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SCIENCE PAPERS AND DISCUSSIONS

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OBSERVATIONS ON THE PAPER PARTITION CHROMATOGRAM AS APPLIED TO THE DETECTION OF ALKALOIDS

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INTRODUCTION

A NUMBER of workers have investigated the application of partition chromatography on paper supports to the separation and identification of alkaloids. Foster *et al.*¹ described the separation of ergot alkaloids in butanol-water-acetic acid, using fluorescence under ultra-violet light as a means of detecting position and as a basis for quantitative estimation. Brindle *et al.*² investigated the resolution of solanaceous alkaloids and insoluble alkaloids of ergot in a variety of solvents running on buffered papers and suggested a quantitative approach. Munier and Macheboeuf³ studied the choice of acidified solvents in relation to the dissociation constants of the alkaloids. Schute⁴ reported the separation of some solanaceous alkaloids in the form of their bases, using an ammoniacal solvent and studied factors which influence the R_f value.

The present contribution makes some observations on practical features of the paper partition method as applied qualitatively to a number of common medicinal alkaloids. Experience with a number of solvents is reported and three butanol-water systems acidified with formic, acetic or propionic acids are suggested for general use. The practical procedure is typified by a detailed description, of a chromatogram of a series of alkaloids and their salts run in the butanol-acetic solvent and using an aqueous iodine spray for revelation of position. The technique of applying the spray is described, some diagnostic implications are discussed and particular reference is made to the limitations of the R_f value as a criterion of identity in relation to alkaloids.

EXPERIMENTAL

Choice of solvents.—Figure 1 shows a chromatogram of a variety of alkaloids and their salts run simultaneously in adjacent positions on a $22\frac{1}{2} \times 18$ inch Whatman No. 1 paper. The solvent in this instance was that advocated by Partridge⁵ for the separation of sugars, namely, the saturated upper layer obtained by shaking 40 volumes of *n*-butanol,

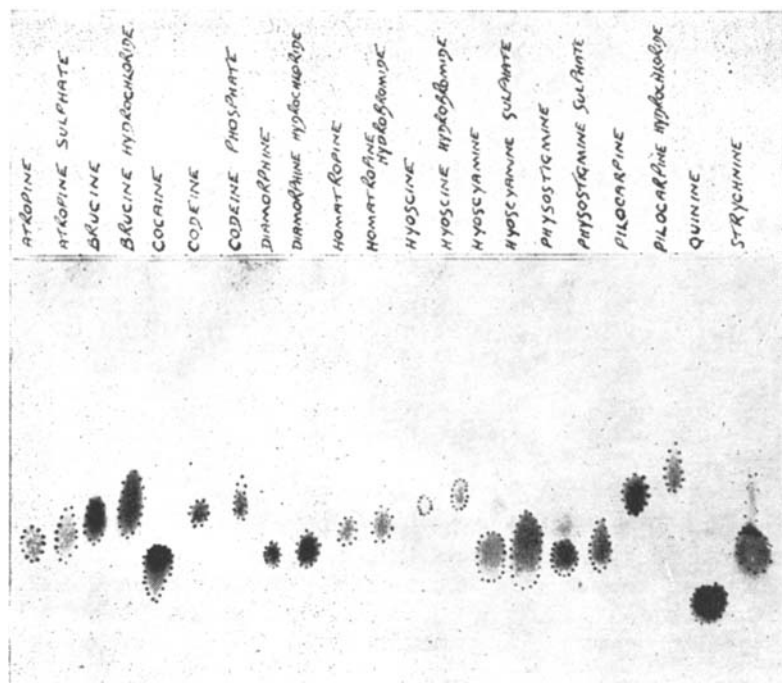


FIG. 1. Paper partition chromatogram of alkaloids.

50 volumes of water and 10 volumes of glacial acetic acid. The water-miscible layer was external to the trough in the usual way, being placed in beakers in the bottom of the tank, the beakers containing a filter paper cylinder to accelerate the attainment of vapour equilibrium. In each case approximately 0.002 ml. of solution (aqueous or organic) containing approximately 100 μ g. of alkaloid or salt (i.e., a 5 per cent. w/v solution) was applied to the starting line on the paper. The solvent was run downwards for 16 hours and removed from the paper in an air draught at 80° to 85° C. The positions of the alkaloids or their salts were revealed by spraying the dry paper with aqueous iodine solution which is referred to in greater detail later. Similarly, the same series of alkaloids and their salts run well in the saturated upper layer obtained by shaking 10 volumes of *n*-butanol, 10 volumes of water and 1 volume of formic acid and again, in the solvent obtained by substituting propionic acid for formic acid in the above formula. The use of hydrochloric, citric and tartaric acids in a *n*-butanol-water system was investigated, but on the whole the resolution was less satisfactory than that obtained with the acetic, propionic or formic solvents and attention was concentrated on the use of these latter 3 systems.

