HPLC-UHPLC Hybrid 2D Platform for LC/MS Analysis of Biological Samples. A New Paradigm.

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Modern UHPLC systems have important advantages over previous generations of HPLC systems. These are (a) higher pressure limits, and (b) considerably smaller delay and post column volumes, which are essential for the development of fast gradient applications. Fast gradient applications by UHPLC/UV systems are easily adopted in QC/QA applications. However, the analysis of complex samples was found to be more challenging and costly, if UHPLC technology was used. There are several reasons for this including;

- Clinical sample analysis usually utilises a large volume injection of a diluted sample, which is suboptimal for a UHPLC autosampler.
- Extracted samples obtained after sample preparation steps, drying and reconstitution may have small particulates that shorten UHPLC column lifetimes.

We developed a cost-efficient hybrid LC platform that's ideal for biological sample analysis by LC/MS. This platform is based on Agilent 1100 and Agilent 1290 series LC devices. The first dimension of the analytical system used a standard pressure range (400 bar max) binary HPLC pump, where the sample was injected using a standard pressure (not UHPLC) autosampler into a pre-analytical column. The sample was then desalted, partially purified, and concentrated using the pre-analytical column.

The fraction containing analytes of interest was transferred through a UHPLC valve to a fused core column. Once the analytes were transferred, a fast gradient was performed by using a UHPLC 1290 pump. The addition of a UHPLC valve and pump to a standard 1100 series LC system (autosampler, pump and column compartment) greatly extends operational flexibility including column selection, while standard LC – which is already available in the lab, performs the initial steps of sample loading and clean-up.

We successfully used this platform for LC/MS analysis. Instead of retiring an entire functioning Agilent 1100 LC system, we just added one UHPLC pump to achieve much greater overall performance, functionality and lower cost, compared to a single pump UHPLC system purchase.

Figure 1. An example of a 2D UHPLC MS/MS system utilising UHPLC pumps in both dimensions.

Simple Column Switching in HPLC

The majority of liquid chromatography systems are based on a single pump and support one column operations. In retrospect – such a 'standard' configuration is more rugged, has simpler operation, requires less skill for method development, troubleshooting and is easy to support. For many years, more complex chromatographic systems were considered unusual or even exotic among the majority of HPLC practitioners. Based on nearly 15 years' experience in our laboratory, we can say that even simple column switching HPLC methods always require higher level method developmental skills, technical support and operator experience. One of the simplest LC column switching platforms consists of 2 gradient pumps supporting alternate column regeneration [1, 2]. In this platform 2 identical columns and 2 gradient pumps are connected via a 2 position/10 port valve. While the first column is used for chromatographic separation, the second column has regeneration and equilibrations steps. Such a configuration usually saves from 1/3 to 1/2 of method run time, compared to the single column/single pump standard configuration.

Another simple type of 2 pump operations

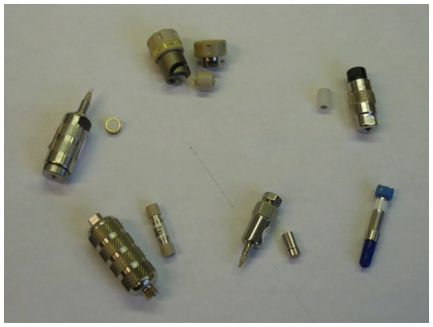

Figure 2. Different guard cartridges and trap columns available for on-line analyte trapping.

was designed for analysis of dirty samples or complex/clinical matrices. The sample (usually untreated) was loaded onto the special online SPE cartridge and large size impurities were excluded/not retained and flushed to waste.

After the cartridge was loaded and washed, it was connected (by valve switching) on the same flow path with the analytical column. Typically, after column switching, the sample preparation cartridge is connected to the analytical column in the reverse direction, compared to the loading step [6, 7]. This is called back flush. This mode offers several benefits, including fast transfer of the analyte of interest to the analytical column, and minimal dispersion of the sample band. With a change in switching valve plumbing, an analyte transfer to the analytical column can be achieved with a forward flush [8, 9]. The combination of forward flush, different column selectivity, and gradient elution – are essentials for multidimensional chromatography. Sample preparation columns however, are not optimal for gradient elution due to their large particle size and/or packing material design. Thus, low resolution sample preparation column or traps, cannot function well as another orthogonal chromatographic dimension. Therefore, the majority of online SPE methods are simple back flush in design. The steps of column switching are as follows: 1. The analyte of interest is transferred to the trap SPE column followed by a wash step. 2. The valve switches, allowing the analytical and sample preparation columns to be connected 3. The valve switches again, and the columns become disconnected. The analytical column undergoes gradient elution and analyte detection, while the sample preparation cartridge undergoes regeneration and equilibration prior to the next sample injection. These concepts are well known but unfortunately are not commonly employed.

