Using Online Sample Preparation with LC-MS/MS to Speed Up the Process of Analysing Pharmaceutical and Personal Care Products

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There is growing environmental concern regarding the health impact of trace levels of pharmaceuticals and personal care products (PPCPs) in water resources. Although the term PPCP was only recently coined, these bioactive chemicals have been in existence for decades, and their effect on the environment is now recognised as an important area of research. PPCPs include prescription and over-the-counter therapeutic drugs, cosmetics, and nutraceuticals such as vitamins. These substances can find their way into water through human activity, residues from pharmaceutical manufacturing and residues from hospitals. Some PPCPs are easily broken down and processed by the human body or can degrade quickly in the environment. Others, however, are not broken down so easily and enter domestic sewers. Until recently individuals' contribution to the combined chemical load in the environment has gone largely unnoticed.

In response to this concern, the U.S Environmental Protection Agency (EPA) recently published Method 1694, which determines dozens of PPCPs in water, soil, sediment and biosolids by high performance liquid chromatography combined with tandem mass spectrometry[1] (HPLC-MS/MS). The method, yet to be promulgated, uses solid phase extraction (SPE) of water samples followed by HPLC-MS/MS analysis using a single transition for each compound to achieve low nanogram/liter (ng/L) limits of quantitation (LOQs). The target analytes in the EPA method are divided into four groups, with each group representing one HPLC-MS/MS run. Three of the groups are extracted under acidic conditions; the fourth is extracted under basic conditions.
These SPE methods can use up to 1 L of sample. Although not sample limited, the storage of large bottles of water requires a great deal of refrigeration space. In addition, manual SPE of 1 L of sample requires several hours of preparation.

One of the opportunities afforded in the analysis of PPCPs in water is to reduce the time required for sample preparation and analysis while maintaining the required sensitivity at the ng/L level and the selectivity to positively identify the analyte of interest. This article will describe a method for online sample preparation and analysis using the Thermo Scientific EQuan system.
This method couples a fast HPLC system with two LC columns – one for pre-concentration of the sample and the second for the analytical analysis – and a high performance

Compound	Class	Compound	Class
Trimethoprim	Antibiotic	4-epi-Chlorotetracycline	Antibiotic, tetracycline
Cefotaxime	Antibiotic, cephalosporin	Demeclocycline	Antibiotic, tetracycline
Norfloxacin	Antibiotic, fluoroquinolone	Chlorotetracycline	Antibiotic, tetracycline
Ofloxacin	Antibiotic, fluoroquinolone	Doxycycline	Antibiotic, tetracycline
Ciprofloxacin	Antibiotic, fluoroquinolone	Anhydrotetracycline	Antibiotic, tetracycline
Lomefloxacin	Antibiotic, fluoroquinolone	Carbamazepine	Anticonvulsant
Enrofloxacin	Antibiotic, fluoroquinolone	Fluoxetine	Antidepressant
Sarafloxacin	Antibiotic, fluoroquinolone	Miconazole	Antifungal
Flumequine	Antibiotic, fluoroquinolone	Thiabendazole	Antihelmintic
Lincomycin	Antibiotic, macrolide	Diphenhydramine	Antihistamine
Azithromycin	Antibiotic, macrolide	Acetaminophen	Analgesic
Erythromycin	Antibiotic, macrolide	Codeine	Analgesic, narcotic
Tylosin	Antibiotic, macrolide	Cimetidine	Antiacid reflux
Anhydroerythromycin	Antibiotic, macrolide	Ranitine	Antiacid reflux
Clarithromycin	Antibiotic, macrolide	Digoxigenin	Antiarrythmic
Roxithromycin	Antibiotic, macrolide	Digoxin	Antiarrythmic
Ampicillin	Antibiotic, penicillin	Diltiazem	Antiarrythmic, benzothiazepine
Penicillin G	Antibiotic, penicillin	Dextromethorphan**	Antitussive
Penicillin V	Antibiotic, penicillin	Atenolol	Beta-blocker
Oxacillin	Antibiotic, penicillin	Metoprolol	Beta-blocker
Cloxacillin	Antibiotic, penicillin	Propranolol	Beta-blocker
Metformin*	Antidiabetic	Albuterol	Bronchodialator
Sulfadiazine	Antibiotic, sulfa	Midazolam	Sedative, benzodiazepine
Sulfathiazole	Antibiotic, sulfa	1-OH Midazolam	Sedative, benzodiazepine
Sulfamerazine	Antibiotic, sulfa	1-OH Alprazolam	Sedative, benzodiazepine
Sulfamethazine	Antibiotic, sulfa	Alprazolam	Sedative, benzodiazepine
Sulfamethizole	Antibiotic, sulfa	Nordiazepam	Sedative, benzodiazepine
Sulfachloropyridazine	Antibiotic, sulfa	1,7-Dimethylxanthine	Stimulent
Sulfamethoxazole	Antibiotic, sulfa	Caffeine	Stimulent
Sulfadimethoxine	Antibiotic, sulfa	Benzoylecgonine	Stimulent
Minocycline	Antibiotic, tetracycline	Cocaine	Stimulent
Oxytetracycline	Antibiotic, tetracycline	Cocaethylene	Stimulent
4-epi-Tetracycline	Antibiotic, tetracycline	Cotinine	Stimulent
Tetracycline	Antibiotic, tetracycline		

