CHAPTER III

PHAMACOGNOSTIC STUDY

Pharmacognostic study is used to characterized, quality control of crude drugs and quantitative analyses. In the macroscopic method, organoleptic sensation is used to determine the size, shape, color, odor, taste, *etc*. of the crude drugs, while the microscopic method revealed plant histology. And the thin-layer chromatographic technique is used to differentiate extracts of different biological origins. Methods for quality control of crude drugs are described in Pharmacopoeia. Furthermore, the quantitative analyses by TLC-densitometer and capillary electrophoresis (CE) using tetrandrine as a marker are described.

Apparatus for microscopic measurements

Microscopic measurements can be carried out using a stage micrometer in conjunction with an eyepiece micrometer and drawing attachment.

Micrometers

Two scales are required, known respectively as a stage micrometer and an eyepiece ocular micrometer. The stage micrometer is a glass slide 7.6 x 2.5 cm (3 x 1 inch) with a scale engraved on it. The scale is usually 1 mm long and is divided into 0.1 and 0.01 parts of a millimeter. The eyepiece micrometer may be a linear scale and the scale 0-10 or it may be ruled in squares. The value of one eyepiece division is determined for every optical combination to be used, a note being made in each case of the objective eyepiece and length of raw-tube.

To calibrate micrometer, unscrew the upper lens of the eyepiece, place the eyepiece micrometer on the ridge inside, and replace the lens. Put the stage micrometer on the stage and focus it in the ordinary way, the two micrometer scales now appear as in Figure 4, when the objective (x4) is in use. It will be seen that when the 7 line of the stage micrometer coincides with the 0 of the eyepiece, the 10 of the stage coincides with 7.7 of the eyepiece. As the distance between 7 and 10 on the stage scale is 0.3 mm, 77 of the small eyepiece division equal 0.3 mm or 300 μm; therefore, 1 eyepiece division of this eyepiece micrometer which is used with the objective (x4) of this microscope equals 300/77 or 3.9 μm (Trease and Evans, 1996).

Photomicrography equipment

Photomicrography equipment is uniquely qualified to be used with the microscope for routine and advanced photomicrography (Figure 5). This equipment is used to take a photograph of specimens, viewing with microscope.

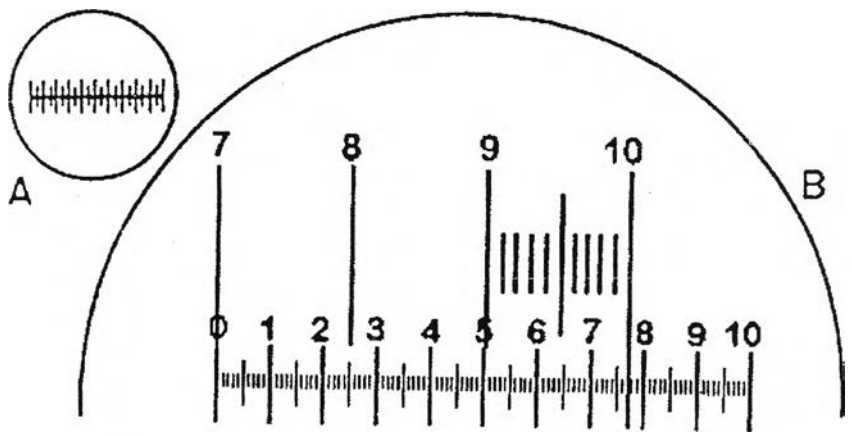

Figure 4. A. Eyepiece micrometer

B. Eyepiece micrometer superimposed on portion of stage micrometer scale.

(Trease and Evans, 1996)

