Application Note: 508

Quantification of EPA 1694 Pharmaceuticals and Personal Care Products in Water at the ng/L Level Utilizing Online Sample Preparation with LC-MS/MS

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Key Words

- EQuan System
- TSQ Vantage
- PPCPs
- Water Analysis

Introduction

There is growing environmental concern regarding the health impact of trace levels of pharmaceuticals and personal care products (PPCPs) in water resources. 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 (HPLC-MS/MS).[1] The method, which is 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 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. We 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, the second for the analytical analysis – and an 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.

Goal

To demonstrate a progressive approach to analyzing 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 Conditions

The EQuan LC-MS/MS experimental setup is illustrated in Figure 1.

Sample Preparation

Aqueous solutions containing 5% – 20% acetonitrile (ACN) and adjusted to pH 2.9, 6.6 or 11.3 were spiked with more than 60 PPCPs at the low ng/L level.

HPLC

Water samples of 0.5 mL were directly injected onto a Thermo Scientific Hypersil GOLD aQ pre-concentration trapping column (2.1 x 20 mm, 12 µm) at 1.5 mL/min with H_2O + 0.2% formic acid. After sufficient washing of the pre-concentration column, the target compounds were transferred to the Thermo Scientific Betasil C18 analytical column (2.1 x 100 mm, 3 µm) for chromatographic separation by gradient elution prior to introduction into the mass spectrometer.

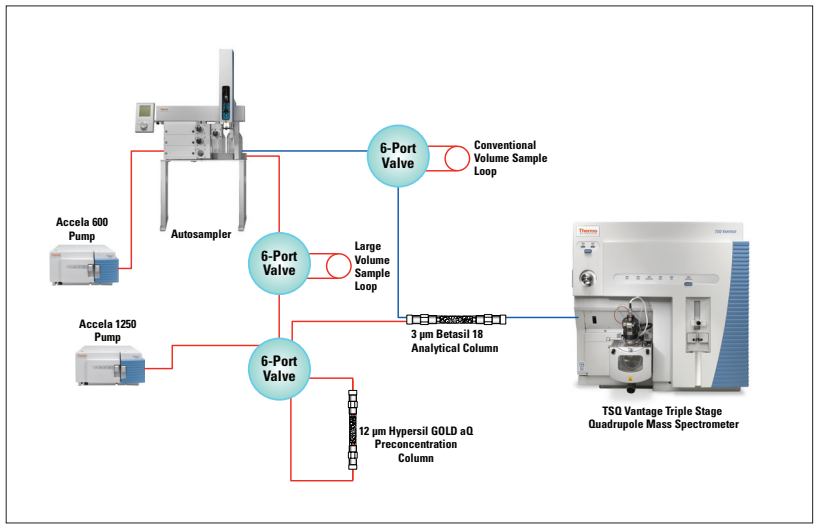

Figure 1. The EQuan pre-concentration LC-MS/MS experimental setup.

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MS

MS analysis was carried out on a Thermo Scientific TSQ Vantage triple stage quadrupole mass spectrometer. Two selected reaction monitoring (SRM) transitions per compound were acquired: one for quantitation and the other for positive confirmation. To maximize 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 ionization (ESI) MS/MS. To simplify the method and reduce the total analysis time, a single 10-minute LC-MS/MS method was developed, which included compounds from additional pharmaceutical classes not included in EPA Method 1694, such as beta-blockers. In total, 67 compounds were analyzed by positive ESI-MS/MS (Table 1). Of these, 54 were from EPA Method 1694 Groups 1, 2, and 4.