**Metformin was analysed using HILIC*
***PPCPs not included in E.P.A 1694*

Table 1. PPCPs analysed

[1]*EPA Method 1694: Pharmaceuticals and personal care products in water, soil, sediment, and biosolids by HPLC/MS/MS, December 2007, EPA-821-R-08-002*

LC-MS/MS instrument. Instead of processing 1 L of water by the manual, time consuming process of SPE described in EPA Method 1694, this alternative approach incorporates online sample preparation in series with LC-MS/MS using smaller volumes of water (0.5 - 20 mL) to achieve ng/L quantitation limits. This article will demonstrate a progressive approach to analysing PPCPs in environmental sources of water at the ng/L level with online sample preparation using small volumes of water, thus saving time and reducing the cost of analysis

Experimental

Water samples of 0.5 mL were directly injected onto a pre-concentration trapping column (2.1 x10 mm, 12 µm) at 1.5 mL/mins with H_2O + 0.2% formic acid. After sufficient washing of the pre-concentration column, the target compounds were transferred to the analytical column (2.1 x 100 mm, 3 µm) for chromatographic separation by gradient elution prior to introduction into the mass spectrometer.

MS analysis was carried out with a Thermo Scientific TSQ Vantage triple stage quadrupole mass spectrometer. Two selected reaction monitoring (SRM) transitions per compound were acquired; one for quantitation with the other for positive confirmation. To maximise the performance of the triple stage quadrupole, time-specific SRM 'windows' were employed at the retention times of the target compounds.

Results and Discussion

The current EPA method 1694 describes three different LC methods for PPCPs from Groups 1, 2 and 4 which are amenable to positive electrospray ionisation (ESI) MS/MS. In order to simplify the method and reduce the total analysis time, a single 10-minute LC-MS/MS method was developed which includes compounds from additional pharmaceutical classes not included in EPA Method 1694, such as beta-blockers. In total, 67 compounds were analysed by positive ESI-MS/MS (see Table 1). Of these, 54 were from EPA Method 1694 Groups 1, 2 and 4.

Such a diverse range of chemical classes meant the challenge was in developing a single LC-MS/MS method without compromising the target ng/L sensitivity. Both sample pH and the %ACN in the sample influenced the response of PPCPs in water when employing the online sample preparation approach with the EQuan system. To determine the best method for

