Super heated water chromatography – a hot topic for LC and LC-MS

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This article discusses the use of high temperature water as a mobile phase and in particular to its application with sub 2 micron phases. A model is discussed which allows for the characterisation of the experimental conditions and is used later to determine the conditions required for an isobaric thermal gradient to be operated. The use of isothermal and thermal gradients are discussed and some example separations are given.

Introduction

Water at room temperature is not commonly used as an elution solvent in reversed phase liquid chromatography, primarily because it does not elute most hydrophobic compounds. As a result organic solvents, such as methanol and acetonitrile, are routinely used to elute hydrophobic compounds from a hydrophobic stationary phase, either isocratically or by using a gradient. These solvents also have the advantage that they possess lower viscosity than water and as a result lower the operating pressure of the HPLC system. This has become of particular importance in recent years, with the advent of sub 2 micron particles.

However, water at elevated temperatures also exhibits a reduced viscosity, and has an increased elutropic strength (reducing the elution time), making high temperature water an ideal substitute for an organic solvent. Examples of these properties have been utilised in the separation of barbiturates [1], herbicides [2], antioxidants [3] and sulphonamides [4]. The advantages of using a purely aqueous mobile phase have also been utilised in coupling with a range of detection systems that are not suitable when using organic solvents e.g. HPLC-NMR and HPLC-ICP applications [5-7].

The use of thermal gradients, comparable to organic gradients, has also been demonstrated where a GC oven is used to heat the column at the required rate [8]. This is particularly applicable to capillary or narrow bore columns where the low thermal mass reduces the temperature gradients occurring radially across larger diameter columns.

So high temperature water appears to be a very useful solvent for HPLC, but its use is very limited for a variety of reasons including;

- Thermal stability of some compounds.
- Thermal stability of HPLC stationary phases.
- And a limited number of HPLC column ovens available that will operate above 100°C.

However,

- Although the thermal stability of the analyte does have to be considered, it would have to be considered for any operating temperature, and there are many compounds that are thermally stable above 100°C.
- The thermal stability of HPLC stationary phases has been improved with the introduction of hybrid phases such as the bridged ethyl hybrid phases from Waters (Massachusetts, USA), and other polymeric columns.
- Fortunately the lack of suitable HPLC column ovens is matched by the availability of GC column ovens, many of which allow ballistic thermal gradients to be applied.

Theory

For effective utilisation of temperature as a parameter in HPLC a simple, predictive model was used, which allows for the characterisation of the chromatographic system. This model is based around two fundamental concepts.

Concept 1

Viscosity is the dominant temperature dependent variable within the system. It is assumed that the temperature only affects the viscosity directly; all other physical parameters are affected indirectly by the change in viscosity. It is also assumed that the viscosity for water has an Arrhenius type or more specifically Andrade [9] relationship with temperature over the range being investigated. More complicated models for the dependency of viscosity on temperature are available but given the added complication of the high pressures and the effect that this has on the viscosity a more simplified approach was taken. Thus the following equation can be written.

$$\eta_T = \eta_0 e^{b/T} \quad \text{(Eq. 1)}$$

where;

η_T – is the viscosity at a temperature T in Kelvin.

b – is a constant which is dependent on the fluid.

T – is the thermodynamic temperature in Kelvin.

η_0 – is the viscosity at 0 Kelvin, this is clearly a theoretical value.

Concept 2

The flow is laminar and the pressure drop can be defined by the Blake-Kozeny [10] equation;

$$\Delta P = \frac{\varphi \eta_T u L}{D_p^2} \quad \text{(Eq. 2)}$$

where

ΔP – is the pressure drop across the column

φ - is a packing term

u – is the superficial velocity of the mobile phase

Dp – the particle diameter

L – the length of the column

Combination of the two equations and rearranging gives the following equation

$$\ln P = Ln\left(\frac{\varphi \eta_0 u L}{D_p^2}\right) + \frac{b}{T} \quad \text{(Eq. 3)}$$

Thus a plot of lnP vs. 1/T will give a linear plot where the gradient is a term relating to the physical variation of viscosity of the fluid to temperature. If the model is correct then this term will be independent of the column characteristics, thus once calculated for one mobile phase can be used in all columns using that mobile phase.

