

Advancement of Chiral Resolution and Separations: Techniques and Applications

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Abstract. Chirality, a ubiquitous phenomenon in nature, denotes the incapability of an entity to superimpose onto its mirror image. This property is inherently manifested in biological systems, where critical biomolecules such as DNA, enzymes, and proteins exist as chiral substances. Notably, proteins often demonstrate enantioselectivity towards their interacting partners, underscoring the critical role of chirality in drug-protein interactions. Consequently, the chirality of pharmaceutical agents significantly influences their efficacy and interaction with targeted proteins, necessitating a profound understanding and ability in chiral separation science to address challenges about chiral drug availability. Despite the complexity of enantiomer separation, the past few decades have witnessed substantial advancements in chiral resolution techniques. This article elucidates several pivotal methods: crystallization-based techniques, chromatographic separation, kinetic resolution, membrane-based separation, etc. Furthermore, the author spotlighted the application of chiral resolution methodologies at various drug research and development junctures, exemplified by a detailed case study on Sotorasib. This discourse aims to accentuate the burgeoning significance and the strides achieved in chiral resolution, paving the way for future innovative developments in this vital scientific domain.

Keywords: Chiral resolution, chirality, chromatographic separation, crystallization.

1. Introduction

Chirality, as the name suggests, is like a person's left and right hands, with the same structure, mirror images of each other, and will not coincide no matter how they rotate. Two chiral molecules mirror each other and cannot coincide. Such two chiral molecules are called enantiomers; they are chemically identical and interact with other chiral molecules or environments differently. Chirality is a universal property of matter, and chiral compounds are widely found in nature. The basic building blocks of life on Earth, amino acids, are almost all L-amino acids, without D-amino acids. The helical conformation of more complex proteins and DNA is right-handed. The receptors or targets that drug molecules play are chiral proteins and nucleic acid macromolecules composed of amino acids, nucleosides, membranes, etc. These locations are mostly chiral environments, so there are certain requirements for the spatial stereoscopic configuration of the drug molecules bound to them, and for this reason, many physical properties (melting point, boiling point, etc.) of enantiomeric drugs are the same. However, the physiological properties are often different, and there are often great differences in pharmacodynamics, pharmacokinetics, and other aspects of *in vivo*.

For example, the antihistamine effect of chlorpheniramine D-isomer is 100 times stronger than that of L-isomer. The intravenous anesthetic ketamine, S- (+)-isomer has a separating anesthetic effect, while the R- (-)-isomer can produce excitement and mental disorders. Another example is thalidomide, one of the most notorious drugs that led to the tragic global medical disaster of limb deformities in the late 1950s. Its (R)-enantiomer has a sedative effect, while the (S)-isomer has a teratogenic effect.

Since the 1990s, the trend towards developing chiral drugs has been evident. The U.S. Food and Drug Administration (FDA) issued guidelines called "Development of new stereoisomeric drugs" in 1992. When a single isomer drug is developed, considering that the transformation and disposal processes of a single enantiomer drug in the organism are different, the relevant data of each enantiomer should be provided during the drug development process. In recent decades, the sales volume of chiral drugs worldwide and their share in the total number of medicines have also increased yearly.

Therefore, the availability of chiral molecules is crucial to human health. Asymmetric synthesis, extraction of natural chiral compounds, and chiral decomposition are the main methods for obtaining chiral compounds. Several chiral resolution methods are described here. Common separation methods of chiral molecules mainly include crystal resolution, chemical fractionation, chromatographic resolution, biological resolution, membrane separation, etc., among which chromatographic resolution can be divided into thin layer chromatography (TLC) chiral resolution, gas chromatography (GC) chiral resolution, high-performance liquid chiral chromatography (HPLC) and supercritical fluid chromatography (SFC).

2. Crystallization Resolution

Louis Pasteur was the first documented person to separate stereoisomers. In 1848, the sodium ammonium tartrate salt could precipitate two mirrored crystals from its saturated solution at low temperatures and separate the crystals with different crystal shapes one by one with forceps under a microscope to obtain two mirror crystals. It was found that the solution containing the half-crystal facing the right crystal showed right rotation, and the half-crystalline left-facing crystal solution showed left-hand optical performance. Pasteur's discovery confirmed the phenomenon of optical isomerism (also known as an enantiomeric phenomenon), officially unveiled the most mysterious of chirality and profoundly impacted the development of stereochemistry. Pasteur's experiment was also rated as one of the "most beautiful chemical experiments in the world". Until now, crystallization resolution is still a very important chiral resolution method [1].

