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HIGH SPEED COUNTER CURRENT CHROMATOGRAPHY: A REVIEW

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ABSTRACT

High speed counter current chromatography (HSCCC) is a technique in which liquid stationary phase is use instead of solid supporting medium. HSCCC is a technique which provides number of advantages over other chromatographic techniques. In this article we focus on the principle, instrumentation and various application based on pharmaceutical science. In this HSCCC technique centrifugal partition technology is used to hold the liquid stationary phase in the column, while the liquid mobile phase is pushed through it that provides high purity and yield. The mechanism of HSCCC is based on the selection of two phase solvent system, partition coefficient, separation column, various solvent system, flow rate of mobile phase and revolution speed. In last few years great progress has been made in the pharmaceutical application of the HSCCC including extraction of medicinal drugs from plants and purification and isolation of active material, purification of dyes and compounds, plant analysis, rare earth elements' separation, analysis of inorganic compounds and elements, drug discovery and development.

KEYWORDS: High Speed Counter Current Chromatography (HSCCC), centrifugal force, solvent system, application.

INTRODUCTION

A liquid-liquid partition chromatographic technique in which use of solid support is not required is known as Counter Current Chromatography. In High-speed counter current chromatography (HSCCC) centrifugal force is used to keep one of the liquid phases in a multilayered coil column while the second liquid phase is pumped through the column. In the design of HSCCC instrument there are lots of potential variations that are especially for column geometries and rotation axes; this variation allows much greater scope for its

application and development than is available with CCC equipment. There is no solid stationary phase is exists thus CCC eliminates irreversible adsorption of samples into the support matrix and the tailing of solute peaks seen with traditional chromatographic columns.[1] HSCCC can provides more efficient mixing and tremendous separation efficiency as compared to the hydrostatic CCC systems with its new hydrodynamic equilibrium system, such as rotation ocular counter current chromatography (RLCCC), centrifugal partition chromatography (CPC), droplet counter current chromatography (DCCC). In recent years there has been seen great advances and blossoming of this kind of CCC technology, with improvement of instrument, method development, increases in the numbers of studies that reports about the basic theory, various application and skills summary of HSCCC. In separation science, this techniques is definitely becomes one of the conventional techniques. It is generally used in fingerprint analysis, screening of a wide variety of bioactive components from natural medicines, and functional food using many different aqueous organic two phase solvent system and for preparative separation, in the last decade.[2-5] For the study of these natural products, all this liquid technique is an attractive proposition, due to its ability to handle complex polar or non-polar crude material extracts with sample quantities from milligrams to tens of grams. Comparatively, now freshly developed (1993) pH-zone-refining CCC was applied from the outset to the separation of multi-gram quantities of dye mixtures such as xanthenes and suoran dyes used as colour additives in food, cosmetics and drugs and as biological stains.

Principle

High Speed Counter Current Chromatography (HSCCC) is depend on the principle, that is of the separation of the solutes between the two immiscible liquid phases. In this chromatography the use of a liquid mobile phase, a support free liquid stationary phase and a centrifugal force field is involves. When the liquid mobile phase is passed through the liquid stationary phase, the centrifugal force field is used to maintain the liquid stationary phase.[3] This kind of liquid partition chromatography is based on the differences, that is between the solubility's of the components of the stationary phase and mobile phase. In each phases, each compound has a different relative solubility. The compound is effectively dispersed between the mobile phase and the stationary phase. The dispersion can be quantified by division of the concentration of the compound in the upper phase and the concentration of the same compound in the lower phase. That is, the ratio of the division of the solute concentration in

the stationary phase and the solute concentration in the mobile phase and this is known as distribution ratio D.

$$D = C_s / C_m$$

Here, C_s is the concentration of the sample component in the stationary phase and C_m is the concentration of the component in the mobile phase.

