Thin Layer Chromatography (TLC)

Paper chromatography, Electrophoresis

III Pharm.D
Department of Pharmaceutical Analysis
SRM College of Pharmacy, Kattankulathur
[1] Preparation of plates

• slurry of adsorbent on glass plate
• spread and dries to make a film over surface
• quantity used to mix slurry depends on:
  – number and size of plates
  – thickness of layer
  – nature of adsorbent
• after activation all plates must be stored in desiccator until used
For five 20 x 20cm plates of 250µ thickness

<table>
<thead>
<tr>
<th>Adsorbent</th>
<th>Slurry</th>
<th>Drying and Activation</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1. Materials with binders (G)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aluminium oxide</td>
<td>30 g + 40 ml H₂O</td>
<td>30 mins at room temperature and then 30 mins at 110° unless otherwise directed.</td>
<td>The binder sets very fast and therefore the whole process should be carried out within 2-3 mins.</td>
</tr>
<tr>
<td>Silica gel</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>2. Materials without binders (H)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aluminium oxide</td>
<td>30 g + 80 ml H₂O</td>
<td>3 hours at room temperature and then 30 mins at 120° unless otherwise directed.</td>
<td>There is no urgency about spreading after slurry production.</td>
</tr>
<tr>
<td>Silica gel</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Spreader

• Several types available commercially
  – eg Desaga Apparatus
    • flat template tray into which glass plates fit
      – must be placed on a flat surface
      – plates are degreased by wiping clean with acetone
    • spreader with rotating chamber and gauge
      – ensure clean and chamber is free moving
      – set gauge to required thickness
  • adsorbent + liquid shaken together in closed flask
    – slurry poured into spreader and lever arm turned to invert rotating chamber
    – spreader pulled slowly across plates
    – air dried in tray and placed on a TLC rack for activation or storage
• NB Many solvents are inflammable! Not near naked flames
[2] Preparation of tanks

- **[i] Solvents**
  - only pure dry solvents used for chromatography
    - redistillation or storage over a drying agent may be needed
  - solvent mixtures should be freshly prepared for analysis
    - these occupy about 1.5 cm depth of tank
  - great care measuring and pipetting
    - if reproducible $R_f$ values are required

- **[ii] Lining tanks**
  - Whatman No.2 chromatography paper of tank height
    - solvent is poured down sides of tank to ensure wetting of lining

- **[iii] Saturation of atmosphere**
  - ground glass lid used to seal tank
  - solvent gently swirled round inside (while holding lid)
  - repeat occasionally for a few seconds during 15 minutes
  - only slide lid back small distance to place plate in

• [i] Determination of spot size
  • spots of various sizes applied to an “end plate” for TLC
    – (or filter paper for paper chromatography)
  • spots by spraying and visualised
  • suitable size selected
    – spots increase in size during chromatogram development
    – hence less colour intensity
    – mixture may be present in unknown solutions
    – overloading leads to streaking
    – trace impurities overlooked if insufficient applied
    – usually 1% solution of reference materials is used
    – 10μl (one spot from 0.5cm diameter capillary) is sufficient
    – (3-4 spots should be applied for paper chromatography)
• [ii] Application
  • remove narrow strip of coating 0.5cm wide from vertical margins of plate
  • measure 3cm from bottom of plate
  • make a small mark 2mm long on the coating of each side of the plate = baseline
  • measure 15cms and make 2 small marks at new height = solvent front
  • place prepared plate on piece of clean drawing paper
  • spot reference solutions each from clean capillary to baseline 1.5cm apart and 2cm from edge
  • spots in centre should be unknown solution
  • or mixture of reference solutions and unknown mixture
  • on paper at the bottom of plate mark off and label positions of spots across plate
  • also mark baseline and solvent line
Application of Spots to a TLC plate

Solv. front

Baseline
[4] Sprays and spraying

- **Sprays**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Heating treatment</th>
<th>Viewed</th>
<th>Solutes used for</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dragendorff's reagent</td>
<td>-</td>
<td>Daylight</td>
<td>Alkaloids</td>
</tr>
<tr>
<td>5% vanillin in $\text{H}_2\text{SO}_4$</td>
<td>-</td>
<td>Daylight</td>
<td>Terpenes</td>
</tr>
<tr>
<td>60% aqueous sulphuric acid</td>
<td>Heat $120^\circ$ 10-15 minutes</td>
<td>Daylight and UV</td>
<td>General spray particularly steroids</td>
</tr>
<tr>
<td>Antimony trichloride 10% in benzene</td>
<td>Heat $120^\circ$ 10 minutes</td>
<td>Daylight and UV</td>
<td>Steroids</td>
</tr>
<tr>
<td>Phosphomolybdic acid 10% in 95% ethanol in water</td>
<td>Heat $110^\circ$ 5 minutes</td>
<td>Daylight</td>
<td>General spray for unsaturated and oxy-compounds</td>
</tr>
<tr>
<td>Aniline hydrogen phthalate</td>
<td>Heat $105^\circ$ 10 minutes</td>
<td>Daylight and UV</td>
<td>Sugars</td>
</tr>
<tr>
<td>Ninhydrin reagent</td>
<td>Heat $10^\circ$ until colour develops</td>
<td>Daylight</td>
<td>Amino acids</td>
</tr>
</tbody>
</table>
• [ii] Spraying
  • eg Shandon spray packs + compressed gas cylinders
  • great care – many sprays
    – toxic (antimony chloride)
    – corrosive (strong acids)
  • use fume cupboard
    – extraction fan working properly
    – sliding window below chest level (only room for forearms)
  • after spraying close window and leave a few seconds
  • hold plate corner/edge with tissue paper
  • may be necessary to heat sprayed plate for visualisation

