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Note

Indirect fluorimetric detection of non-electrolytes in thin-layer chromatography

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Thin-layer chromatography (TLC) is one of the oldest and perhaps the simplest chromatographic separation techniques still in widespread use. In recent years, emphasis has been on high-performance liquid chromatography (HPLC) to separate complicated samples because of its high separation efficiency and advanced instrumental detection system. Therefore, TLC is far behind HPLC, even though a lot of developments in sorbent materials, particle sizes and detection instruments have been achieved[1–5]. However, TLC has certain advantages because it is an open bed while HPLC is a closed bed systems. Since each plate is used only once, there is no possibility of poisoning a TLC plate, which is far too easy to do with an HPLC column. This may result in permanent damage to the column if samples cannot be removed. TLC plates, however, are free from this problem and highly retained components can still be detected. Simultaneous sample separation and detection[6] and two-dimensional separation[7–9] can be used. TLC is also quick, simple, convenient, inexpensive and disposable. TLC can complement HPLC as a pilot technique for the optimization of separation conditions[10] and can be used as a sample preparation technique for further separation[11–12].

Detection in TLC, unlike HPLC, is a static process, being completely separated from chromatographic development. So, a detector that works well for HPLC may not work for TLC. Detection of colored compounds, in general, causes no problem. The same holds true of compounds which exhibit fluorescence or phosphorescence under UV light. For colorless compounds, one usually adopts densitometry[13], directly if the compounds are UV-absorbing, or after color reaction[14] for which a specific spray reagent is required. Even though these methods are sometimes quite sensitive, they cannot be used to detect analytes which do not absorb in the UV region or have no appropriate spray reagents. The use of fluorescence layers[15–17] has been successfully demonstrated for detecting colorless compounds. However, this still requires that the analytes absorb either at the absorption or the emission wavelength of the fluorophore on the plate.

Recently, indirect fluorimetric detection of anions and non-electrolytes in HPLC has been demonstated[18–21]. Briefly, a fluorophore in the mobile phase generates a large uniform background fluorescence. When the analytes elute, displacement of the fluorophore causes a change in the fluorescence intensity to allow detection. Following the same principles, the indirect fluorimetric detection of anions in TLC has also been achieved[22]. This article will present an indirect fluorimetric detection method for

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non-electrolytes (alkanols, bile acids and digoxin series) in TLC. Not only are detection limits improved, but the method is also much simpler and analysis time is much shorter compared with other detection methods in TLC for the same compounds[23,24].

EXPERIMENTAL

Reagents

HPLC grade acetonitrile and methanol, and certified grade methyl ethyl ketone were obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.). HPLC grade chloroform was obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). HPLC grade tetrahydrofuran and analytical grade salicylic acid were obtained from Baker (Phillipsburg, NJ, U.S.A.). Alkanols, digoxin, digitoxin, taurocholic acid and taurodeoxycholic acid were purchased from Aldrich (Milwaukee, WI, U.S.A.). Digoxigenin, glycocholic acid and glycodeoxycholic acid were purchased from Sigma (St. Louis, MO, U.S.A.). 2-(1,1'-Biphenyl)-4-yl-5-phenyl-1,3,4-oxadiazole (PBD) was purchased from Eastman-Kodak (Rochester, NY, U.S.A.). 2-(1-Naphthalenyl)-5-phenyloxazale from Lambda Physik (Acton, MA, U.S.A.). Deionized water further purified on a Milli-Q system (Millipore, Bedford, MA, U.S.A.) was used to prepare pretreating solutions or eluents.

Materials

K_6 silica gel plates and KC_{18} reversed-phase plates were purchased from Whatman Chemical Separation (Clifton, NJ, U.S.A.). Adsorbosil RP-HPTLC plates were obtained from Alltech Assoc. (Deerfield, IL, U.S.A.). UV lamps (312 nm, 254 nm) were purchased from Cole Palmer (Chicago, IL, U.S.A.). A microsyringe calibrated to 0.1 μl units was purchased from Hamilton (Reno, NV, U.S.A.).

