THE SEPARATION AND IDENTIFICATION OF SOME BARBITURATES BY PAPER PARTITION CHROMATOGRAPHY

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When barbiturates are extracted from biological materials, micro-methods are usually required for their identification. Many workers have contributed to the technique of micro-identification of the barbituric acid derivatives that are used in therapeutics. Among the more recently described methods should be mentioned optical crystallography[1] and the observation of X-ray diffraction powder patterns.[2] The purpose of the present paper is to report some results obtained by application of the technique of paper partition chromatography[3] to the problem of micro-identification of barbituric acid derivatives. In view of the success of this technique in other fields, there was *a priori* good reason to believe that results of interest would emerge. Moreover, the difficulties which may occur in crystallographic analysis or in melting-point determinations because of the polymorphism of some barbituric acid derivatives, should be eliminated by the use of partition chromatography. As far as the chemical literature is known to the authors, the paper chromatographic separation of the barbiturates commonly used in therapeutics has not as yet been made the subject of a general experimental study. A short outline of the paper partition chromatography of barbiturates and other pharmaceutical products has been given by Alvarez de la Vega[4] and Raventós[5] has described the separation of barbiturates from thiobarbiturates by chromatography on alumina column and successive elution.

The authors have developed chromatograms of 8 commonly used barbiturates and 2 thiobarbiturates with different organic solvents saturated with water. When chromatograms of barbiturates are run with a neutral solvent and with water as the stationary phase, no distinct spots can be obtained because of the ionisation of the acids. This difficulty, well known from previously reported paper chromatographic studies of other organic acids, may be overcome by adding a stronger acid[6,7,8] or ammonia[9,10,11] to the solvent system. To eliminate the ionisation effect in the chromatograms of barbituric acid derivatives, we have used formic acid or acetic acid, ammonia or pyridine. Most of the solvent systems, acid or alkaline, which have been examined in the present work, prove to give a quite unsatisfactory separation of the barbituric acid derivatives. Some of these unsuccessful solvent systems are shown in Table I, as the negative results should be considered useful for further investigation in this field. A good separation of the barbituric acid and thiobarbituric acid derivatives that have been subjects for this study, is obtained in toluene-acetic acid-water (100 : 40 : 50), and in chloroform-10 per cent. ammonia (100 : 50); the separation is fairly good for some of the substances in *n*-butanol—10 per cent. ammonia (100 : 35). The R_F values observed

TABLE I

Solvent system, alkaline mobile phase	Toluene-water-pyridine 100 : 30 : 50	Toluene, 10 per cent. ammonia 100 : 50	Chloroform (B.P.)-water-pyridine 100 : 20 : 40
R_F values at 18° to 20° C. for substances 1, 2, 3, 4, 9, 10	All about 0·95	All about 0, except No. 4	All about 0·92
Solvent system, acidified mobile phase	*n*-Butanol-water-acetic acid (95 per cent.). 40 : 50 : 10	Chloroform (B.P.)-methanol-water-formic acid (90 per cent.). 100 : 80 : 60 : 20	Chloroform (B.P.)-water-formic acid (90 per cent.). 100 : 50 : 20
R_F values at 18° to 20° C. for substances 1, 2, 4, 5, 6, 8	All about 0·90	All between 0·85 and 0·96	All between 0·75 and 0·96

TABLE II

Substance number	Barbituric acid derivatives. Substituents			R_F values at 19° ± 1° C.		
	5	5	1	Toluene-water-acetic acid 100 : 50 : 40	*n*-Butanol-10 per cent. ammonia 100 : 35	Chloroform-10 per cent. ammonia 100 : 50
1	Allyl	Allyl	—	0·52	0·64	0·07
2	Allyl	*iso*propyl	—	0·61	0·69	0·18
3	Ethyl	*Cyclo*hexenyl	—	0·69	0·69	0·22
4	Methyl	*Cyclo*hexenyl	Methyl	0·90	0·75	0·90
5	Ethyl	Ethyl	—	0·26	0·50	0·03
6	Ethyl	Phenyl	—	0·50	0·61	0·06
7	Ethyl	1-Methylbutyl	—	0·74	0·79	0·64
8	Ethyl	Phenyl	Methyl	0·90	0·75	0·82
	Thiobarbituric acid derivatives. Substituents					
9	Allyl	*iso*propyl	—	0·86	0·77	0·36
10	Ethyl	*Cyclo*hexenyl	—	0·89	0·80	0·50

on chromatograms developed with these three solvent systems are shown in Table II. The R_F values reported in Table II are mean values from series of chromatograms where 25 μg. of the barbiturates have been employed and the temperature has been maintained at 18° to 20° C. during the experiments. For the detection of the spots 4 different spraying reagents have been used. Thiobarbituric acid derivatives will appear as black spots on spraying with ammoniacal silver nitrate and heating, and as green spots on spraying with a solution of diethylamine and copper sulphate in methanol. Barbituric acid derivatives with unsaturated substituents will reduce a dilute solution of potassium permanganate sprayed on the chromatogram. The positions of the saturated barbituric acid derivatives have been revealed by means of a precipitating reagent, mercuric sulphate solution.

In some cases two barbiturates, whose R_F values under identical conditions do not differ sufficiently to make it possible to confirm their separation, may be differentiated by spraying with the various reagents described in this paper.

For the unambiguous identification of the barbiturates listed in Table II replicate chromatograms should be run with at least 2 of the different

solvent systems and further replicate chromatograms should be sprayed with the various reagents. Since absolute R_F values are not given in this paper, measurements of R_F values should not be considered as sufficient for the definite identification of a barbituric acid derivative, but reference substances should be included in the chromatograms of the unknown substance.