A similar design, that utilises a simple trap/guard column instead of a sample preparation cartridge, is well known in capillary/nano chromatography [10]. At submicroliter/min flow rates, sample loading and washing can be very prolonged. To accelerate this time consuming step, sample loading and washing are performed at high flow rates to the trap cartridge, minimising the impact of pump and autosampler delay volume. After the sample load and wash steps, the trap column is connected to the capillary column via the switching valve, similar to online SPE, and followed by analyte(s) transfer from trap to column.

As we can see, these trap loading applications are usually beneficial to protect the analytical column or the mass spectrometer [11] when dirty/complex samples are used or with low flow rate applications. For analysis of adequately pure samples, such as for QC/QA, there is no need for additional system complexity beyond the single pump/single column configuration. It is an unwritten but axiomatic rule for standard HPLC applications, that simple HPLC systems are more robust and easier to support compared to more complex column switching platforms.

Benefits and Limitations of UHPLC

In this report, we question the validity of this axiom as applied to UHPLC applications. Initial UPLC/UHPLC systems, similar to standard [400-600 bar pressure] HPLC, have a similar design: single pump, single autosampler and single column; and typically – single detector. We would like to emphasise, that UHPLC is not a system that is only capable of operating at higher pressures. UHPLC is optimised to work with sub 2 micron ultra –high resolution columns to produce and detect very narrow chromatographic peaks. To ensure high efficiency of sub 2 micron particle size columns, all components of the LC system were redesigned and optimised. UHPLC –in contrast to conventional HPLC, is a low dwell volume system designed for fast runs at high pressure. I will not discuss in this paper changes in column technology, pump, detector and column compartment design that lead from HPLC to UHPLC – this could be the subject of a separate series of lectures. We will only highlight a few critical aspects. UHPLC columns are optimal for sample injection volumes of a few microliters. Therefore, typical UHPLC autosampler injection volume is reduced to around 25 µl maximum. Utilisation of smaller injection loops minimises system large delay volumes typical of larger sample loops, and is essential for UHPLC efficiency. Unfortunately, there is a major problem if large volumes of diluted sample have to be injected. Injection of diluted samples is frequently necessary in bioanalytical applications, such as dilute and shoot or analysis of ultra-filtrates. Samples after SPE contain high concentrations of organic solvent, which should be removed prior to injection onto reverse phase columns. A common approach to removing the solvent is to dry the SPE eluate, however certain biological compounds do not redissolve in the injection solvent buffer, either due to adsorption or due to solubility