Table 1. PPCPs analyzed

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	Ranitidine	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	Stimulant
Sulfamethoxazole	Antibiotic, sulfa	Caffeine	Stimulant
Sulfadimethoxine	Antibiotic, sulfa	Benzoylecgonine	Stimulant
Minocycline	Antibiotic, tetracycline	Cocaine	Stimulant
Oxytetracycline	Antibiotic, tetracycline	Cocaethylene	Stimulant
4-epi-Tetracycline	Antibiotic, tetracycline	Cotinine	Stimulant
Tetracycline	Antibiotic, tetracycline		

**Metformin was analyzed using HILIC*

***PPCPs not included in EPA 1694*

With such a diverse range of chemical classes, 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 affected the response of PPCPs in water when employing the online sample preparation approach with the EQuan system. To determine the best method for achieving ng/L sensitivity on the TSQ Vantage™ mass spectrometer, the effects of sample pH and %ACN were investigated.

Effects of Sample pH

Sample pH was found to affect 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 2, chlorotetracycline (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 due to 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 anhydroerythromycin at pH 2.9.

The pH also affected the solubility of some PPCPs, even within the same compound class. Figure 3 displays the area response for cloxacillin and penicillin. For cloxacillin, the area response at pH 2.9 and 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 in the bottom right. The same trends were observed with LC-MS/MS (5 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 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 4. However, this pH effect was not observed when using a 5 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 4. This difference 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. With a 5 µL injection directly onto the analytical column, the partitioning 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% ACN, ranitidine and other basic PPCPs, such as cimetidine, codeine, and lincomycin, yielded quantitative trapping recovery using the EQuan system.

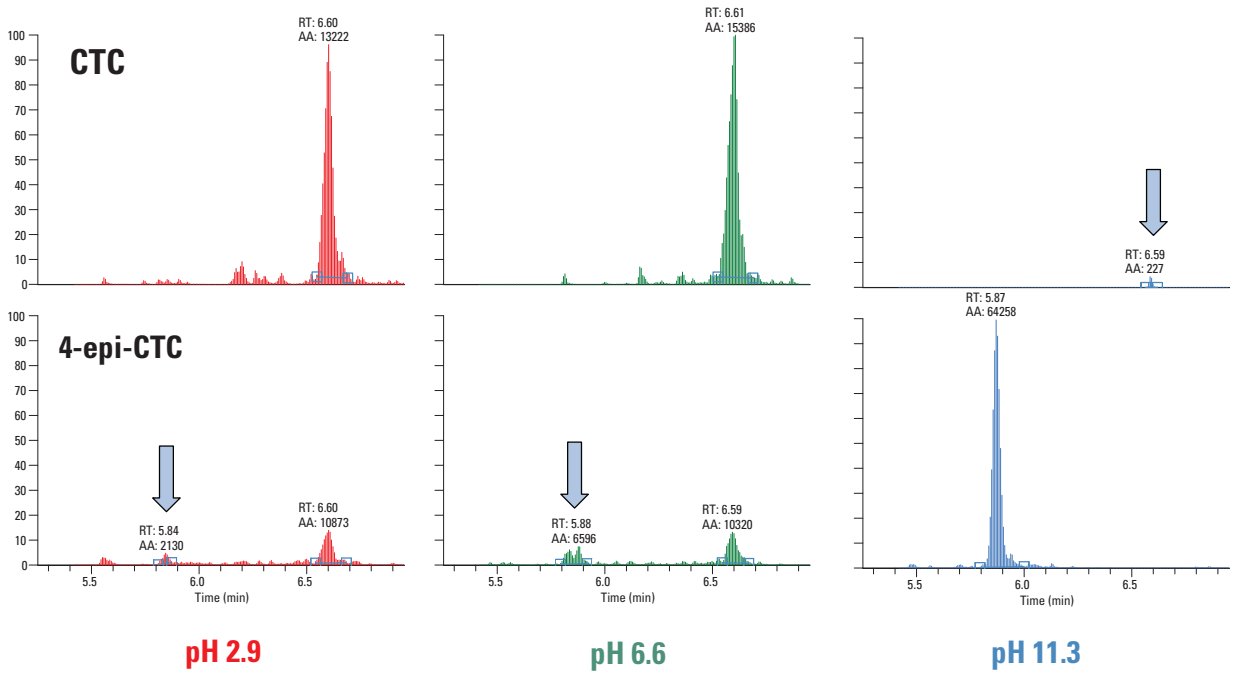

Figure 2. Chromatograms showing the pH effect on chlorotetracycline (CTC).

