produces a curved segment at lower pH values.[3] In calculating the purity and ionization constant, only the linear portion of each plot was used. Nevertheless, it may be seen (Table II) that the intercept obtained (and therefore the purity evaluated) is not markedly influenced by the choice of activity coefficient. It is rather the ionization constant that reflects the difference, as may be seen by the two slopes in Fig. 1. A plot of pKa′ *versus* $\sqrt{\mu}$ demonstrated an essentially linear relationship for both sets of data. These were extrapolated to infinite dilution to evaluate a thermodynamic ionization constant. The results (Table II) indicate that the pKa obtained with both values are consistent with the literature (5–7). Therefore, since the two sets of data are the same, the type of activity coefficient employed for calculations appears to be a matter of personal choice.

[3] In this area of the titration, hydrogen ion makes its greatest contribution to Z' values, so that an error in the activity coefficient would be most evident at these higher acidities. This is comparable to Benet and Goyan's type A curve in Fig. 1 (2) for the case where high erroneous pH values are substituted. In all cases the Kielland values are higher than the $\gamma\pm$, and would result in a lower hydrogen ion concentration.

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Identification of Some Barbiturates by Paper and Thin-Layer Chromatography

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Paper and thin-layer chromatographic procedures are described which can serve to separate a multiple mixture of 12 different barbiturates of toxicological interest.

THE APPLICATION of paper and thin-layer chromatography seems, so far, to be the most promising approach in the identification of barbiturates (1–9). The different procedures adopted leave much to be desired and a simple method for the separation and identification of a multiple mixture of barbiturates is of great value in medico-legal analysis.

A simple, rapid method of separation and identification of 12 barbiturates encountered either alone or in a mixture during the toxicological studies in the National Centre of Criminological Research is reported in this paper. The procedures adopted are based on the application of both paper and thin-layer chromatographic techniques to the following barbiturates: phenobarbital U.S.P., cyclobarbital,[1] barbital U.S.P., diallylbarbituric acid N.F., allylisopropylbarbituric acid,[2] butobarbital,[3] amobarbital U.S.P., secobarbital U.S.P., methylphenobarbital,[4] ethyl-*n*-hexylbarbituric acid,[5] pentobarbital U.S.P., and hexobarbital.[6]

EXPERIMENTAL

Paper Chromatography

The earlier attempt of Kybing (3) and Ledvina (4) for the chromatographic separation of barbiturates on formamide paper gave promising results. It was, therefore, decided to find out the most appropriate system of formamide and the developing solvent which fulfills speedy and efficient separation.

The following systems were investigated: (*A*) paper impregnated with formamide, (*B*) solvent containing formamide, and (*C*) formamide included in both the paper and the solvent.

Paper.—Sheets of Whatman No. 1 filter paper were impregnated with 20–30% formamide in acetone for about 10–15 min. The air-dried sheets were kept in a dark place away from dust. It is recommended that the paper be freshly impregnated.

Solvents.—Chloroform–benzene–ammonium hydroxide, concentration 13:3:6, was employed for system *A* (paper impregnated with formamide). Chloroform–*n*-butanol–formamide–5 *N* ammonium hydroxide, concentration 5:3:1:3, was employed for system *B* (solvent containing formamide). Chloroform–benzene–formamide–5 *N* ammonium hydroxide, concentration 12:2:1:5, was employed for system *C* (formamide included in both the paper and the solvent).

Reagent.—Silver reagent: (*a*) Silver nitrate, A. R., 0.5% methanolic solution. (*b*) Methanol–ammonium hydroxide, concentration 9:1. (*c*) Sodium hydroxide, A. R., 5% methanolic solution. The reagent is prepared by mixing solutions (*a*), (*b*), and (*c*) in the ratio 5:1:2. The reagent has to be freshly prepared.

Standard Solution of Barbiturates.—The above-mentioned barbiturates were used in a chloroform solution of a concentration of 1.5 mcg./μl.

Procedure.—The sheets were spotted in duplicate with 3–4 μl. of the chloroformic solution of the barbiturates and placed into a chamber previously saturated with the stationary phase. The solvent front de-

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[1] Marketed as Phanodorn by Winthrop Laboratories.
[2] Marketed as Alurate by Roche Laboratories.
[3] Marketed as Soneryl by Specia.
[4] Marketed as Prominal by Winthrop Laboratories.
[5] Marketed as Hebaral by Parke Davis & Co.
[6] Marketed as Evipal by Winthrop Laboratories.

