1.14.1 Thin-layer chromatography

In thin-layer chromatography the adsorbent is a thin, uniform layer (usually about 0.24 mm thick) of a dry, finely powdered material applied to a suitable support, such as a glass plate or an aluminium or plastic foil. The mobile phase is allowed to move across the surface of the plate (usually by capillary action) and the chromatographic process may depend upon adsorption, partition, or a combination of both, depending on the adsorbent, its treatment, and the nature of the solvents used. During the chromatographic procedure the plate is contained in a chromatographic chamber (usually made of glass to permit observation of the movement of the mobile phase up the plate), which is usually saturated with the solvent vapour. Solid supports frequently used are silica gel, kieselguhr, alumina, and cellulose; to these may be added suitable substances, for example, calcium sulfate to promote adhesion to the support. The prepared layer may be impregnated with buffering materials to afford acidic, neutral, or basic layers, or with other material, such as silver nitrate, designed to modify its properties. In certain cases the layer may consist of an ion-exchange material. This wide range of possible layers, used in conjunction with different solvent systems allows an almost infinite variation of separating power that makes thin-layer chromatography such a useful technique in pharmaceutical analysis.

As an adjunct to identification, thin-layer chromatography may be used by comparing the behaviour of the material to be identified with that of a standard substance, usually an authentic specimen of the substance being examined. If the two substances move identical distances during the chromatographic process and if the two substances, when mixed together and then subjected to chromatography, move as a single substance, it may be presumed that the two substances are identical. This presumption may be strengthened by repeating the procedure using a different system of chromatography; in general, if two substances behave identically in as many as three fundamentally different systems the presumption of identity becomes very strong.

For identification purposes it convenient to define the relative distance that the unknown material moves in relation to the distance moved either by the solvent front or by a standard reference material. On a developed chromatogram, the ratio of the distance travelled on the adsorbent by a given compound to that travelled by the leading edge of the solvent (or mobile phase), both measured from the point of application of the test substance, is referred to as the $R_f$ value of the substance in the given chromatographic system. The ratio of the distances moved by the compound and a stated reference substance is referred to as the $R_f$ value. In practice $R_f$ values may vary considerably according to the exact experimental conditions so that the $R_f$ value determined against the reference substance subjected to chromatography on the same surface gives a more reliable numerical value. Even more reliable, however, is comparison with an authentic specimen as described above and this is the procedure usually used for pharmacopoeial purposes.

To determine the position of a colourless substance on a developed chromatogram it is usually necessary to treat the chromatogram with a reagent that will either char the separated substances or convert them to coloured or fluorescent derivatives. A convenient alternative that is frequently applicable is to carry out the chromatography on a surface impregnated with a substance that fluoresces strongly when examined under short-wave ultraviolet light. Areas of the plate occupied by substances absorbing at the same wavelength show as dark spots on the fluorescent background. In special cases, other means of recognition may be used, for example, the detection of radioactivity where labelled compounds are being separated or a microbiological response where antibiotics are concerned.

The most valuable use of thin-layer chromatography in pharmaceutical work is to provide a means of assessing low levels of impurities in medicinal substances. For this purpose the substance is applied to the chromatographic surface and, after chromatography, any secondary spots to be seen in the chromatogram after appropriate visualization are compared for size and intensity with those of low loadings of expected impurities that have simultaneously been subjected to chromatography on the same plate. Such a procedure requires that the expected impurities be available and in certain monographs the use of authentic specimens of impurities is called for. Frequently, such impurities are not available and in such cases it is often possible to compare secondary spots arising from trace impurities with the spot obtained by carrying out chromatography on the same plate using an appropriately low loading of the substance being examined. This expedient is not always possible since impurities and the substance being examined may respond in different ways to the method of revelation used, but it often provides an acceptable criterion by which the level of impurity in the substance may be judged. A third procedure that is sometimes advocated is to apply such an amount of the substance being examined that, after chromatography, no secondary spots will appear if the sample is acceptably pure. This is the least satisfactory of the three methods since ability to see a secondary spot is a subjective matter and because the intensity of spots on a chromatogram may vary considerably from one occasion to another depending on the exact conditions of chromatography.

For quantitative procedures the spot may be removed from the plate, the substance eluted with a suitable solvent and then determined by a sufficiently sensitive method, such as a spectrophotometric measurement, either directly or after a chemical reaction. In certain cases, quantitation can also be achieved by measurement of the spot intensity with the aid of a scanning densitometer and subsequent comparison of the intensity with the intensities obtained from standard amounts of the same substance similarly treated.