Effects of %ACN

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 in others if the percentage was too high (Figure 5). This effect, observed for ciprofloxacin, trimethoprim, fluoroquinolones, and sulfa drugs, was attributed to a loss of compound retention on the trapping column, where 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 fall-off in MS response was observed with a 5 µL injection onto the analytical column.

The effect of decreased analyte retention with increasing %ACN in the water sample was also observed with cotinine using a 5 µL injection on the analytical C18 column. As Figure 6 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 virtually unchanged, even at 20% ACN. This is likely due to the fact that 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, but 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 very high polarity. Hence, 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.

EQuan Method Summary

Despite all of the challenges in the development of one single LC/MS method for this diverse group of components, a balance was found that allowed the measurement of the 67 PPCPs in water by the 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 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 7 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 2.9, and the blue chromatograms were the water samples at pH 11.3. In general, basic conditions were preferable for analyzing the smaller, more polar compounds, and acidic conditions were preferable for analyzing the larger, more lipophilic compounds.

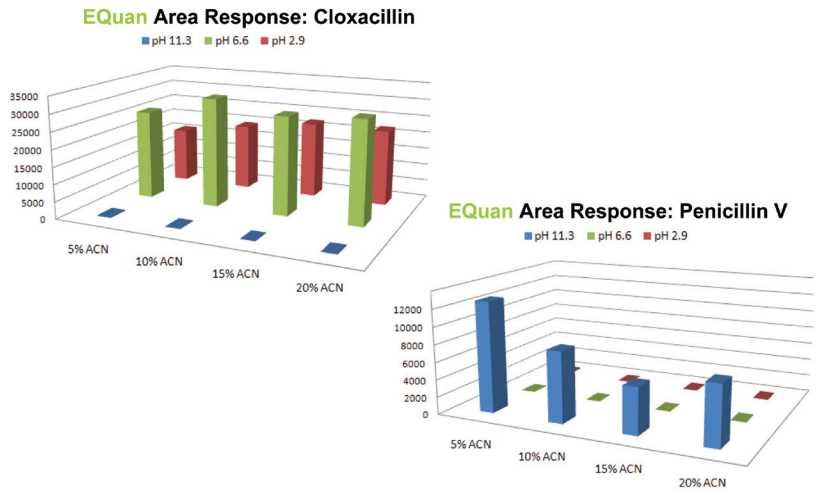

Figure 3. Area response plots demonstrating the pH effect on the sample solubility.

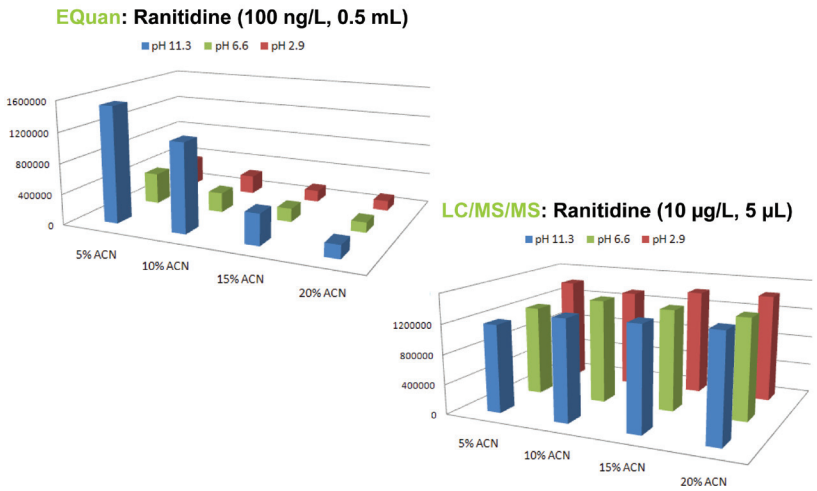

Figure 4. Area response plots for ranitidine demonstrating the pH effect on the preconcentration column.