Sample preparations

Alcohol mixtures were prepared by dissolving decanol, dodecanol, tetradecanol and hexadecanol in acetonitrile- toluene (90:10). Bile acids mixtures were prepared by dissolving taurocholic acid (TC), taurodeoxycholic acid (TCD), glycocholic acid (GC), glycodeoxycholic acid (GDC) in methanol. Digoxin series mixtures were prepared by dissolving digoxin, digitoxin and digoxigenin in chloroform–methanol (2:1).

Chromatography and detection

Alcohols. (a) The Adsorbosil RP-HPTLC plate is immersed in acetonitrile–water (40:60) containing $2 \cdot 10^{-5}$ *M* PBD for 2 h. The plate is then dried with a heat-gun. Samples are spotted by a micro-syringe. The plate was developed by acetonitrile–water (98:2) containing $4 \cdot 10^{-3}$ *M* PBD for 6 min. After drying the plate again, the plate was placed under a UV lamp (312 nm). A series of negative spots can be observed.

(b) The samples were spotted on a KC_{18} reversed-phase plate directly (no pretreatment) by a microsyringe and the plate was developed by acetonitrile water (98:2) containing $4 \cdot 10^{-5}$ *M* α-NPO for 6 min. The plate is then dried and visualized under the UV lamp (312 nm). A series of positive spots can be observed.

Bile acids. A K_6 silica gel plate was treated by chloroform–acetonitrile (50:50)

containing $1 \cdot 10^{-4}$ *M* salicylic acid for 1 h and was then dried. After spotting the bile acid mixture on the plate by a microsyringe, the plate is developed with chloroform–methanol–methyl ethyl ketone (46:46:8) containing $3 \cdot 10^{-3}$ *M* salicylic acid for 24 min. The plate was then dried and visualized under a UV lamp (312 nm). A series of positive spots can be observed.

Digoxin series. (a) An Adsorbosil RP-HPTLC plate was pretreated with acetonitrile–water (40:60) containing $1 \cdot 10^{-5}$ *M* α-NPO for 1 h and was dried with a heat-gun. Samples were spotted with a micro syringe, and the plate was developed by methanol–water (7:3) containing $4 \cdot 10^{-5}$ *M* α-NPO for 8 min. The plate was then dried and visualized under a UV lamp (254 nm). A series of negative spots can be observed.

(b) After spotting the samples on a KC_{18} RP-TLC plate directly (no pre-

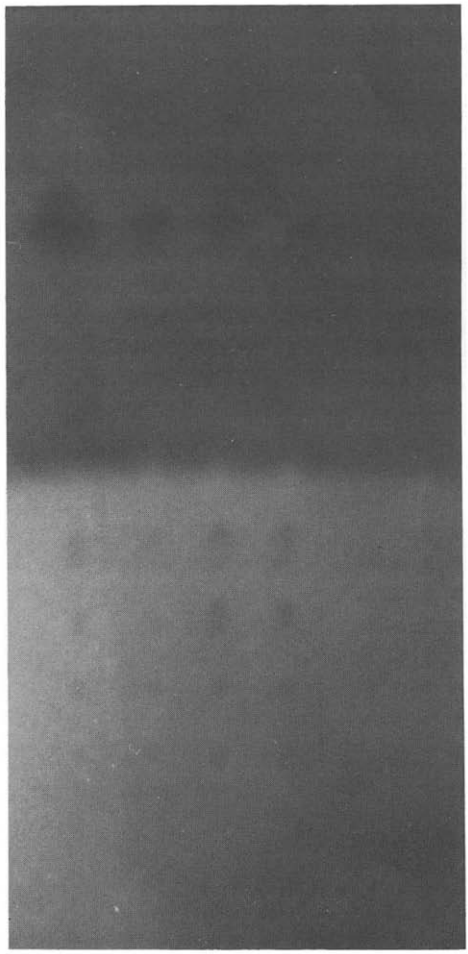

Fig. 1. Indirect fluorometric detection of alkanols on an Adsorbosil RP-HPTLC plate using procedure (a) in the text. Development is from bottom to top, with the front midway up the plate. The sample at the right contains 20 nmol each of decanol (C_{10}), dodecanol (C_{12}), tetradecanol (C_{14}) and hexadecanol (C_{16}), showing as lower intensity spots from top to bottom of the picture. The other three samples contain the same components as the right one but at concentrations of 1/3, 1/10 and 1/30 of that at the right.

