Monolithic Silica Columns for Simple and Fast LC-MS Analysis of Antibiotics in Mammalian Tissue and Body Fluid and in Pharmaceutical Formulations

Stephan Altmaier, Merck Millipore, Frankfurter Str. 250, 64293 Darmstadt, Germany

This work describes fast and straightforward high performance liquid chromatography methods with UV or mass spectrometry detection for the analysis of antibiotics in mammalian tissue or urine and in pharmaceutical formulations. The monolithic and very robust silica structure of the columns used in this work allows for short sample preparation procedures. All samples were separated on C18 reversed phase columns via gradient elution profiles and directly transferred to UV or MS for the analysis of all components. This setup enabled the identification of antibiotics in samples such as urine or liver within very short analysis times and with a sample preparation step kept as short as possible.

Antibiotics are natural products of metabolism of bacteria, fungi or organisms such as plants and amphibians, generated in order to fight an infection or to gain advantage in genetic selection. Examples for such natural antibiotics are allicin from garlic, Echinacea plant extract, honey containing antimicrobial enzymes or cinnamon.

From a more medical point of view, antibiotics are a class of drug substances blocking metabolic processes of microorganisms. As a result, reproduction and viability of these microorganisms is prevented. Antibiotics are utilised in the treatment of a vast number of infections caused by bacteria. Depending on their activities, they can be referred to as bacteriostatic (stopping bacteria from reproducing, but not crucifying them) or bactericide (stopping reproducing and crucifying). The most important classes of antibiotics are penicillin, cephalosporins, carbapenems, fluoroquinolones, macrolides, aminoglycosides and tetracyclines, differing in their effectiveness towards various types of bacteria. Antibiotics used for therapy are nowadays produced fully or partially synthetic or via biotechnological processes.

One drawback of antibiotics is their (ab)use in intensive mass animal farming. Here, these drugs are often applied in a preventive manner against various illnesses and in order to improve the results in animal fattening. As a result antibiotic resistant bacteria are selected. These and antibiotic compounds are then released into the environment via manure spreading, and the biological activity of antibiotics in ground water and soil further increases the number of resistant bacteria. With respect to humans, the unspecific or unnecessary prescription and taking of antibiotics also leads to the development of bacterial resistance. In addition, the excretion of excess antibiotics via urine or faeces leads to the same problems as described above for animal farming. Such approaches in both human and veterinary medicine clearly foil antibiotic stewardship, i.e., a responsible case-to-case indication utilising antibiotics.

As a consequence of their lax handling, antibiotics are nowadays omnipresent in the environment and in the food chain, namely in meat and dairy products, but also in vegetables. This makes a reliable, fast and robust analysis of antibiotics in various complex matrices necessary. In this work several straightforward methods combining reversed phase liquid chromatography (LC) separation and ultraviolet (UV) and mass spectrometry (MS) detection are presented for the analysis of four different antibiotics in food and urine *(Figure 1)*. Robust monolithic silica column technology was utilised, enabling fast chromatographic runs and the detection of all analytes without the need for complex sample preparation procedures and costly UPLC systems.

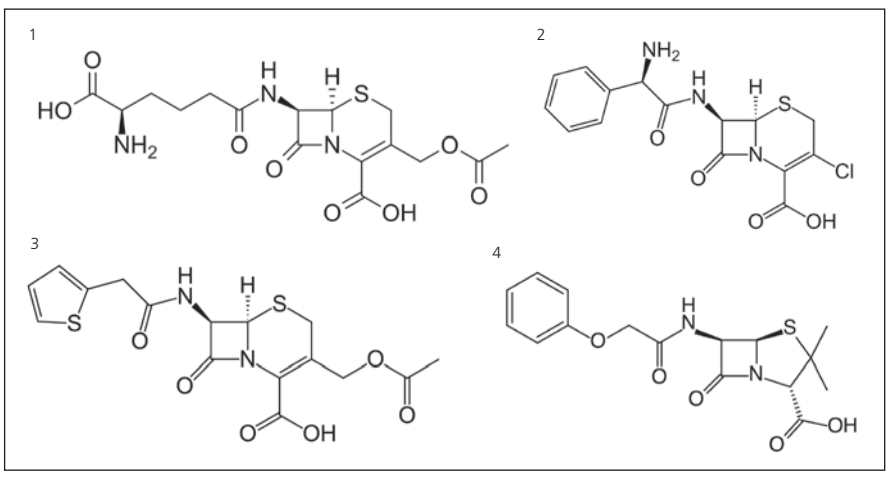

Figure 1. Chemical structures of antibiotics analysed in this work. 1. Cephalosporin c, 2. Cefaclor, 3. Cefalotin, 4. Phenoxymethylpenicillin.

