Liquid Chromatography Problem Solving and Troubleshooting

Question

Would you provide some insight into what I should consider in selecting an internal standard?

Answer

In answering your question it is helpful to understand why internal standards are used. These are compounds that are added to the sample as a means of compensating or minimizing experimental error that arises as the result of work up, dilution, injection, detection, and any other steps in the assay that can vary. As such, it is important to select a compound that has similar physical characteristics to those of the analyte. For cases in which the analysis involves the quantitation of one or only a few components, typically only a single internal standard is used. However, for more complex samples (i.e., samples that contain a variety of different types of analytes or when the chromatogram is relatively long, or both) the addition of more than one internal standard may be necessary.

An important first consideration in minimizing problems that may arise from sample work-up procedures, such as extraction differences or variability in the chromatographic elution behavior, is to choose an internal standard that has similar solution properties to those of the analyte. For neutral solutes one would select an internal standard that contains no ionizable groups and is similar in size and structure to the analyte. Likewise, compounds containing acidic or basic groups or a combination of both, which is the case for the majority of analytes, it is important that the equilibrium properties (protonation/deprotonation) be comparable between the analyte and the internal standard. For example, an internal standard that contains a carboxyl group would be selected for analytes such as fatty acids, many of the common nonsteroidal anti-inflammatories, nonflavanoids and related phenolic acids, and others. Additional information about the effect of pH on the chromatographic characteristics of analytes can be found in a previous troubleshooting note (1).

Under ideal circumstances, it is often possible to find a closely related compound such as a homologue or an isomer of the analyte. In the case of homologues, selection of the compound to be used as the internal standard depends upon where one wishes it to elute in the chromatogram in relation to the analyte (i.e., before or after). Under reverse-phase conditions, the addition of simple nonpolar groups (such as a methylene carbon in an alkyl chain or methyl and halogen groups in the analyte) will result in the internal standard being retained longer than the parent structure. Conversely, if a closely related compound can be found that has one less carbon or halogen atom it will elute before the analyte. The opposite chromatographic behavior is observed when a polar substituent such as a hydroxyl group is added to the analyte.

This strategy is useful when measuring a single component or a set of structurally related analogs. However, in many cases one is faced with carrying out a simultaneous measurement on a mixture of several analytes that are structurally unrelated and contain both acidic and basic compounds (i.e., a combination product such as cough–cold medication). When analyzing this type of mixture, it is generally useful to find a compound that will elute somewhere between the analytes (assuming there is sufficient chromatographic resolution). This will provide the best compromise between measurement reliability and speed of the analysis.

In addition to the above considerations, it is also advisable to select an internal standard that has similar detection properties. If a simple UV monitor is being used, the molar absorptivity and λ_{max} should be similar between the analyte and the internal standard. For example, if the analyte contains a simple aromatic ring, then one would normally choose an internal standard that also has the same aromatic structure. Likewise, for electrochemical detection an internal standard with similar REDOX characteristics would be important. If these are not known it is useful to measure the

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electrochemical behavior of the compounds being studied, because in some instances relatively small structural differences such as the addition or removal of a hydroxyl group from the parent compound may result in significantly different detection characteristics. For other more exotic detection systems, simple rules are sometimes more difficult. For example, HPLC in combination with electrospray ionization–mass spectrometry is becoming an increasingly more important technique. In this latter instance, variations in the ESI introduction system (e.g., inline versus off-axis spraying and capillary introduction design) as well as operating parameters (e.g., flow rate and buffer concentration) can result in significant differences in the mass-spectrometric profile between what appears to be structurally similar analytes. These result in differences in fragment, parent, adduct, and dimer ion production, which is beyond the scope of the current discussion. However, because of the importance of HPLC–ESI–MS, it will be considered in a future troubleshooting note.

References

1. R.K. Gilpin. Liquid chromatography problem solving and troubleshooting. *J. Chromatogr. Sci.* **39:** 77–78 (2001).