A Q&A

Reversed-Phase Liquid Chromatography using Surrogate/ Additional Stationary Phases

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Neuland's approach to chromatography has applications in laboratories faced with improving efficiency and throughput using existing technology.

Liquid chromatography has several important applications in the pharmaceutical realm. The technique can be used to identify or to quantitate an analyte or active pharmaceutical ingredient (API). Another important function of liquid chromatography is as a preparative method to separate an analyte or active pharmaceutical ingredient (API) from their related substances. Techniques that can increase the resolution between related substances and the API are vitally needed for establishing the identification, assay, and preparative output.

Pharmaceutical Technology recently spoke with Mohmed (Mike) K. Anwer, PhD, vice president and head of Neuland Laboratories Limited in Hyderabad, India, and Christopher Cimarusti, PhD, a consultant with CMC Development, about the use of a surrogate/additional stationary phase bound to C18/C8 reversed-phase media. The pair also discussed the effect of this set-up on resolution parameters such as retention, efficiency (i.e., number of theoretical plates), tailing (i.e., peak asymmetry), and selectivity.

Pharmaceutical Technology: What is a surrogate stationary phase? How does it differ from an additional stationary phase?

Cimarusti: We use the terms surrogate stationary phase (SSP) and additional stationary phase (ASP) interchangeably. Hydrophobic quaternary ammonium salts, such as tetrabutylammonium hydrogen sulfate, bind to the C18 chains of a reversed-phase column and act as additional stationary phase sites for the analyte to bond. It is well documented that the inclusion of quaternary ammonium salts in mobile phases in classical ion-pair reversed-phase HPLC will achieve superior resolution of analytes.

There is, however, a fundamental difference between a tetrabutylammonium ion acting as an ion-pairing agent and a tetrabutylammonium ion acting as an SSP. When it acts as an ion-pairing agent, it affects the interaction of an unmodified stationary phase with a modified analyte. When it acts as an SSP, it affects the interaction of a modified stationary phase with an unmodified analyte.

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Pharmaceutical Technology: How is an SSP-coated C18 column different from a standard C18 reversed-phase column?

Anwer: The SSP binds to C18 chains and to residual silanols. These additional binding sites increase the loading capacity of a reversed-phase column. Generally, the optimized loading capacity of a standard C18 reversed-phase column is about 1% of the total column volume. Due to additional and more exposed hydrophobic sites from the ASP/SSP, the column's loading capacity or preparative output is increased by seven to 10 times. The optimized preparatory output of a normal silica column is about 7% of its total column volume at about seven times the preparatory output of an equal volume of a reversed-phase column.

Pharmaceutical Technology: Do all SSPs interact similarly with C18 chains?

Cimarusti: Essentially, yes. The binding of the SSP to the C18 chains of the reversed-phase column depends on the number of carbons comprising the alkyl chains of the quaternary ammonium salt. SSP, such as tetrabutylammonium salt, would bind to the C18 chains at a lower concentration of the organic solvent (e.g., below 10% aqueous acetonitrile) and elute at a higher concentration of the organic solvent (e.g., greater than 20% aqueous acetonitrile).

Hydrophobic quaternary ammonium salts containing more than six carbon atoms in the alkyl portion of the quaternary ammonium salt require a higher concentration of acetonitrile for desorption from the C18 chains. Therefore, such chains are tightly bound to the C18 chains of the reversed-phase stationary phase, even at 60% aqueous acetonitrile. These latter SSPs allow a higher concentration of organic modifiers to be used as eluents.

Pharmaceutical Technology: Can you describe the general features of an SSP?

Anwer: Any quaternary ammonium or phosphonium salt that has alkyl or other lipophilic moieties that can adhere to the C18 and C8 chains of the reversed-phase column can function as an SSP. Weak binders such as tetrabutylammonium salts readily desorb from the column and must be replenished with fresh SSP by using a tetrabutylammonium base buffer as eluent. It is important that the quaternary ammonium salt remain bound to the C18 chains of the reversed-phase column under the conditions of chromatography. If the organic modifier is at a concentration high enough to desorb the SSP from the C18 chains, then the SSP should be replenished by including it in the mobile phase.

Pharmaceutical Technology: How do you improve separation and resolution between two analytes?

Anwer: The resolution equation is the "brain of chromatography." The separation between any two components of interest is expressed by the "resolution equation" that is impacted by three factors: retention, efficiency, and selectivity. The general approach to improving resolution between any two analytes involves optimizing these three parameters. The *retention factor* is the residence time of the analyte in the column. The longer the analyte spends time in the column interacting with the stationary and mobile phases, the better the separation between the two components will be. Increasing k or k' requires patience.

The *efficiency factor*, or the number of theoretical plates, says that the smaller the diameter of particle, the greater is its efficiency in separating two closely eluting components. This varies as the "square of the diameter of particles," meaning a column packed with 5-micron particles has four times more theoretical plates than a column packed with 10-micron particles. Note that the particle diameter of reversed-phase media used in preparative HPLC is typically 10–20 microns.

Increasing the number of theoretical plates is expensive. Analytical columns with 1.7-micron diameter particles are now available from commercial sources such as Waters Corporation. The theoretical plate number is usually about 166,000 plates per meter, which is very good.

Finally, the *selectivity factor* is the ratio of the two peaks' retention factors. When the two peaks co-elute, the alpha or the *selectivity factor* is one. The alpha should be higher than one. The higher the value, the better is the separation between the analytes. Increasing alpha requires an understanding of the analyte's interactions with the stationary phase and mobile phase.

Pharmaceutical Technology: Which resolution factor is impacted the most by the SSP?

Anwer: SSP addresses the selectivity term, which is most impacted by the SSP. This is a broadly and generally applicable way of changing alpha, which is the desired feature of this kind of chromatography.