Waters Lab Highlights

An Internal Communication of Applications and Techniques

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DP/LS/ED/AA/PH

Eluent Selection for Reverse Phase Chromatography of Peptides

In recent years reverse phase gradient chromatography by HPLC has become the principle method employed by research groups, quality control labs and pharmaceutical manufacturers, for the purification and analysis of peptides. Sample types range from simple mixtures of a peptide and a single contaminant to complex protein digests containing hundreds of components. The method is sensitive, rapid and reproducible, but individual choices of buffer, mobile phase additives, pH and gradient slope make comparisons of separations difficult. We have evaluated several eluent systems for cleanliness, selectivity and baseline slope in peptide analysis and will discuss buffer choice in both general and specific terms. The effects of ion pairing eluents on selectivity in peptide chromatography have been discussed in Lab Highlights 0090, 0127 and 0197 and in recent work by P. Young and T. Wheat of the Waters Life Science Marketing Lab (J. Chromatogr., in press).

Since most peptides are hydrophobic to some degree they bind well to reverse phase columns and are eluted from the column using a gradient from aqueous to increasingly organic mobile phase. For the organic solvent, acetonitrile has been shown to be superior in its selectivity and UV transparency in the separation of small (less than fifty amino acid residue) peptides, while alcohols such as 1-propanol or isopropanol are useful for eluting large, more hydrophobic peptides. Alcohols have the drawback of relatively high viscosity and are commonly used in combination with acetonitrile to reduce back pressure.

Two factors impose significant limitations on the choice of the aqueous mobile phase:

1. For most peptide separations, the quality and consistency of the analysis is better at acidic rather than neutral or basic pH.
2. For micro-preparative work (peptide mapping and isolation), a completely volatile mobile phase system is preferred to facilitate lyophilization of collected fractions.

Within the set of possible volatile acidic mobile phases the most common are trifluoroacetic (TFA), hydrochloric (HCl) and heptafluorobutyric (HFBA). Weaker acids such as acetic and formic have to be used in high concentrations in order to maintain the pH such that all peptides carry a positive charge. Unfortunately, these concentrations preclude the use of low UV wavelengths (e.g. 214 nm) and detection must be done at higher wavelengths, typically 280 nm.

In high sensitivity applications (less than 1 μg of sample injected) UV absorbance of the mobile phase in the range from 200-220 nm can make detection difficult.

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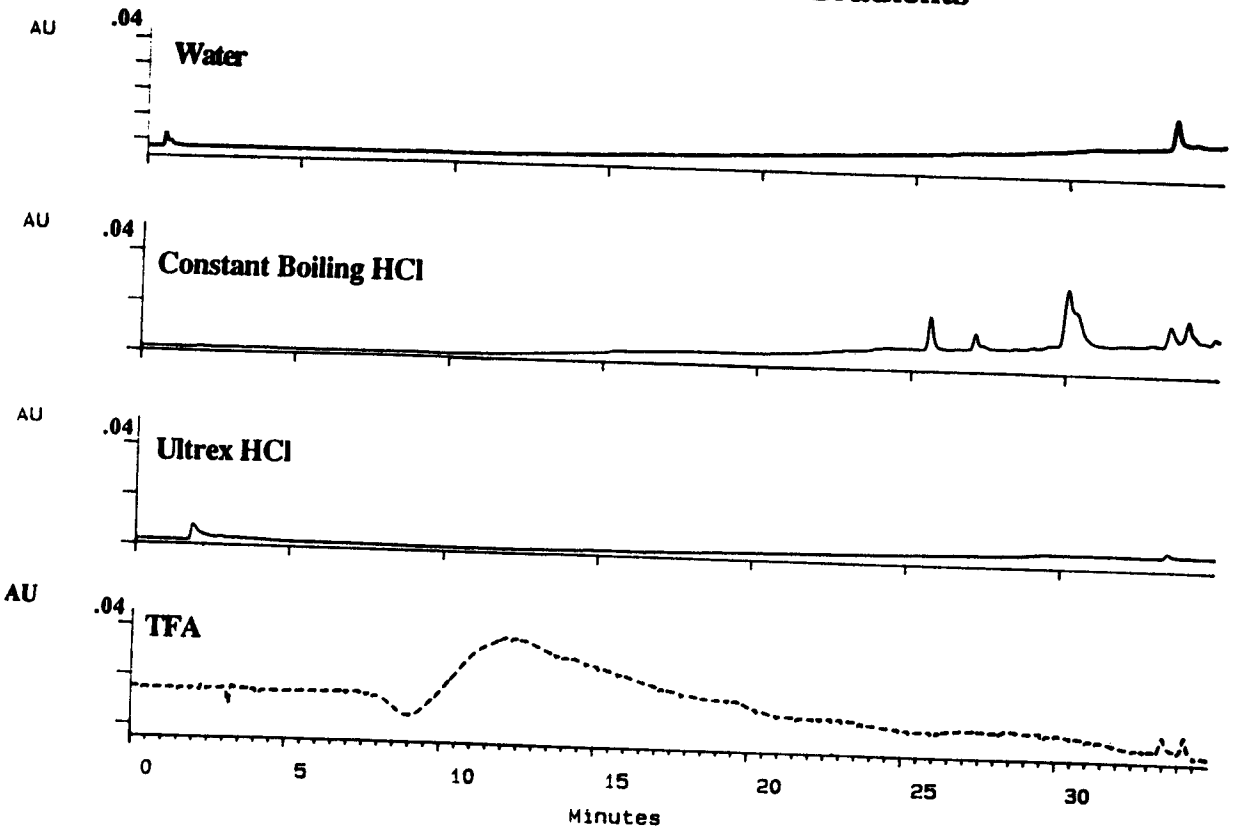
HCl is the most advantageous choice in this respect as TFA will cause a severe baseline drift towards lower absorbance as the gradient progresses towards higher organic content. This effect can be somewhat offset by "balancing" the organic eluent with TFA. This procedure is inexact and can be tricky depending on the concentration used and variability of TFA from various sources and batches, although many laboratories do this successfully. The purity of HCl used in aqueous mobile phases may also affect baseline performance or introduce reagent peaks in the chromatogram. Figure 1 is a compare plot of UV absorbance at 214 nm comparing the absorbances over a linear gradient run on a Delta Pak™ C_{18} column (3.9 x 150mm) from 5 to 65 percent acetonitrile in 30min at a flow rate of 1ml/min of four mobile phases; water, 6mM Constant Boiling HCl (Pierce Chemical Co.), 6mM Ultrex grade HCl (J.T. Baker) and 0.1% sequanal grade TFA (Pierce) (the acetonitrile contained .08% TFA for this run). It is apparent that the type and quality of the acid can affect baseline performance.

Figure 1: Comparison of Blank Gradients



The question of corrosion of stainless steel caused by low pH, chloride-containing mobile phases is a potential concern and for this reason the use of a non-metallic mobile phase delivery system such as the Waters 625 and non-metallic column hardware are recommended. If a stainless steel containing system is to be used with HCl, flushing the acid from the system with water before shutdown will minimize the deleterious effects of the acid.

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