Table I records the average R_f values of the selected alkaloids and their salts in the 3 solvents, obtained from a large number of chromatograms prepared in the manner described in connection with Figure 1. Any

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apparent inconsistency between the relative positions of alkaloids in Figure 1 and the R_f values in Table I is due to the solvent front at the end of the run of the particular chromatogram illustrated not being parallel with the starting line, a frequent circumstance in downward runs with such broad fronts. The R_f values for codeine phosphate in the butanol-formic and butanol-propionic solvents do not appear in Table I. Certain apparent anomalies occurred here which are referred to in the discussion.

TABLE I
Average R_f VALUES

Alkaloid or salt	<i>n</i> -Butanol	40	<i>n</i> -Butanol	10	<i>n</i> -Butanol	10
	Acetic acid	10	Formic acid	1	Propionic acid	1
	Water	50	Water	10	Water	10
Atropine	0.73		0.58		0.68	
Atropine sulphate	0.72		0.56		0.64	
Brucine	0.67		0.44		0.58	
Brucine hydrochloride	0.64		0.39		0.53	
Cocaine	0.76		0.72		0.79	
Cocaine hydrochloride	—		0.66		0.69	
Codeine	0.65		0.42		0.51	
Codeine phosphate	0.61		—		—	
Diamorphine	0.75		0.57		0.65	
Diamorphine hydrochloride	0.74		0.55		—	
Homatropine	0.68		0.52		0.57	
Homatropine hydrobromide	0.66		0.51		0.54	
Hyosine	0.63		0.42		0.51	
Hyosine hydrobromide	0.62		0.41		0.49	
Hyoscyamine	0.75		0.57		0.66	
Hyoscyamine sulphate	0.73		0.55		0.60	
Physostigmine	0.76		0.63		0.68	
Physostigmine sulphate	0.74		0.61		0.68	
Pilocarpine	0.58		0.36		0.47	
Pilocarpine hydrochloride	0.54		0.33		0.40	
Quinine	0.84		0.68		0.81	
Quinine hydrochloride	0.83		0.69		0.87	
Strychnine	0.74		0.58		0.70	
Strychnine hydrochloride	0.73		0.56		0.69	

Figure 2 shows a chromatogram obtained in precisely the same manner as that illustrated in Figure 1, when extract of jaborandi and tincture of nux vomica were applied to the paper with no preliminary treatment other than a simple evaporation to one-fifth and one-tenth of the original volumes respectively. The spots due to pilocarpine, strychnine and brucine were clearly defined. The use of *n*-butanol or *isobutanol* saturated with water was investigated without success. Many of the alkaloids tended to move in a diffuse manner and, as would be expected on theoretical grounds, the position of the spots was very capricious, particularly in the presence of strong negative radicals; so much so that chromatograms obtained with these solvents appeared to be quite useless for practical purposes. The use of papers previously soaked in buffer solutions had the effect of stabilising the rates of travel, but in general the spots tended to be elongated to an extent which made the measurement of R_f values very indefinite. Water-saturated phenol was considered to have possibilities in relation to the alkaloids, but the results with this solvent with or without the addition of acetic acid or ethanol were disappointing. All the alkaloids and salts in the present series ran in a well-defined manner in these phenolic solvents, but with very high mobilities which brought

them all near the solvent front. Solvents based on phenol appeared to hold very little promise for the purpose of separating alkaloids.

Choice of method.—The descending flow method is well adapted to accommodate the running of large numbers of chromatograms at one time; often a very desirable feature. Also, there is no limitation on the distance the solvent front may be allowed to travel and this is sometimes an advantage for increasing the resolution of substances of low mobility. The tank and troughs used in obtaining the chromatograms typified by Figure 1 were very similar in design to those described by Woiwod.⁶ Where these two advantages do not apply, experience in the present investigation confirms the opinion of Brindle *et al.*² that the capillary ascent technique originally advocated by Williams and Kirby⁷ gives very compact spots with the alkaloids. From experience in this and other investigations the ascending flow method is to be preferred. The solvent front is usually very uniform and generally the reproducibility of the R_f values is superior to that with downward flow methods, at least when these are on the tank scale. The slower attainment of vapour equilibrium in the latter case may be a partial factor.