TABLE I.—R_f VALUES OBTAINED USING STANDARD SOLUTIONS OF BARBITURATES[a]

Barbiturate Used	Solvents A	B	C
Barbital	0.06	0.65	0.09
Phenobarbital	0.07	0.58	0.06
Secobarbital	0.13	0.80	0.56
Diallylbarbituric acid	0.15	0.68	0.12
Cyclobarbital	0.18	0.70	0.23
Allylisopropylbarbituric acid	0.19	0.69	0.29
Butobarbital	0.24	0.73	0.35
Amobarbital	0.31	0.72	0.46
Pentobarbital	0.41	0.83	0.64
Ethyl-*n*-hexylbarbituric acid	0.58	0.79	0.78
Methylphenobarbital	0.63	0.71	0.90
Hexobarbital	0.77	0.76	0.85

[a] R_f values given here represent the average of 6 determinations.

TABLE II.—R_f VALUES OF BARBITURATES OBTAINED BY USING SILICA GEL G AND KIESELGUHR[a]

Barbiturate Used	Mobile Phase[b] (a)	(b)	(c)
Phenobarbital	0.20	—	—
Cyclobarbital	0.30	0.46	...
Barbital	0.31	0.13	...
Diallylbarbituric acid	0.34	0.24	...
Allylisopropylbarbituric acid	0.50		...
Butobarbital	0.53	...	...
Amobarbital	0.58	...	...
Secobarbital	0.63	...	0.43
Methylphenobarbital	0.64	...	0.90
Ethyl-*n*-hexylbarbituric acid	0.64	...	0.67
Pentobarbital	0.66	...	0.50
Hexobarbital	0.77	—	—

[a] R_f values given here represent the average of 6 determinations. [b] (*a*) for Silica Gel G; (*b*) and (*c*) for kieselguhr.

scended to the proper height (30 cm.) within 2–2.5 hr. After drying at room temperature in a stream of air for 10–15 min., the barbiturates were developed as white spots using the standard method of spraying with silver reagent (10). Table I shows the typical results obtained using the standard solution of barbiturates.

Thin-Layer Chromatography

Apparatus.—The apparatus used was essentially the one designed by Stahl (11) using 20 × 20 cm. glass plates.

Adsorbent.—Silica Gel G (Merck); kieselguhr (Merck) impregnated with formamide.

Mobile Phase.—(*a*) Ethyl acetate *n*-hexane–ammonium hydroxide, concentration 20:9:10, was employed for the Silica Gel G. (*b*) Carbon tetrachloride–chloroform, concentration 1:2, was employed for kieselguhr. (*c*) Carbon tetrachloride–chloroform, concentration 1:1, was employed for kieselguhr.

Reagent.—Silver reagent (10).

Standard Solution of Barbiturates.—The above-mentioned barbiturates were used in a chloroformic solution of a concentration of 1.5 mcg./μl.

Procedure.—Each plate was covered to a thickness of about 250 μ with a paste consisting of 4 Gm. of silica gel in 12 ml. of distilled water (or 4 Gm. kieselguhr in 16 ml. of 20% formamide in acetone). Precautions were taken to prevent air bubbles. The chromatoplates were dried in air for 15 min. at 105° (for silica gel) and 1 hr. at 60° (for kieselguhr).

The plates while still hot were spotted in duplicate with 3–4 μl. of the chloroformic solution of the barbiturates, and placed into a chamber containing the mobile phase. The solvent front ascended to the proper height (15 cm.) within 45 min. (in case of silica gel) and 20 min. (in case of kieselguhr). After drying at room temperature in a stream of air for 15 min., the barbiturates were developed as white spots against a grayish brown background using the standard method of spraying with silver reagent (10).

DISCUSSION

The 2 systems, *viz.*, paper impregnated with formamide and formamide included in both the paper and the solvent afforded satisfactory means of separation of the multiple mixture of barbiturates. The only 2 instances in which the separation failed when applying these 2 systems were phenobarbital–barbital and cyclobarbital–allylisopropyl barbituric acid mixtures as shown in Table I.

Regarding the thin-layer chromatography, silica gel gave 5 distinct ranges of R_f values, *viz.*, phenobarbital (R_f 0.2), cyclobarbital–barbital–diallylbarbituric acid (R_f 0.32), allylisopropylbarbituric acid–butobarbital–amobarbital (R_f 0.54), secobarbital – methylphenobarbital – ethyl - *n* - hexylbarbituric acid–pentobarbital (R_f 0.64), and hexobarbital (R_f 0.77). When applying kieselguhr as adsorbent, the cyclobarbital–barbital–diallylbarbituric acid mixture as well as secobarbital–methylphenobarbital–ethyl-*n*-hexylbarbituric acid–pentobarbital mixture could be effectively separated as shown in Table II.

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