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Quantitative TLC Determination of Primidone, Phenylethylmalonediamide, and Phenobarbital in Biological Fluids

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Abstract □ A rapid and reproducible TLC determination of primidone and its metabolites in plasma and urine is described. The method consists of extracting the drugs into chloroform, evaporating the organic phase to dryness, dissolving the residue in an accurately measured volume of chloroform–acetic acid (9:1), and spotting this solution on a thin layer of silica gel. Quantitation is achieved by comparing the areas under the peaks obtained from scanning the TLC plates in a spectrodensitometer. The limit of detection is 1 mcg./ml. plasma for primidone and phenobarbital and 2 mcg./ml. plasma for phenylethylmalonediamide. Mean recoveries obtained from spiked plasma samples were: primidone, 93%; phenylethylmalonediamide, 97%; and phenobarbital, 102%.

Keyphrases □ Primidone—TLC separation and quantitation with phenylethylmalonediamide and phenobarbital in biological fluids □ Phenylethylmalonediamide—TLC separation and quantitation with primidone and phenobarbital in biological fluids □ Phenobarbital—TLC separation and quantitation with primidone and phenylethylmalonediamide in biological fluids □ TLC—separation and quantitation of primidone, phenylethylmalonediamide, and phenobarbital in biological fluids

Several procedures have been published on the quantitation of primidone and two of its metabolites, phenylethylmalonediamide and phenobarbital, in biological fluids. One chemical method used oxidation of primidone to phenobarbital (1). The other procedures used either TLC (2–4) or GLC (5–11). The TLC procedures were semiquantitative, since they relied on measuring spot size and intensity or colorimetric estimation after elution of compounds from the plates. The GLC methods were generally reproducible, accurate, specific, and sensitive but were relatively time consuming. This disadvantage cannot be neglected when analysis time is important as in a bioavailability study involving several hundred assay samples. This paper describes a fast and quantitative TLC procedure for estimating primidone, phenylethylmalonediamide, and phenobarbital in plasma and urine.

EXPERIMENTAL

Apparatus—A spectrodensitometer[1] equipped with a density computer[2] was used. The light source consisted of a xenon–mercury 200-w. lamp[3]. The instrument was operated in the reflection mode, using the dual-beam system. Samples were scanned at 220 nm. (density computer set at 0.2 o.d.). Scanning and chart speeds were set at 10.2 cm. (4 in.)/min.

Materials and Chemicals—Silica gel TLC plates[4] (20 × 20 cm.) were employed. The plates were divided into 20 equal channels, each 1 cm. wide, with a scoring device[5]. The plates were heated at 100° for 1 hr. before use. Stock solutions of primidone[6], phenylethylmalonediamide[6], and phenobarbital[7] were prepared by dissolving accurately weighed amounts in methanol. Working standards were made by appropriate dilutions of the stock standards. All solvents used were reagent grade. Samples were applied to the plates with 10-, 25-, or 50-μl. syringes[8].

Developing Systems—For primidone and phenylethylmalonediamide analysis, the following developing system was used: ethyl acetate–benzene–acetic acid (90:20:10) (System A). For phenobarbital analysis, the system consisted of benzene–ethyl acetate–acetone–acetic acid (100:25:15:10) (System B). These systems require saturated tanks and an environment where the relative humidity is maintained at less than 25%.

Procedure for Determining Drugs in Plasma—Two-milliliter plasma samples spiked with appropriate amounts of all three drugs were extracted with 30 ml. chloroform in 50-ml. conical tubes. The tubes were shaken on a mechanical device[9] for 15 min. Unspiked plasma samples served as blanks. After extraction, the tubes were centrifuged and 25 ml. of the extracts were transferred to a clean 50-ml. conical tube and evaporated under nitrogen[10]. The sides of the tubes were washed with 1-ml. portions of chloroform, and the solutions were evaporated to dryness. The residues were dissolved in 100 μl. of a solution of chloroform–acetic acid (9:1).