FIG 2.

In addition to the advantages mentioned, the capillary ascent technique has the virtues of being extremely easy to operate and makes no demands for specially designed equipment. The arrangement for upward runs made in the present work was very simple. Samples were applied to a sheet of chromatographic paper 14×9 inches on a line parallel with and $1\frac{1}{2}$ inches from one of the short ends. The paper was then bent into the form of a cylinder with the edges just overlapping and secured with paper clips. The cylinder, samples downward, was placed in a petri dish concentric with the rim of the dish and a beaker standing in its centre, the whole standing upon a ground glass plate. The water-immiscible solvent layer was poured into the dish and the water-miscible layer into the beaker which also carried a paper cylinder to accelerate the attainment of vapour equilibrium. A glass cylinder with ground ends was quickly placed in position and closed at the top with a second ground glass plate. A run is most conveniently commenced at the end of the day and allowed to continue overnight, which applies also to the descending flow method.

Choice of paper.—Whatman No. 1 paper gave very satisfactory results. The coarser No. 4 grade supplied also for chromatographic purposes allows faster runs and is sometimes convenient for this reason, but

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there is a corresponding loss of precision and definition and the No. 1 grade paper is to be recommended for general purposes.

The iodine spray.—Brindle *et al.*² and Schute⁴ reported some difficulty in obtaining a satisfactory response to the use of iodine in revealing the positions of alkaloids on the paper. In the present investigation it was found that the success of this reagent is a function of the concentration of iodine used in the solution, which is fairly critical, and, in particular, to the character and manner of application of the spray. A concentration of 0.2 per cent. w/v of iodine obtained by dilution of the B.P. Appendix reagent was found to be just sufficiently strong to give a good reaction with the alkaloids without producing more than a faint transient staining of the rest of the paper, so obtaining a maximum contrast. Early experience showed that in order to obtain a satisfactory result it was absolutely essential to apply the solution fairly forcefully in a fine mist free from coarse droplets, and that to achieve this a well-designed atomising device, operated by a sustained force of compressed air, is required. The hand-operated intermittent puffer type of atomiser was found to be almost useless. Suspending the paper sheet in front of a frosted glass screen illuminated from behind is a considerable aid in observing the course of wetting, thus facilitating the uniform and systematic application of the reagent solution which is essential for success. The photograph from which Figure 1 was reproduced was taken 3 minutes after spraying, during which interval the borders of the spots were permanently outlined by quickly and lightly dotting with ink.

Brindle and co-workers² reported that the colour of the iodine stain was brown with hyoscyne and tinged with blue in the case of atropine. This is confirmed in the present investigation with the added information that the reaction with hyoscyamine is similar to that of atropine. The colours of the stains from the alkaloids in the present series were otherwise generally brown. The relative rates at which the stains from the various alkaloids faded were very reproducible. It is not suggested that much could be made of this from the diagnostic angle, but it is interesting to note the more extreme cases. The stains from the solanaceous alkaloids fade rapidly, particularly the hyoscyne stain, whilst the quinine, brucine and strychnine stains are very persistent and in the case of the latter pair may be observed many months later on papers kept in the dark. Another very reproducible and curious feature concerns the stain from homatropine and salts. As the spots fade a characteristic speckled appearance develops. This and other features mentioned above are probably of some diagnostic value.

DISCUSSION

The methods and techniques described can be used to indicate the presence of the alkaloids mentioned and probably many others, in solutions applied to the paper, provided due regard is paid to their concentration in the sample solution which may be a biological fluid or extract or one which is the result of a preliminary separation and may be aqueous or organic. As a working guide something of the order of 100 $\mu\text{g.}$ of an

alkaloid will need to be present to ensure its detection by the iodine spray. This would apply to the solanaceous group in particular. Some others will be detected in much smaller proportion, e.g., pilocarpine, strychnine and brucine as shown in Figure 2 when as little as 25 μ g. were present. It may be that two or more alkaloids are present at very different concentrations and if this possibility has to be taken into account it is desirable to run a number of chromatograms, in adjacent positions on the sheet, of dilutions and concentrations of the sample solution. As an alternative to concentration, two or more applications can be superimposed on the same sample spot.