treatment) by a microsyringe, the plate is developed by methanol–water (7:3) containing $2 \cdot 10^{-3}$ *M* salicylic acid for 8 min. Then, the plate is dried and visualized under a UV lamp (312 nm). A series of positive spots can be observed.

RESULTS AND DISCUSSION

Figs. 1 and 2 demonstrate the indirect fluorometric detection of alkanols by reversed-phase TLC. Nanomole levels of each alkanol can be detected by our eyes. The dark spots in Fig. 1 are not due to the presence of an absorbing species. Rather, less of the fluorophore exists locally because it has been transferred out of the stationary phase by the analyte. It should be mentioned that the positive (higher intensity) spots observed in Fig. 2 are not due to the fluorescence of alkanols. The reason for this

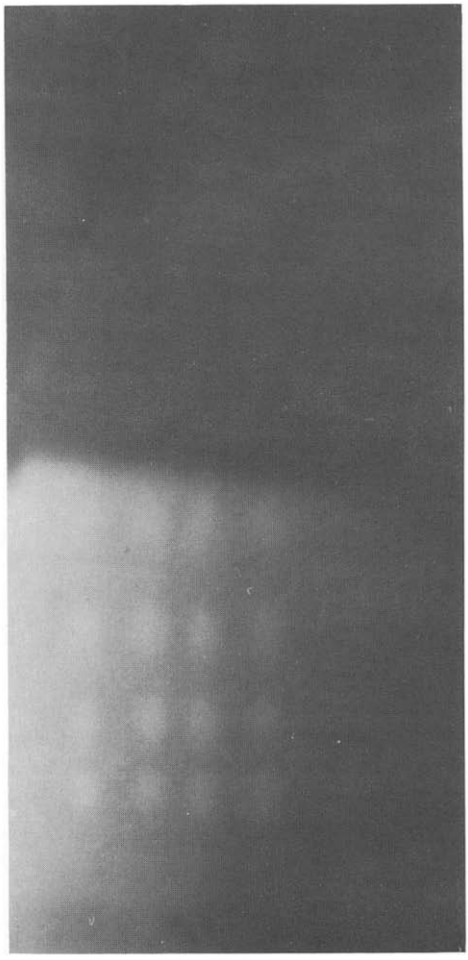

Fig. 2. Indirect fluorometric detection of alkanols on a KC_{18} reversed-phase TLC plate using procedure (b) in the text. Development is from bottom to top, with the front midway up the plate. The analytes show up as higher intensity spots. The sample components and concentration range are the same as those of Fig. 1.

phenomenon is that alkanols transfer visualizing reagent (α-NPO here) from the mobile phase to the stationary phase. So, where the sample spot is, more of the fluorophor exists, and a stronger fluorescence signal is observed. An earlier paper[25] had a detailed discussion on this. The choice of fluorophore determines whether positive or negative (lower intensity) spots are observed, even though the same developing solution is used. This clearly shows that differences in partition into the stationary phase between the fluorophore and the analytes is responsible for the signal.