Separations effected by thin-layer chromatography may sometimes be improved by multiple development (when the chromatogram is allowed to dry and then subjected again to the same system of chromatography), by continuous development (when the mobile phase is allowed to evaporate continuously from the upper end of the adsorbent surface), or by two-
dimensional chromatography (when the chromatographic plate is allowed to dry, turned at right angles, and then subjected to further chromatography, frequently in a different solvent system from that used for the initial chromatography). Caution should be exercised in interpreting the results of chromatograms where such intermediate drying processes are used, however, since decomposition, such as oxidation, of the substance being chromatographed may occur on the plate. The process of two-dimensional chromatography is especially valuable in judging whether any chemical change is taking place during the development process. If a mixture of substances is developed first in one direction and then at right angles with the same solvent the separated substances will lie in a diagonal line across the plate if no artefacts are being produced.

In thin-layer chromatography the adsorbent is usually spread in a thin even layer on a support plate. This may be undertaken in the analytical laboratory but it is also possible and convenient to obtain commercially prepared chromatographic surfaces that are attached to glass or to plastic or metal foil. Unfortunately, so sensitive may the chromatographic process be to minor changes in conditions that these various commercially available materials are not always interchangeable one with another or with an apparently similar laboratory-coated plate. A factory-coated silica gel plate prepared by a given manufacturer may give different separation characteristics from a laboratory-coated plate prepared using the same manufacturer's coating substance, and instances exist where a perfectly satisfactory method of separation devised using laboratory-prepared plates fails when using precoated plates and vice versa. Great caution must, therefore, be exercised when changing from one type of chromatographic plate to another and the suitability of a given type of plate should always be assessed before reliance is placed upon it.

The chromatographic chamber in which chromatography takes place should be protected from light if it is suspected that the materials to be examined may be unstable in light. In any case, the chromatographic chamber should always be in a position where the direct rays of the sun cannot fall on it since the rays may be refracted to different degrees owing to imperfections in the glass walls of the chamber. This may give rise to areas of elevated temperature on the chromatographic plate and result in erratic flow of the mobile phase.

**Recommended procedure**

The method given below assumes the use of a laboratory-prepared chromatographic plate but a precoated plate, activated if necessary, may be used provided that it has been shown to be suitable for the particular application.

The equipment consists of:

- a device for spreading on plates a uniform layer of coating substance of the desired thickness;
- plates 200 mm long and wide enough to accommodate the required number of solutions to be examined and the reference solutions;
- a chromatographic chamber of transparent material, usually glass, with a tightly fitting lid, of a size suitable for the plates used.

Prepare a slurry of the coating substance and, using the spreading device, coat the carefully cleaned plates with a layer about 0.25 mm thick, unless otherwise specified in the monograph. Allow the coated plates to dry in air and heat to activate, unless otherwise specified in the monograph, at 110 °C for 30 minutes, then allow to cool. If the plates are not to be used immediately, store them in a desiccator containing silica gel, desiccant, R. Remove a narrow strip (2-5 mm) of the coating substance from the vertical sides of the plate.

Unless otherwise specified in the monograph, work under saturated chamber conditions. To achieve such conditions, line the chromatographic chamber with filter-paper and pour into the chamber a sufficient quantity of the mobile phase to saturate the filter-paper and form a layer about 5 mm deep. Close the chamber and allow to stand for at least 1 hour at room temperature.

**Method**

All operations during which the plate is exposed to the air should preferably be carried out at a relative humidity of 50-60%. Apply the volume of the solution as specified in the monograph as a compact spot, preferably not more than 4 mm in diameter. Application may be made using a micropipette, a syringe, or other suitable means. The spot should be placed about 1.5 cm from the lower edge and not less than 2 cm from the vertical sides of the plate. Where more than one chromatogram is run on the same plate, the spots should be placed not less than 1.5 cm apart and form a line parallel with the lower edge of the plate. When the solvent has evaporated, place the plate in the chromatographic chamber, ensuring that the plate is as nearly vertical as possible and that the starting points are above the level of the mobile phase. Close the chamber and maintain it at a constant temperature. Allow the mobile phase to ascend, usually 10-15 cm, remove the plate, mark the position of the solvent front and dry as specified in the monograph.