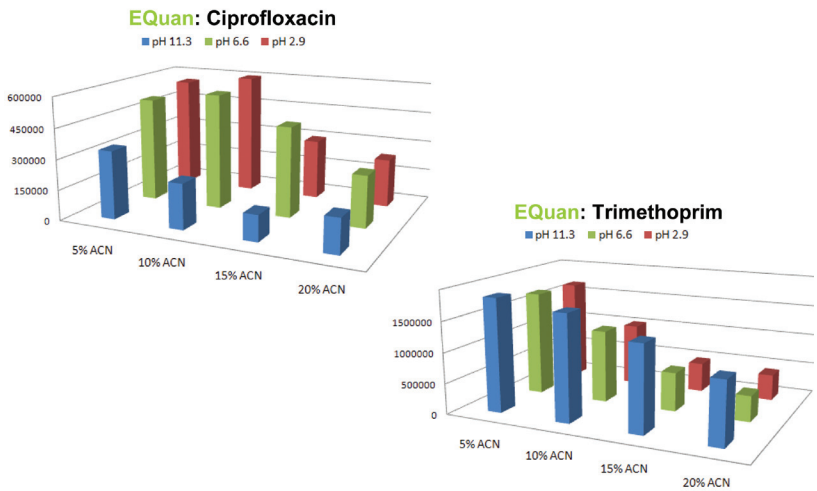

Figure 5. Area response plots showing effect of decreased retention with increasing %ACN.

