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Note

A simple combination of R_F value and melting-point determination for the identification of barbiturates

C. POLCARO

Laboratorio di Cromatografia del C.N.R., Via Romagnosi 18/A, Rome (Italy)

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Thin-layer chromatography is used extensively for drug screening either for drugs of abuse or to detect cases of poisoning.

Chromatographers are well aware that one single R_F value cannot be considered conclusive evidence for the presence or absence of a substance and the use of a wide range of confirmatory techniques, such as gas chromatography of the substance eluted from the spots, infrared or ultraviolet spectra, or even gas chromatography–mass spectrometry, has been proposed.

The following interesting method was recently suggested by Berezkin *et al.*[1]. A compound to be identified is partitioned between two immiscible solvents and both solutions are injected into a gas chromatograph. By using the retention time and the ratio of the two peaks (which indicates the partition coefficient between the two solvents), it is possible to identify it or distinguish it from others.

Thus the identification of a compound does not present problems if suitable instrumental techniques (and technicians) are available. It does, however, present a problem in a small hospital laboratory or similar unit that is not equipped with modern instruments.

A simple technique is reported here, which has been developed as a possible aid in the identification of barbiturates in urine and which seems to be of general application.

It is based on the determination of the melting point of the substance after separating it on a thin layer. This procedure was proposed earlier by Martinek[2], who eluted the substance from the adsorbent and recrystallized it under a microscope, thus employing microchemical techniques and skills that cannot be expected to be within the competence of a laboratory technician. The method described below is simpler in that it is based on a simple sublimation procedure[3,4]. The only remarkable feature of this technique is that it can be effected with the small amounts (several micrograms) that are available in spots on thin layers.

EXPERIMENTAL

Materials

The following pre-coated thin-layer plates were employed: silica gel 60 F_{254} (E. Merck, Darmstadt, G.F.R.), and Polygram Cell 300 DEAE, Polygram Cell 400

UV_{254} and Polygram Ionex-25 SB-Ac, which were obtained from Macherey, Nagel & Co. (Düren, G.F.R.). Desaga development jars, 26 × 11 × 20 cm, were used. The following reagents were prepared: 0.05% aqueous potassium permanganate; 0.2% diphenylcarbazone in 95% ethanol and 2% mercury(II) chloride in 95% ethanol, equal volumes of which were mixed before use.

All chromatograms were developed with the solvent chloroform–isopropanol–25% ammonia (45:45:10).

The various barbiturates were pharmaceutical products as obtained from the manufactures, but in some instances were extracted from tablets. Their origin and proprietary names are listed in Table I.

TABLE I

R_F VALUES FOR AND SYNONYMS OF A RANGE OF COMMONLY USED BARBITURATES (R_F VALUES ARE RELATIVE TO PENTOBARBITAL = 1)

Solvent: chloroform–isopropanol–25% ammonia (45:45:10).

Barbituric acid	*Commercial name*	*Proprietary name*	*Pre-coated silica gel G 60 F_{254}*	*Polygram Cell 300 DEAE*	*Polygram Cell 400 UV_{254}*	*Polygram Ionex-25 SB-Ac*
5-Phenyl-5-ethyl	Phenobarbital	Luminal	0.53	0.35	0.69	0
5,5-Diethyl	Barbital	Veronal	0.73	0.52	0.70	0.3
5,5-Diallyl	Diallylbarbital	Dial	0.76	0.62	0.77	0.3
5-(1-Cyclohexenyl)-5-ethyl	Cyclobarbital	Phanodorm	0.80	0.69	0.90	0.54
5-(1-Cycloheptenyl)-5-ethyl	Heptabarbital	Medomin	0.88	0.75	0.95	0.70
5-Allyl-5-isobutyl	Itobarbital	Sandoptal	0.91	0.80	0.93	0.70
5-Ethyl-5-(3-methylbutyl)	Amobarbital	Amytal	0.96	0.92	1	1
5-Allyl-5-(1-methylbutyl)	Secobarbital	Seconal	1	1	1	1
5-Ethyl-5-(2-pentyl)	Pentobarbital	Nembutal	1	1	1	1
5-Methyl-5-(1-cyclohexenyl)-N-methyl	Hexobarbital	Evipan	1.03	1.03	1	1.20

Chromatographic development

The chromatography chambers were lined with filter paper and 100 ml of the solvent mixture placed in a chamber, which was closed and allowed to equilibrate for 1 h. All chromatograms were developed at a room temperature of 22–25°.

With the Cell 300 DEAE and Cell 400 UV_{254} layers the samples to be chromatographed have to be placed behind the liquid front, that is, the thin layer is allowed to develop up to a distance of 3.5–4 and 1.5 cm, respectively, and only then are the samples applied to it. All four thin layers are then allowed to develop in the same jar simultaneously for 1 h and 15 min. The R_F values obtained are shown in Table I. We used the following five standard mixtures of barbiturates in solution in diethyl ether at a concentration of 1 mg/ml for each barbiturate: phenobarbital–itobarbital, barbital–cyclobarbital–amobarbital, diallybarbital–secobarbital, cyclobarbital–pentobarbital and heptabarbital–hexobarbital.