Fig. 3 shows the indirect fluorometric detection of bile acids. Subnanomoles of each can be detected. The reason for the presence of positive spots is the same as that for alkanols. Touchstone *et al.*[24] had previously demonstrated the separation of

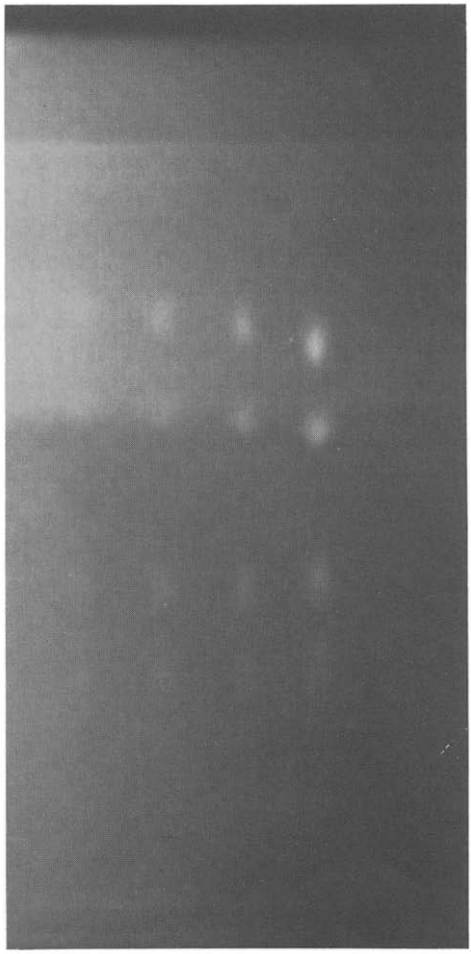

Fig. 3. Indirect fluorometric detection of bile acids on a K_6 silica gel plate. Development is from bottom to top, with the front near the top edge of the plate. The sample at the right contains 10 nmol each of taurodeoxycholic acid (TDC), taurocholic acid (TC), glycodeoxycholic acid (GDC) and glycocholic acid (GC), showing up from top to bottom of the picture as higher intensity spots. The other three samples contain the same components as the right one but at concentrations of 1/3, 1/10 and 1/30 of that at the right.

conjugated bile acids on reversed-phase TLC and detection by a spectrodensitometer in the transmission mode. But the method is time consuming and one needs to search for a specific spray reagent. By our approach, separation is faster and detection is simpler. Similar detection limits can be achieved even by our naked eyes. With a scanning laser beam for excitation, one would expect even better detection due to signal averaging and intensity enhancement.

The indirect fluorometric detection of digoxin series is shown in Figs. 4 and 5. The positive spots in Fig. 5 occur for the same reasons as the alkanols in Fig. 2. Comparing the results from a previous paper[23], the detection limits for digoxin,

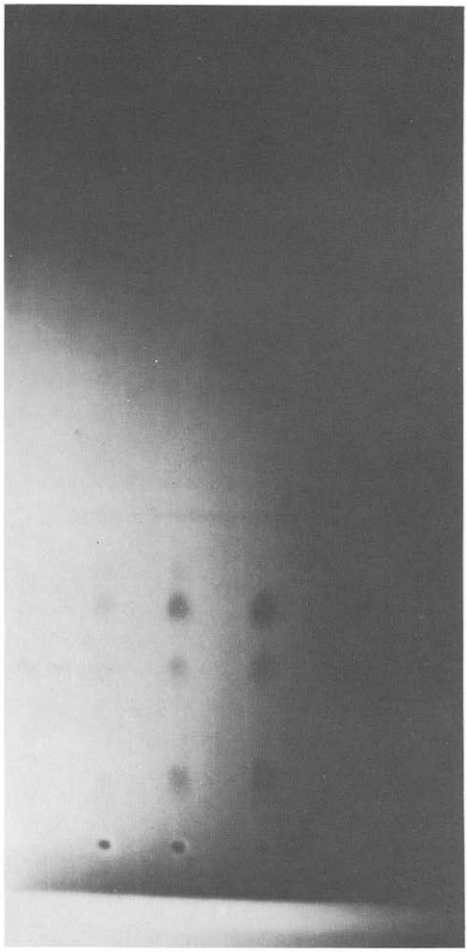

Fig. 4. Indirect fluorometric detection of digoxin series on an Adsorbosil RP-HPTLC plate using procedure (a) in the text. Development is from bottom to top, with the front midway up the plate. The sample at the right (barely illuminated) contains 15 nmol of digoxigenin and 7.7 nmol each of digoxin and digitoxin, showing up from top to bottom of the picture as lower intensity spots. The other three samples contain the same components as the right one but at concentrations of 1/3, 1/10 and 1/30 of that at the right. Two impurities are present, one above and one below the set of three analytes.

