Optimized Separation of Angiotensins in Acidic and Basic Conditions by HPLC with ELSD

Application Note

Small Molecule Pharmaceuticals

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Introduction

PLRP-S reversed-phase material is a macroporous polymeric medium that is stable at pH 1-14. Due to this enhanced chemical stability, it is possible to optimize peptide separations using not only the conventional acidic ion pairing agents, such as trifluoroacetic acid (TFA), but alkaline pH as well. This can be a significant advantage when the macromolecule has limited solubility at acid pH, but is readily soluble under alkaline conditions. The eluent pH can also be exploited for the control of selectivity of a reversed-phase separation of peptides and proteins by altering the charge distribution due to the presence of acidic and basic amino acid residues in the macromolecules.

The separation of an angiotensin mixture is suitable for an investigation of the changes in selectivity that can be obtained in relation to the concentration of mobile phase modifier and pH. Angiotensin is a peptide hormone in the blood that causes vasoconstriction, increased blood pressure and release of aldosterone from the liver. Three angiotensins are very closely related as can be seen from their amino acid sequences.

Angiotensin I	Asp-Arg-Val-Try-Ile-His-Pro-Phe-His-Leu
Angiotensin II	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe
Angiotensin III	Arg-Val-Tyr-Ile-His-Pro-Phe

Coupling PLRP-S with the Agilent ELSD evaporative light scattering detector is an excellent system for separating angiotensins. The Agilent ELSD offers greater sensitivity than UV detection. Solvent peaks are absent and excellent baseline stability is present. The Agilent ELSD is renowned for its rugged design and ability in delivering high performance for demanding HPLC or GPC applications. PLRP-S 100Å columns are ideally suited to the analysis of low molecular weight compounds such as angiotensins because the small pore sizes have extremely high surface areas available to the solutes.

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Instrumentation

For structural analysis of peptides and proteins, LC-MS is employed. However, with this technique, the eluents must be volatile and so a volatile pH eluent was evaluated for the separation of angiotensins. The Agilent ELSD was used as this has the same eluent requirements as an MS detection system.

Column: PLRP-S 100Å 5 µm, 250 x 4.6 mm (p/n PL1512-5500)

Detector
Figure 1: Agilent ELSD (neb=85 °C, evap=75 °C, gas=1.0 SLM)
Figure 2: Agilent ELSD (neb=85 °C, evap=80 °C, gas=1.0 SLM)

Materials and Reagents

Figure 1
Eluent A:TFA (various %) in Water
Eluent B:TFA (various %) in ACN
Figure 2
Eluent A:NH_4OH (various mM) in Water
Eluent B:NH_4OH (various mM) in ACN

Conditions

Flow Rate:	1.0 mL/min
Gradient	
Figure 1:	Linear 12-40% B in 15 min
Figure 2:	Linear 10-100% B in 15 min

Results and Discussion

The most common ion pairing agent for reversed-phase analysis at acidic pH is TFA. It is normally used at a concentration of 0.1%, which is considered sufficient to pair with the positively charged and polar residues in the peptides, and it gives good peak shape. However, at this concentration Angiotensin II and III co-elute (Figure 1).

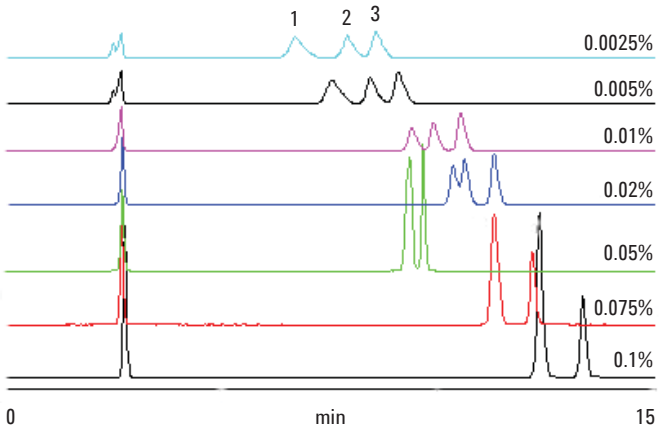

Figure 1. Elution of angiotensins at differing concentrations of TFA (1. angiotensin III 2. angiotensin II 3. angiotensin I).

As the concentration of TFA is reduced, the retention time decreases and resolution of the two peptides is obtained. However, as the concentration is reduced from 0.01%, band broadening occurs (Figure 1).

In Figure 2, separation of the three angiotensins is shown as a function of ammonium hydroxide concentration.

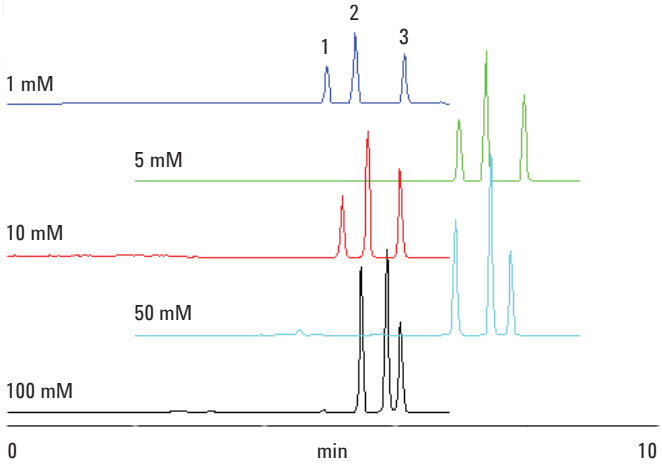

Figure 2. Separation of angiotensins at differing concentrations of ammonium hydroxide (1. angiotensin II 2. angiotensin I 3. angiotensin III).

Conclusion

The Agilent ELSD and a PLRP-S column successfully separated a mixed sample of angiotensins.

PLRP-S columns are the preferred choice for the analysis of many small molecules. These columns are more retentive for small molecules than the majority of alkyl bonded silicas. PLRP-S media possess a much greater surface area than alkyl bonded silicas and therefore even polar molecules may be retained for much longer, resulting in greater resolution.

PLRP-S columns used with the Agilent ELSD is an ideal combination that allows management of the eluent pH to control the selectivity of a reversed-phase separation of peptides and proteins.

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