The volumes applied to the thin layers were as follows; for the silica gel layer, 10 μl; Cell 300 DEAE layer, 2.5 μl; and Cell 400 and Ionex-25 SB-Ac layers, 5 μl. The samples to be analysed were placed on the layer as follows: one (containing about the same amount of barbiturates as the standards) to each layer except on the silica gel which four spots were placed side by side, two of 10 μl and two of 40 μl (in the order 10, 40, 40 and 10 μl).

Preparation of spiked urine samples

Urine samples (50 ml) were spiked with 0.5 ml of an aqueous solution containing 0.5 mg of a barbiturate (if necessary 0.1 *N* sodium hydroxide was used to dissolve the barbiturates that are insoluble in water). The spiked urine samples were then extracted according to the method of Berry and Grove[5]. The urine was acidified to pH 1–2 with 1 *N* sulphuric acid and extracted with an equal volume of chloroform by shaking the mixture for several minutes in a Vortex shaker or for 10 min by hand. After centrifugation, the chloroform layer was filtered through a porous glass filter G 4 and evaporated to dryness under vacuum. The residue was then dissolved in 0.4 ml of diethyl ether.

Detection of spots after development

The thin layers, while still moist with the developing solvent, are viewed under an ultraviolet lamp at 254 nm and the spots marked with a pencil. As the eluent contains ammonia the spots are visible even on the DEAE-cellulose thin layers, although rather weakly, and on the Ionex layer (without a fluorescence indicator). The chromatograms are then allowed to dry in an oven at 60° until all the ammonia has volatilised.

The Cell 400, Ionex and Cell 300 DEAE layers are then sprayed with the $HgCl_2$–diphenylcarbazone reagent. The barbiturates appear as rather weak rose-coloured spots on a violet background, which become a bright rose colour on an almost white background on heating the layers in an oven at 80° for about 10 min or exposing them to sunlight. The chromatograms on the silica gel layer are divided into zone A containing a 10-μl spot, zone B the two 40-μl spots and zone C the other 10-μl spot. Zones A and C are sprayed with $HgCl_2$–diphenylcarbazone and permanganate reagents, respectively, as described by Lehmann and Karamustafaoglu[6]. While all barbiturates react with the former reagent, only those with double bonds give spots with the permanganate reagent (*i.e.* diallylbarbital, cyclobarbital, heptabarbital, itobarbital, secobarbital and hexobarbital).

Determination of melting points after chromatography

After development, there are two chromatograms for 40 μl of sample on the silica gel layer (in zone B, in which the spots visible under UV light were also outlined). Some of these spots also yielded positive reactions with the $HgCl_2$–diphenylcarbazone reagent on the adjacent 10-μl samples. These zones corresponding to positive reactions are scraped off from one of the chromatograms (the other being kept as a reserve) and the silica gel containing the spot is inserted into a micro-column, 2.5 mm I.D. and about 40 mm long, drawn out to a fine tip and with a small wad of cotton wool (previously washed with chloroform) at its lower end as described by Martinek[2]. The substance is eluted from the silica gel with chloroform (diethyl ether was also

TABLE II

MELTING POINTS DETERMINED FOR BARBITURATES

Barbiturate	*Melting point (°)*	
Phenobarbital	174	(by sublimation the low-melting form is obtained)
Barbital	176	(by sublimation the low-melting form is obtained. Transition to the higher-melting form (183°) is sometimes observed during heating)
Diallylbarbital	173	
Cyclobarbital	166–173	
Heptabarbital	174	
Itobarbital	138–139	(Merck Index)
Amobarbital	157	
Pentobarbital	129	
Hexobarbital	146	
Secobarbital	—*	

* The melting point of secobarbital (98–100°) was not obtained because no crystals suitable for a melting-point determination were formed. Secobarbital was therefore identified by applying the reagent of Wagenaar, as described by Davis[7], to the residue on the slide; oily droplets were formed, which subsequently became rosette-shaped crystal agglomerates.

tried but was less satisfactory) and the eluate evaporated to dryness on a microscope slide. The slide is then placed on a Kofler bank and a cover slip (supported on both sides by two other cover slips) placed over the residue so as to collect the sublimate that is formed, the cover slip being held a fraction of a millimetre above the residue. By slowly applying heat one can see whether a sublimate forms. If so, the cover slip is placed in a Kofler melting-point microscope and the melting point of the sublimate recorded.

RESULTS

We carried out melting-point determinations as described above with all the pure samples and with two spiked urine samples (Table II). For the pure samples we used the five mixtures of barbiturates described under Experimental and placed sufficient solution on the silica gel plates to give 20 μg of each barbiturate, which seems to be the smallest amount from which one can still obtain sufficient sublimate to enable its melting point to be determined.

The two spiked samples of urine contained: (a) a mixture of barbital, cyclobarbital and amobarbital and (b) secobarbital. The extraction of the acidified urine with chloroform was, of course, found to be only partial and consequently there is always much less material available than with the corresponding standard solution. It was for this reason that 40-μl samples were used instead of the 20 μl that suffice for the standards.

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