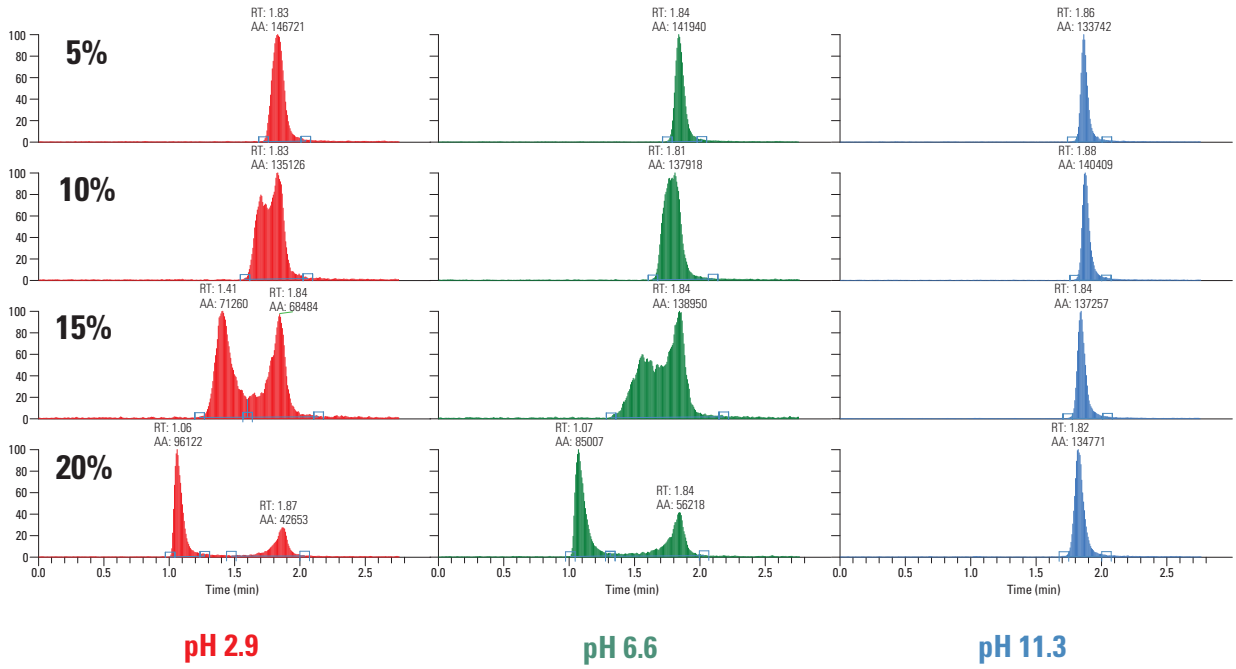

Figure 6. Chromatograms showing the %ACN effect on LC column retention for cotinine.

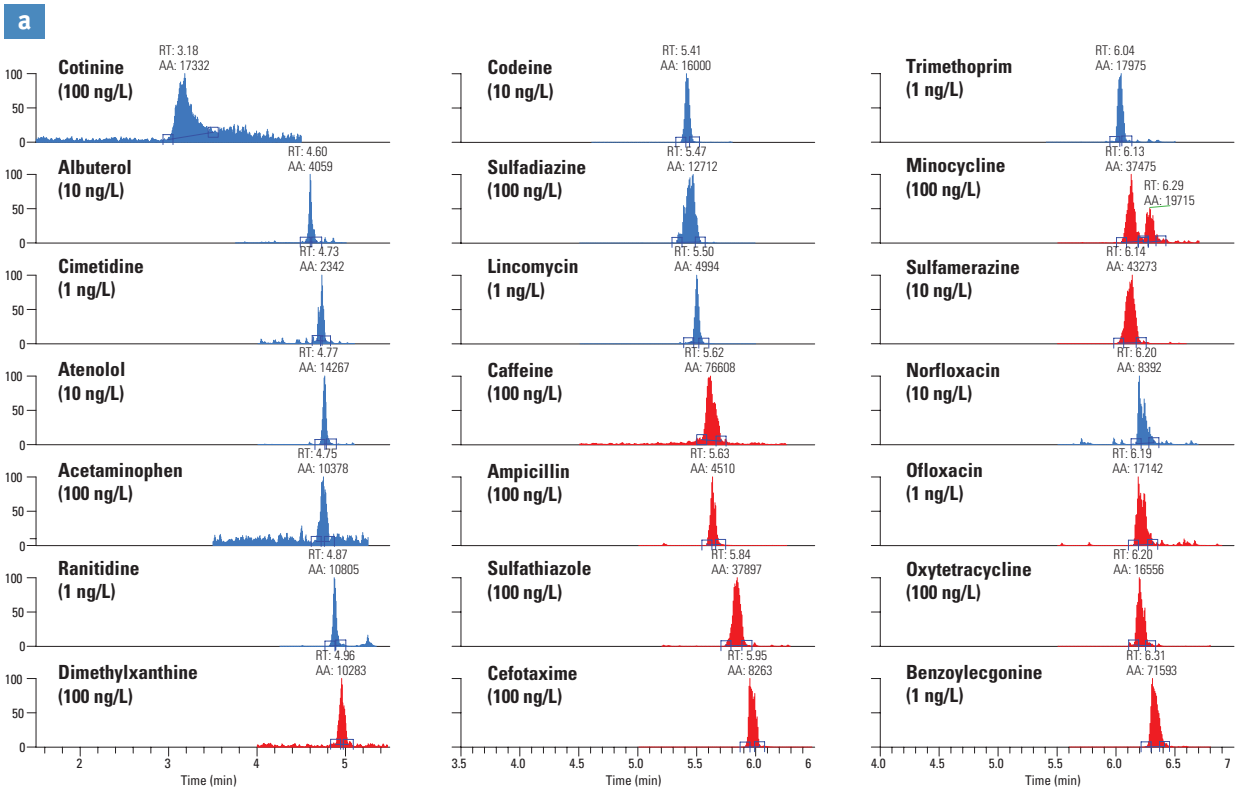

Figure 7 (a,b,c). Example chromatograms of the PPCPs in water at the ng/L level. The LLOQ for each compound is listed in parentheses.