Table I: List of antibiotics analysed in this work: Names, corresponding monoisotopic mass, relevant MS peaks (calculated) and molecular ion formulas.

Antibiotic compound	Monoisotopic mass / g/Mol	Relevant MS peak / m/z
Cephalosporin C	415.1	416.1
Cefaclor	367.0	368.0
Cefalotin	396.0	337.0
Phenoxymethylpenicillin	350.1	351.1, 160.0

Experimental

Materials and Methods

The HPLC system used was a Dionex Ultimate 3000 (Thermo Scientific Dionex Corporation, Sunnyvale, California, USA) equipped with a UV detector (detection wavelength 254 nm), a Chromolith® FastGradient RP-18 endcapped 50-2 mm analytical monolithic silica column and a Chromolith® RP-18 endcapped 5-2 mm guard cartridge (both Merck Millipore, Darmstadt, Germany). The data acquisition was performed with Chromeleon software.

A Bruker Esquire 6000plus mass spectrometer with an ion trap and an on-line electrospray ionisation (ESI) source operated in positive mode was utilised in the m/z range scan from 100-500. Flow and temperature of the dry gas was set to 12 L/min and 365°C, respectively, nebuliser gas pressure was 2.8*105 Pa. Trap conditions: Max. accu time 100 ms, target 200.000, averages: 2.

Sample Preparation

Bovine liver was purchased at a local butcher. Detailed information about sample preparation or fortification can be found in the different sections below.

Antibiotics Stock Solution

Stock solutions were prepared by ultra wave supported dissolution of three antibiotics in 10 mL water and a filtration of the resulting solutions using a Millex® syringe filter driven unit PTFE 0.45 µm. The final concentrations of the single compounds in each solution were: Cephalosporin C (CPS) 217 mg/L, cefaclor (CFC) 2343 mg/L, cefalotin (CPT) 158 mg/L. The final stock of all three antibiotics ('CCC') was then prepared by combining 9.52, 198.1 and 652.4 µL of the three antibiotic stock solutions of CFC, CPT and CPS.

Antibiotic Formulation

An antibiotic formulation containing phenoxymethylpenicillin (penicillin V) and various preservatives (sodiummethyl-4-hydroxybenzoate, sodiumpropyl-4-hydroxybenzoate, sorbic acid) and additives (saccharose, sodium cyclamate, saccharin, citric acid, carmine (E120)) was utilised to spike human urine. The stock solution was prepared by dissolving 34.9mg of the dry powder in 50 mL water and filtering of the resulting solution using a Millex® syringe filter (0.45 µm pore diameter). The concentration of phenoxymethylpenicillin in the final solution was 69.8 mg/L.

Urine Spiked with Antibiotics

Human urine was first centrifuged at 4500 rpm for 45 minutes and filtered utilising a Millex® syringe filter (0.45 µm pore diameter). Urine was then combined with the antibiotic formulation in a 1:1 volume ratio.

Bovine Liver Spiked with Antibiotics

One blank and one bovine liver sample spiked with the CCC mixture was prepared. 0.98 g of liver were broken up and mixed with 0.6 mL of CCC solution. In parallel, a blank sample (1.02 g of liver) was prepared in a similar manner but without the addition of antibiotics. After one hour both samples were combined with 10 mL of ACN/water 80:20 (v:v), homogenised using an Ultra-Turrax T25 (IKA) and centrifuged at 4500 rpm for 15 minutes. The clear and yellowish supernatant was decanted off into two glass tubes and ACN was evaporated during 60 minutes at a gas flow of 1 bar and a temperature of 40°C with a TurboVap instrument (Biotage). The residue was transferred to a LiChrolut® RP-18e 500 mg cartridge preconditioned with 5 mL of ACN and two times 5 mL water. The yellow band was eluted into a centrifuge tube with 5 mL ACN and residual eluent was vacuumed. 1 mL water was added to the solution and after shaking for 30 seconds the mixture was again concentrated with a TurboVap (120 minutes, 40°C, 1 bar compressed air). The samples were then filtered via a Millex® syringe filter (0.45 µm pore diameter) and transferred to HPLC vials.

