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FACTORS AFFECTING THE ION-PAIR REVERSED-PHASE THIN-LAYER CHROMATOGRAPHY OF ORGANIC ACIDS ON PARAFFIN-COATED AND C_{18} BONDED SILICA GEL

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SUMMARY

Factors affecting the reversed-phase thin-layer chromatography of a range of organic acids have been investigated for C_{18} bonded and paraffin-coated silica gel using six quaternary ammonium ion-pair reagents. For strongly acidic compounds such as 2,5-dihydroxybenzoic acid, 2,6-dihydroxybenzoic acid and gentisic acid satisfactory reversed-phase thin-layer chromatography was only achieved in the presence of ion-pair reagents. For weaker acids, such as isoxepac, the presence of ion-pair reagents is not essential for good chromatography and the effects of ion-pair reagents in reducing the R_F are less marked. With most of the ion-pair reagents investigated the precoating of the plate with ion-pair reagent was found to be essential to ensure that ion-pair formation occured. The effects of ion-pair concentration, mobile phase composition and pH were investigated, and the application of the technique to the chromatography of drug metabolites is discussed.

INTRODUCTION

Reversed-phase thin-layer chromatography (RP-TLC) is a useful alternative to normal-phase (NP) TLC on silica gel for the separation of polar compounds (review see ref. 1). Several types of chemically bonded RP-TLC plates are available commercially, and RP-TLC may also be performed on ordinary silica gel TLC plates pre-coated with paraffin or octanol[2]. We have successfully used both bonded and coated plates for the RP-TLC of a range of compounds of widely differing structure[3–6]. However, a limitation of RP-TLC in our laboratory has been the poor chromatography obtained with very polar ionic compounds[6]. Examples of such compounds include the acidic metabolites of aspirin, a number of mono- and dihydroxybenzoic acids, and the major hydroxy-metabolites of the drug antipyrene. Usually on

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RP-HPLC these compounds chromatographed at the solvent front, even when solvents of low eluotropic strength were employed. The addition of acids to suppress ionisation in order to achieve lower R_F values for these compounds was without effect[6].

In high-performance liquid chromatography (HPLC) the retention of polar ionic compounds is often achieved with ion-pair reagents and some applications of the use of such compounds in RP-TLC have been described[7–11]. This paper describes the results of investigations on the use of ion-pair reagents for the RP-TLC of a variety of polar organic acids. The compounds investigated include a range of hydroxybenzoic acids, together with metabolites of aspirin and antipyrene. Altogether we have examined the properties of six commonly used ion-pair reagents on both C_{18} bonded and paraffin coated RP-TLC plates.

EXPERIMENTAL

Preparation of TLC plates

Paraffin coated plates were prepared by dipping 20 × 20 cm plastic backed silica gel TLC plates (Macherey-Nagel, Camlab, Cambridge, U.K.) in a solution of heavy refined paraffin oil (Nujol) in dichloromethane (7½%, v/v). Ion-pair reagents (Fisons, Loughborough, U.K.) were of HPLC grade and were added to the coating solution at concentrations of between 0.001 and 0.1 *M*. The C_{18} bonded RP-TLC plates (E. Merck and Co., BDH, Poole, U.K.) were coated with ion-pair reagents by dipping in solutions of the appropriate ion-pair reagent from 0.001 to 0.1 *M* in dichloromethane. In order to completely dissolve some of the ion-pair reagents the addition of ethanol (up to 15%, v/v) to the coating solution was required.

The ion-pair reagents used in these studies were as follows: tetramethylammonium chloride (TMA), tetrapropylammonium bromide (TPA), tetra-*n*-butylammonium bromide (TBA), cetrimide (tetradecyltrimethylammonium bromide), cetyltrimethylammonium bromide (CTA) and dodecyltrimethylammonium bromide (DTA).

Chromatography

Chromatography was performed in glass TLC tanks 20 × 20 × 5 cm using solvent systems composed of mixtures of water and methanol.

Test compounds were applied to the plates as solutions in methanol using 1-µl glass capillaries. Following development the test compounds were visualised under UV light at 254 nm. The following test compounds were used in these studies: isoxepac (6,11-dihydro-11-oxodibenz[*b,e*]oxepin-2-yl acetic acid), methylisoxepac [2-(6,11-dihydro-11-oxodibenz[*b,e*]oxepin-2-yl) proprionic acid], acetylsalicylic acid, *p*-aminosalicylic acid, 5-methoxysalicylic acid, salicylic acid, 2,5-dihydroxybenzoic acid, 2,6-dihydroxybenzoic acid, gentisic acid, antipyrene, 4-hydroxyantipyrene, methyl[3]-hydroxyantipyrene and ecdysone.

