27. Liquid Chromatography

Liquid Chromatography is a method to develop a mixture injected into a column prepared with a suitable stationary phase by passing a liquid as a mobile phase through the column, in order to separate the mixture into its components by making use of the difference of retention capacity against the stationary phase, and to determine the components. This method can be applied to a liquid or soluble sample, and is used for identification, purity test, and quantitative determination.

A mixture injected into the column is distributed between the mobile phase and the stationary phase with a characteristic ratio (k) for each component.

$$k = \frac{\text{amount of compound in the stationary phase}}{\text{amount of compound in the mobile phase}}$$

The ratio k represents the mass distribution ratio (or the capacity factor) k' in liquid chromatography.

Since the relation given below exists among the ratio (k), the time for which the mobile phase is passed through the column (t_0: time measured from the time of injection of a compound with $k = 0$ to the time of elution at the peak maximum), and the retention time (t_R: time measured from the time of injection of a compound to be determined to the time of elution at the peak maximum), the retention time for a compound on a column has a characteristic value under fixed chromatographic conditions.

$$t_R = (1 + k)\, t_0$$

Apparatus

Basically, the apparatus required for the liquid chromatographic procedure consists of a pumping system for the mobile phase, a sample injection port, a column, a detector and a recorder. A mobile phase component regulator, a thermostat for the column, a pumping system for reaction reagents and a chemical reaction chamber are also used, if necessary. The pumping system serves to deliver the mobile phase and the reagents into the column and connecting tube at a constant flow rate. The sample injection port is used to deliver a quantity of the sample to the apparatus with high reproducibility. The column is a tube with a smooth interior, made of inert metal, etc., in which a packing material for liquid chromatography is uniformly packed. A column with a stationary phase chemically bound on the inside wall instead of the column packed with the packing material may be used. The detector is used to detect a property of the samples which is different from that of the mobile phase, and may be an ultraviolet or visible spectrophotometer, fluorometric detector, differential refractometer, electrochemical detector, chemiluminescence detector, electric conductivity detector, mass spectrophotometer, etc. The output signal is usually proportional to the concentration of samples at amounts of less than a few μg. The recorder is used to record the output signals of the detector. As required, a data processor may be used as the recorder to record or output the chromatogram, retention times or amounts of the components. The mobile phase component regulator is used to vary the ratio of the mobile phase components in a stepwise or gradient fashion.

Procedure

Fix the detector, column and mobile phase to the apparatus, and adjust the flow rate and the column temperature to the values described in the operating conditions specified in the individual monograph. Inject a volume of the sample solution or the standard solution specified in the individual monograph with the sample injector into the column through the sample injection port. The separated components are detected by the detector, and recorded by the recorder as a chromatogram. If the components to be analyzed have no readily detectable physical properties such as absorbance or fluorescence, the detection is achieved by changing the components to suitable derivatives. Usually, the derivatization is performed as a pre- or post-column labeling.

Identification and purity test

Identification of a component of a sample is performed by confirming agreement of the retention time of the sample with that of an authentic specimen, or by confirming that the peak shape of the sample is unchanged after mixing the sample with an authentic specimen.

In general, the purity of the sample is determined by comparing the sample solution with a standard solution which is prepared by diluting the sample solution to a concentration corresponding to the specified limit amount of the impurity, or by the peak area percentage method. Unless otherwise specified, if a sample is separated into isomers in the chromatogram, the isomer ratio is calculated by using the peak area percentage method.

The peak area percentage method is a method to calculate the proportion of the components from the ratio of the peak area of each component to the sum of the peak areas of every peak recorded in the chromatogram. In order to obtain accurate results in evaluating the proportion of the components, it is necessary to correct the area of each component based on the relative sensitivity to the principal component.

Assay

(1) Internal standard method—In the internal standard method, choose a stable compound as an internal standard which shows a retention time close to that of the compound to be assayed, and whose peak is well separated from all other peaks in the chromatogram. Prepare several kinds of standard solutions containing a fixed amount of the internal standard and several graded amounts of the authentic specimen specified in the individual monograph. Based on the chromatogram obtained by injection of a fixed volume of individual standard solutions, calculate the ratio of peak area or peak height of the authentic specimen to that of the internal standard, and prepare a calibration curve by plotting these ratios on the ordinate against the amount of the authentic specimen or the ratio of the amount of the authentic specimen to that of the internal standard on the abscissa. The calibration curve is usually obtained as a straight line passing through the origin. Then, prepare a sample solution containing the internal standard in the same amount as in the standard solutions used for the preparation of the calibration curve according to the method specified in the individual monograph, perform the liquid chromatography under the same operating conditions as for the preparation of the calibration curve, calculate the ratio of the peak area or peak height of the objective compound to that of the internal standard, and read the amount of the compound

from the calibration curve.