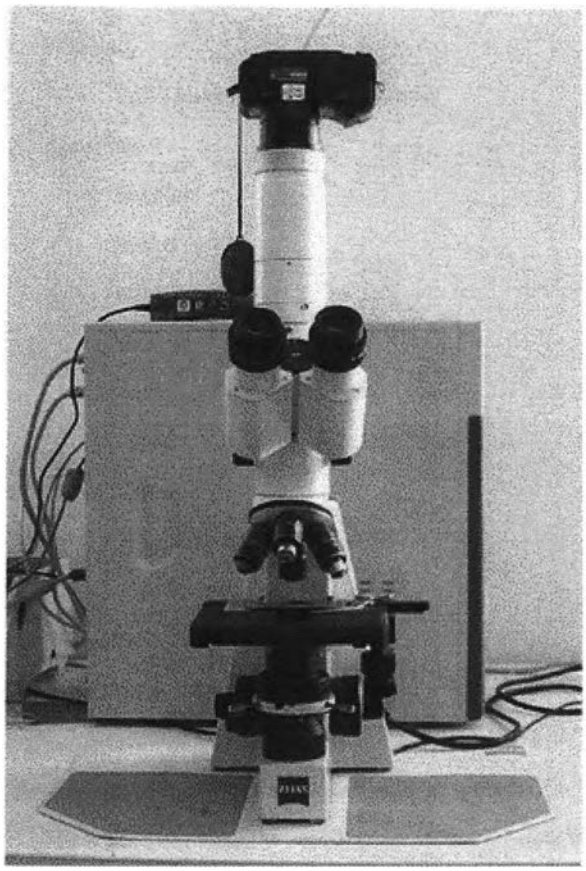

Figure 5. Compound microscope Zeiss model Axiostor attached with digital camera Sony Cyber-short model DSC-S85.

Quality Controls

One possible problem in devising standards for crude drugs concerns the requirement for an assay of the active constituents when the latter may not have been precisely ascertained. Furthermore, one of the tenets of the herbal medicine is that the maximum effectiveness of the drug derives from the whole drug or its crude extract rather than from isolated components. In cases where an assay is lacking it is therefore of paramount importance that the crude drug is properly authenticated, its general quality verified and all formulations of it prepared in accordance with good manufacturing practice. Although official standards are necessary to control the quality of drugs their use doses raise certain problems. Of necessity, to accommodate is necessary to set relatively low standards which allow the use of commercial materials available in any season. There are a number of standards, numerical in nature, which can be applied to evaluation of crude drugs either in the whole or the powdered condition (Trease and Evans, 1996). For this investigation, these are following:

Loss on drying

This is employed in the EP, BP, USP and TP. Although the loss in weight, in the samples so tested, principally is due to water, small amounts of other volatile materials will also contribute to the weight loss. For materials, which contain little balance combines the drying, process and weight recording; it is suitable where large numbers of samples are handled and where a continuous record of loss in weight with time is required (Trease and Evans, 1996).

Moisture content

The "loss on drying" methods can be made more specific for the determination of water by separating and evaluating the water obtained from a sample. This can be achieved by passing a dry inert gas through the heated sample and using an absorption train (specific for water) to collect the water carried forward; such method can

be extremely accurate, as shown in their use for the determination of hydrogen in organic compounds by combustion analysis. Method based on distillation has been widely used for moisture determination. This method is employed in the *USP*, *BP* and in *TP* and *EP* for some volatile oil-containing drugs (Trease and Evans, 1996).

Ash content

The determination of ash is useful to detecting low-grade products, exhausted drugs and excess of sandy or earthy matter; it is more especially applicable to powder drugs. Different types of ash figures are used such as total ash, acid-insoluble ash and water soluble ash. A total ash usually consists mainly of carbonates, phosphates, silicates and silica. If the total ash were treated with dilute hydrochloric acid, the percent of acid-insoluble ash may be determined. This usually consists mainly of silica and a high acid-insoluble ash in drug (Trease and Evans, 1996).

Extractive value

The determination of water-soluble or ethanol-soluble extractives is used as a means of evaluating drugs the constituents of which are not readily estimated by other means. In certain cases extraction of the drug is by maceration, in others by a continuous extraction process. For the latter the Soxhlet extractor is particularly useful and has been in use for many years, not only for the determination of extractives but also for small-scale isolations (Trease and Evans, 1996).