As the separation occurs, mixing zones are formed and that leads to the separation between different solutes. The amount of liquid stationary phase present in the column can be changed. If the amount of stationary phase present in the column decreases, the chromatographic peak resolution can decrease. It has been found that there should be a good maintenance of the stationary phase and the solutes must have partition ratio that is between 0.5 and 2 for most favourable separation.[4]

MECHANISM

Selection of the two-phase solvent system

Usually a single phase is used to elute the analytes released from the adsorptive or liquid phase coated on the solid support in liquid chromatography. Comparatively, the CCC technique uses a two-phase solvent system which is made up of a pair of equally immiscible solvents, one used as the stationary phase and the other as the mobile phase. [5] The best pair of mobile and stationary liquid phase should be selected for effective HSCCC separation and for getting the best peak resolution and retention of the stationary phase. A minor change in composition of the mobile phase can have an effect on the composition of the stationary phase; hence it is a very important step. Initially it must form two immiscible phases. Here, non-reactive and innocuous solvents must be used. The factors which must be satisfied while selecting a solvent system are as follows: (1) Stability of the sample in the solvent system, (2) Solubility of the sample in the solvent system, (3) Settling time of the solvent system, (4) Satisfactory retention of the stationary phase in the column, (5) Partition coefficient of the sample.

Partition coefficient

The ratio of the distribution of solute between the two mutually equilibrated solvent phases is known as the partition coefficient, K. As in conventional liquid chromatography, generally it is expressed by the division of the amount of solute in the stationary phase and the amount of solute in mobile phase. Here, the better resolution of peaks will obtain if the partition

coefficient of the stationary phase will be higher. When the upper phase is used as the mobile phase, it should be pumped into the column in the tail-to-head elution mode but in case of lower phase it should be pumped into the column in the head-to-tail elution mode. The low flow rate of mobile phase will give a high retention of the stationary phase, which results in better peak resolution. The sample may be dissolved either in one phase or in a mixture of the two phases, and the volume of injection should be less than 5% of the total column capacity.[8] For the determination of the proper elution mode, consideration should be given properly to the settling time of the solvent, because higher correlation with the retention of the stationary phase has given by it.[9] The mobile phase viscosity, the interfacial tension, and the liquid-phase density difference affect the retention of the liquid stationary phase.[10]

Separation column

A multilayer coil separation column is used in High Speed Counter Current Chromatography uses a multilayer coil separation column, prepared from twisting a long piece of Polytetrafluoroethylene (PTFE) directly tubing around the spool shaped column holder making various layers. When this coiled column is subjected to a planetary motion, it produces a familiar effect which drives all objects of different density towards the end of the coil, one is called 'head' and the other is called 'tail', this effect is called the 'Archimedean screw'.

The following three size of Polytetrafluroethylene (PTFE) tubing is generally used in preparation of the multilayer coil (separation column) of the High Speed Counter Current Chromatography is,

a. 0.85 - 1.0 mm (analytical),
b. 1.6 mm (semi-preparative),
c. 2.6 mm (preparative).

Various solvent systems

The solvent system consists of 1-butanol/acetic acid/water, it is used for the polar compounds, for moderately hydrophobic compounds, hexane/ethyl acetate/methanol/0.1 M HCl is used, and for hydrophobic compounds, hexane/ethanol/water or hexane/acetonitrile can be used.[11] Earlier as a solvent system Carbon Tetrachloride and Chloroform are used because of their properties such as, low viscosity and high density, but detection of their carcinogenic activity stopped their use. Also the Dichloromethane and Diethyl ether can be used, but it leads to a vapour-lock that forces the stationary phase from the system and stops

the chromatography. The solvent system containing hexane, ethyl acetate, methanol, and water are most commonly used for the four component system. In four component system lower phase consists of methanol and water and the upper phase consists of hexane and ethyl acetate. The chance of emulsification in the column due to the presence of low polarity organic solvents like ethyl acetate. For that prevention a T-split with a small split ratio is used, through which only 1/20 of the flow stream can be introduced into the HPLC system.[12] The maintaining same ionisation states during the chromatographic separation for the solvent system have to be for ionisable compounds. A solvent system can be affected by addition of a small amount of acid or base or a low concentration of buffer as an aqueous component.[13] Two-phase solvent systems containing organic solvents are not suitable for the purification of proteins because of the property of denaturation of proteins in organic solvents. So, the aqueous polymer two-phase systems are used as they provide non-denaturing media to the enzymes, sub-cellular particles and cells.[2]