RESULTS AND DISCUSSIONS

The use of ion-pair (IP) RP-TLC for both C_{18} bonded and paraffin-coated TLC plates was investigated. A number of factors, including ion-pair concentration, pH, and the methanol content of the solvent were examined to determine their im-

portance for IP-RP-TLC. In addition the effects of coating the TLC plates with ion-pair reagent were compared with simply including the ion-pair in the mobile phase. The results of these experiments are detailed below.

Use of ion-pair reagents

The use of all six ion-pair reagents in the solvent was examined for both paraffin coated and C_{18} bonded RP-TLC plates. With paraffin-coated plates the R_F values of 2,5-dihydroxybenzoic acid, 2,6-dihydroxybenzoic acid, gentisic acid, and *p*-aminosalicylic acid were unaffected by the inclusion of any of the ion-pair reagents in the mobile phase (0.05 *M*). A similar result was observed for the C_{18} bonded plates when used with ion-pair reagents containing a long alkyl chain such as cetrimide. However, with short chain ion-pair reagents, such as TMA and TBA, a significant decrease in the R_F of the test compounds compared to the controls was obtained, indicating that ion-pair formation had occurred. For example the R_F values of 2,5- and 2,6-dihydroxybenzoic acids were reduced from 0.81 and 0.75 to 0.58 and 0.36, respectively, following the inclusion of TMA in the mobile phase. In some instances two spots were observed, one at the solvent front corresponding to non-ion-paired test compound and a second well retained, suggesting that a limited amount of ion-pair formation had occurred.

The lack of ion-pair formation observed when some ion-pair reagents were present in the mobile phase appears to be because the reagent was more retarded by the adsorbent than the solvent. The solvent therefore eluted the test compounds before the reagent reached them and ion-pair formation did not occur. When examined under UV light it was generally possible to distinguish two bands on the TLC plate when long chain ion-pair reagents had been used in the mobile phase. One of these corresponded to the solvent front, whilst the second which had a lower R_F, corresponded to a zone of ion-pair reagent. This was confirmed by rotation of the plate through 90°, and re-spotting gentisic acid along the whole length of the new origin. The plate was then re-chromatographed and it was observed that the R_F of gentisic acid was reduced from 0.9 to 0.6 up to the point where the ion-pair reagent had migrated in the first development, after which the R_F reverted to 0.9. This result is similar to that recently reported by Szepesi *et al.*[11] using cetrimide.

The degree to which ion-pair formation occurs is therefore dependant on the rate of migration of the ion-pair reagent relative to the solvent front, which in turn is related to the hydrophobic properties of the reagent. Thus, the relatively hydrophilic TMA migrates sufficiently close to the solvent front to allow ion-pair formation. However TBA and TPA migrate more slowly and ion-pair formation was incomplete resulting in two spots corresponding to ion-paired and non-ion-paired compounds. The longer chain reagents are so efficiently retarded in this system that ion-pairs are not formed.

The effect of precoating the TLC plates with ion-pair reagent was then investigated as a method of overcoming the lack of effect seen when using the longer chain ion-pair reagents in the solvent. In these investigations the effect of ion-pair concentration in the coating solution on the subsequent chromatography was also examined using TBA.

In the case of the paraffin-coated plates the ion-pair reagent was included in the paraffin coating solution, enabling simultaneous impregnation of the plate with

both paraffin and ion-pair reagent. The C_{18} bonded plates were dipped in a solution of ion-pair reagent in dichloromethane (containing up to 15% ethanol to dissolve the reagent).

The effect of TBA concentration on the R_F of the model compounds for the paraffin-coated plates is illustrated in Fig. 1A. At the lowest concentration examined (0.001 *M*) there was little effect on the R_F of the test compounds (*p*-aminosalicylic acid, 2,5-dihydroxybenzoic acid, and gentisic acid). With increasing ion-pair concentration a significant reduction in R_F was obtained, with the maximum effect being observed at concentrations of 0.01 *M* and above. A similar effect was seen on the C_{18}-bonded plates (Fig. 1B). Based on these results a concentration of 0.05 *M* ion-pair reagent in the coating solution was chosen for further studies in order to ensure that the maximum effect was obtained.