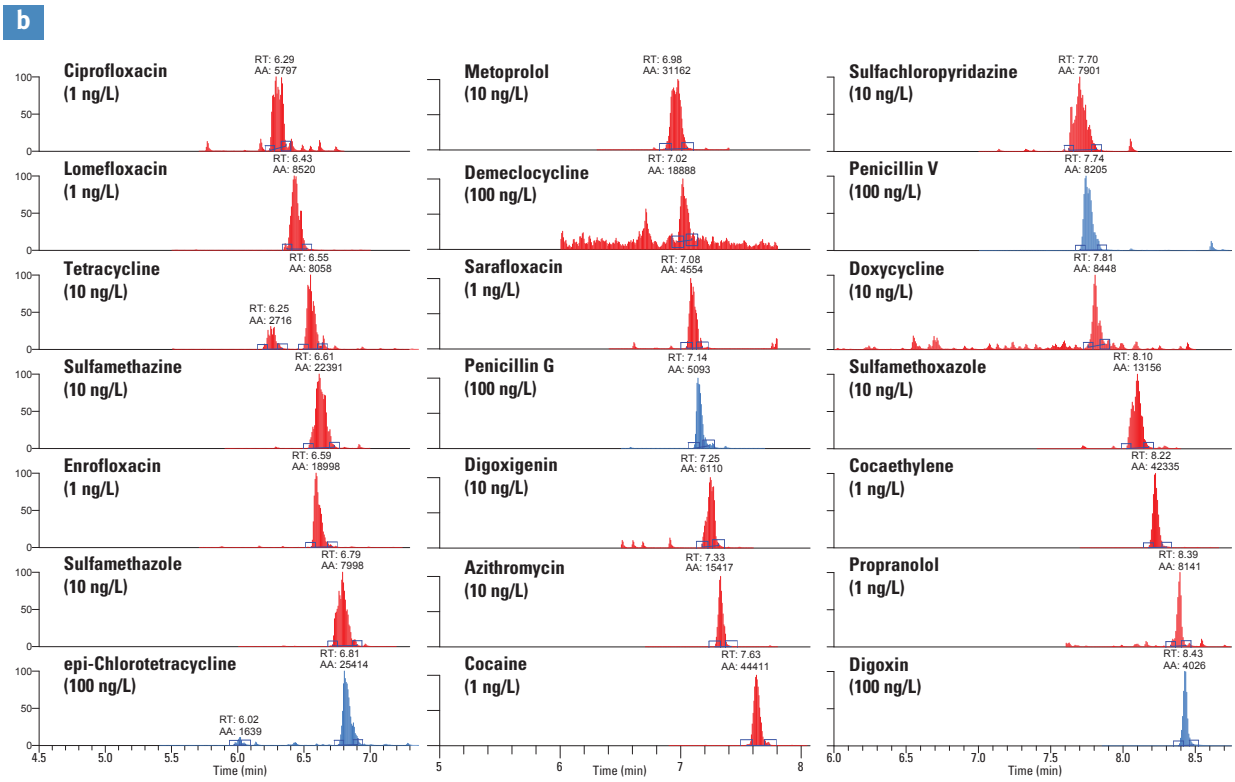

Figure 7. Example chromatograms of the PPCPs in water at the ng/L level. The LLOQ for each compound is listed in parentheses. (continued)