Salt-forming crystal resolution is also a more classic chiral resolution method, the mechanism of which is to add a chiral resolution agent to the system to be separated, and the resolution agent can selectively form salt with only a certain enantiomer or form a pair of diastereomeric salts with the racemate, and then realize crystallization separation according to the difference in solubility between the isomers. For the current drugs, 75% of drugs are weak bases, and 20% are weak acids. So, it is possible to develop pharmaceutical salts to resolve the isomers. For the resolution procedure development of a given racemic compound, the first step is to find the most suitable resolving agent, which could be time-consuming but the most important step [2].

Natural alkaloids, such as brucine, cinchonine or quinine, can resolve racemic acids. A disadvantage of these alkaloids is their toxicity; only one enantiomer is available. Other basic resolving agents are phenylethylamine, ephedrine, etc. The disadvantage of the resolution method is that the resolution yield is low, the theoretical yield is only 50%, and another half of the products are unwanted enantiomers. Discarding unwanted enantiomer is significant waste; it is a waste of resources and environmental pollution. If the unwanted enantiomers can be racemic, reconverted into racemates, and then can be recycled and resolved. The recycling process of resolution-racemization is a direct and effective way to remedy this disadvantage. In recent years, with the deepening of the research on eutectic systems, eutectic systems have been increasingly used in chiral separation. The operation and mechanism of eutectic resolution and salt-forming resolution are similar, except that the force between the resolution agent and the component to be separated in salt-forming separation mainly relies on ionic bonds. In contrast, the force in eutectic resolution is mainly manifested as hydrogen bonds.

3. Chromatographic Resolution

In 1966, Gil-AV and colleagues first used GC for direct enantiomeric separations [3]. It is a landmark event for chiral chromatography. Chiral chromatography has been a key technique for analyzing or separating enantiomers for five decades and is now widely used for the direct separation of racemic mixtures. There are usually three methods for the principle of chromatography in separating enantiomers. (1) the use of chiral reagents and the fractionated substance for derivatization reaction to generate diastereomers, which can be resolved by traditional chiral chromatography; (2)

Chiral additives are added to the mobile phase and resolve by using the non-chiral stationary phase; (3) Chiral Stationary Phase (CSPs) for resolution. The most effective methods are the CSP method. The reason why CSP can resolve the enantiomer is that when the chiral stationary phase interacts with the racemate, one of the enantiomers and the stationary phase form an unstable transient enantiomer complex, resulting in different retention times during column elution to achieve the purpose of resolution. The application of chromatographic resolution is divided into analytical level and preparative level.

3.1. Chiral TLC Resolution

Using TLC plates with chiral selective stationary phases, chiral substances can be separated by those TLC plates. This method is simple to operate, convenient and economical, but because the sensitivity of its quantitative analysis is not high, it is now mainly used for qualitative analysis. The CSP sources used in this method include β -cyclodextrin, chiral amino acid ligands, chiral ion-pairing reagents, etc. [4]. Chiral TLC is an alternative to chiral separation, especially when rapid analysis is required with simple equipment.

3.2. Chiral GC Resolution

The first try of chromatographic resolution is the chiral GC method, which is also effective for the chiral separation of volatile compounds. GC has the advantages of high effective energy, high sensitivity, strong selectivity, fast speed, wide application and easy operation, which samples detected should have high volatility and thermal stability. β -cyclodextrin is also commonly used as the CSP for chiral GC. In recent years, porous organic materials have received more and more attention as CSP for chiral GC, which is porous materials assembled from discrete organic molecules with shape-durable and permanent cavities through weak intermolecular forces [5].

3.3. Chiral HPLC Resolution

Chiral HPLC is widely used as a chiral identification and resolving tool for racemic mixtures of various structures, especially in separating photoactive substances prone to racemate, and has unique advantages over stereoselective synthesis [6]. Chiral stationary phases are the most efficient HPLC resolution method. The CSPs-HPLC method can resolve the enantiomer because when the chiral stationary phase interacts with the racemate, one of the enantiomers and the stationary phase form an unstable transient enantiomer complex, resulting in different retention times during column elution and achieving the purpose of resolution.

