Identification of Unknowns in Ground and Surface Water by LC/Q-TOF

Abstract

Accurate mass analysis by LC/Q-TOF with a series of accurate mass tools is used in this study to find target, suspect, and unknown pharmaceuticals and their degradation products in surface and groundwater along the South Platte River near Denver, Colorado. The ability to detect unknown pharmaceuticals is used to examine removal and transport of pharmaceuticals from a major metropolitan area (Denver) to the South Platte River and eventually to alluvial groundwater nearby. This study is a good example of using unknown identification to understand transport and removal of pharmaceuticals in groundwater and surface water systems.
Introduction

The importance of wastewater as a source of pharmaceuticals in surface water has been studied extensively since the late 1990s with a famous review of the problem reported in 1998\(^1\). This review was followed by a study of pharmaceuticals in surface water by the U.S. Geological Survey\(^2\). Both studies found that sewage wastewater was the major source of pharmaceuticals in water samples. Since then, thousands of papers have been published on the occurrence of pharmaceuticals in surface water and wastewater, including many review articles\(^3,4\). However, the occurrence of pharmaceuticals in groundwater is much less studied or reviewed\(^4\), despite the earliest documented report of pharmaceuticals in water being for groundwater impacted by sewage\(^3\).

This study describes the analytical workflow and the set of analytical tools with accurate mass that have successfully been used to identify pharmaceuticals and their degradants using liquid chromatography and quadrupole time-of-flight mass spectrometry (LC/Q-TOF-MS). In addition, using these workflows, both suspect and unknown pharmaceuticals found in the South Platte River near Denver, Colorado were reported. Finally, these pharmaceuticals and their degradants in alluvial groundwater wells affected by the South Platte River were measured using LC/Q-TOF-MS analysis to better understand transport and removal of pharmaceuticals. This Application Note is a compilation of the many studies carried out using the Agilent 6500 Series of LC/Q-TOF-MS for the discovery of pharmaceuticals in surface and groundwater.

Experimental

Samples

Ten alluvial wells along the South Platte River were sampled multiple times over a three-month period in 2016. Standard ground and surface water sampling methods were used, with both a peristaltic and bladder pump\(^5\). Sampling of the South Platte River and two alluvial wells was also carried out in 2009–2010, sampling monthly over one year. All of the alluvial wells were in hydrologic contact with the South Platte River. The South Platte River itself was sampled by grab sample. Figure 1 shows the location of the alluvial wells along the river.

Analytical methods

The separation of the analytes was carried out using an Agilent 1290 Infinity II LC equipped with a reversed-phase C8 analytical column of 150 mm x 4.6 mm and 3.5 µm particle size (Agilent ZORBAX Eclipse XDB-C8). Column temperature was maintained at 25 °C. The injected sample volume was 10 µL. Mobile phases A and B were water with 0.1 % formic acid, and acetonitrile, respectively. The optimized chromatographic method held the initial mobile phase composition (10 %A) constant for five minutes, followed by a linear gradient to 100 %A after 30 minutes.

The HPLC system was connected to an Agilent 6545 LC/Q-TOF equipped with an Agilent Jet Stream source. The data recorded were processed using Agilent MassHunter software.

Figure 1. Location of groundwater wells along the South Platte River approximately 30 miles north of Denver, Colorado\(^5\).
Sample preparation
Samples were concentrated using an offline solid phase extraction (SPE) technique that allowed the sample to be analyzed multiple times for MS analysis, MS/MS analysis, and to reach the required ng/L concentration level. A 100 mL water sample was processed using SPE on a Gilson robotic system. The sample was eluted with 5 mL of methanol, and was then evaporated under nitrogen to 0.5 mL for a final concentration factor of 200 fold. This method is a generic method that has been used successfully for antidepressants in surface waters and wastewaters.

Results and discussion
Analytical workflow and tools used
The analysis of groundwater and surface water samples required the adoption of a workflow method that allows for both suspect and unknown detection of pharmaceuticals. Table 1 shows the set of tools used for suspect and unknown analysis, while Figure 2 shows the workflow system that allows for a thorough analysis of unknown samples using an iterative system. The four types of tools include:

- Hardware
- Software
- Ion chemistry
- Physical, chemical, and biological tools related to surface and groundwater chemistry

This approach is highly effective at the process of suspect and unknown identification in surface and groundwater samples for pharmaceuticals.