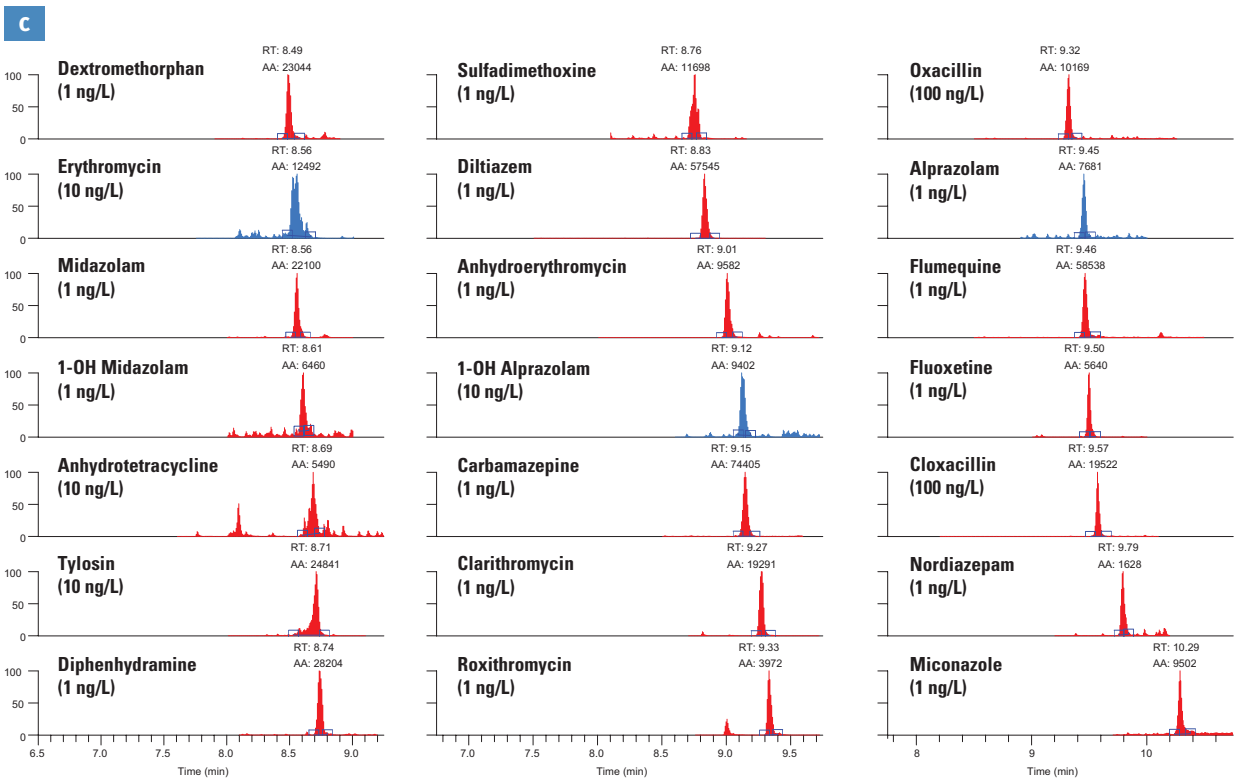

Figure 7. Example chromatograms of the PPCPs in water at the ng/L level. The LLOQ for each compound is listed in parentheses. (continued)

Conclusion

The current EPA Method 1694 describes three different LC methods for PPCPs from Groups 1, 2, and 4, which are amenable to positive ESI-MS/MS. To simplify the method and reduce total analysis time, a single 10-minute LC-MS/MS method was developed on the EQuan system including compounds from additional pharmaceutical classes not included in the EPA method, such as beta-blockers and benzodiazepines.

The EQuan system significantly reduced sample preparation and analysis time while providing quantification of PPCPs in water at low ng/L levels. Online sample preparation of the water samples eliminated the need to use two different offline SPE methods on 1 L of water. This reduced the total analysis time from hours to minutes. The sensitivity of the TSQ Vantage mass spectrometer, using time-dependent SRMs to maximize detector duty cycle, provided low- or sub-ng/L limits of quantitation for the targeted PPCPs in water.

References

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.
2. Loftin, K.A.; Adams, C.D.; Meyer, M.T.; Surampalli, R. "Effects of Ionic Strength, Temperature, and pH on Degradation of Selected Antibiotic" *J. Environ. Qual.*, **2008**, 37, 378-386.

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