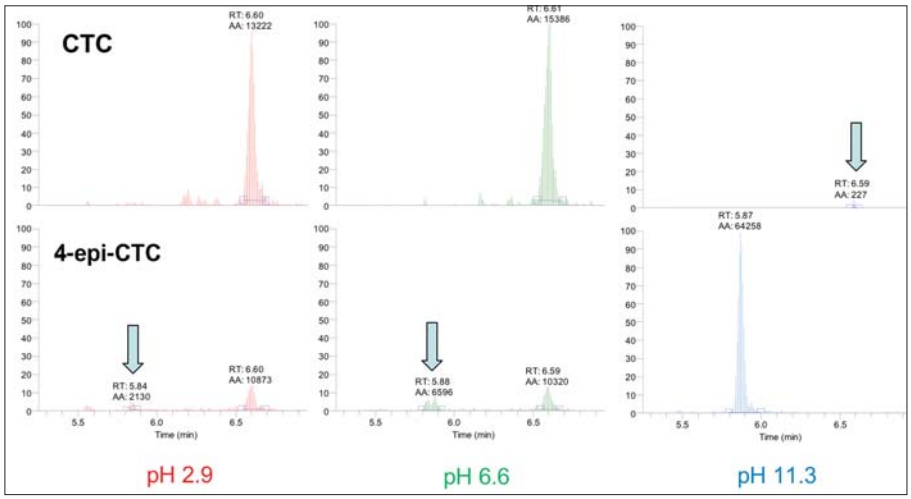

Figure 1. Chromatograms showing the pH effect on chlorotetracycline (CTC).

achieving ng/L sensitivity on the mass spectrometer, the effects of sample pH and %ACN were investigated.

Effects of Sample pH

It was found that sample pH affected the response of some PPCPs in water based on chemical reactivity. During the method development, PPCPs were added to aqueous solutions at three different pHs: 2.9, 6.6 and 11.3. As shown in the chromatograms in Figure 1, chlortetracycline (CTC) was readily observed at pH 2.9 and pH 6.6. However, at pH 11.3, CTC completely disappeared, being converted to 4-epi-CTC. It is important to note that no 4-epi-CTC was added to the water samples prior to LC-MS/MS analysis. All of the 4-epi-CTC detected was a result of the conversion of CTC, which has been shown to have a short half life in solutions at pH 11.2. A similar effect was observed with erythromycin, which reacted quickly in acidic solution and converted to anhydroerythomycin at pH 2.9.

The pH also affected the solubility of some PPCPs, even within the same compound class. Figure 2 displays the area response for cloxacillin and penicillin. For cloxacillin, the area response at pH 6.6 is evident in the bar chart at the top left; whereas at pH 11.3, cloxacillin was not observed. A similar effect was seen for ampicillin, oxacillin, cefotaxime and diltiazem. However, the opposite effect was observed for penicillin V (and G), as seen in the bar chart. The same trends were also observed with LC-MS/MS (5.0 µL injection) as with the EQuan method (0.5 mL injection) indicating that this is a sample solubility effect.

The pH effect on the MS response was also observed with several other PPCPs when using the EQuan system. Using ranitidine as an example, the MS response was observed with several other PPCPs when using the system. The MS response was much greater at pH 11.3 than at pH 2.9 or 6.6, as shown in the chart at the top left of Figure 3. However, this pH effect was not present when using a 5.0 µL injection of the water samples directly onto the analytical column at the same mass loading of ranitidine, as seen in the bar chart in the lower right of Figure 3. This difference

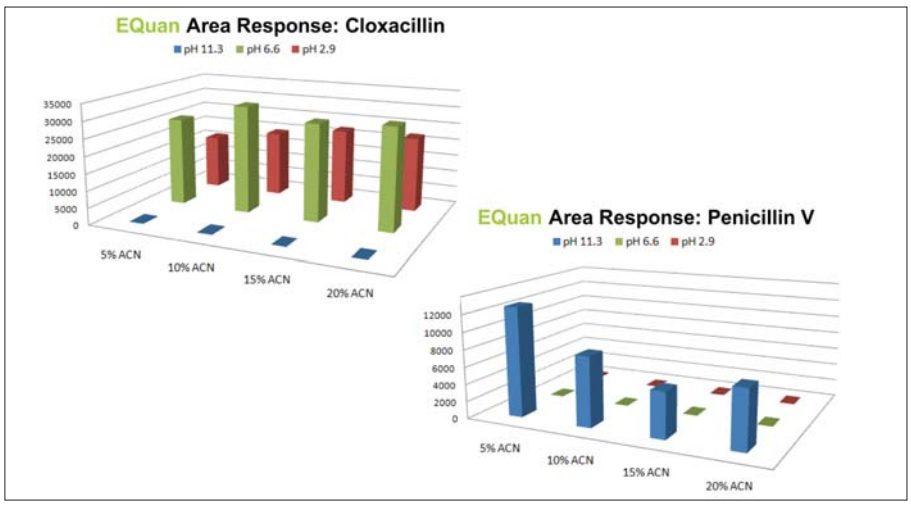

Figure 2. Area response plots demonstrating the pH effect on the sample solubility.

