High Speed Resolution of Nucleosides in UHPLC

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Introduction

Modified Nucleosides in urine samples can be an indication of carcinogenesis, so analytical methods able to determine these compounds in biological fluids are of high importance. We show an application using UHPLC in order to provide a fast, precise, high resolution separation and quantification of nine modified nucleosides.

UHPLC is particularly suited to this analysis as it is simple, precise, fast and selective, meaning that accurate results can be obtained in minutes rather than hours. High sensitivity is also another improvement that UHPLC tends to offer over traditional HPLC, meaning lower LOD and lower LOQ's can be achieved.

We highlight how choice of stationary and mobile phase are important variables when developing analytical methods and their subsequent transfer to UHPLC. Making the correct selection allows the analyst to combine selectivity with efficiency to ensure that optimisation of the separation really occurs.

We show how the separation of the nucleosides can be achieved in under 3 minutes with the correct use of stationary phase and mobile phase combination.

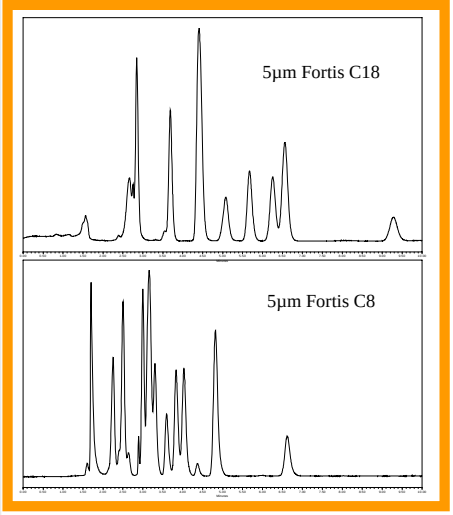
Methodology

Using a standard 12 nucleosides test mixture from Sigma-Aldrich the performance of two stationary phases was evaluated in order to select the best column for analysis of the modified nucleosides. Next the choice of organic modifier was studied to obtain the best selectivity to allow full resolution of the nucleosides.

Stationary Phase Selection

Conditions:
Mobile Phase: 95% H_2O 5% Acetonitrile
Flow rate: 1ml/min
Wavelength: 254nm
Column size: 150 x 4.6 mm

FIGURE 1. C18 vs C8 Selectivity in ACN



Test Mixture Composition:

Sodium formate, 10mg/ml
Pseudouridine, 25ug/ml
Cytidine, 50ug/ml
3-Methylcytidine, 100ug/ml
Uridine, 25ug/ml
1-Methyladenosine, 25ug/ml
2-Thiocytidine, 10ug/ml
5-Methylcytidine, 100ug/ml
7-Methylguanosine, 25ug/ml
2'-O-Methylcytidine, 20ug/ml
Inosine, 25ug/ml
Guanosine, 25ug/ml
Ribothymidine, 50ug/ml

Mobile Phase Selection

Conditions:
Mobile Phase: 95% H_2O 5% Methanol
Flow rate: 1ml/min
Wavelength: 254nm
Column size: 150 x 4.6 mm

FIGURE 2. C18 vs C8 Selectivity in MeOH

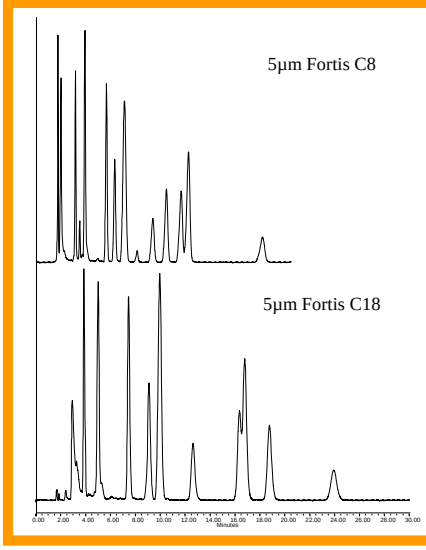
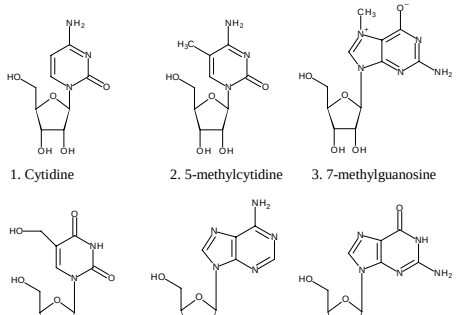


Figure 1 shows that the Fortis C8 provided resolution of more of the 12 nucleosides when compared to the Fortis C18 phase. From figure 2 we can see that both stationary phases gave improved chromatography with methanol as the organic modifier compared to acetonitrile.