Thin-Layer Chromatography (TLC)

TLC, which together with paper chromatography comprises "planar" or "flatted" chromatography, is the simplest of all of the widely used chromatographic methods to perform. A suitable closed vessel-containing solvent and a coated plate are all that are required to carry out separations and qualitative and semi-quantitative analysis. With optimization of techniques and materials, highly efficient separations and accurate and

precise quantification can be achieved. TLC can be used also for preparative scale separations by employing specialized apparatus and techniques.

Basic TLC is carried out as follows. An initial zone of mixture is placed near one end of the stationary phase, a thin layer; the sample is dried; and the end of the stationary phase with the initial zone is placed into a mobile phase, usually a mixture of pure solvents, inside a closed chamber. The components of the mixture migrate at different rates during movement of the mobile phase through the stationary phase, which is termed the development of the chromatogram. When the mobile phase has moved an appropriate distance, the stationary phase is removed, the mobile phase is rapidly dried, and the zones are detected by application of a suitable visualization reagent.

Differential migration is the result of varying degrees of affinity of the mixture components of the stationary and mobile phases. Different separation mechanisms are involved, the predominant forces depending on the exact nature of the two phases and the solutes. The interactions involved in determining chromatographic retention and selectivity include hydrogen bonding, electron-pair donor/electron pair acceptor (charge transfer), ion-ion, ion-dipole, and van der Waals interactions. Among the latter are dipole-dipole, dipole-induced dipole, and instantaneous dipole-induced dipole interactions.

Sample collection, preservation, and purification are problems common to TLC and all other chromatographic methods. For complex samples, the TLC development will usually not completely resolve the analyze (the substance to be determined) from interference unless a prior purification is carried out. This is most often done by selective extraction and column chromatography. In some cases, substances are converted, prior to TLC, to a derivative that is more suitable for separation, detection, and/or quantification than the parent compound.

Detection is most simple when the compounds of interest are naturally colored or fluorescent or absorb ultraviolet (UV) light. However, application of a location or visualization reagent by spraying or dipping is usually required to produce color or

fluorescence for most compounds. Absorption of UV light is common for many compounds, e.g., aromatics and those with conjugated double bonds. This leads to a simple, rather universal detection method on layers impregnated with a fluorescence indicator (fluorescence quench detection).

Compound identification in TLC is based initially on R_f values compared to authentic standards. R_f values are generally not exactly reproducible from laboratory to laboratory or even in different runs in the same laboratory, so they should be considered mainly as guides to relative migration distances and sequences. Factors causing R_f values to vary include: dimensions and type of the chamber, nature and size of the layer, direction of mobile phase flow, the volume and composition of the mobile phase, equilibration conditions, humidity, and sample preparation methods preceding chromatography (Sherma, 1991).

Two-Dimensional Thin-Layer Chromatography

In many TLC process, and even with several solvent steps, the separation of mixtures along a single line do not always give a clear resolution of the individual components. However, this problem can often be overcome by using two-dimensional TLC, even for closely related compounds (Mendham, 2000)

It must be stressed that the factors that determine the reproducibility of results in the one-dimensional method have an even greater effect in the two-dimensional method (Randerath, 1968). The major point of variation in this technique is what is done to the solvent system or to the layer between the two developments. In order to obtain reproducible results, the layer must always be treated in exactly the same way before development in the second direction. Thus, for instance, the conditions of the intermediate drying must never be altered (Randerath, 1968).

If very small quantities of substance have to be detected, it should be noted that the lower limits of detection are higher for two-dimensional than for one- dimensional

chromatograms, since diffusion effects cause greater dilution of the substance in the longer development time of the two-dimensional method. Nevertheless, the detection sensitivity is still considerably greater than on a two-dimensional paper chromatogram. A further substantial advantage is that two different separation principles (e.g. adsorption and partition chromatography or partition chromatography and electrophoresis) can be combined in the two-dimensional technique (Randerath, 1968).