The intercept gives a lumped parameter term, which includes the particle size, the linear velocity of the fluid, a viscosity term, and a packing coefficient. This term is clearly dependent on the physical characteristics of the column and will vary from one column to another.

Equation 3 can be rearranged in terms of the experimental parameter being varied, temperature, flow or pressure. This allows experiments to be designed where either the pressure, temperature or flow is kept constant and the remaining two parameters are varied. This can be effectively utilised to allow for isobaric separations where the temperature and flow are varied to maintain a constant pressure across the column.

Experimental

Chemicals

The model compounds used in this study were antipyrine, aminohippuric acid, paracetamol, hydroxyantipyrine, aminoantipyrine, atenolol, aminobenzoic acid, theophylline, phenacetin, and caffeine (Sigma, Poole, Dorset). These compounds were chosen to represent typical polar drug molecules. The ultrapure water used as mobile phase was obtained from a Millipore MilliQ system.

A real application metabolite study sample was also analysed using a thermal gradient to determine if this technique could be applicable to the analysis of some known thermally labile compounds.

Chromatography

Up to three 100 x 2.1mm, 1.7 µm Acquity columns (Waters, Massachusettes), connected in series, were used for the chromatography. The chromatographic system comprised of a Prominence Sil-20A/C autosampler and a Sil-20 AB binary pump which was used in conjunction with a GC-2014 GC oven (Shimadzu, Milton Keynes, UK) which was employed to heat the column, with precolumn heating and post column cooling to ensure optimal performance. This oven was capable of operating at temperatures up to 450 °C. Where pressures in excess of 6000psi were required an Acquity HPLC system (Waters, Massachusetts) was used in place of the Shimadzu autosampler and binary pump.

The eluent from the column went directly into an API365 MS/MS (Applied Biosystem, Manchester, UK). Contact closures were used to connect the LC – GC oven - MS/MS systems together. The source conditions were set as 450°C, with an ion spray voltage of 5000V, and gas settings of 12 for the nebuliser gas, 10 for the curtain gas, and 3 for the CAD gas. All the compounds were tuned in positive mode using M+1, as the m/z.

Results

Testing the theory

To test the theory four different columns were used;

- 50 x 4.6mm, 5µm Gemini column (Phenomenex, Macclesfield, UK),
- 50 x 2.1mm, 3.5µm Zorbax column (Agilent, Manchester, UK),
- 50 x 2.1, 3.5µm Xterra column and a 100 x 2.1mm, 1.7 µm Acquity column (Waters, Milford, MA, USA).

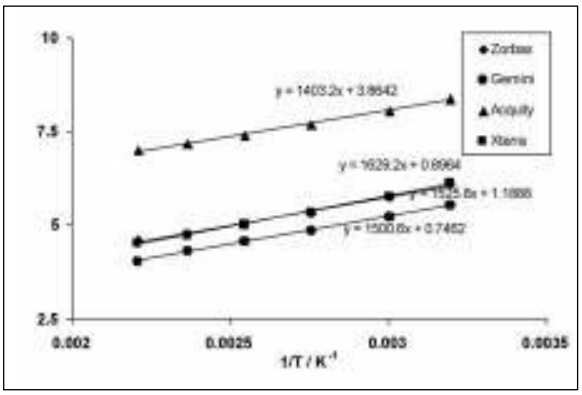

Figure 1. Graph showing the linear relationship that exists between the reciprocal temperature and the log of the pressure for four different columns (♦ - Zorbax, ● - Gemini, – ▲ Acquity and ■- Xterra). From ref [8].

Water was passed through the columns at different temperatures and plots of ln P vs. 1/T were drawn, shown in Figure 1. In all cases the gradients are comparable, suggesting that the concepts proposed here are valid. The intercepts are different; again this would be explained by the model which states that different columns will give different intercepts as the intercept relates to the physical characteristics of the column with regard to the packing efficiency, size of packing material, length of the column etc.

With a model developed, which allowed for the various physical parameters to be determined, a series of experiments were performed, initially under isothermal conditions, to see the effect that temperature has on the separation process.

