Laboratory exercise to advanced practice of analytical chemistry – JS 2012/2013

Supervisor Ing. Blanka Vrbková

High-performance liquid chromatography – the inosine, adenosine and their 2´-deoxy-forms determination – optimizing and validation

THEME GOALS – find out the validation parameters and verify characteristics of liquid chromatography in exercise.

Conditions:

- basic knowledge of theory behind HPLC (written test)
- knowledge of the theme (written test)
- laboratory coat, appropriate foot-wear
- eventual lunch one after one ;-)

Definition of the analytical problem

The acquisition and processing of experimental data affect the final results in the terms of precision and accuracy. Verification of the functionality of the selected analytical procedure is necessary. Suitability of the method for solving the analytical problem is statistically proven of its reliability – precision and accuracy of method - and examination of border of its practical usability. This process called validation of method.

The validation goal: find out the selectivity, accuracy, precision (repeatability, intermediate precision and reproducibility), linearity, dynamic range of method, limit of detection, limit of quantification and robustness.

Definition of theme

Determination of validation parameters, such as precision, linearity, LOD and LOQ, using the model standards mixture.

Samples

Standards of nucleotide's bases (store solution with 10 mM concentration):

2´-deoxyinosine-5'-monophosphate (dIno),
inosine-5'-monophosphate (Ino),
2´-deoxyadenosine-5'-monophosphate (dAde),
adenosine-5'-monophosphate (Ade).

Library of UV-VIS spectra of purines, pyrimidines, nucleosides and nucleotides.

Thiourea (0.002% aqueous solutiuon).

Mobile phase (MP): 15 mM phosphate buffer, pH 3.8, 10% methanol.

Apparatus

HPLC system **10 AVP** by SHIMADZU (degasser GT-154, system control unit SCL-10AVP, 2x pump LC-10AVP, oven CTO-10ASVP, PDA detector SPD-M10AVP, control software Class-VP 5.02), colony 2x100x4.6 mm, Onyx C18, monolithic, particle size: 13 nm mesopores, 2 μm macropores.

OPERATING SEQUENCE

Analysis of test mixture (acetone-benzene-toluene) in stored column condition (MP: 70% methanol)

1. Chromatographic analysis of solution of test mixture – important to determine functionality of column status. We observe the peaks asymmetry (tailing, dozing), capacity factor (calculated from retention time).

Analysis of inert analyte – thiourea (MP: 15 mM phosphate buffer, pH 3.8, 10% methanol)

1. Chromatographic analysis of thiourea – important for capacity factors of each nucleotides.

Analysis of nucleotides standards (MP: 15 mM phosphate buffer, pH 3.8, 10% methanol)

1. Linearity.

Verify the linearity by measuring of 7 standards concentrations. The calibration curve will be constructed in the range 1.5-0.0004 mM. Each solution analyze one time. Construct the calibrate curves; determine the dynamic range of method for each of nucleotides. Test the significance of both parameters (slope, intercept) of linear equation (by Excell, Statistica,…), determine the coefficient of determination (statistical confirmation of linear dependence).

2. Precision.

Prepare mixture of two nucleotides (Ino, dIno) in three concentration levels – 0.5; 0.05 and 0.005 mM. Each solution measure five times. Calculate the standard deviation, relative standard deviation and confidence intervals from retention times of each of nucleotides.

3. LOD, LOQ

Find out the LOD and LOQ by both methods: i) from calibration curve and baseline at equilibration of mobile phase, and ii) numerical method from chromatogram at low nucleotides contents and baseline noise.

First calculation: we can utilize the values of signal of blank and calibration curve at separation techniques. From chromatogram of blank (chromatogram at mobile phase equilibration) we determine the maximum variation of baseline (h_{max}) in the 20-times area at half of weight of analyzed analyte. The response of detected analyte content then apply: $y_d = 3 \times h_{max}$ and for concentration at limit of detection: $x_d = y_d/b_1$, where "b_1" is slope of calibration curve and must be from dependence $y = b_1x$, where "y" is the height of peak, not area!

Second calculation: find out the limits from method of gradual dilution, we dilute the sample until the signal has height 3times higher than average noise. The baseline noise is evident at this measured concentration. For calculation the follow formula:

$$LOD = \frac{3 \cdot N \cdot c}{S}$$

Where “N” is the height of noise at baseline, “S” the height of analyte signal at “c” concentration of analyte.

Chemometric evaluation

1. The outlier test for a small set of results (Dean-Dixon or Dixon’s test), arithmetic mean, standard deviation (SD), and relative standard deviation (RSD) calculate for each results measured more than twice. The results given in a confidence interval.

2. Calculate the capacity factor for test mixture (benzene, toluene relative to acetone), for nucleotides (n = 7, retention times from linearity measurement; relative to thiourea).

3. Linearity – testing the hypothesis of slope (b = 1), and intercept (a = 0), and their confidence interval. Determine the dynamic range for each of nucleotides and the coefficient of determination. Construct the graphical dependence of height peaks on their concentrations.

4. Precision. Testing for deviations for each levels of nucleotides concentrations (T-test; widely used test for detecting a deviation of a test object from a standard by comparison of the means).

5. LOD, LOQ – calculated by both methods, mentioned above.