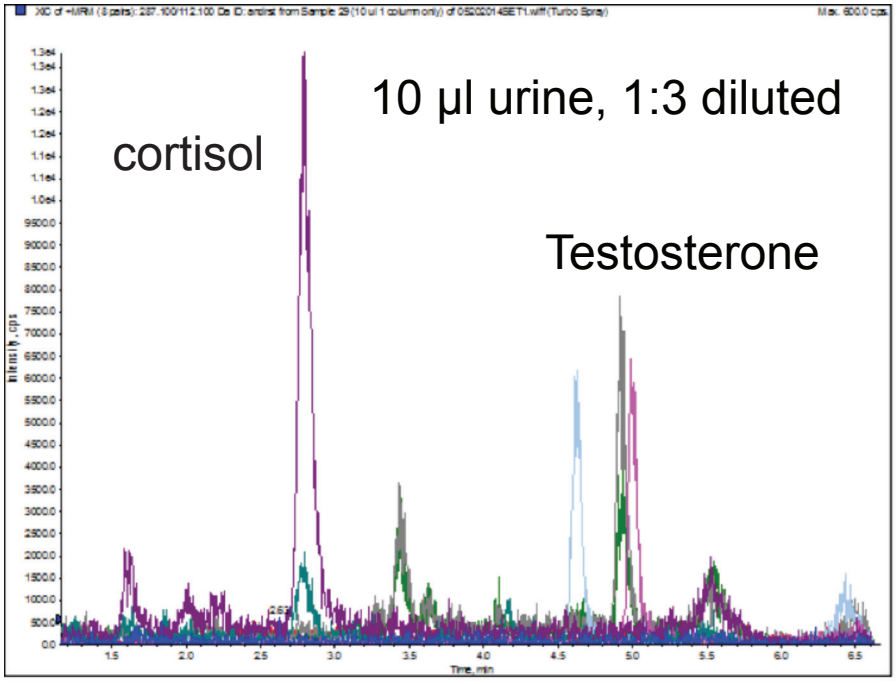

Figure 3. Analysis of urinary cortisol and testosterone by single column UHPLC-MS. Retention times of 2.8 and 5.0 mins, respectively. 2x50 mm C18 Ascentis Express column (Supelco). 30%B-60%B water/methanol linear gradient containing 0.4% formic acid over 4.2 minutes, flow rate 0.4 ml/min.

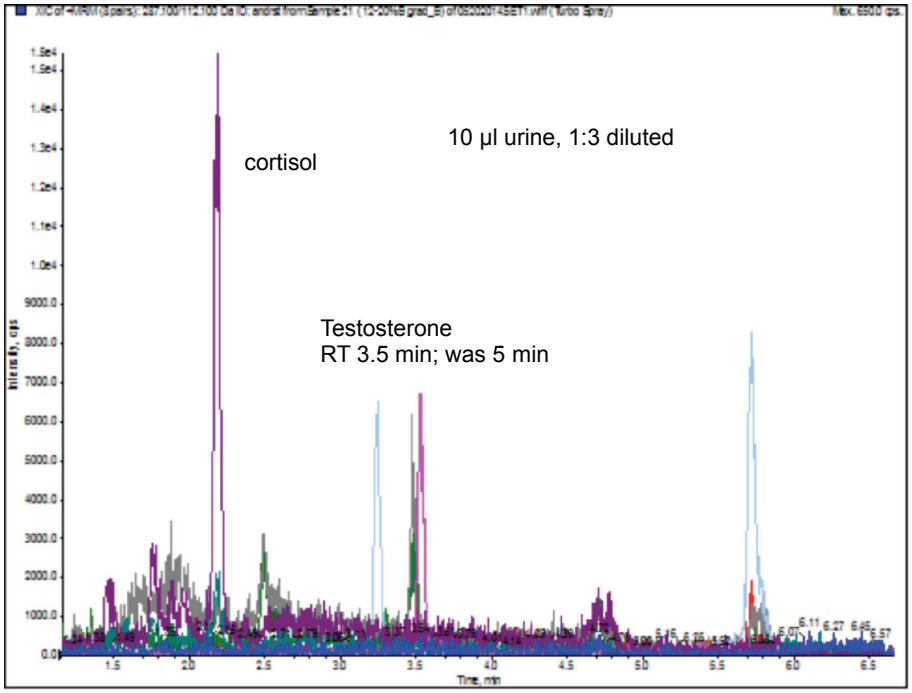

Figure 4. Reduction in elution time of urinary cortisol and testosterone from 2.8 to 2.1 and from 5.0 to 3.5 min by implementation of a loading trap with a HPLC-UHPLC combination.
Loading trap: 2x20 mm C8 Ultra guard column (Restek). Water/acetonitrile containing 0.1% TFA step gradients at flow rate 0.4 ml/min. 0.25 min sample load at 10%B followed by 0.15 min wash at 20% B. Trap to column transfer from 0.4 min to 2.5 min.

issues. There is also the problem that direct injection of the redissolved SPE eluate vastly reduces column lifetime, unless sample filtration or centrifugation are performed. We observed that partial drying of biological samples, followed by dilution prior to injection, is safer for a column frit; however, a large sample volume needs to be injected. On the other hand, installation of a large injection loop to a UHPLC autosampler is highly counterproductive, since this contributes large delay volume to the system, which should be avoided when using UHPLC columns.