Appropriate aliquots (25–50 μl.) were spotted on scored TLC

[1] Model SD 3000, Schoeffel Instrument Corp.
[2] Model SDC 300, Schoeffel Instrument Corp.
[3] Hanovia Lamp Division, Camrand Precision Ind.
[4] Sil G-25-22, Brinkmann Instruments Inc.
[5] SDA 320, Schoeffel Instrument Corp.
[6] Pharmaceuticals Division, Imperial Chemical Industries Ltd.
[7] USP powder, Mallinckrodt Chemical Works.
[8] Hamilton Co.
[9] Eberbach, Ann Arbor, Mich.
[10] N-Evap, Organomation Association.

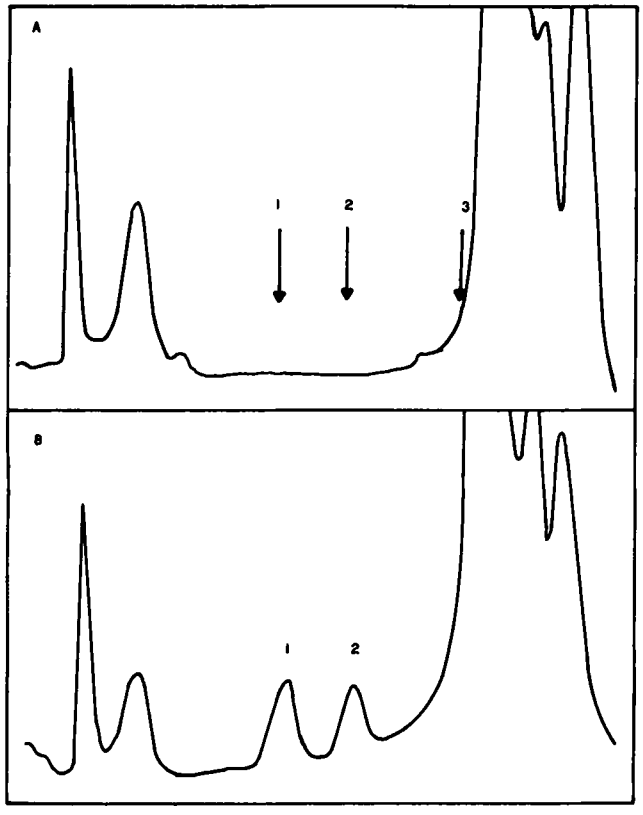

Figure 1—*(A) Chromatogram of a blank plasma extract showing* R_f *values for: 1, primidone; 2, phenylethylmalonediamide; and 3, phenobarbital. (B) Chromatogram of an extract from a plasma sample spiked with primidone (1) and phenylethylmalonediamide (2) at 5-mcg/ml. concentration. Extra peaks represent extractable plasma impurities.*

plates, generally in duplicate. Each channel on which a sample was spotted had to its immediate left a nonspotted reference channel. Standards of at least two different concentrations were also spotted so that a calibration line was included on each plate. The plates were then developed, dried, and read as described. Drug concentration was determined by comparing the areas under the peaks obtained from the standards to those obtained from the spiked samples. The procedure is also applicable to the determination of primidone, phenylethylmalonediamide, and phenobarbital in urine. Specimens are adjusted to pH 7.0 before extraction.

***In Vivo* Experiment**—To assess the applicability of the method, six beagle dogs were given 192 mg. of primidone intravenously (drug dissolved in 4 ml. dimethylformamide). Heparinized blood samples were collected prior to injection and at 0.25, 0.50, 0.75, 1, 2, 3, 4, 6, and 8 hr. after injection. The blood samples were centrifuged immediately, and the plasma was frozen until analyzed.