Chiral stationary phases are divided into the following categories: i) Chiral polymer bonded stationary phases. It is divided into natural glycan derivatives (cellulose, starch derivatives, etc.) and synthetic chiral polymers; ii) Protein and glycoprotein bonding stationary phase. The unique primary and tertiary structure of proteins plays a very important role in chiral recognition, especially the hydrophobic grooves formed by the tertiary structure, which are important for forming diastereotype complexes; iii) Price type. It is prepared by covalently bonding a monolayer of chiral organic molecules to a mechanically good carrier (such as silica gel); iv) Macrocyclic class. This chiral stationary phase is prepared by bonding or coating a chiral macrocyclic molecule to the surface of silica gel, including chiral crown ether, cyclodextrin, and the newly emerging macrocyclic antibiotic; v) Molecular imprinting. Since the resolution of enantiomers of receptor-blocking drugs was reported in 1991, many meaningful molecules, including drugs, peptides, and nucleic acids, have been separated. More than 700 chiral stationary phases have been studied, and many analytical columns have become marketable commodities. Chiral enantiomer separation using chiral columns is a very efficient and straightforward method.

3.4. SFC Chiral Resolution

In the 1960s, supercritical fluids were introduced into chromatography as mobile phases. This is called supercritical fluid chromatography [7]. In its early years, SFC was conceived as a hybrid of

HPLC and GC. SFC is considered a rapid separation tool comparable to HPLC, and it can be used to separate high boiling point substances, which is impossible in GC. Recently, some companies have invested time and resources in developing powerful, user-friendly SFC instruments. Those advances promote the possible further use of SFC in the pharmaceutical industry. SFC has many practical advantages.

Since CO₂ replaces most mobile phases, one advantage of SFC is the low amount of solvent. This advantage may not be apparent in analytical-level separations but is significant in preparative-level separations. Many purification laboratories spend considerable time removing solvents from fractions, greatly limiting the efficiency of obtaining the desired product or result after complete compound purification. In SFC, CO₂ in the mobile phase is removed by step-down, leaving only a small amount of co-solvent. The resulting fraction also has a higher product concentration, shortening the time required for solvent removal and product separation. The fraction can also be used directly for analysis, eliminating the need for sample enrichment or concentration. This is critical for compounds that degrade under normal, prolonged drying conditions.

Other advantages of the lower SFC organic solvents include cost savings, safety (in terms of flammability and toxicity), and lower environmental impact. Regarding solvent purchase and disposal, SFC's cost advantage is considerable. In addition, further cost savings are achieved due to the lower energy consumption required to remove solvents. SFC also avoids using toxic solvents such as acetonitrile used by RPLC and aliphatic hydrocarbon and chloride solvents used by NPLC. As a by-product of other industrial processes, CO₂ is relatively inexpensive and can be recycled.

SFC efficiency is improved by the low viscosity and high diffusion of the mobile phase for chromatographic speed and efficiency. So that chiral separation can achieve a good separation effect even at high flow rates. When the sample returns to normal pressure after the SFC separation is completed, the CO₂ in the fluidity is directly vaporized, which greatly shortens the post-processing time due to the lower energy consumption required to remove the solvent and reduces the heat load during spinning, further saving costs. Since the mobile phase is CO₂, good separation results can be achieved when separating acidic compounds without adding other reagents.

3.5. SMB Chiral Resolution

SMB separation technology is based on chromatographic adsorption separation, originated in the petrochemical industry, was first proposed by the United States Global Oil Products Company and separated paraxylene [8]. From the 60s of the 20th centuries to the present, simulated moving beds have been extended to fine chemicals, pharmaceuticals, food and other industries. SMB technology combines adsorption and chromatography and is widely used in chemical, sugar, and biopharmaceutical industries because of its advanced technology. Its core principle is to simulate the countercurrent contact between solid and liquid and use the force between maximizing mass transfer to achieve binary separation, which reduces material consumption compared to traditional chromatographic separation methods.

The SMB originated in chromatographic separation technology, which is based on the difference in the partition coefficient of the separated components between the mobile and stationary phases, and the mobile phase is mixed into a column filled with the stationary phase to separate the mixture. According to the operation mode, liquid chromatography separation can be divided into a batch and two continuous batches; the operation is simple and easy to implement, but because of the low throughput, large mobile phase consumption, low adsorbent utilization rate and other shortcomings, it is gradually replaced by continuous chromatographic separation technology. For example, SMB technology can isolate 10 Ton (R)-3-chloro-1-phenylpropanol from racemates per year, an intermediate of antidepressant (R)-fluoxetine. Increasing the separation amounts of chiral drugs while maintaining high efficiency is the main problem SMB faces in chiral drug fractionation.

4. Membrane Chiral Resolution

The membrane separation method is to make chiral molecules pass through the biofilm, and the pore size of the biofilm and the biomolecules on the surface of the biofilm will recognize it so that the required enantiomers pass through the membrane pores, while others cannot pass through, to achieve the purpose of separation. The advantages of the biofilm separation method are low pollution, low energy consumption and effective consumption. Membrane separation is a promising high-efficiency chiral separation method. According to the membrane's morphology, chiral membranes are usually divided into liquid films and solid membranes. The liquid film exists between the two liquid phases, and chiral molecules are resolved in the liquid film. Therefore, one of the enantiomers is preferentially transferred to achieve the separation of the racemate. However, the liquid film's poor mechanical stability and durability limit its usage. Since solid films are unaffected by these drawbacks, they have more potential for large-scale chiral resolution.