Quantitative Controls

TLC-densitometry

Methods for the quantitative measurement of separated solutes on a thin-layer chromatogram can be divided into two categories. In the more generally used *in situ* methods, quantitation is based on measurement of the photo-density of the spots directly on the thin- layer plate, preferably using a densitometer. The densitometer scans the individual spots by reflectance or absorption of a light beam; the scan is usually along the line of development of the plate. The difference in intensity of the reflected (or transmitted) light between the adsorbent and the solute spots is observed as a series of peaks plotted by a chart recorder. The areas of the peaks correspond to the quantities of the substances in the various spots. This type of procedure requires comparison with spots obtained using known amounts of standard mixtures, which must be chromatographed on the same plate as the sample. Improvements in the design of densitometers have considerably increased the reliability of quantitative TLC determinations.

Commercial instruments for *in situ* quantitative analysis based on direct photometric measurements have played an important role in modern TLC. Both double- and single-beam instruments are available. They are of particular value in HPLC where the quality and surface homogeneity of the plates are generally very good. The densitometers scan the individual spots by reflectance or absorption of a light beam. In reflectance densitometry the chromatogram is scanned by the moving beam of light and the intensity of the reflected light from the surface is measured. The differences in the light intensity between the adsorbent and any spots are observed as a series of peaks in the scan display. The areas of the peaks correspond to the quantities of the materials in the spots. Alternative procedures produce a similar record by measuring the light transmitted through the plate. With photo-densitometers the transmitted or reflected radiation is used to produce a photograph of the chromatogram revealing dark and light zones for the areas of the

separated compounds. The standard deviation for quantitative TLC determinations by densitometry is better than 5 %(Mendham *et al.*, 2000).

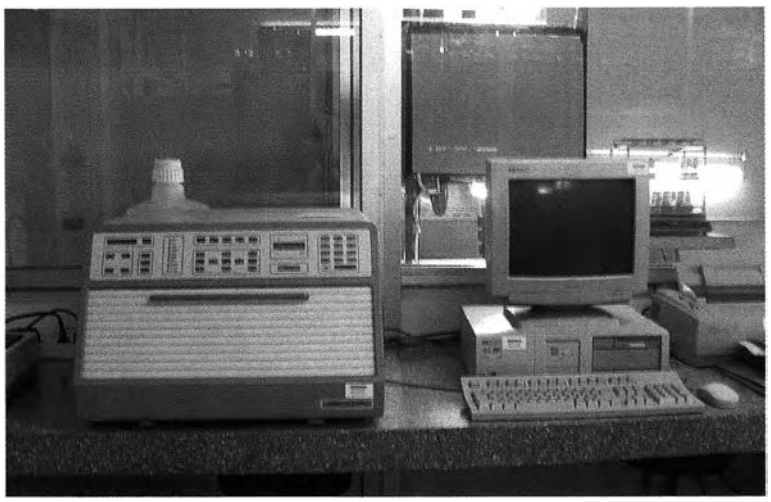

Figure 6. TLC-scanner II (CAMAG)

Capillary Electrophoresis (CE)

Traditional electrophoresis involves the movement of a colloidal or dissolved substance relative to a buffer solution as a result of an applied field. This leads to migration of the particles or ions towards either the cathode or the anode, depending on the effective charge of the particles. The term "zone electrophoresis" has been applied to those systems in which ionic mobilities are studied on strips of paper, cellulose acetate or acrylamide. These systems have been used extensively over many years for studying biological and bio chemical systems, especially separation of proteins. The more recent application of DNA fingerprinting in forensic science laboratories and for parental identification is typical of the value of modern electrophoretic methods.

More recently the big expansion in electrophoresis has been through the use of capillaries, work begun by Mikkers *et al,* who used capillary tubes with 200 μm internal diameter to achieve rapid separations. This has been advanced even further by Jorgenson and Lukacs, who reduced the capillary diameter to 75 μm using Pyrex tubes and led to the development of commercial instruments. By using capillaries, as distinct from fiat bead systems, it was possible to minimize zone spreading, improve heat dissipation, shorten separation times and increase efficiencies so they were comparable with values for high- performance liquid chromatography. Because of these factors, capillary electrophoresis (CE), also called capillary zone electrophoresis (CZE), has rapidly developed into a widely used process, and modern equipment can achieve the separation of complex steroid or drug mixtures within 10 to 15 minutes (Mendham *et al.*, 2000).

