Liquid Chromatography

Liquid Chromatography is designed to analyze individual components in a mixture, using a column packed with a suitable material as a stationary phase and a liquid as a mobile phase. In the Liquid Chromatography, a test sample injected into the column is separated into individual components by flowing the liquid through the column under pressure using a pump. This method utilizes the difference in the retention capacity of individual components in the immobile phase. This method is applicable to liquids or substances which can be made into solutions, and used for identification tests, purity tests, assays, and other tests.

Apparatus The apparatus consists generally of a pumping system, a sample injection device, a chromatographic column, a detector, and a recorder. The chromatographic column is kept at a constant temperature by a thermostat, if necessary. The pumping system delivers the mobile phase to the column and joint tube, and others at a constant flow rate.

The detector detects components which are different in property from the mobile phase, and gives signals in proportion to its concentration for the substances of a few micrograms or less. Usually, ultraviolet- and visible-range spectrophotometer, differential refractometer, or spectrophotofluorometer are used. The recorder records intensity of signals obtained by the detector.

Procedure Condition the apparatus previously, adjust the mobile phase, the column, the detector, the flow rate of the mobile phase to the specified operating conditions as directed in the individual monograph, equilibrate the column at the specified temperature. Inject the test solution or the standard solution or control solution, prepared as directed in the individual monograph, into the sample injection device, using a sample valve or a microsyringe. The separated components are detected by the detector and recorded as a chromatogram on the recorder. Identification of the substances is carried out by confirming that the same retention time as for the standard solution is obtained, or that the retention time does not change nor does the peak width widen when the standard sample is added. Determination is generally performed according to either of the methods below, using the peak height or peak area, .

(1) Internal Standard Method Prepare several standard solutions containing a constant amount of the specified internal standard and known, graded amounts of the standard object component. With each of the chromatograms obtained by injecting a constant volume of each standard solution, calculate the ratio of the peak height or peak area of the standard objection component to that of the internal standard. Prepare a calibration curve by plotting these ratios on the ordinate and the

ratios of each amount of the standard object component to the amount of the internal standard, or the amounts of the standard object component on the abscissa. The calibration curve is usually a straight line through the origin. Then, prepare the test solution containing the same amount of the internal standard by the method prescribed in the individual monograph, record a chromatogram under the same conditions as for the preparation of the calibration curve, calculate the ratio of the peak height or peak area of the object component to that of the internal standard, and perform the determination, using the calibration curve.

(2) Absolute Calibration Curve Method Prepare standard solutions containing graded amount of the standard object component, and inject a constant volume of each standard solution, exactly measured. With the chromatograms obtained, prepare a calibration curve by plotting the peak heights or peak areas of the standard object component on the ordinate and the amounts of the standard object component on the abscissa. The calibration curve is usually a straight line through the origin. Then, prepare the test solution by the method prescribed in the individual monograph, record a chromatogram under the same conditions as in the preparation of the calibration curve, measure the peak height or peak area of the object component, and perform the determination, using the calibration curve.

For either method above, the peak height or peak area is generally measured using an appropriate one of methods (1) and (2) below.

(1) Peak Height Use either of the two methods.

Peak height method Measure the distance between the peak maximum and the intersection of a perpendicular line drawn from the peak maximum to the horizontal axis of recording paper and a line linking the both side inflection points of the lower end of the peak.

Automatic peak height method Determine the signal from the detector as the peak height, using a data processor system.

(2) Peak Area Use either of the following methods.

Width at half-height method Multiply the peak width at half-height by the peak height.

Automatic integration method Measure the signal from the detector and determine the peak area, using a data processing system.