The membrane separation technology has attracted great research interest because of the following advantages: 1) The membrane separation is highly efficiency with low energy consumption. 2) It is easy to achieve continuous operation, and the process is relatively simple and suitable for large-scale chiral resolution. 3) Membrane separation has low requirements for production and operation conditions. 4) The process does not require heating, improving safety and reducing unnecessary losses. So, membrane separation technology is increasingly used for drug separation [9].

5. Case Study

For those resolution methods, GC is very useful for small quantities of preparative separations and the preparation of thin-layer chromatography. LC has been proven to be the technique of choice in drug discovery to achieve a few milligrams to kilograms of material, which can cover the preclinical API needs. Over the past few years, other enantiomeric separation methods, such as SFC, have been improved to prepare kilogram-sized samples. SMB, on the other hand, can prepare samples from hundreds of kilograms to tons. The use of membranes is still limited to a few drugs, and more research is needed, but due to its relatively low cost and ease of manipulation, it is noted as a promising method for preparative-grade scale enantiomeric separation. Traditional salt-forming crystallization can be used for large-scale production. Like Sotorasib's research process, different stages of research and development require different chiral separation methods to ensure the smooth progress of research and development [10].

Sotorasib is an axial chiral compound, a star of new drugs in recent years. During the development of single atropisomer Sotorasib, chiral HPLC was used to isolate isomers in the drug discovery stage. The first 2 kg batch of Sotorasib API for clinical studies relies on chiral chromatography separation. A subsequent 10 kg clinical batch was performed using SMB chromatography, which reduced time and solvent usage compared to conventional chromatographic separations. For commercial batches of Sotorasib APIs, the research team turned to find a solution based on the traditional crystallization method. In developing the classical resolution of key intermediate rac-6, the team used high-throughput experiments to determine the appropriate separating agent and solvent system to achieve selective crystallization of the (M)-6 isomer. The success of this separation process enabled the preparation of multi-metric tons of (M)-6 while avoiding the initial need for large-scale chiral chromatography. At the same time, to avoid the waste of other isomers of the resolution, the efficiency of the recovery process is further improved by developing and optimizing the recovery and recycling process, providing a more environmentally friendly method for Rac-6. The key intermediate rac-6 is separated by the traditional crystallization separation method to obtain the required tonnage (M)-6, and the (P)-6 racemic recovery and recycling process was developed too. Secured the availability of commercial batches of the Sotorasib API.

From this case, preparative chromatography is the key for early preclinical and clinical study and has played a key role in delivering pure Sotorasib in early development activities, but it is not the first choice for commercialization. In addition to the previously mentioned drawbacks (high solvent usage,

low throughput, etc.), this approach is complicated for the supply chain and needs more cycle times due to the need for a third party for chromatographic separations. The traditional crystallization method is time-consuming, but a suitable crystallization is key for the drug's commercial batches.

6. Conclusion

Chirality is one of the most common phenomena in nature and living systems. Chiral substances, especially chiral drugs, play a very important role in many fields, such as life sciences, medicine, and chemistry, and closely affect human health. Chiral drugs have high efficacy, less toxicity, broad application prospects, and market potential in medicine. There are significant differences in the biological activity of chiral drugs and the pharmacological effects, metabolic processes and toxicity of enantiomers in the human body, and some even produce diametrically opposite effects. With the deepening of people's research on chiral drugs, drug regulatory authorities have also put forward clear regulations on drug research and development. At present, the newly approved drugs are chiral. Therefore, exploring innovative ideas to obtain single chiral compounds more efficiently has become an important research direction for more and more scientists and pharmaceutical companies.

In recent years, with scientists' research on the separation of chiral compounds, chiral separation technology has achieved certain achievements. However, there are still limitations in the separation of chiral compounds in industry, such as limited application range and high cost of different separation technologies. These methods described above have advantages, limitations, and certain complementarity, and it is these complementarities that allow us to obtain more single enantiomers with high efficiency. The separation and analysis of chiral drugs is a hot area in research, and future research of chiral separation technology will develop in the direction of low cost, high separation efficiency, simplicity, speed and green production. After the continuous research of the majority of researchers, chiral separation technology will become more and more mature, better assist and accelerate drug research and development.

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