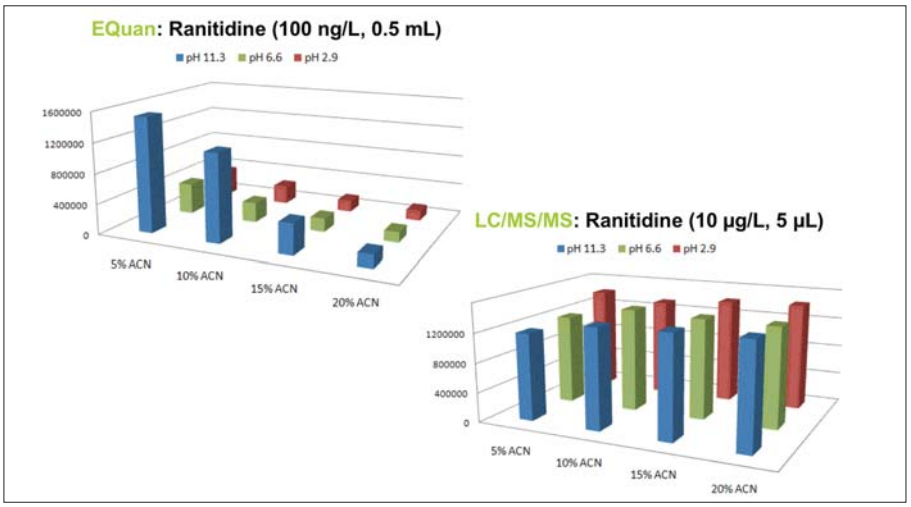

Figure 3. Area response plots for ranitidine demonstrating the pH effect on the preconcentration column.

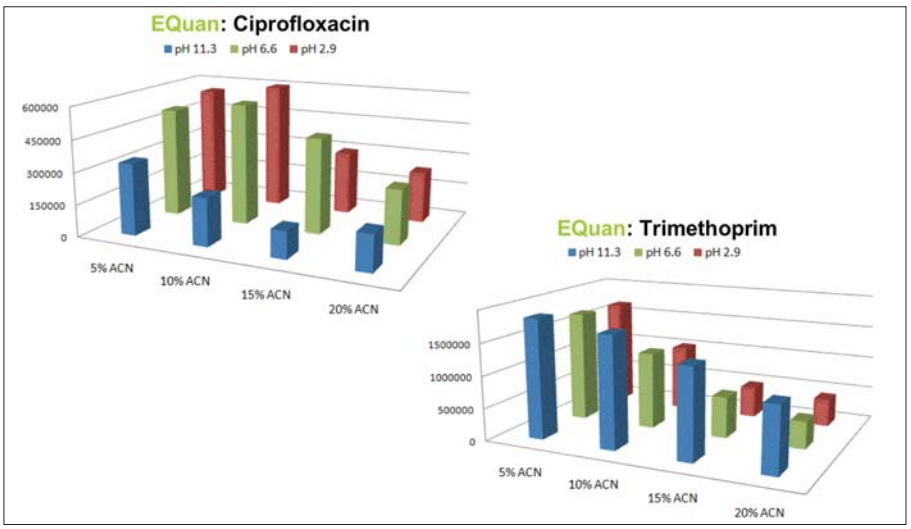

Figure 4. Area response plots showing effect of decreased retention with increasing %ACN.