RESULTS AND DISCUSSION

With developing System A, primidone (R_f 0.42) can be separated from its metabolites phenylethylmalonediamide (R_f 0.53) and phenobarbital (R_f 0.67) and from extractable plasma impurities (Fig. 1). Both primidone and phenylethylmalonediamide can be quantitated when System A is used, but phenobarbital cannot be estimated due to interfering plasma components. However, the system can be used for the determination of all three drugs in urine, because interfering impurities are absent. To quantitate phenobarbital in plasma, developing System B must be used (Fig. 2). The use of two developing systems may make the method less appealing, but they are necessary only when phenobarbital needs to be determined in plasma. This method was developed for application in bioavailability studies. When a single dose of primidone is administered, phenobarbital determination is not required since it is not detectable in plasma following such a dose (12). However, phenobarbital determination is indicated in studies involving multiple dosing of primidone.

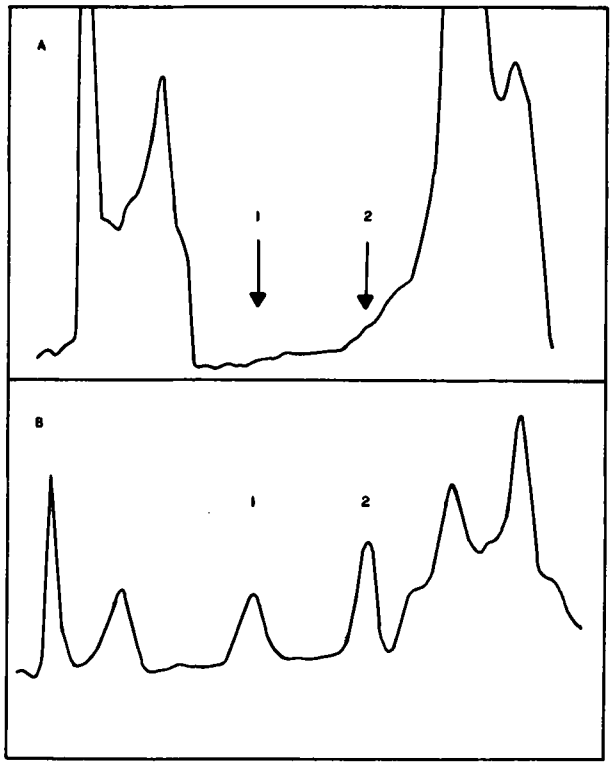

Figure 2—*(A) Chromatogram of a blank plasma extract showing* R_f *values for primidone (1,* R_f *0.34) and phenobarbital (2,* R_f *0.58). Phenylethylmalonediamide has same* R_f *as primidone in this system. (B) Chromatogram of an extract from a plasma sample spiked with primidone (1) and phenobarbital (2) at 5-mcg./ml. concentration. Extra peaks represent extractable plasma impurities.*

Typical calibration lines are shown in Fig. 3. Correlation coefficients are: primidone, 0.996; phenylethylmalonediamide, 0.993; and phenobarbital, 0.990 (n = 5). Although the range of linearity is rather narrow for phenobarbital (about 1–6 mcg.), it is not a problem as long as a calibration line covering the concentration range of the unknowns is included on every plate.

A summary of the recovery studies performed in plasma is given in Table I. Mean recoveries were: primidone, 93% (n = 21); phenylethylmalonediamide, 97% (n = 18); and phenobarbital, 102% (n = 21). Mean recoveries from spiked urine samples were: primi-

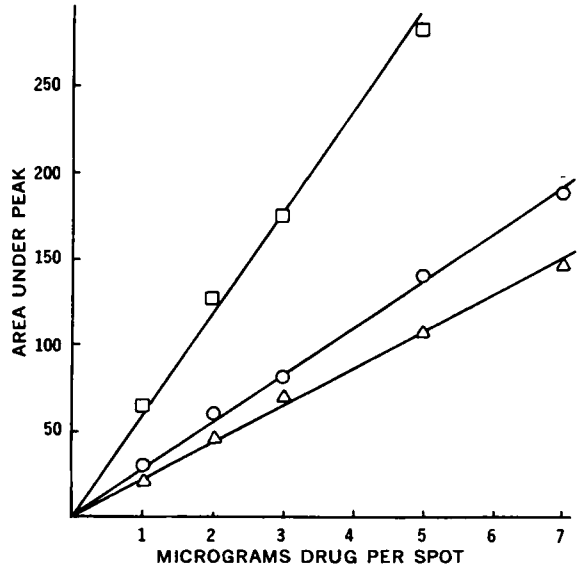

Figure 3—*Typical calibration lines for primidone (○), phenobarbital (□), and phenylethylmalonediamide (△).*