Table 1. The tools used for unknown and suspect analysis.

<table>
<thead>
<tr>
<th>Tools used</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hardware tools</td>
<td>High resolution and accurate mass, All Ions, Auto MS/MS, Pseudo MS</td>
</tr>
<tr>
<td>Software tools</td>
<td>Databases, libraries, Kendrick mass, mass structure correlator, elemental forcing routines, isotope calculator</td>
</tr>
<tr>
<td>Ion chemistry tools</td>
<td>Adduct formation (rule of 5), fragmentation tools (diagnostic and fragment ions), rules of fragmentation (N-rule and odd and even electron ion rules)</td>
</tr>
<tr>
<td>Physical and biochemical tools—thinking outside the box</td>
<td>Biochemical pathways, Biodegradation pathways, UV degradation and chemical oxidation, Correlation pathways</td>
</tr>
</tbody>
</table>

Tools used
The workflow in Figure 2 begins with a suspect list being searched in the accurate mass file acquired in TOF mode. The suspect list differs from a targeted list in that only the exact mass is known for a suspect (sometimes a retention time may be known but no standard is currently available, which is the de facto definition of a suspect or nontarget list). A targeted compound will have retention time and several confirmation ions using accurate mass as a confirmation based on a concept of identification points. The suspect/nontarget list used was a list of 100 common pharmaceuticals that have been found in surface water and wastewater.

Figure 2. Analytical workflow chart for unknown and suspect identification.
Two approaches are possible, either an Excel database (CSV file) or an Agilent database search, called a Personal Compound Database (PCD.cdb) file. A personal compound database and library (PCDL.cdb) file will also contain MS/MS spectra, in the library portion. The PCD.cdb file may also include a structure as a mol file that can readily be imported into the Molecular Structure Correlator software for fragment verification. Both are easy to make and use.

The 22 compounds detected and confirmed in this study include:

- Beta blocker pharmaceuticals for blood pressure control
- Antibiotics
- Antidepressants
- Caffeine
- Sucralose, a low-calorie sweetener

The beta blockers, antibiotics, and antidepressants were:

- Atenolol
- Azithromycin
- Bupropion
- Clarithromycin
- Codeine
- Dextrorphan
- Diltiazem
- Diphenhydramine
- Erythromycin anhydrate
- Gabapentin
- Lamotrigine
- Metoprolol
- Oxycodone
- Sulfamethoxazole
- Thiabendazole
- Trimethoprim
- Venlafaxine
- Various metabolites of these pharmaceuticals

In addition to using the tools in Table 1 and the workflow in Figure 2, metabolites were found using different tools, such as diagnostic ions (Box 3, Figure 2). The concept of diagnostic ions means that pharmaceuticals of a specific chemical family will often have similar fragment ions or ions that are diagnostic of their structure. The publications by Ferrer and Thurman list approximately three fragment ions that may be used as diagnostic ions for the identification of related pharmaceuticals and pesticides either in the same family or metabolites of detected compounds.

A good example of this tool is the discovery of lamotrigine glucuronide, which was found in the South Platte River using several tools, including both a chlorine filter and diagnostic fragment ion. The diagnostic ion in this case was the m/z 256 ion, which is the same mass as the protonated molecule of the parent compound, lamotrigine. This compound was eventually identified after MS/MS and standard matching.

The Molecular Structure Correlator shown in Box 5 in Figure 2, is another useful tool. Before a standard is purchased, it is possible to carry out MS/MS. The accurate mass fragments can then be considered using the structure correlator, which, if possible, assigns the correct accurate mass to each of the fragments. This tool is a favorite for suspect analysis, since it is rare that two compounds have the same fragment ions, though it does occasionally happen, as was the case with an identification of tramadol and a metabolite of venlafaxine.