In an individual monograph, generally one of the standard solutions with a concentration within the linear range of the calibration curve and a sample solution with a concentration close to that of the standard solution are prepared, and the chromatography is performed with these solutions under fixed conditions to determine the amount of the objective compound. Generally, the relative standard deviation (variation coefficient) is calculated to confirm the reproducibility of the ratios of the peak area or peak height of the objective compound to those of the internal standard, which are obtained by repeating the injection of a fixed volume of the standard solution.

(2) Absolute calibration curve method—Prepare standard solutions with several graded amounts of the authentic specimen, and inject accurately a fixed volume of these standard solutions. With the chromatogram obtained, prepare a calibration curve by plotting the peak areas or peak heights on the ordinate against the amount of the authentic specimen on the abscissa. The calibration curve is generally obtained as a straight line passing through the origin. Then, prepare a sample solution according to the method specified in the individual monograph, perform the liquid chromatography under the same conditions as for the preparation of the calibration curve, measure the peak area or peak height of the objective compound, and read the amount of the compound from the calibration curve.

In an individual monograph, generally one of the standard solutions with a concentration within the linear range of the calibration curve and a sample solution with a concentration close to that of the standard solution are prepared, and the chromatography is performed with these solutions under a fixed condition to obtain the amount of the component. In this method, all procedures, such as the injection procedure, must be carried out under a strictly constant condition. Generally, the relative standard deviation (variation coefficient) is calculated to confirm the reproducibility of the peak areas of peak heights of the objective compound which are obtained by repeating the injection of a fixed volume of the standard solution.

Method for peak measuring

Generally, the following methods are used.

(1) Peak height measuring method

(i) Peak height method: Measure the distance between the maximum of the peak and the intersecting point of a perpendicular line from the maximum of the peak to the horizontal axis of recording paper with a tangent linking the baselines on both sides of the peak.

(ii) Automatic peak height method: Measure the signals from the detector as the peak height using a data processing system.

(2) Peak area measuring method

(i) Width at half-height method: Multiply the peak width at the half-height by the peak height.

(ii) Automatic integration method: Measure the signals from the detector as the peak area using a data processing system.

Terminology

Reproducibility of test: Reproducibility of test is used as a method to ensure that the results obtained by a given procedure truly meet the requirements of the test described in the individual monograph. It is given as the relative standard deviation (S_R(%)).

Symmetry factor: Symmetry factor shows the degree of symmetry of a peak in the chromatogram, and is defined as S in the following equation.

$$S = \frac{W_{0.05\,h}}{2f}$$

$W_{0.05\,h}$: Width of the peak at one-twentieth of the peak height,

f: Distance between the perpendicular from the peak maximum and the leading edge of the peak at one-twentieth of the peak height,

where $W_{0.05\,h}$ and f have the same unit.

Relative standard deviation: Generally, it is given as S_R (%) defined by the following equation.

$$S_R\,(\%) = \frac{100}{\bar{X}} \times \sqrt{\frac{\sum_{i=1}^{n}(x_i - \bar{X})^2}{n-1}}$$

x_i: Measured value

$\bar{X}$: Mean of measured values

n: Number of repeated measurements

Complete separation of peak: Complete separation of the peak means that the resolution between two peaks is not less than 1.5.

Separation factor: Separation factor shows the relation between the retention times of peaks in the chromatogram, and is defined as α in the following equation.

$$\alpha = \frac{t_{R2} - t_0}{t_{R1} - t_0}$$

t_{R1}, t_{R2}: Retention times of two compounds used for the resolution measurement ($t_{R1} < t_{R2}$).

t_0: Time of passage of the mobile phase through the column (time measured from the time of injection of a compound with $k = 0$ to the time of elution at the peak maximum).

The separation factor (α) is a characteristic of the thermodynamic difference in partition of two compounds. It is basically the ratio of their partition equilibrium coefficients or of their mass-distribution ratios, and is obtained from the chromatogram as the ratio of the retention times of the two compounds.

Resolution: Resolution shows the relation between the retention time and the peak width of peaks in the chromatogram, and is defined as R_S in the following equation.

$$R_S = 1.18 \times \frac{t_{R2} - t_{R1}}{W_{0.5\,h1} + W_{0.5\,h2)}}$$

t_{R1}, t_{R2}: Retention times of two compounds used for measurement of the resolution ($t_{R1} < t_{R2}$),

$W_{0.5\,h1}$, $W_{0.5\,h2}$: Peak widths at half peak height,

where t_{R1}, t_{R2}, $W_{0.5\,h1}$ and $W_{0.5\,h2}$ have the same unit.

Number of theoretical plates: Number of theoretical plates is generally defined in terms of the following equation to indicate the extent of the band broadening of a compound in the column.

$$N = 5.55 \times \frac{t_R^2}{W_{0.5\,h}^2}$$

t_R: Retention time of compound,
$W_{0.5\,h}$: Width of the peak at half peak height,

where t_R and $W_{0.5\,h}$ have the same unit.