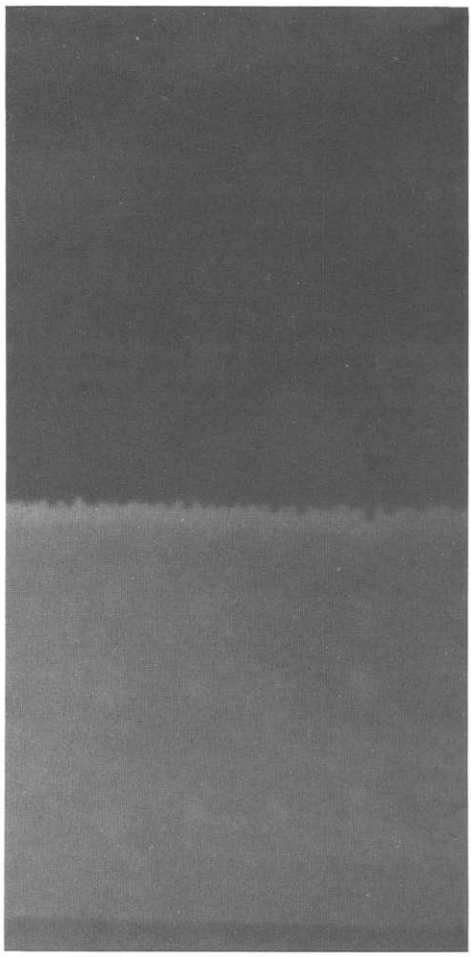

Fig. 5. Indirect fluorometric detection of digoxin series on a KC_{18} reversed-phase TLC plate using procedure (b) in the text. Development is from bottom to top, with the front midway up the plate. The sample components and concentration range are the same as those of Fig. 4.

digitoxin and digoxigenin are at least two orders of magnitude lower by our approach. Also, spray reagents are omitted in our method.

In indirect fluorimetric detection methods, the following equation can be used to estimate the limit of detection of non-electrolytes[26]

$$C_s > \frac{1}{D_R(R/C_m + V_s)} \quad (1)$$

where C_s is the molar concentration of the analyte detected, D_R is the dynamic reserve (which is defined as the ratio of the background signal to the noise level), R is the displacement ratio (which is defined as the number of visualizing molecules transferred

by one analyte molecule), C_m is the molar concentration of visualization reagent and V_s is the volume of 1 mol of the analyte. The equation tells us that the dynamic reserve, concentration of the visualization reagent and the displacement ratio all play important roles in the sensitivity of indirect fluorometric detection.

In order to improve the detection limit or sensitivity of indirect fluorometric detection further in TLC, the background signal should be decreased as much as possible. This can be achieved by decreasing the concentration of the visualization reagent in the pretreating solution or in the developing solution. However, if the background signal is too low, the displacement ratio may also decrease and contrast will be lost.

The visual (or photographic) observation in this work is obviously poor compared to instrumental detection methods with respect to D_R in eqn. 1. Therefore, further improvements in detectability should be possible. Fluorescence with high-frequency modulation should allow D_R to increase by 2 to 3 orders of magnitude[18]. The high sensitivity of laser-excited fluorescence should make it compatible with much lower fluorophore concentrations, if the natural fluorescence of the TLC substrate can be discriminated against.

CONCLUSION

Indirect fluorometric detection of non-electrolytes (alkanols, bile acids and digoxin series) has been presented. Nanomolar detection limits were achieved. The selection of the visualization reagent and the chromatographic conditions is important because of the dependence of displacement ratio and spot character (negative spot or positive spot) on it. With minor modifications of the recipes given in this work, one can now essentially combine indirect fluorometric detection with any TLC separation.

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