The essential CE system consists of a fused silica capillary filled with an aqueous buffer electrolyte; the two ends dip into containers of the electrolyte, one holding the anode, the other holding the cathode. The sample is introduced by inserting the anode end of the capillary into the sample vial and then applying an electric field to the sample vial, leading to electrokinetic injection, or by using pressure on the vial to produce hydrodynamic injection. Once a few nanoliters of the sample are in the capillary, it is replaced in the anode electrolyte container and the high- voltage power supply is used to bring about the separation. Auto samplers are now commonly available for handling large numbers of samples one after the other. The nature and consistency of the buffer solution must be constantly monitored, as it is likely to be affected by the migration and accumulation of solutes. CE development has occurred very rapidly, but most features in design of the components are now fairly standard. The various parts of the instrument are now described in more detail.

Capillaries

Although glass and Teflon capillary tubes have been used fused silica capillaries 30-100 cm long are now virtually standard equipment. They possess an internal diameter of 50-100 μm and external diameters of 200-400 μm. They are coated externally with polyimide for protection. Sometimes the capillary may have an internally bonded chemical surface, such as glycerol, in order to reduce interaction between the moving molecules and the capillary walls.

Applied field

Capillary electrophoresis needs high-strength electric fields to achieve rapid and efficient separation. The high-voltage power supply needs to provide voltages between 20 and 100 kV at constant voltage a typical current would be between 50 and 200 μA. In the ideal system it should be possible to operate either under constant or constant-voltage conditions and to reverse the polarity of the system.

Detector

Any CE instrument is only as good as its detector, and detectors continue to attract a great deal of study. The detector should aim to avoid causing any effect that might increase band broadening or the dead volume. Because of this, the most commonly used systems are on-column systems where the UV absorbance or fluorescence is measured while the material flows through a short length of the capillary. The standard approach for detection is to remove a short stretch of the polyimide plastic covering from around the capillary tube and to use it as the detector cell for a beam of light on one side of the capillary passing through to a detector on the other side. The usual absorption laws then apply, and the signal from the photo detector can be coupled to a recorder. As not all substances absorb at a single fixed wavelength, the system should be able to operate at a

range of wavelengths or it should incorporate a photodiode array to obtain complete spectral data as the zones pass through.

Detection may also be carried out using laser-induced fluorescence, benefiting from the way in which laser beams can be accurately directed to very specific sites in the capillary sample window. The fluorescence is observed at right angles to the laser beam and is passed through a filter to the photomultiplier. Other detection systems use the properties of chemiluminescence, thermo-optical absorbance and conductivity. CE may also be coupled with mass spectrometry. Forming a highly sensitive and specific detector system. In the CE/MS detector the capillary outlet is directed into an electrospray ionization interface with the mass spectrometer; this is achieved by passing the capillary outlet into a stainless steel sheath which completes the electrical circuit for the electrophoresis system. Detection limits for CE detectors are normally in the range of 10^{-16} to 10^{-20} mol.

Applications

Capillary electrophoresis is still a rapidly expanding field; new developments and applications are regularly published for both qualitative and quantitative analyses. As the use of migration times cannot be totally conclusive for qualitative analysis, it is in quantitative analysis that the greatest application occurs, based upon integration of detector signals and comparisons with the necessary standards. However, the great value of CE is its very reliable application to the analysis of inorganic ions and to organic anions. As with other separative procedures, samples can be spiked with reference standards. But in qualitative analysis, errors of peak assignments occur due to migration variation that arises from applied field fluctuations, changes in the capillary wall lining, pH alterations and progressive modifications in the buffer composition. To help overcome these problems, it is common to incorporate a migration time marker in the sample as a reference point for the calculations of relative migration times.

In quantitative analysis it is common to use the same calibration techniques as other analytical procedures: internal and external standards and standard addition methods. They greatly improve the level of reproducibility in CE quantitative analysis. But always take great care with respect to maintaining the quality of the capillary and the purity of the buffer system.

Figure 7. Capillary Electrophoresis, P/ACE System 5000 Beckman.