Finally, Box 6 in Figure 2 shows the power of iterative use of the workflow chart. There can be correlation, or lift, between compounds. Lift is a marketing term indicating sales increase with a specific advertisement. Here, we use lift to mean that a compound is present due to the sale of this compound as either a mixture or given with another compound. These compounds do not correlate in water samples by concentration due to different chemistries and removal processes during water treatment and transport. Thus, the presence of one may add a lift or give an opportunity for the other compound to be present. This is the case where two compounds are related by use but do not necessarily correlate in a 1:1 sense. A good example is the detection of sulfamethoxazole in the South Platte River (Table 2), which is commonly taken as an antibiotic for urinary infections, and is combined with trimethoprim. When the accurate mass for trimethoprim is searched, it is found at a low intensity, below the cutoff of the suspect search by database, but the compound is present at trace levels. It is preferentially removed by wastewater treatment but may still be present at just above the limit of detection of the analysis method, which shows the power of using the workflow chart, for each compound detected in the suspect analysis or unknown identification.
Pharmaceuticals in alluvial groundwater
Eight compounds were frequently found in the alluvial groundwater 100–500 m from the South Platte River. These compounds were:
- Bupropion
- Caffeine
- Carbamazepine
- Gabapentin, a metabolite of carbamazepine
- Lamotrigine
- Sucralose
- Sulfamethoxazole
This was a decrease from the 22 compounds confirmed in the South Platte River. The 14 compounds that were removed by bank filtration or diluted below detection levels as the river water flowed to the alluvial wells included:
- Atenolol
- Azithromycin
- Clarithromycin
- Codeine
- Dextrophan
- Diltiazem
- Diphenhydramine
- Erythromycin anhydrate
- Metoprolol
- Oxycodone
- Thiabendazole
- Trimethoprim
- Venlafaxine and its metabolite
In a 2009–2010 sampling of a groundwater well much closer to the South Platte River, approximately 50 m, atenolol, diphenhydramine, venlafaxine, and its metabolite were also detected, which shows minimal transport for these compounds.

Table 2. LC and MS and MS/MS instrument conditions used in this study.

<table>
<thead>
<tr>
<th>LC conditions for the 1290 Infinity II LC</th>
<th>MS conditions positive ion mode</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Column</strong></td>
<td>ZORBAX Eclipse XDB-C8 150 mm × 4.6 mm and 3.5 μm (p/n 963967-906)</td>
</tr>
<tr>
<td><strong>Column temperature</strong></td>
<td>25 °C</td>
</tr>
<tr>
<td><strong>Injection volume</strong></td>
<td>10 µL</td>
</tr>
</tbody>
</table>
| **Mobile phase** | A) Acetonitrile  
B) 0.1 % formic acid in water |
| **Run time** | 30 minutes |
| **Flow rate** | 0.6 mL/min |
| **Gradient** | 90 %B at time 0, hold for 5.0 minutes, gradient to 100 %B at 30 minutes, then 10 minutes post run time |
| **Sheath gas temperature** | 350 °C |
| **Sheath gas flow** | 11 L/min |
| **Gas temperature** | 250 °C |
| **Desolvation gas flow-rate** | 10 L/min |
| **Nebulizer pressure** | 45 psi |
| **Capillary voltage** | 3,500 V |
| **Nozzle voltage** | 0 V |
| **Skimmer voltage** | 65 V |
| **Octopole RF** | 750 V |
| **Accurate mass spectral range** | 50–1,000 m/z |
| **Fragmentor voltage** | 190 V |
| **Auto MS/MS conditions** |  |
| **Quadrupole isolation width** | 1.3 m/z |
| **Collision energies** | 15, 30 eV |
| **MS mass range** | 100–1,000 m/z |
| **MS acquisition rate** | 4 spectra/second |
| **MS/MS mass range** | 40–700 m/z |
| **MS/MS acquisition rate** | 4 spectra/second |
| **Threshold** | 25,000 counts |
| **Relative threshold** | 0.01 % |
| **Active exclusion** | Enabled, excluded after 1 second, released after 0.2 minutes |
| **Static exclusion** | 118–123 m/z  
700–1,000 m/z |
| **Model** | Common organic molecules, only singly charged precursors, sort precursors by abundance, scan speed varied on precursor abundance, with a target of 50,000 counts/spectrum |
| **Purity** | 100 % with a purity cutoff of 30 % |
Figure 3 shows a good example of conservative transport (that is, nonremoval), which includes both a pharmaceutical and a sweetener. They are gabapentin and sucralose. The concentration of these two compounds in the South Platte River were either identical or decreased only by 50 % when moving through alluvial groundwater, even at distances of up to 500 m from the river.