Following upon an indication of the presence of one or more alkaloids by the methods described a measurement of the R_f value should in theory provide positive evidence of identity. In practice, however, there are a number of factors which influence the mobility of alkaloids in the solvent. Some of these factors apply generally to the paper partition method and were discussed in the original publication by Consen *et al.*⁸ It is very doubtful if the reproducibility of an R_f value can be kept within 2.5 per cent. either side of the average of a number of determinations. Superimposed upon this are influences which apply to basic substances such as the alkaloids. The most important point here is the effect of the negative ions present.

It was seen in the present investigation that the alkaloids were much more mobile in saturated butanol than the corresponding salts. This factor was almost but not quite eliminated when an acid was introduced into the solvent as will be seen by reference to Figure 1 and to the average R_f values reported in Table I. The R_f value of the salts are, however, almost without exception consistently less than those of the bases. In natural extracts and the like, the radicals with which an alkaloid may be associated are not known with certainty and its apparent R_f value may therefore be slightly different from that obtained with the pure base or a known salt. For these reasons the determination of an R_f value when the sample solution is a natural extract or where the nature of the negative radical is otherwise unknown, can only be regarded as supplying rough presumptive evidence of the identity of the alkaloid or alkaloids in question. Also, for the same reasons, no more definite information will be obtained by running a chromatogram of the pure alkaloid or a salt alongside that of the test sample as a means of confirmation, although it can be said that the salt will generally provide the more reliable indication. A better procedure is to load the sample solution with the suspected alkaloid. Much more reliable information will of course be obtained by some preliminary separation of any alkaloids present using the usual procedure, when they can be applied either in their basic form in an organic solvent, or in the form of a known salt in aqueous solution. The corresponding reference chromatogram can then be selected accordingly.

A further point arose in the present work in connection with codeine phosphate. In the butanol-acetic solvent one spot was obtained in the normal manner the R_f value of which is indicated in Table I. In the butanol-formic solvent 3 spots (average R_f values 0.25, 0.33, 0.41) and

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in the butanol-propionic reagent 2 spots (average R_f values 0.19 and 0.37) were found. This obviously warrants further investigation, but meanwhile draws attention to the false indications which may arise from the presence of phosphate in a sample solution with the two solvents in question.

The foregoing suggests that the R_f value cannot be accepted as a reliable criterion of identity of alkaloids and that some confirmatory evidence should always be sought. As already mentioned, the iodine stains are to some limited extent diagnostic and the use of more specific spray reagents merits investigation. For example, morphine on a chromatogram gives the expected colour reactions when sprayed with 0.1 N sulphuric acid, followed by saturated potassium iodate and ammonia solutions.

Whilst acknowledging the limitations of the paper partition chromatogram it is nevertheless a most valuable expedient to the analyst confronted with the detection of alkaloids in extracts, pharmaceutical preparations and the like. Especially is this so in the exploratory stages of an analysis when information is required as to the presence of alkaloids, their probable number and some indication of identity. The economy of time and labour, the trifling expenditure of sample material and the immense amount of information which can often be obtained overnight on one sheet of paper are the special attributes of this elegant and simple method.

The authors' thanks are due to the Directors of Beecham Research Laboratories, Ltd., for permission to contribute this paper, to Miss E. C. Russell for much of the earlier practical work and to Mr. D. F. Lawson for the photographs from which the two figures were taken and numerous others in connection with this investigation.

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DISCUSSION

The paper was presented by MR. D. N. GORE.

MR. J. E. CARLESS (Manchester) said that the paper would have been more valuable if solvents could have been found to give greater differences between the R_f values of the alkaloids studied. In Table I, corresponding to R_f values between 0.73 and 0.76 of the first solvent, there were at least 5 alkaloids. Identification of the individual alkaloids in that group would be impossible, due to overlapping. The difficulty might be overcome by the use of specific spot tests and smaller spots. The use of buffered filter paper would offer an advantage in those cases where R_f values were close together. Tailing of spots when using buffered filter

paper gave trouble. Tailing of zones on cellulose columns had been overcome by arranging a pH gradient down the column. For the identification of alkaloids by paper partition chromatography, the plotting of R_f values against the pH might give characteristic curves of diagnostic importance.

DR. W. MITCHELL (London), commenting on the method of applying the iodine, referred to a device known as the "Aerograph Artists' Spray Brush" which he had found to be of value in obtaining a uniform spray.

MR. D. N. GORE, in reply, said that whilst agreeing with Mr. Carless's criticism concerning the solvents, the paper was not intended to convey the impression that the three solvents used would necessarily cover everything. The efficiency of the atomiser was of particular importance where the spots were of a transient nature.