Flow rate of the mobile phase

The separation time, the amount of stationary phase retained in the column, and the peak resolution is determined by the flow rate of the mobile phase. Even if a lower flow rate is requiring a longer separation time, it generally gives a higher retention level of the stationary phase[22], which improves the peak resolution. The flow rates for the commercial multilayer coil are as follows,

a. 1 ml/min for an analytical column with 0.85 - 1.0mm i.d. PTFE tubing (1000 - 1200 rpm) (up to 50 mg sample load),
b. 2 - 3 ml/min for a semi-preparative column with 1.6 mm i.d. PTFE tubing (800 - 1000 rpm) (up to 500 mg sample load),
c. 5 - 6 ml/min for a preparative column, with 2.6 mm i.d. PTFE tubing (600 - 800 rpm) (up to 1 g sample load).

The above range of flow rates should be modifying according to the settling time of the two phase solvent system as well as other factors. The use of a lower flow rate is suggested if the settling time is around 20 second and K value of the analyte is small.

Revolution speed

For the High Speed Counter Current Chromatography, for preparative separation the most favourable revolution speed (revolution and planetary rotation speeds are always the same)

ranges between 600 and 1200 rpm (40 – 160 X g) with 10 cm revolution radius as per the i.d. of the separation coil. The volume of the stationary phase taken in the column will reduce by the use of a lower speed which leads to the lower peak resolution.