Sucralose was 50 % of the concentration found in the South Platte River, while gabapentin reached the same concentration as in the river. These two compounds can then be compared to the other pharmaceuticals found by LC/Q-TOF-MS analysis. For example, Figure 4 shows the removal of atenolol and diphenhydramine relative to the South Platte River. There is 90 % removal of atenolol, a blood pressure regulator, and 99 % removal of diphenhydramine, an over-the-counter antihistamine medication. Both compounds are thought to be adsorbed to the sediments of the alluvial aquifer.

Figure 3. Conservative tracers of alluvial groundwater flow with a pharmaceutical, gabapentin, and a sweetener, sucralose.

Figure 4. Removal of atenolol and diphenhydramine in alluvial groundwater relative to the South Platte River.
Although it is not clear why there is removal, it appears both sorption caused by the hydrophobic effect and by cation exchange could be important mechanisms in the removal of these pharmaceuticals as they transport through groundwater. Figure 5 shows another example of this removal process of pharmaceuticals from alluvial groundwater for venlafaxine and its metabolite, desmethylvenlafaxine.

In this example, venlafaxine and its major metabolite are 60% removed during transport to alluvial wells close to the South Platte River. The amine group present may be partially protonated at the pH of groundwater (~7.5). Thus, cation exchange and the hydrophobic effect are in play to remove this antidepressant and its metabolite from groundwater. These two compounds are also important in that they are sources for N-nitrosodimethylamine (NDMA) during water treatment when using chloramines for water purification\(^\text{12}\). Apparently, the dimethylamine group reacts with chloramine to form NDMA\(^\text{12}\).

**Figure 5.** Venlafaxine and desmethylvenlafaxine in alluvial groundwater within 50 m of the South Platte River.
Conclusions

The identification of unknowns requires a complex set of tools, which are described in this Application Note and are shown in Table 1. There are four major categories of tools: hardware, software, ion-chemistry, and physical and biochemical tools. The reference set included in this Application Note and shown in Figure 2 are a guide for examples of how to apply these various tools. Both hardware and software tools continue to evolve with improved resolving power and mass accuracy. The use of auto MS/MS is useful since the evolution of accurate mass databases. The application of ion chemistry tools and physical and biochemical tools is an area of research on accurate mass that develops around the goal of the user. The type of problem to be solved, for example, a pharmaceutical class such as opiates, will dictate the ion chemistry and biochemical pathways. These tools are enhanced by software, but are driven by the user’s application. Finally, the study of unknowns will continue to develop as more applications and problems are addressed by LC/Q-TOF analysis.

Figure 6 shows three pharmaceuticals that were nearly always found in alluvial groundwater 100–500 m from the South Platte River. The compounds include carbamazepine, lamotrigine, and sulfamethoxazole. They are two antidepressants and one antibiotic, respectively. The compounds may vary radically in concentration over time, with lamotrigine always being of the highest concentration, a trend noted in the literature for wastewater\textsuperscript{10}. These three compounds are definitely important for future studies of groundwater and pharmaceuticals. It is also important to realize that concentration levels may vary quickly because of the river water source, which is affected by wastewater output from the Denver area, in this case, and by the seasonal uses of many pharmaceuticals.

Finally, one of the important uses of alluvial groundwater is as a drinking water source, especially along rivers in the arid western United States. In fact, the South Platte River is a good example since it flows from Denver to near Kansas City, MO before joining the Missouri River. Along this 600 mile stretch, alluvial groundwater is a drinking water source for many communities in Nebraska and eastern Colorado. Thus, the results reported here have important impact for drinking water standards, and shows how important the analysis of suspect and unknown compounds are for water quality in the arid west where water re-use is important. Water re-use in this sense means that wastewater may be purified by infiltration to groundwater and later consumed as a drinking-water source.
References