Isothermal Separations

As discussed in the introduction, increasing the temperature at which the chromatography was performed reduces the column back pressure and gives shorter retention times for the analytes on the Acquity columns. One method for representing these data can be seen with the use of a Van't Hoff plot [11], Figure 2, which shows some examples of the Arrhenius relationship that exists between the retention time and the absolute temperature. The linearity of the Van't Hoff plots indicates that the retention mechanism is not changing over the temperature range investigated, in this case 40 to 180°C. All of this data was obtained on a 100 x 2.1mm, 1.7µm Acquity BEH C18 column.

One point of interest, illustrated in Figure 2, is the effect that temperature can have on the

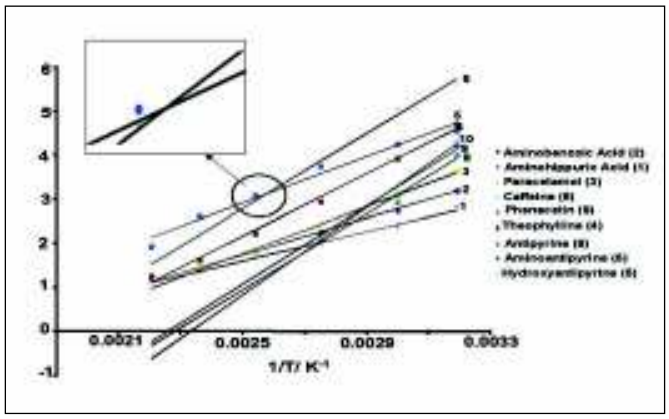

Figure 2. Van't Hoff plots for the test probes run on the Acquity column. The inset shows the point at which the elution order is reversed for aminoantipyrine and caffeine.

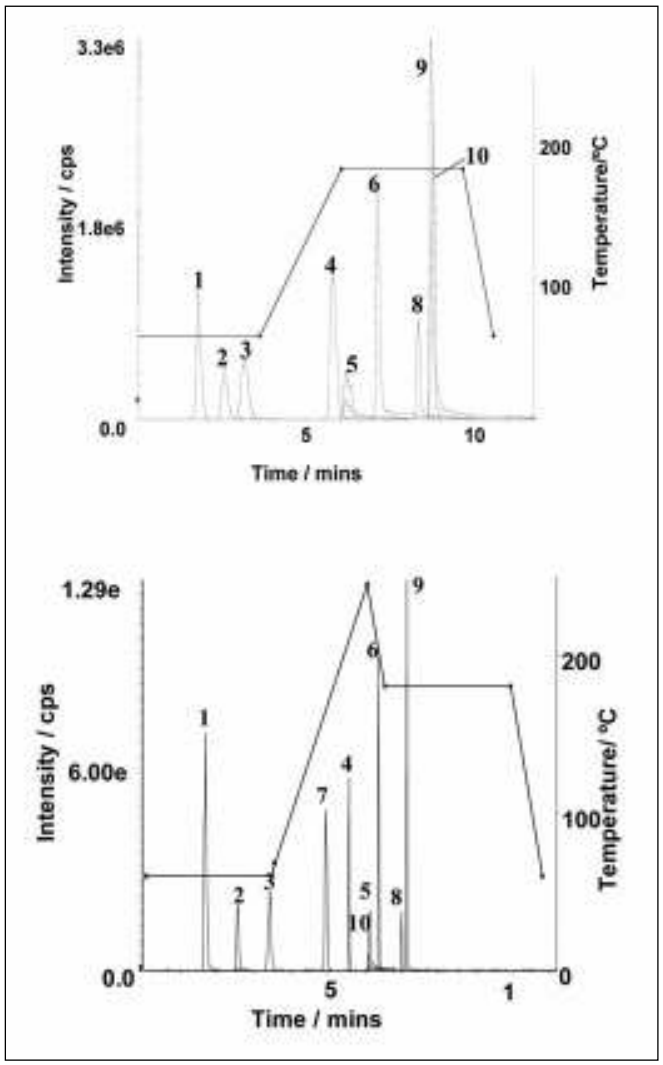

Figure 3. Separation of test compounds:
(a) Initial thermal gradient separation obtained on 2.1 x 100mm 1.7µm Acquity column.
(b) Separation obtained after modifications to the experimental arrangements, using the same column.
From ref [12].

