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Biomolecule Separation by HPLC

Practical 8 - Bench practical

Aim

To separate and quantify a supplied biomolecule preparation by an appropriate chromatographic mode.

Principle

An analyte distributes between a stationary phase and flowing mobile phase. Retention and separation depend on the column chemistry, mobile-phase composition, flow, temperature and analyte ionisation.

A detector response is related to concentration using a qualified calibration or comparison with a reference preparation. Retention agreement supports identification but needs the selectivity evidence required for the intended use.

Materials and Requirements

- HPLC system with suitable column and detector
- Method-specified mobile phase and diluent
- Reference material, blank and placebo
- Qualified filters, vials and volumetric glassware

Procedure

1. Record the column identity, mobile-phase composition, flow, temperature, detector settings, injection volume and method version.
2. Prepare mobile phase and solutions using compatible grades and the adopted filtration and degassing process. Record solution stability and reference potency.
3. Check the system for leaks and equilibrate using the instrument procedure. Establish a stable baseline before the analytical sequence.
4. Run a blank, reference preparations and system-suitability injections. Evaluate retention, resolution, peak shape and repeatability against predefined method criteria.
5. Inject independently prepared test solutions bracketed by appropriate check standards. Include a blank after high concentrations when carryover is a concern.
6. Review peak identification and integration consistently. Retain the original chromatograms and document any justified integration changes.
7. Calculate concentration or assay, reverse dilutions and review the complete sequence before reporting. Preserve audit-trail and processing information.

Observations and Record

Enter actual measurements or the supplied teaching data. Record the method, material identity, conditions and units.

Injection	Retention time (min)	Area / area ratio	Resolution or comment
Blank	Record	Record	Interference / carryover
Reference replicates	Record	Record	Suitability
Test preparation 1	Record	Record	Assay
Test preparation 2	Record	Record	Preparation precision

Calculations

• Resolution, Rs = 2(tR2 - tR1) / (w1 + w2), using baseline peak widths
• Theoretical plates, N = 16(tR / w)^2, using baseline width
• For matched dilutions in a valid external-standard comparison: assay (%) = test area / standard area x reference concentration / nominal test concentration x reference potency (%)

Worked Example

Illustrative adjacent peaks: retention times 5.00 and 5.40 min; baseline widths 0.20 and 0.24 min.
Rs = 2(0.40) / 0.44 = 1.82. The first peak gives N = 16(5.00/0.20)^2 = 10000.
For equal nominal concentrations, test and standard areas of 49000 and 50000 with reference potency 99.7% give assay = 49000/50000 x 99.7 = 97.706%.
These values illustrate calculations; use the adopted method criteria to decide whether a sequence is acceptable.

Biomolecule-specific controls

• Select size-exclusion, ion-exchange or reversed-phase conditions appropriate to the molecule and intended attribute.
• Assess recovery and adsorption. A protein concentration result does not establish native structure or biological activity.

Result and Interpretation

• Report assay or concentration together with the observed suitability results.
• A resolved, reproducible peak must also be shown to be specific for the intended analysis.
• Investigate failed suitability or carryover before using affected test results.

Precautions

• Use compatible solvents and the column pressure and temperature limits.
• Collect solvent waste in designated containers and keep incompatible wastes separate.
• Prevent particles and bubbles entering the flow path.
• Avoid selective reintegration or reinjection to obtain a preferred result.

Viva Questions and Answers

Why equilibrate the column?

A stable stationary-phase environment improves retention and response reproducibility.

Does a single retention time prove identity?

No; coelution is possible and identity requires the intended method evidence.

Why bracket test injections?

Check standards can reveal response drift during the run.

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