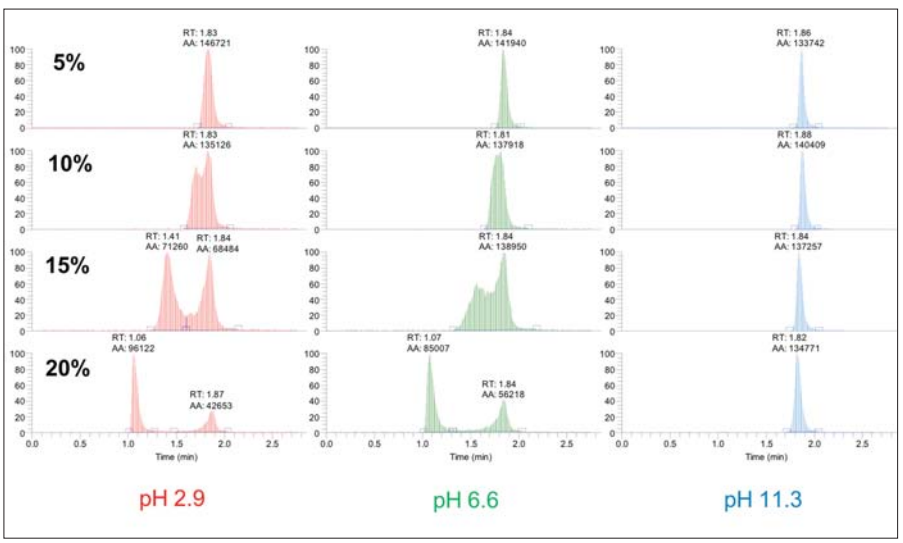

Figure 5. Chromatograms showing the %ACN effect on LC column retention for cotinine.

in response is believed to be attributed to the change in the local partitioning chemistry between ranitidine and the stationary phase of the pre-concentration column; the portioning chemistry was not affected for a long enough period to change the retention of ranitidine. Nevertheless, under the right sample solution conditions, namely pH 11.3, and 5 - 10% acetonitrile (ACN), ranitidine and other basic PPCPs, such as cimetidine, codeine and lincomycin, yielded quantitative trapping recovery using the EQuan system.

Effects of varying %Acetontrile

The effect on the LC-MS/MS response for the PPCPs was examined as a function of the %ACN in the water samples. Many of the larger, more lipophilic compounds, such as the macrolide antibiotics, showed a significant increase in area response as a function of increasing %ACN in the water sample. For tylosin and roxithromycin, the increased response was most dramatic between 5% and 10% ACN at pH 2.9. The area response increased by a factor of 3 for roxithromycin and a factor of 10 for tylosin when the %ACN was increased from 5% to 10%. The same trend was observed with LC-MS/MS (5 µL injection) as with the EQuan system indicating that this is a sample solubility effect due to the compounds' lipophilic nature.

Although increasing the %ACN in the water sample helped the response of certain PPCPs, it caused a significant decrease in response if the percentage was too high (Figure 4). This effect, observed for ciprofloxacin, trimethoprim, fluoroquinolones, and sulpha drugs, was attributed to a loss of compound retention on the trapping column. Here, compounds have a greater affinity for the solvent than the trapping column stationary phase. This effect is similar to compound "break through" on an SPE cartridge. No reduction in MS response was observed with a 5 µL injection onto the analytical column.

The effect of decreased analyte retention with increased %ACN in the water sample was also observed with cotinine using a 5 µL injection on the analytical C18 column. As Figure 5 shows, the LC peak splitting for cotinine was readily observed in acidic (red) and neutral (green) water samples. However, at pH 11.3 the cotinine peak was largely unchanged, even at 20% ACN. This is probably because the basic compound cotinine is uncharged at pH 11.3, which increases its affinity for the C18 stationary phase.

As seen with cotinine, the biggest challenge in developing an EQuan method for PPCPs was the small, highly polar organic compounds. Different trapping columns and mobile phases were tested. However, as expected, compromises had to be made to

allow the largest breadth of PPCPs in one LC-MS/MS run. Metformin was the clearest example. Despite many approaches, no satisfactory reverse-phase LC method could be discovered because of its high polarity. Therefore, as described in EPA Method 1694, hydrophilic interaction liquid chromatography (HILIC) was used for the successful LC separation of metformin in water. Again, pH had a dramatic effect on the response of metformin (and other Group 4 PPCPs). The best response for metformin was with the water sample adjusted to pH 11.3 prior to injection on the reverse-phase EQuan trapping column.