Note: Avoid the use of authentic specimens, internal standards, reagents or solvents containing substances that may interfere with the determination.

Among the operating conditions specified in the individual monograph, inside diameter and length of the column, particle size of the column packing material, column temperature, composition ratio of the mobile phase, composition of buffer solutions in the mobile phase, pH of the mobile phase, concentration of ion pair-forming agents in the mobile phase, ionic strength of the mobile phase, numbers of condition changes, timing of such changes, gradient program, composition and flow rate of derivative-producing reagents, reaction time and temperature of reaction chamber and flow rate of mobile phase may be modified within limits which allow the required elution order, resolution, symmetry factor, and relative standard deviation to be obtained.

28. Loss on Drying Test

The Loss on Drying Test is a method to measure the loss in mass of the sample, when dried under the conditions specified in each monograph. This method is applied to determine the amount of water, all or a part of water of crystallization, or volatile matter in the sample, which is removed during the drying.

The description, for example, "not more than 1.0% (1 g, 105°C, 4 hours)" in a monograph, indicates that the loss in mass is not more than 10 mg per 1 g of the substance in the test in which about 1 g of the substance is accurately weighed and dried at 105°C for 4 hours, and "not more than 0.5% (1 g, in vacuum, phosphorus (V) oxide, 4 hours)," indicates that the loss in mass is not more than 5 mg per 1 g of the substance in the test in which about 1 g of the substance is accurately weighed, transferred into a desiccator (phosphorus (V) oxide), and dried in vacuum for 4 hours.

Procedure

Weigh assurately a weighing bottle that has been dried for 30 minutes according to the method specified in the monograph. Take the sample within the range of ±10% of the amount directed in the monograph, transfer into the weighing bottle, and, unless otherwise specified, spread the sample so that the layer is not thicker than 5 mm, then weigh it accurately. Place the loaded bottle in a drying chamber, and dry under the conditions specified in the monograph. When the size of the sample is large, convert it to small particles having a size not larger than 2 mm in diameter by quick crushing, and use the crushed sample for the test. After drying, remove from the drying chamber, and reweigh accurately. When the sample is dried by heating, the temperature is within the range of ±2°C of that directed in the monograph, and, after drying the bottle, the sample is allowed to cool in a desiccator (silica gel) before weighing.

If the sample melts at a temperature lower than that specified in the monograph, expose the sample for 1 to 2 hours to a temperature between 5°C and 10°C below the melting temperature, dry under the conditions specified in the monograph. Use a desiccant specified in the monograph, and renew frequently.

29. Loss on Ignition Test

The Loss on Ignition Test is a method to measure the loss in mass when the sample is ignited under the conditions specified in each monograph. This method is usually applied to inorganic drugs which lose a part of the components or impurities during ignition.

The description, for example, "40.0 – 52.0% (1 g, 450 – 550°C, 3 hours)" in a monograph, indicates that the loss in mass is 400 to 520 mg per g of the substance in the test in which about 1 g of the substance is weighed accurately and ignited between 450°C and 550°C for 3 hours.

Procedure

Previously ignite a crucible or a dish of platinum, quartz or porcelain to constant mass, at the temperature directed in the monograph, and weigh accurately after cooling.

Take the sample within the range of ±10% of the amount directed in the monograph, transfer into the above ignited container, and weigh it accurately. Ignite under the conditions directed in the monograph, and, after cooling, reweigh accurately. Use a desiccator (silica gel) for the cooling.

30. Mass Variation Test

Mass Variation Test is the test to determine the uniformity of dosage units by mass variation. This test is not applied to the dosage forms to which *Content Uniformity Test* is applied. Apply the following test unless otherwise specified in the individual monograph. The *Content Uniformity Test* can be employed for this test when the method directed in the *Assay* is used for determination.

Select 30 units, weigh the first 10 units individually and calculate the acceptance value. The requirements are met if the acceptance value is less than or equal to 15.0%. When the acceptance value is greater than 15.0%, test the next 20 units. The requirements are met if the final acceptance value of the 30 dosage units does not exceed 15.0% and no unit shows a deviation that exceeds 25.0% of the label claim.

$$\text{Acceptance value} = |M - A| + ks$$

M: Label claim (100%), unless otherwise specified in the individual monograph.
A: Content of active ingredient (% of label claim) determined under Assay.

$$x_i = w_i \times A/\overline{W}$$

$x_1, x_2 \cdots x_i \cdots x_n$: Individual estimated contents of the units tested.
$w_1, w_2 \cdots w_i \cdots w_n$: Individual masses of the units tested.
$\overline{W}$: Mean of individual masses ($w_1, w_2 \cdots w_i \cdots w_n$).
n: Sample size (number of units in a sample).
k: Acceptability constant, $k = 2.2$ when the sample size is 10, and $k = 1.9$ when the sample size is 30.
s: Standard deviation of the sample.