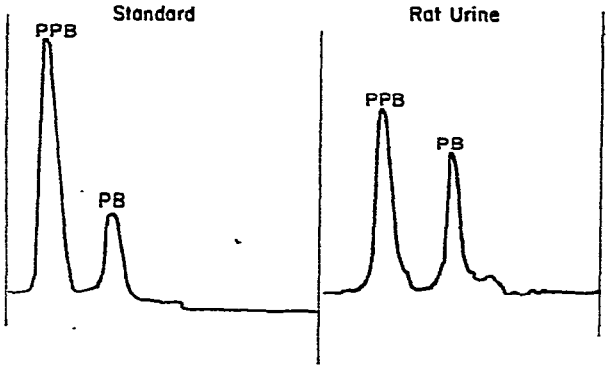

Fig. 1. Results of a scan obtained after TLC separation of PB and PPB from standard solutions and urine extracts from the rat induced with PB.

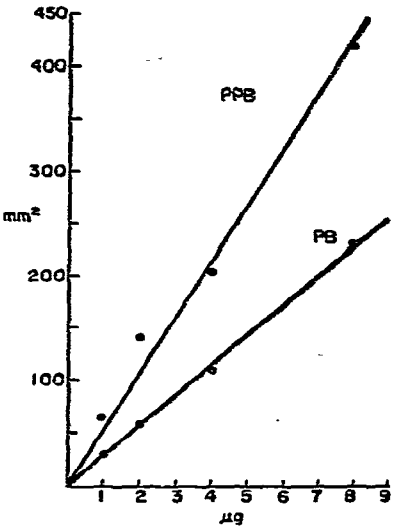

Fig. 2. Calibration curves for 0–8.0 μg of PB and PPB from an Analtech silica gel GF plate after development and scanning at 255 nm.

shows peaks of PB and PPB in chromatograms containing standard and rat urine. The resolution illustrated is evidence for the effectiveness of the method. Calibration curves were linear from 0–8.0 μg in both cases at wavelength 255 nm. Fig. 2 shows different calibration curves for PB and PPB. Values for daily PB and PPB excretion rates were calculated from data obtained by quantitative TLC and are given in Table I. Determination of PB levels in several tissues was also possible, although no occurrence of PPB was observed (see Table II).

TABLE I

CONCENTRATIONS OF PB AND PPB IN THE URINE OF AN INDUCED RAT OVER A 13-DAY INTERVAL DETERMINED BY DENSITOMETRY OF THIN-LAYER CHROMATOGRAMS

Day	*Urine output (ml)*	*PB per day excreted (mg)* *	*PPB per day excreted (mg)*
1	26	2.73	0.77
2	36	4.90	1.30
3	37	6.29	1.67
4	61	6.00	1.65
5	58	4.12	1.28
6	56	3.81	0.92
7	59	4.43	1.83
8	74	3.81	0.89
9	59	4.48	0.89
10	62	4.90	0.78
11	64	4.74	1.02
12	64	4.22	2.94
13	74	4.51	1.33

* See text for method of induction with PB.

TABLE II

PB LEVELS IN THE RAT TISSUE AFTER SACRIFICE, DETERMINED BY THIN-LAYER DENSITOMETRY

Tissue extract	*PB Level (μg PB per mg tissue)*
Kidney	0.064
Liver	0.090
Brain	0.044

Quantitative TLC in our studies provided us with a rapid, simple and accurate methodology. It offered an alternative to gas chromatography, thereby avoiding derivatization. It supplied substantial information regarding enzyme activity and metabolism in the rat induced with PB. [^{14}C]PB added to urine or blood and extracted by the method described have consistently given recoveries of 96% or more.

Multiple 2-μg samples of PB on thin-layer chromatograms gave an average peak area of 118.8 mm^2. The standard deviation was 11.6 with a coefficient of variation of 9.8%. The coefficient of variance for 1-, 2-, 4- and 8-μg samples of phenobarbital per scan over a period of 15 months varied from 7 to 10%. Comparable results were obtained for PPB.

REFERENCES

1 J. C. Touchstone, M. F. Schwartz, S. S. Levin and D. Y. Cooper, *Abstracts Pittsburgh Conf. on Analytical Chemistry and Allied Spectroscopy, Cleveland, 1977.*
2 T. C. Butler, *J. Pharmacol. Exp. Ther.*, 116 (1956) 326.
3 S. K. Levy and T. Schwartz, *Clin. Chim. Acta*, 54 (1974) 19.
4 U. Hezel, in A. Zlatkis and R. E. Kaiser (Editors), *High Performance Thin-Layer Chromatography*, Elsevier, Amsterdam, 1977, p. 147.
5 J. C. Touchstone, S. S. Levin and T. Murawec, *Anal. Chem.*, 43 (1971) 858.