Conclusion

Despite all of the challenges in the development of one single LC/MS method for this diverse group of compounds, a balance was found that allowed the measurement of the 67 PPCPs in water by the Thermo Scientific EQuan system with a large majority being quantified at or below 10 ng/L using a 0.5 mL injection volume with detection on the triple stage quadrupole TSQ Vantage™ mass spectrometer. The best compromise for the online sample preparation method was to run an acidified and a basified water sample containing 10% ACN. Figure 6 shows example chromatograms for the PPCPs in water at the ng/L level using this approach. The red chromatograms were the water samples at pH 2.0 and the blue chromatograms were the water samples at pH 11.3. In general, basic conditions were preferable for analysing the smaller, more polar compounds and acidic preferable for analysing the larger, more lipophilic compounds. Use of this faster method will enable improved analysis of PPCPs and contribute to improving the environment.

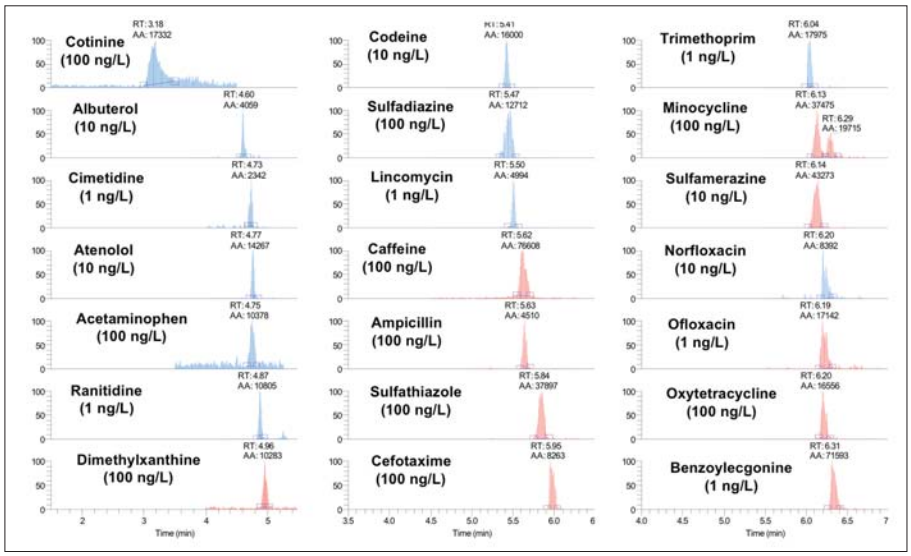

Figure 6. (a,b,c). Example chromatograms of the PPCPs in water at the ng/L level. The LLOQ for each compound is listed in parentheses.

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Agilent Technologies announce the launch of the 7200 Quadrupole Time-of-Flight gas chromatography/mass spectrometry (Q-TOF GC/MS) The Agilent 7200 is the world's first Q-TOF GC/MS built specifically for GC that delivers a new level of chromatographic and spectral resolution to a wide range of biological and chemical GC/MS analyses. The instrument was showed in Singapore at an event for the first time, and generated a lot of interest from the conference attendees. Agilent's 7200 Q-TOF GC/MS is well-suited for analyzing target compounds and unknowns in challenging applications such as metabolomics, food safety, natural products research, environmental analysis, sports-doping detection, and energy research. With the 7200 Q-TOF GC/MS, customers are able to coax more qualitative and quantitative information out of increasingly complex samples. Also at the event, Agilent introduced the 6550 iFunnel Quadrupole Time-of-Flight liquid chromatography mass spectrometry system (Q-TOF LC/MS). The 6550 iFunnel Q-TOF offers 10 times more sensitivity than its previous model, while delivering fast acquisition to 50 spectra/sec, up to five orders of intra-scan dynamic range, and robust performance and operation. The system is well suited for highly challenging, qualitative and quantitative analyses in applications such as metabolomics, food-safety screening, early-stage drug metabolism and pharmacokinetics, and protein identification.

For more information email terry_sheehan@agilent.com