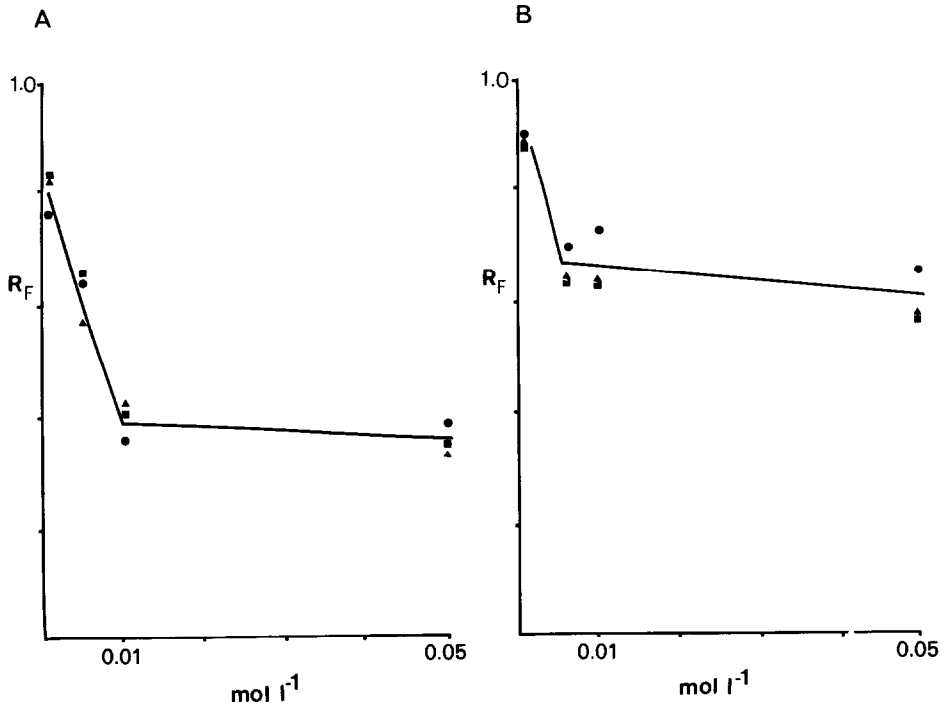

Fig. 1. Effect of TBA concentration used for coating C_{18} bonded (A) and paraffin-coated (B) RP-TLC plates on the R_F values of *p*-aminosalicylic acid (●); 2,5-dihydroxybenzoic acid (■) and gentisic acid (▲).

Effect of pH on IP-RP-TLC

The effect of solvent pH was also investigated as in RP-HPLC this factor is an important determinant of retention behaviour. Paraffin-coated plates impregnated with 0.05 *M* TBA were chromatographed in a solvent system consisting of methanol–phosphate buffer (0.5 *M*) (1:1), at pH values of 2, 4, 6, 8, and 10. The effect of these pH values on the R_F values of 2,5-dihydroxybenzoic acid, *p*-aminosalicylic acid, salicylic acid, and 2,6-dihydroxybenzoic acid was studied. No signifi-

cant change in the R_F of any of these compounds was discernible over the pH range examined. For example the R_F of salicylic acid was 0.44 at pH 2 and 0.43 at pH 10.

Effect of organic modifier composition on chromatography

The effect of the organic modifier content of the mobile phase was investigated using paraffin-coated plates impregnated with 0.05 *M* TBA. For the same solvent composition paraffin-coated plates gave higher R_F values than the C_{18} bonded TLC plates. The methanol content of the solvent was varied between 0 and 80% and the change in R_F values for a range of test compounds studied. These results were compared for the same compounds and solvent systems with paraffin coated-TLC plates which had not been impregnated with TBA.

For very polar compounds such as gentisic acid, *p*-aminosalicylic acid, and 2,5-dihydroxybenzoic acid a linear relationship between R_F and the methanol content of the mobile phase was observed when TBA was present, (Fig. 2). In the absence of ion-pair reagent changing the composition of the solvent had no effect on the chromatography of these compounds which ran at or near the solvent front in all the systems tested. With weaker acids such as isoxepac and its methyl analogue a linear relationship between R_F and the methanol content of the mobile phase was seen both in the presence of TBA and in its absence. However, when the ion-pair reagent was present on the TLC plate the R_F values of isoxepac and methylisoxepac were reduced from 0.49 and 0.39 to 0.2 and 0.16 respectively [using methanol–water (1:1) as solvent].