• [i] Making permanent record
  • most sprays produce coloured spots
  • check for extra spots under UV light
    – mark with needle point
  • trace all plates with pencil
    – use tracing paper same size as plate
    – allow heated plates time to cool
  • transfer data to paper record
  • each drawing should be labelled with
    – coating substance and activation period
    – thickness of layer
    – solvents used
    – spray used
• mark the colour and D or U beside each spot
  D = daylight observation
  U = UV observation

• file record in results section
R_f values

• qualitative results of TLC
  – expressed as fractions of 1.0
  – can be expressed from Rf values (eg Rf x 100)
  – no more than two decimal places
    • due to inaccuracy of physical measurement

• may not be reproducible
  • only give an indication of possible nature of unknown
  • complete identification only obtained if spot is eluted and micro-scale physical measurements done (MS, UV, IR)

• standard references should always be used on same plate for comparison
  • most sprays produce differential colours of fluorescence
  • colour test provides extra evidence with distance migration
\[ R_f = \frac{\text{Distance from centre of solute spot (cm) to the baseline}}{\text{Distance from solvent front to baseline (cm)}} \]

- \( R_x \) value
  - migration of solute with internal standard
  - attempt to overcome variability in \( R_f \) values
  - internal standard is a solute added to the mixture
    - has similar chemical nature
    - \( R_f \) value in middle range of unknown compounds

\[ R_x = \frac{\text{Distance from the centre of unknown spot to baseline}}{\text{Distance from the centre of standard spot to the baseline}} \]

- hoped ratio would eliminate variations due to differences in conditions between analyses
Paper chromatography

[1] Preparation of paper
   - cut to 35cm x 43cm
   - pre-treatment may be necessary

[2] Preparation of tanks
   - large round tanks used
   - 150ml solvent to give 1.5cm depth
   - tanks not lined but atmosphere must still be pre-saturated
     • running solvent
     • snug fitting lid
- quantity to be applied determined as for TLC
- baseline drawn in lead pencil approx 1” from long side
- spots
  - applied 5cm apart and from edge
  - as small as possible (each allowed to dry before next applied)

[4] Running the paper
- shorter sides stapled to make a cylinder (3mm gap)
- stood in tank to elute
- solvent front marked after elution
- stood upside down in fume cupboard to dry
- further treatment may be necessary
Electrophoresis

• Principle
  – charged ion group will migrate towards one of the electrodes when placed in an electric field

• Practice
  – mixture placed in a narrow band or zone at a suitable distance from each electrode
  – various components draw away from one another at different rates in different directions
  – to fix substances at positions a stabilising medium is needed (paper) to hold solution for electric field
  – dried on termination of run
  – (solutions will also separate)
• Migration velocities (M)
  – of a substance is defined as distance travelled from the origin per second at a field strength of 1 volt/cm (constant voltage)
  – is a very small term
  – \( M = \frac{\text{cm}^2}{\text{volt sec}} \)
  – eg If an amino acid migrates roughly 12cm in 45 mins when a potential of 500 volt is applied across a paper strip 32cm long...
    \[
    M = \frac{12}{45 \times 60} \times \frac{32}{500} = 2.5 \times 10^{-4} \text{ cm}^2/\text{volt sec}.
    \]

• Relative mobilities
  – calculated by reference to migration of a fast component
    • ornithine is a convenient reference standard at < pH7
    • aspartate used above pH7
  – at low voltage rel mob fairly constant for a given buffer
• Methods

• Setting up paper
  – 20 x 32cm sheets of paper cut from larger Whatman No.1
    » (longer side cut in direction of flow of paper)
  – 32cm sides bisected with a pencil line and origins marked
  – 1μl volumes of standards are applied
    » 4cm from edge and 2cm apart
    » dried in warm air stream
  – spot kept as small as possible
  – wear gloves
    » avoids making papers with ninhydrin-positive substances from hand perspiration
  – paper is wetted with appropriate buffer solution up to origin
    » then blotted between filter papers to remove excess
    » and placed carefully in tank
• switch on power supply, allow few seconds to stabilise

• switch off when run is complete

• carefully remove paper from tank

• dry at 110°, spray evenly with ninhydryin reagent, heat at 110° to get maximum colour