chromatographic selectivity. In this plot, two of the test probes are highlighted, caffeine and aminoantipyrine, and it can be seen that the plots actually cross, indicating that there was a reversal in the elution order. Thus, whilst separated at low temperatures, the peaks coalesce at 113 °C, but performing the chromatography at a higher temperature separation of the peaks is once again obtained, but with a reversed elution order. This relates to the differences in the enthalpy and entropy of adsorption of the compound with the stationary and mobile phases. Since only one of these parameters has a significant temperature dependency, it is common for temperature to affect the elution order.

Thermal Gradient Separations

The application of an organic gradient is commonly used within HPLC as a means to focus peaks, obtaining better sensitivity, and also reducing retention times. Applying a thermal gradient would mimic the application of an organic gradient in a binary solvent HPLC system.

The separation obtained using a thermal gradient on the Acquity column was initially disappointing, due to the broad peak shapes and significant tailing observed with many of the test compounds, Figure 3a. Modifications were made to the experiment to improve peak shape and separation. These modifications concentrated on ensuring that the mobile phase remained in a liquid and under isobaric conditions throughout the run.

In particular;

- Using the model to vary the flow rate to obtain nominally isobaric conditions, the flow rate varied from 0.4mls/min at the start temperature of 60 °C flows to 1.1mls/min at highest maintained temperature 180 °C. The overshoot of the final temperature is due to the thermal lag existing between the temperature experienced within the column and that set for the GC oven.
- the use of a cooling bath to cool the eluent to room temperature, prior to it entering the MS ensured effective ionisation of the analytes.
- the use of a post column restrictor to maintain the mobile phase in a liquid state throughout the column.
- the use of an algorithim to offset for the thermal lag between the GC oven and the temperature being experienced inside the HPLC column

Figure 3b shows the optimal separation achieved using the hot water chromatography system for a 10cm Acquity column. It shows that very sharp peaks can be obtained and comparison with the earlier plot demonstrates the importance of post column cooling, post column restrictor and the use of an isobaric separation.

The notion of using high temperatures to reduce the viscosity and hence back pressure across the column has been demonstrated. To further test these ideas a series of sub 2 micron particle, 2.1mm i.d. columns were coupled together to form a column 1 meter in length. Running at a temperature of 320 °C and with a flow rate of 0.6 ml/min the same sample set were analysed. Figure 4 shows the resulting chromatogram. Investigation of the efficiencies for the peaks shows

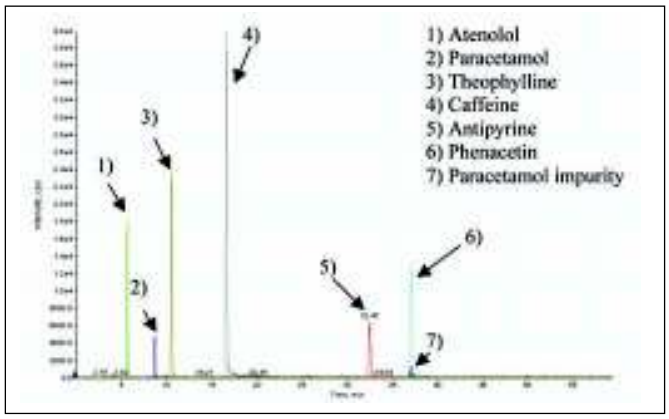

Figure 4. Separation of test compounds. 0.6ml/min 14000 psi 180°C on 2.1x 1000mm, 1.7µm Acquity column. Peak 6 has an efficiency of 104,000 plates