Effect of different ion-pair reagents on IP-RP-TLC

The chromatography of 2,5-dihydroxybenzoic acid, 2,6-dihydroxybenzoic acid, *p*-aminosalicylic acid, gentisic acid, 5-methoxysalicylic acid and salicylic acid was investigated for each of the six ion-pair reagents. Both paraffin coated and C_{18} bonded plates (impregnated with 0.05 *M* ion-pair reagent) were employed in these studies. Initially a mobile phase of methanol–water (1:1) was used, and good chromatography was obtained for the short chain ion-pair reagents such as TMA, TPA and TBA. Thus with 2,6-dihydroxybenzoic acid R_F values of 0.4 (TMA), 0.51 (TPA) and 0.37 (TBA) were achieved on paraffin-coated TLC plates, whilst R_F values of 0.25 (TMA), 0.2 (TPA), and 0.48 (TBA) were obtained on the C_{18}-bonded plates.

With the longer chain ion-pair reagents such as cetrimide, CTA, and DTA the methanol–water (1:1) solvent system was insufficiently eluotropic, and R_F values of less than 0.2 for all the test compounds were obtained. However, with increased amounts of methanol in the mobile phase it was also possible to obtain satisfactory chromatography with these reagents.

Applications of IP-RP-TLC

The use of IP-RP-TLC provides a convenient and simple method for retaining and separating very polar ionic compounds which would otherwise chromatograph at the solvent front in normal RP-TLC. In drug metabolism studies two types of application are immediately obvious. Firstly the chromatographic separation of very polar acidic metabolites such as glucuronide and sulphate conjugates, or acidic metabolites such as gentisic acid, in order to obtain metabolite profiles. As an example the major hydroxy-metabolites of antipyrene are easily chromatographed and

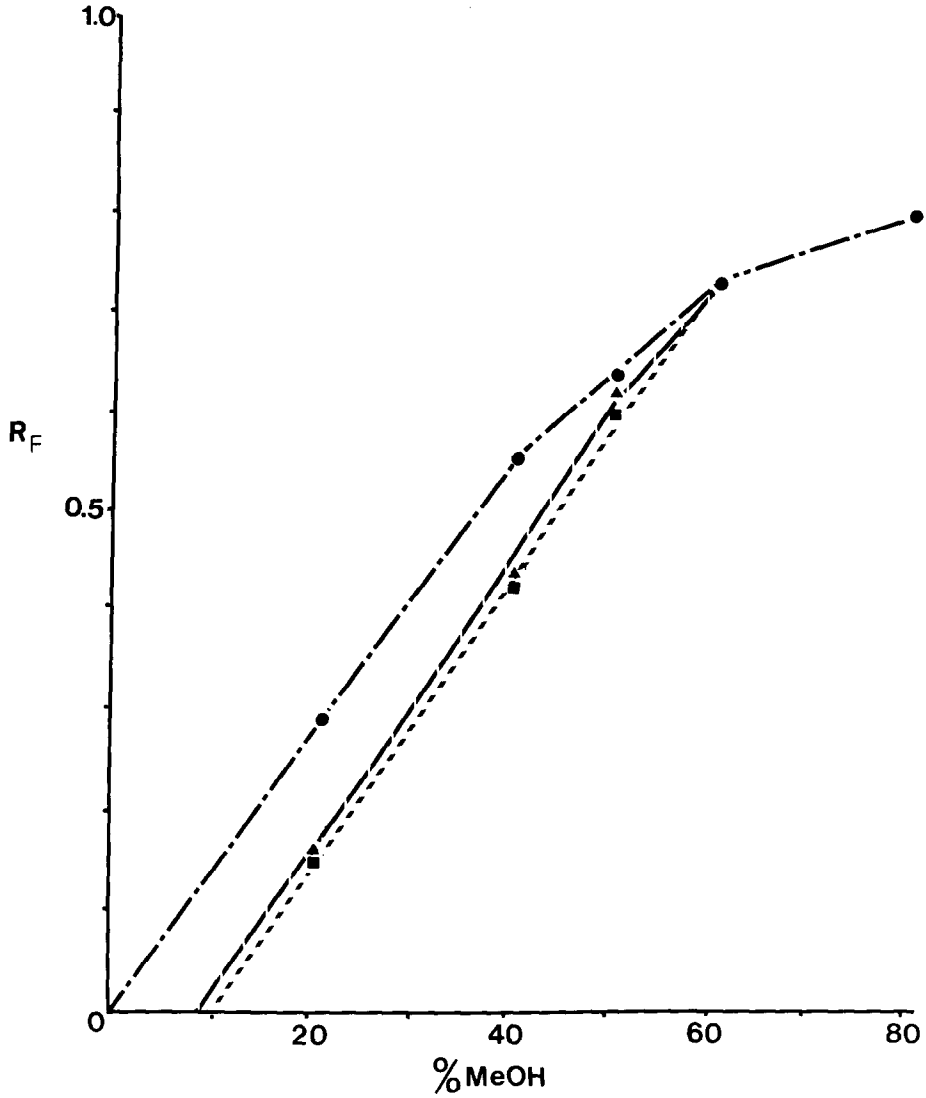

Fig. 2. Effect of mobile phase composition on the R_F values of gentisic acid (■); *p*-aminosalicylic acid, (●) and 2,5-dihydroxybenzoic acid (▲) on paraffin-coated plates concomitantly coated with 0.05 *M* TBA.