Using Fortis C8 as stationary phase and methanol in the mobile phase we were able to resolve the most nucleosides contained in the mixture in a run time of 20 minutes

Separation of Modified Nucleosides

Using the previously recommended stationary phase and mobile phase combination nine modified nucleosides were then analysed….



1. Cytidine
2. 5-methylcytidine
3. 7-methylguanosine
4. 5-hydroxymethlyuridine
5. Adenosine
6. Guanosine
7. 5-methlyuridine
8. Thymidine
9. N^2-methylguanosine

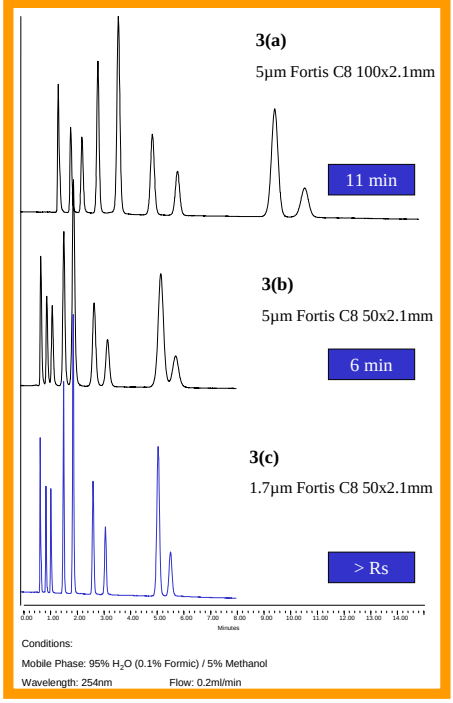
HPLC to UHPLC Method Development

The nucleosides were first resolved using a Fortis C8 analytical column, as can been seen in figure 3(a) we obtain good baseline resolution of all nine nucleosides in under 12 minutes using a 5µm 100x2.1mm column.

To shorten further the analysis time the nucleosides were tested using a 5µm 50x2.1mm column. As can be seen in figure 3(b) although the analysis time has been shortened to 6 minutes we are just starting to lose baseline resolution.

In order to regain baseline resolution the particle size was reduced by moving to a 1.7µm UHPLC column. Figure 3(c) shows how resolution has been regained whilst still giving identical retention profile for the nucleoside peaks.

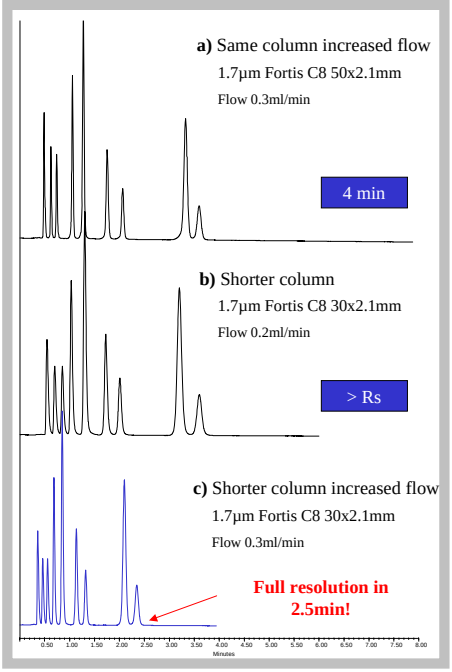
FIGURE 3. Scaling HPLC to UHPLC



Optimizing the UHPLC Method

With the move to 1.7µm UHPLC particles we have gained resolution which can be used to further increase the speed of analysis, either by increasing flow rate or by moving to a shorter column. Both of these options are shown in figure 4.

FIGURE 4. Optimizing UHPLC Conditions



Discussion

In this poster we have shown that it is important to select the correct stationary and mobile phases in order to gain optimum resolution of a modified nucleoside mixture.

We show that by the application of UHPLC column technology full resolution of 9 modified nucleosides is possible in under 3 minutes using a simple mobile phase.