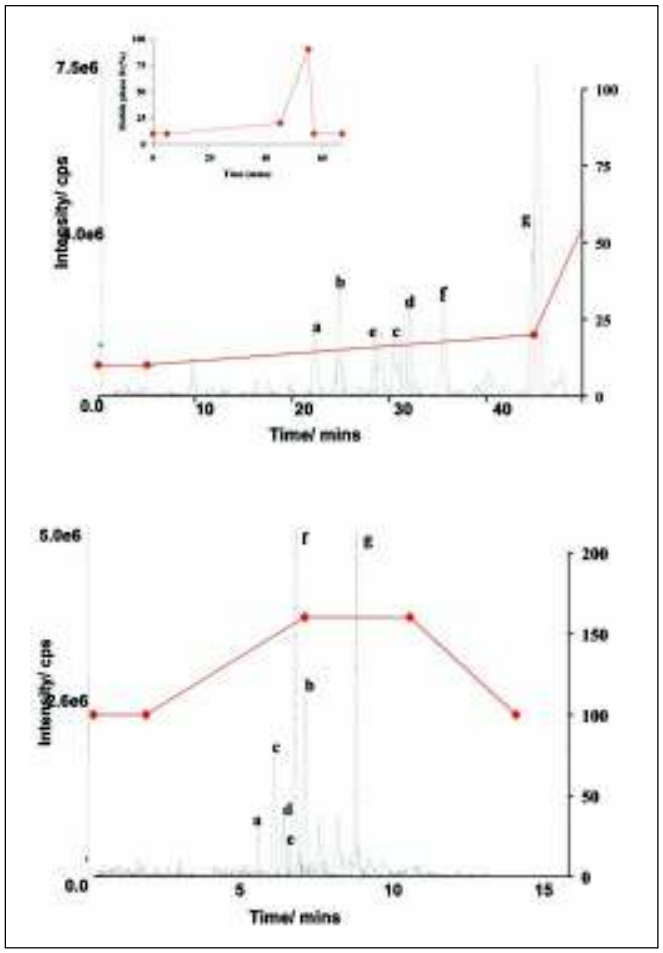

Figure 5. Chromatograms obtained using
(a) a generic HPLC method: a = hydroxy-O-glucuronide, c = hydroxyl, d = hydroxyl, e = di-glucuronide, f = parent, b= hydroxy-O-glucuronide and g = hydroxyl.
(b) a thermal gradient on an Acquity column.

that phenacetin, peak 6, has an efficiency of 104,000 theoretical plates. This demonstrates the possibilities that can be achieved when using high temperature HPLC, although clearly there are financial limitations to using such long columns.

Metabolite study

Figure 5 shows two chromatograms obtained using a generic HPLC method, and that obtained using a thermal gradient on a UPLC column. This optimised thermal gradient showed a reduction in the analysis time of nearly 30 minutes compared to the generic HPLC method. Using this thermal gradient, thermally labile metabolites were also detected. There was a reduction in the signal response observed for some of the components under these conditions, in particular peak e, the di-glucuronide, and some thermal degradation under these conditions cannot be ruled out. However, as this type of metabolite profiling/ identification is qualitative rather than quantitative analysis, it is the ability to detect and identify a metabolite that is critical.

Conclusions

Many authors have demonstrated the applicability of high temperature chromatography to the analysis of a wide range of organic compounds. The environmental benefits, though minimal, are obvious but also the advantages of being able to utilise higher flow rates, often a prohibitive factor in the use of capillary columns ensure that the science of chromatography will be hotting up. However for effective use of this technology manufacturers have to be heavily involved to allow for high temperature ovens to be used as standard, both isothermally, and also with thermal gradients. The benefits of operating under a constant pressure when utilising these thermal gradients have been demonstrated here.

The current article has only discussed the use of high temperature liquid chromatography with purely aqueous mobile phases. There are, however, benefits of applying this technology to the use of mobile phases with organic components. Not only does it reduce the amount of organic required, but the selectivity of the chromatographic system is changed when compared to room temperature separations, this makes it a very powerful tool in separation optimisation.

The advent of more stable stationary phases has allowed the use of high temperature separations for liquid chromatography. Thermal stability of the analytes does have to be considered, but the benefits of reduced analysis times coupled with lower back pressures and differing selectivity offer the separation scientist a powerful tool for the discrimination of complex mixtures. The potential for high temperature LC is still largely untapped, with the obvious benefits still to be realised. The future is genuinely starting to heat up for high temperature liquid chromatography.

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