Instrumentation

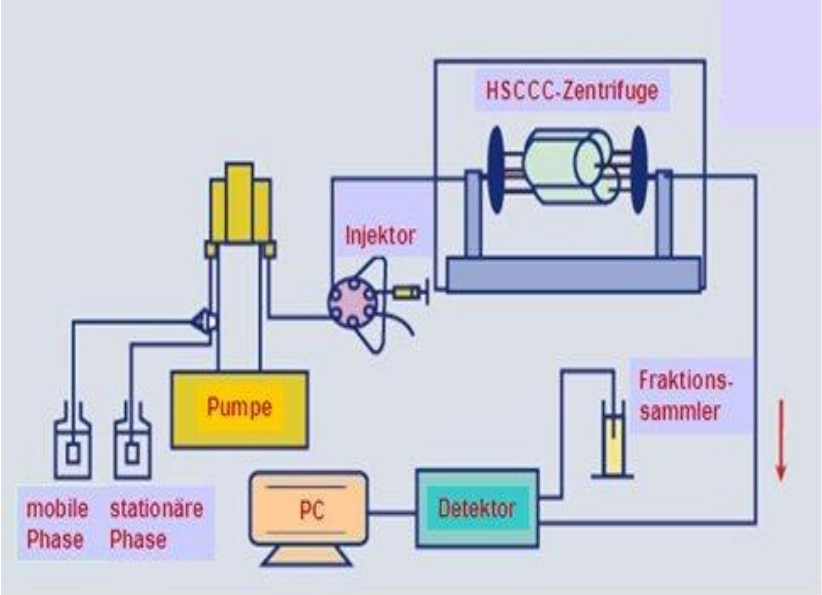

Figure-1: Schematic diagram of HSCCC unit

Above figure-1 shows the schematic diagram of "The High Speed Counter Current Chromatography". This HSCCC instrument consist of a column containing liquid stationary phase, a mobile phase reservoir, pump, injection valve, detector, a fraction collector and a recorder. Here the column consists of inert tubing that is made up of stainless steel or teflon, which is helically coiled and rotates on its own axis. The gear assembly is arranged in such a way that the helical coils revolves around a central axis and the planetary motion is produced. An oscillating force field is produced when the helical coil rotates on its own axis and revolves around a central axis at each given point along with the length of tubing.[23] Due to the planetary motion, the solvent in the column is depends on various velocities and centripetal acceleration which helps the stationary phase to remain in column and the mobile phase is pumped with the sample components. Thus the alternating zones of mixing is obtained from these velocities and centripetal acceleration. It also forms the partition of the sample material occurs between the mobile phase and the stationary phase, resulting in the separation of different components of the sample. A coil mounted on each planetary axis and it contains the coils of continuously wound Teflon tubing. The helix of the rotating coil tube produces a force known as the Archimedean screw, that pushes the liquids towards the head

and the tail, the higher pressure end and the lower pressure end of the tube respectively. The direction and intensity of the Archimedean screw force is depends on the direction and the rotational speed and helical pitch of the coiled tube.[24] In HSCCC, when the lower phase is preferred as the mobile phase the elution occurs in 'head-to-tail' mode and in opposite case the elution occurs in 'tail-to-head' mode. In order to improve the tracing, ethanol is mixed at a volume ratio of 1:5 to the effluent at the inlet of the detector. The column contents are collected in graduated cylinder by pressured air for determining the volume of the stationary phase retained column. By the division of the volume of the retained stationary phase and the total volume, the retention of the stationary phase in the column can be calculated.[11] Here, the HSCCC columns operate more rapidly than DCCC, the Craig and gravity based columns. Moreover, the spiral disk assembly consisting of multiple layers of plastic disks with spiral channels can be used for the improvement of the stationary phase retention, that works on the principle of utilizing a radially acting centrifugal force for retaining a sufficient amount of stationary phase of highly polar solvent system such as polymer phase system.[25-28] The important function of the spiral disks is to improve the column efficiency and to reduce the dead space in the transfer tubes. Here a special concern must be given towards leakage of the solvent from each of the spiral disk. The tubing damage which caused by vibration under the fluctuating centrifugal force field is protected by the spiral tube assembly that fixed in polyethylene glycol (MW 3350), which improves the separation of proteins with polyethylene glycol (PEG). This equipment is generally used for the separation of proteins and dipeptieds.[22,29-30]

Detectors for HSCCC

The detection of the separated components obtained by HSCCC is due to the high-performance liquid chromatography-diode array detection (HSCCC-HPLC-DAD). The effluent from the outlet of HSCCC is divided into two parts, from which one part is collected and the other part is introduced directly into an HPLCDAD system, for purity analysis, through a switch valve. The purities of the obtained fractions from HSCCC are monitored and fractions with high purities are collected.[31] In the HSCCC separation techniques, a UV detector with Hg lamp 254-280 nm is generally used. Other detectors can also be used such as mass spectrometric detector, pH detector, evaporative light scattering detectors (ELSD), conductivity detector. For example, ELSD is used for systematic separation and purification of non-chromophoric chemical components from Chinese medicinal herb Adenophora

tetraphlla. The 2996 photodiode array detector is used for the purification and isolation of ginkgo flavonol glycosides from Ginkgo biloba leaves by HSCCC.

Applications

Extraction of medicinal drugs from plants and purification and isolation of active material