Table I—Summary of Recovery Experiments in Plasma

Primidone				Phenylethylmalonediamide				Phenobarbital			
Amount Spiked, mcg./ml.	Amount Recovered, mcg./ml. Run 1	Run 2	Run 3	Amount Spiked, mcg./ml.	Amount Recovered, mcg./ml. Run 1	Run 2	Run 3	Amount Spiked, mcg./ml.	Amount Recovered, mcg./ml. Run 1	Run 2	Run 3
1.0	1.0	1.0	1.0	—	—	—	—	2.0	2.0	2.1	2.1
2.5	2.3	2.3	2.2	2.5	2.3	2.4	2.6	4.0	4.0	4.0	3.9
5.0	4.3	4.6	4.6	5.0	5.0	5.1	4.8	6.0	6.0	5.4	6.0
7.5	6.6	6.5	6.8	7.5	6.9	7.2	7.9	8.0	8.5	8.4	8.2
10.0	9.0	8.9	9.0	10.0	10.4	10.2	10.0	10.0	10.7	10.2	10.3
15.0	12.7	15.2	15.6	15.0	13.6	14.4	15.3	20.0	20.8	21.2	21.7
20.0	18.8	16.7	16.6	20.0	18.4	17.7	16.9	30.0	31.6	29.8	30.2
Mean recovery		92.6%		Mean recovery		97.0%		Mean recovery		102.5%	
SD		6.5%		*SD*		6.4%		*SD*		4.1%	
Range		83.3–104.1%		Range		84.9–105.9%		Range		90.0–107.0%	

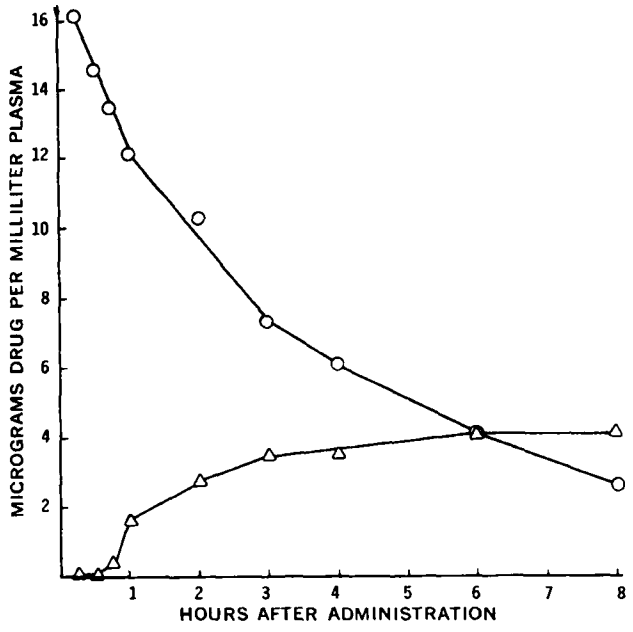

Figure 4—*Mean plasma levels of primidone* (○) *and phenylethylmalonediamide* (△) *following intravenous injection of primidone in dogs.*

done, 85%; phenylethylmalonediamide, 92%; and phenobarbital, 93%. The procedure is simple and rapid. One chemist can easily analyze 20–25 samples in a working day.

An *in vivo* study was performed to verify the applicability of the method. Plasma levels of primidone and phenylethylmalonediamide obtained following a single intravenous injection of primidone to dogs are shown in Fig. 4. Phenobarbital was not detected.

In summary, a quick, reproducible, and specific quantitative TLC method for the determination of primidone and its metabolites in plasma and urine was described. The method is especially useful when a large number of samples must be assayed.

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