The next important topic is the reduced lifetime of the rotor seals by higher pressure in the autosampler and other switching valves employed by the UHPLC system. For example, the dump valve – a valve that selects flow from the column either to waste or to the mass spectrometer source, operates basically at low pressure. In our oldest LC/MS system, this valve has not required maintenance for 12 years. UHPLC autosampler seals are replaced every few months with moderate 600-800 Bar pressure applications. It is important to note that rotor seal wear debris, notable as tiny black dust, does not disappear nor is flushed out, and steadily accumulates on top of the column frit [12]. To prevent excessive rotor seal wear in column switching applications, it is essential to decrease system pressure. Therefore use of core-shell (fused-core) technology columns is more attractive instead of sub 2 micron UHPLC columns. In addition, we have noticed a significant decrease in the performance of the UHPLC mixer above 800 bar due to increased solvent viscosity at these higher pressures. This is an additional factor in favour of choosing core-shell columns. In our experience ruggedness of system operation is maximised when the UHPLC system operates in the 600-700 bar range, and preferably not exceeding approximately half of the system maximum.

As mentioned previously, an increase in UHPLC complexity with a 2D UHPLC platform (Figure 1) increases the expense and complexity of operations and support. After a few years of 2D UHPLC LC/MS use, we started to downgrade and modify it by adding standard HPLC components. With experience, we understood that some UHPLC column switching applications do not require solely UHPLC chromatographic equipment. In parallel, we conducted hardware research and modifications of our old 2D LC/MS system, constructed from Agilent 1100 modules.

Combining HPLC-UHPLC Components

Our old standard 2D LC/MS system was designed for routine analysis of peptide hormones from SPE-pre-purified human plasma, and utilised 2 different chromatographic columns, peak heart cut analyte transfer with simultaneous in-line dilution [13, 14]. Currently our human C-peptide assay, utilises this 2D platform, and is considered a reference method registered with the International Federation for Clinical Chemistry. In the second dimension we now use a short, 3 cm length 2 mm i.d. fused core C18 column (Ascentis Express peptide) from Supelco [15]. The method operating pressure was under 250 bar and the performance of a standard HPLC was still acceptable after the major gradient delay volume reduction and optimisation of solvent mixing.

Recently manufactured HPLC and UHPLC modules are network ready under Windows 7 and 8 OS, while older Agilent 1100 modules are only network supported via a special network adapter. We took a stand alone Agilent 1290 UHPLC pump and initialised a network with the computer and communications via Chemstation as the master device. Other 1100 series HPLC modules were networked to each other, and at the end – to the new UHPLC pump. This network configuration works with Windows XP, 7 and 8. This was the first unexpected benefit of the addition of a new module to our old system.

However, if a UHPLC pump is connected to a standard pressure –large delay autosampler, this does not allow the benefits of UHPLC to be realised within the system. We purchased a 6 port -2 position MXT715 series Rheodyne UHPLC valve, and synchronised via contact closure with the HPLC system. Using a 6 port valve it is easy to establish a typical "online SPE" platform. A standard pressure HPLC autosampler connected to a standard HPLC pump can initiate sample loading to a trap column, followed by washing the trap to waste. Typically, biological samples contain salts which interfere with sample ionisation; salts may also form adducts with the analyte or suppress analyte ionisation. For hydrophobic analytes which are retained on the trap, a washing step is very beneficial. The wash step can easily be performed at 1-2 ml/min. This helps wash out the autosampler sample loop and valve, which assists in reducing carryover. A trap is connected to the UHPLC valve. Valve switching changes the plumbing from trap to waste, to trap to UHPLC column. In a second valve position, a trap to fused core column to UHPLC pump are disconnected from the autosampler and its large gradient delay volume; this allows fast undistorted gradients to be performed.

There are special trap columns/cartridges available on the market. In addition standard guard cartridges can also be used as traps (Figure 2). Typically, HPLC guard hardware is stable up to 400 bar with some exceptions up to 600 bar. UHPLC guards are also available, however in fewer dimensions