HSCCC is a technique used for a large range of compounds that includes peptides, synthetics, and natural products.[38] For obtaining pure form of major kavalactones kavain and demethoxyyangonin, HSCCC is the best method.[39] This method is useful for the isolation of the natural product from the raw material as well as from antibiotics and isomers.[40],[41] For example, Indole auxins can be saperated and Flavonoids can be separated from a crude ethanol extract of sea buckthorn (Hippophae rhamnoides), obtained with a two-phase solvent system composed of chloroform-methanol-water (4:3:2, v/v/v). By using a salt free solvent system such as ethyl acetate-n-butanol-water (3:2:5), tetracycline derivatives can be separated. Indole auxins can be separated and obtained by using a two-phase solvent system composed of n-hexane-ethyl acetate-methanol-water (1:1:1:1, v/v/v/v). The separation of DNP amino acid is done by using a two-phase solvent system composed of chloroform-acetic acid-0.1 M hydrochloric acid (2:2:1, v/v/v). Sulforaphane can be isolated from Broccoli Seed Meal using a two-phase solvent system composed of n-hexane / ethylacetate/methanol/water (1:5:1:5,v/v/v/v).[42] Isolation of alkaloids palmatine, berberine, epiberberine, and coptisine from Coptis chinensis Franch can be done by using a system of chloroform-methanol-HCl solution (0.3 – 0.1 mol/L) at different volume ratios.[43] By using an n-hexane-ethanol-water (6:5:5) system bioactive lignans schisanhenol and its acetate can be separated in a multilayer coil column.[44]

Purification of dyes and compounds

HSCCC is used for the purification of dyes. Quick purification of target compounds from crude samples or semi-pure fractions or complex mixtures such as triterpenoic acids can be done by it.[45] Compounds with a wide range of polarities in natural product research can be separated by it.[46],[47] Ranging of sample quantities from sub milligram to 100 mg are successfully split in few hours. Partition efficiencies of up to several thousand theoretical plates are provided by it. It becomes difficult to isolate many complex mixtures because the main constituents are present in a minor quantity in such a complex mixtures, such as plant extracts and microbial fermentation products, but liquid stationary phase in HSCCC proved

to be favourable in separation of bioactive compounds from natural sources like animal tissues, plant extracts, microbial fermentation products.[5],[6] for example, the isolation of the components of bacitracin, which consists of a group of peptides with a bactericide activity. It contains the major active component, BC-A, its oxidation product, BC-F, and over 20 other minor components, as shown by a reverse-phase HPLC analysis. The analysis of wide range of wine constituents is done by use of it. The problem of direct extraction and separation of some organic substances from a sewage sludge medium is resolved by HSCCC.[49] It is used in the purification of recombinant proteins directly from a crude E. coli lysate. A variety of labile wine aroma precursors, antioxidants, and pigments are studied. The fractionation of polymeric wine constituents helps in analytical research.[48] HSCCC is used in the separation and purification of Quercetin-3-O-sambubioside from the leaves of Nelumbo nucifera.[32]

Plant analysis

HSCCC is used in the separation of small synthetic molecules from large bio molecules. Biological activity is preserve by the help of liquid-liquid partition in solute retention. It provides a fast and effective separation of compounds with separation having problematic solubility or difficult separation in the existing reverse phase chromatography purification, it has been effective in the analysis of natural pigment such as carotenoids, anthocyanins, betalains and chlorophyll-related pigments. Anthocyanins can be isolated from elderberries (Sambucus nigra L.), blackberries (Rubus fruticosus L. agg.), Purple Heart (Tradescantia pallida Rose), red wine, purple corn (Zea mays L.). [50] HSCCC can analyse various polar and non polar natural pigments, for example red wine, black carrot juice, blood orange juice, tayberry and so on successfully.[51] A solvent system composed of ethyl acetate-10 mM potassium phosphate (1:1, v/v) at pH 6.5 is used to separate esculin, coumarin, 2- and 3- or 4-hydroxycinnamic acids.[52] It helps in the identification of bioactive lignans from plant natural products.[53] The study of plant alkaloids is possible when this technique is coupled with mass spectrometer. For the separation of labile substances like aroma compounds or their respective precursors (polyols and glycocongugates); flavor analysis is also important applications, from complex natural mixtures. Such as, the analysis of reactive flavour precursors from Rosa damascene flowers has been reported.[55] The separation of cell fragments and fractionation of complex mixture becomes easy.[54] It is also useful in validating the presence of a specific compound in a mixture and in the analysis of non-volatile or thermally unstable molecules and in the determination of an unknown impurity.