separated using IP-RP-TLC, but are extremely difficult to chromatograph using ordinary RP-TLC or NP-TLC. Secondly, by using a combination of IP-RP-TLC and RP-TLC the partial characterisation of metabolites into acid and non-acidic species is possible. A simple method of achieving this is to chromatograph the same sample in parallel tracks on the same RP-TLC plate with one track impregnated with ion-pair reagent and the other normal. Acidic metabolites will be selectively retained in the presence of the ion-pair reagent, whilst non-ionic compounds will be unaffected. An example of this is illustrated in Fig. 3, where a mixture of ecdysone, 2,5-dihy-

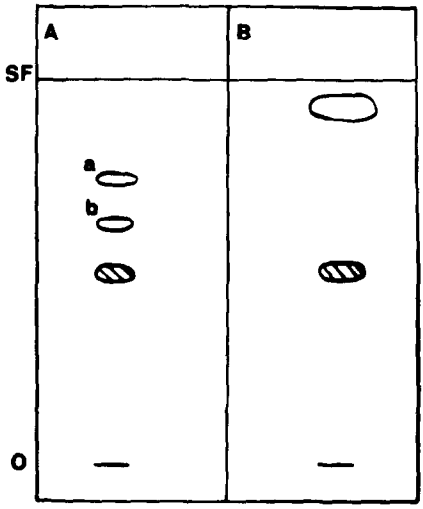

Fig. 3. Effect of the presence (track A) or absence (track B) of TMA on the RP-TLC of a mixture of 2,5-dihydroxybenzoic acid (a), 2,6-dihydroxybenzoic acid (b) and ecdysone (shaded) on paraffin-coated TLC plates.

droxybenzoic acid and 2,6-dihydroxybenzoic acid have been chromatographed on an RP-TLC plate partially coated with TMA. In the absence of TMA the acidic compounds chromatographed at the solvent front, whilst ecdysone was well retained (R_F 0.5). In the presence of TMA both acids were well retained with R_F values of 0.75 (2,5-dihydroxybenzoic acid) and 0.63 (2,6-dihydroxybenzoic acid) whilst the R_F of ecdysone was unaffected. This type of experiment, when combined with specific enzymic hydrolysis for conjugates should allow non-ionic, acidic and conjugated metabolites to be easily distinguished.

CONCLUSIONS

On the basis of these results the most important factors determining chromatographic properties in IP-RP-TLC are ion-pair concentration, ion-pair chain length, the organic modifier content of the solvent and the requirement to precoat the TLC plate with the ion-pair reagent before use. The pH of the solvent had no effect on the IP-RP-TLC of the test compounds. Certainly for paraffin-coated plates pre-chromatographic impregnation of the plate is essential in order to achieve ion-pair formation. This is also true for C_{18} bonded plates used with the longer chain, more hydrophobic ion-pair reagents (cetrimide, CTA, DTA), and similar results have been found by Szepesi *et al.*[11] using cetrimide. However with short chain ion-pair reagents such as TMA IP-RP-TLC may also be performed on the C_{18} bonded plates by including the reagent in the mobile phase. However, the R_F values obtained using 0.05 *M* TMA or TBA in the solvent were significantly higher than those obtained when plates were coated with a solution containing 0.05 *M* ion-pair reagent. Pre-chromatographic impregnation of the plates therefore leads to more efficient ion-pair formation, and in our laboratory we have adopted a 0.05 *M* ion-pair concentration for this process. This is similar to that used by other groups (0.05–0.1 *M*)[7,9].

In previous studies we have compared paraffin-coated and C_{18} bonded RP-TLC plates and found both types performed well. This is also the conclusion from the present study on IP-RP-TLC. However, given that with most of the ion-pair reagents studied pre-chromatographic coating of the plates with reagent is required then the paraffin coated plates have the advantage of economy. Where speed is important non-ion-pair impregnated C_{18} bonded plates with a short chain ion-pair reagent such as TMA present in the solvent may be used.

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