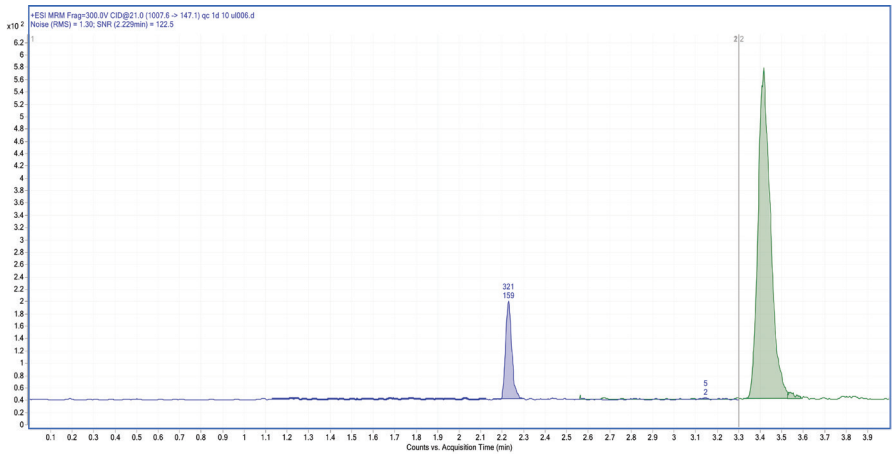

Figure 5. Increase in ionisation efficiency of peptide analyte: Plasma human c-peptide after SPE extraction. Samples contained high salt and phosphate concentrations. Peak 1 (blue) single column method. Peak 2 (green) trap-column method.
1D method: 2x30 mm C18 Ascentis Express Peptide column (Supelco). 20%B-30%B water/acetonitrile linear gradient containing 0.4% formic acid over 4.0 minutes, flow rate 0.4 ml/min. Loading trap 2x10 mm Zorbax StableBond 300A C18 (Agilent). 85% water/15% acetonitrile containing 0.1% TFA appled for loading and washing steps at 0.75 ml/min.

and chemistries, have lower capacity, and are considerably more expensive. The UHPLC guard column hardware is specially designed to have less void volume. This feature however, is less important in a 6 port valve column switching applications, since the trap column should not be connected for the entire run to the fused core column; it is only essential for a brief analyte transfer from the trap to the column. This transfer can be performed at a slower flow rate to keep pressure below 400 bar for a short time, and then flow rate is increased again after valve switching. The disconnected trap can be regenerated and equilibrated by a standard HPLC pump. With dirty biological samples and moderately hydrophobic analytes, the cartridge retains phospholipids and other hydrophobic bulk impurities, as well as potentially large particulates, thus keeping the fused core column clean.

A commonly accepted 'rule' of HPLC method development suggests that the guard column should be made from the same material as the main analytical column. That's true, if there is no valve between the guard and column. The optimal trap choice however, is an interesting and intriguing part of method development. Trap dimensions and chemistry, mobile phase composition (not necessary identical with the analytical column), back flush or forward flush, loading and washing flow rates, are mostly analyte-dependent key factors that may not be optimal when generic single column methods are applied.

During our research we found that our HPLC-UHPLC platform is more economical, rugged and allows larger volume to be injected compared to UHPLC alone. For analysis of urinary cortisol and testosterone by HPLC-UHPLC-MS we achieved earlier steroid elution compared to a single column UHPLC method, reducing method run time (Figures 3 and 4). The impact of sample quality on the ionisation efficiency of peptides can be unpredictable; LC/MS analysis of peptides are very analyte and instrument dependent. In LC/MS analysis of complex biological samples, use of a trap allows more efficient and faster eliminations of salts, thus keeping the mass spectrometer cleaner – a key factor for mass spectrometer sensitivity and ruggedness (Figure 5).

In Conclusion

Based upon our experience we believe that hybrid HPLC-UHPLC is a more rugged, economical and versatile column switching system as compared to a pure 2D UHPLC configuration. For the latter, sample volume is limited and for longer columns due to pressure limitations the washing/equilibration flow rate cannot as high as for shorter 50 mm length columns.

Sample loading to trap, using a standard HPLC pump and autosampler is more convenient and cost efficient. Loading and wash steps using a trap greatly improve ruggedness of mass spectrometer operations. We successfully used a HPLC-UHPLC platform for LC/MS analysis. Instead of retiring an entire functioning Agilent 1100 LC system, we just added one UHPLC pump to achieve much greater overall performance, functionality and lower cost compared to a new UHPLC system purchase. The addition of a UHPLC valve and pump to a standard 1100/1200 series HPLC system (autosampler, pump and column compartment) greatly extends operational flexibility including column selection, while standard HPLC – which is already available in the lab, performs the initial steps of sample loading and clean-up. In the future, when HPLC practitioners gain confidence with HPLC-UHPLC MS method development, this will facilitate further implementation of immunoaffinity LC/MS analysis. Immunoaffinity columns typically do not tolerate high pressures, and the analyte transfer from immunoaffinity column to trap will be the right analytical solution.

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