Separation of rare earth elements

Rare earth elements are essential for nutrient uptake, biomass, quality, chlorophyll formation, plant root development and resistance against stress. HSCCC is not only used for the recognition of rare earth elements and compounds in mixtures, but also for their separation for example, yttrium,[56] thorium,[57] and lanthanides.[58] HSCCC is used for the successfully separation of Lanthanides like La^{+3}, Pr^{+3} and Nd^{+3}.It is also used for the separation of lighter rare earth metal ions.[59] It is also used in the purification of xanthanolide.[60] By HSCCC aqueous PEG-Na2SO4 biphasic system containing acetylacetone is used to separate La(III), Ce(III), Nd(III), and Yb(III) by HSCCC. [61] For the metal-ion enrichment the multilayer coil system is used. It can concentrate metal ions in very small quantities thus it is superior to other analytical techniques. It is generally used for the recognition of metal ions at trace levels. The trace quantity of zinc in natural mineral water can be determined by the enhancement separation through an HSCCC compounds.

Preparative-scale separations of chiral compounds

The preparative-scale separations of chiral compounds are done by a multilayer coil planet centrifuge.[63] By using HSCCC, the (+) and (-) enantiomers of a racemic compound mixture can be separated. This can be done by the addition of a chiral selector to a first liquid phase of two pre-equilibrated immiscible liquid phases and then charging a counter current chromatographic centrifuge column with that liquid phase. Thus the result obtained is, the formation of a counter current chromatographic centrifuge column charged with a chiral selector, and the racemic compound mixture is introduced into the centrifuge column afterwards, then after a second liquid phase is passed through the centrifuge column, thus charging it with a mixture, chiral selector, and first liquid phase, to elute the (+) enantiomer and (-) enantiomer from the counter current chromatographic centrifuge column.

Analysis of inorganic compounds and elements

The separation of inorganic substance is different from those of organic substance because inorganic substances contain complexing agents. As this process is a non-equilibrium process, the separation competence is depending on the type of complexation process, the mass transfer rate of the process and rate of complexation process.[34], [64] The effective separation of Copper, Magnesium, Nickel, Cobalt, Iron and other inorganic elements is done by HSCCC. Concentrations of Sm and Nd isotope are also determined in rock samples.

Drug discovery and drug development

A major application of HSCCC is product development and discovery of drug, where increasing from milligrams to grams and then to kilogram is easily possible. Development of method for a drug is possible due to 100% recovery of the sample is obtained by HSCCC.[13] Additionally, it is a high resolution separation method and hence the isolation of a large range of stable and unstable compounds is possible which helps in drug discovery. By using this technique, fractionation of natural products is obtained that leads to the discovery of drug, moreover separation of two compounds having similar polarity can be done by this method. In this technique the liquid stationary phase is used that analyse natural metabolites successfully. The performance of the HSCCC column is depends on the amount of stationary phase taken in the column. The separation of complex natural extracts can be done by it. The separation of organic compounds is nicely done by a multiple-layer coiled column. Here, the planetary motion is also used to set- up a hydrodynamic equilibrium between two immiscible solvent phases that enables short separation times. Hence, it helps in the development in the separation methods of existing drugs and new drugs discovery.

CONCLUSION

High speed counter current chromatography (HSCCC) is a superior preparative separation technique. It is better technique in comparison to the other separation technique because in this technique the utilization of support free liquid stationary phase and there is no loss of sample with high separation effectiveness and resolution by using the centrifugal field. So, the using of liquid phase, it is free from the adsorption of solutes to the column and also the recovery of samples and reagents are without contamination or decomposition. There is a possibility to use same column frequently for separation using with the different stationary phase. In drug development and drug discovery the quantity of the product will be easily scale up from milligrams to grams and then to kilogram by using HSCCC. Another advantage is an exceptional technique for the separation of natural pigments and bioactive constituents, which are available in very small quantity.

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