EXPERIMENTAL

Apparatus and Technique. The apparatus for descending chromatograms and the technique utilised in the present work, have been described in detail by Partridge[12] and later by other authors.[8] The same technique has been employed in chromatographic work previously published by this laboratory.[13,14]

Each barbituric acid derivative is dissolved in chloroform to obtain a 0·5 per cent. solution. Melting-point determinations under the microscope are used to control the purity and identity of the samples employed. 5 μl. or 10 μl. of the 0·5 per cent. solutions, corresponding to 25 or 50 μg. of the barbiturates were applied on the "starting line," drawn on Whatman filter paper No. 1, cut in rectangular sheets, 20 × 56 cm. Two of the sheets thus prepared are then suspended from the steel trough (without solvent), which is supported in the glass chamber. At the bottom of the chamber is placed a liberal volume of each phase of the solvent system, that is to be used. The paper and the atmosphere within the carefully closed, airtight chamber are now brought into vapour equilibrium with the solvent system, by allowing it to stand for 15 to 20 hours (over-night) at 18° to 20° C. After this equilibration period the non-aqueous phase of the solvent system is added to the trough and this mobile phase is allowed to flow down the paper till about 45 cm. from the "starting line." This movement will require at 18° to 20° C. about 3 hours for toluene, 18 hours for *n*-butanol, and 4 hours for chloroform. The solvent systems are prepared by shaking for 1 minute in a separating funnel the following mixtures, which are then allowed to separate by standing for 15 to 20 hours at 18° to 20° C.: (1) Toluene (b.pt. 109° to 110° C.)—water—acetic acid (95 per cent.) (100 : 50 : 40 by weight). (2) *n*-Butanol (b.pt. 116° to 177° C.)—ammonia (10 per cent.) (100 : 35 by weight). (3) Chloroform—ammonia (10 per cent.) (100 : 50 by weight).

Commercial chloroform, stabilised with ethanol, may be used, if reference substances are included in the chromatograms. The observed R_F values will then depend upon the ethanol content of the sample of commercial chloroform used in the experiment. A greater reproducibility of the experiments is obtained when ethanol-free, anhydrous chloroform is used. The R_F values reported in Table II are obtained by using alcohol-free, anhydrous chloroform, which should be freshly prepared, since alteration products of this unstabilised chloroform may undergo reactions with ammonia. It should be mentioned that the chloroform-ammonia system seems to be very sensitive to temperature variations during the equilibration period for the chamber atmosphere, and a shorter equilibration period (4 to 6 hours) may be used before running the chromatograms,

if temperature variations may thus be prevented. The chromatograms are dried in the air at room temperature and the spots are then revealed by the following spraying reagents.

Spraying reagents. (1) Thiobarbiturates will appear as black spots with ammoniacal silver nitrate. The paper is sprayed with a mixture (10 ml.) of equal volumes of 0·1N silver nitrate and 10 per cent. ammonia, and dried for 15 minutes at 95° C. Barbiturates may now appear as white spots (silver salts) on a yellow background; thio-barbiturates will usually not appear. The paper is then sprayed with more of the same reagent. Thiobarbiturates will now appear as black spots, immediately or after 5 minutes at 95° C.

(2) The paper is sprayed with a mixture of equal volumes of diethylamine 10 per cent. in methanol (99 per cent.) and a saturated solution of anhydrous copper sulphate in 90 per cent. methanol. Green spots indicate the position of the thiobarbiturates. The colour is best observed in daylight and will be visible for at least 20 hours. This colour-reaction has been described as fairly specific for thiobarbiturates by Raventós.[5]

(3) The paper is sprayed with a 0·02N solution of potassium permanganate. Yellow spots on a red background will appear immediately for barbituric acid derivatives with substituents containing a double carbon to carbon bond in open chain. If the reducing effect is produced by the hexenyl group, the spots will appear 1 to 2 minutes after spraying and will be well developed after 3 minutes.

(4) The saturated barbituric acid derivatives will appear as white spots (precipitation) when the paper is irrigated with a solution of mercuric sulphate. 5 g. of mercuric oxide is dissolved in 100 ml. of water and 20 ml. of concentrated sulphuric acid (the mercuric sulphate solution of the Danish Pharmacopeia IX). This solution is diluted with an equal volume of water before use. The reagent should not be sprayed on the chromatogram, since inhalation of the finely dispersed drops of this reagent will be dangerous. The spots are therefore revealed by the following method. A sheet of filter paper (Whatman No. 1) is dipped into the reagent and then placed on a clean glass plate. Another sheet of filter paper is pressed gently against it to absorb any excess of the reagent. The chromatogram is then placed between the two irrigated paper sheets on the glass plate and pressed gently with the hand until the reagent has penetrated it completely. The saturated barbituric acid derivatives (substances No. 5, 6, 7, 8) will appear as white spots that are well defined and easy to observe. The unsaturated barbituric acid derivatives, except substance No. 3, will not appear, probably because of the greater solubility of their mercuric complex in excess of the reagent.

Other spraying reagents which have been tried, failed to give satisfactory results. Detection of spots by means of an indicator proved to be difficult because of the weak acid character of the barbituric acid derivatives. The colour reaction of barbiturates with cobalt acetate and ammonia or an amine was examined under varying conditions. A spraying reagent based upon this reaction, that will give distinct coloured spots with less than 100 μg. of barbiturate in the chromatograms, has not as yet been found.

SUMMARY

1. Separation of 8 barbituric acid derivatives and 2 thiobarbituric acid derivatives by paper partition chromatography with 3 different solvent systems is described and the observed R_F values are reported.

2. The preparation of various spraying reagents, which may be used to differentiate substances showing approximately the same R_F value under identical conditions is described.

3. The results are considered useful for micro-identification purposes, when barbiturates are isolated from biological materials.

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