Results and Discussion

A prerequisite in modern LC-MS analysis is high sample throughput, or in other words: A fast LC-MS method in order to avoid a bottleneck in the system sample preparation-separation/detection-analysis. Hence, a mixture of the three antibiotics cephalosporin, cefaclor and Cefalotin was prepared and a fast gradient LC method was developed in order to achieve baseline separation of all analytes. Detection was performed by both UV and MS and a monolithic silica column was chosen as a stationary phase to allow for a low backpressure separation at a comparably high flow rate *(Figure 2)*. All compounds eluted within less than two minutes and a baseline separation of the narrow peaks was achieved.

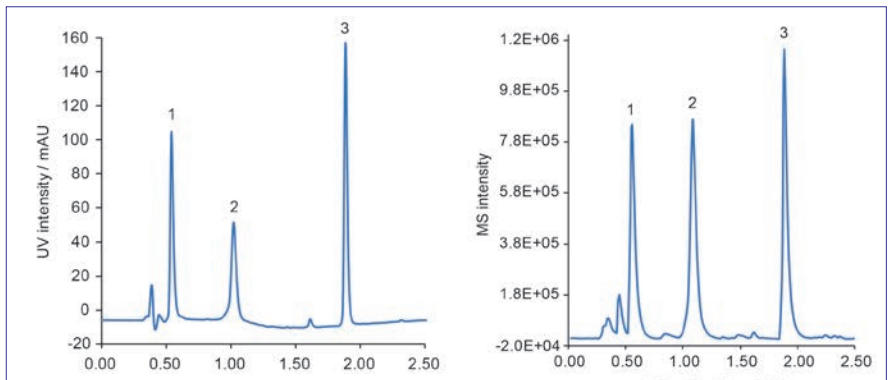

Figure 2. LC-MS chromatogram of a mixture of a standard solution of three antibiotics separated on Chromolith® FastGradient® RP-18 endcapped 50-2 mm analytical monolithic silica column. Detection: UV (254 nm, left) and positive ESI-MS (m/z 100 – 500), base peak chromatogram (BPC, right). Mobile phase A: acetonitrile + 0.1% formic acid, mobile phase B: water + 0.1% formic acid; flow rate 0.4 mL/min; gradient: 0' 5% A, 2' 95% A, 5' 95% A; pressure drop 23 bar. Sample: 1 Cephalosporin c, 2 Cefaclor, 3 Cefalotin.

In a second example the same mixture of antibiotics was utilised to spike homogenised bovine liver. After spiking and centrifugation a typical solid phase extraction (SPE) protocol was performed. Undissolved particulate matter as well as salts were removed applying a pre-packed SPE cartridge containing reversed phase stationary phase material.
LC-MS analysis of the resulting extract as well as of an unspiked blank control sample was achieved utilising the same gradient as applied in *Figure 2*. A 50-2 mm reversed phase monolithic silica column was used in these runs. In order to protect the analytical column a 5-2 mm RP-18e guard column was additionally installed. MS data of the analysis of the blank sample displays a bunch of matrix peaks in the range of 0.5 – 3 minutes, with several unassigned polar compounds eluting mainly in the void volume and possibly interfering with the cephalorsporin signal (*Figure 3* top). Hence, the gradient run provides a trade-off between speed of analysis and resolution. The analysis of the spiked bovine liver extract confirmed some peak overlay in the < 1 min region, nevertheless all antibiotics were identified via MS detection (*Figure 3* bottom). Later eluting analytes cefaclor and Cefalotin were detected as baseline separated peaks. No or only small peaks are eluting in the range of 3-5 minutes, revealing the excellent suitability of the applied sample preparation procedure in the analysis of antibiotic contamination in mammalian tissue.

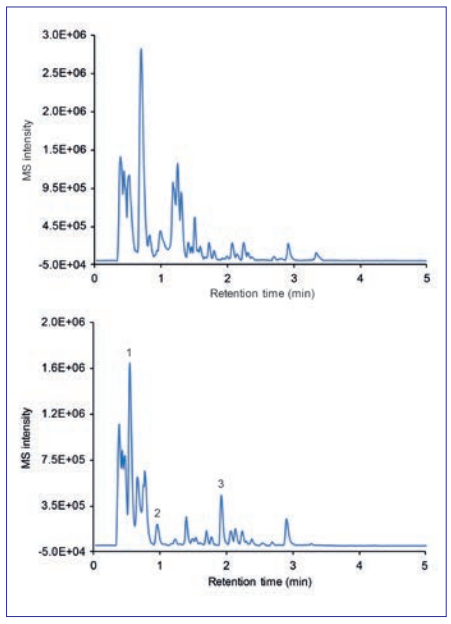

Figure 3. LC-MS chromatograms of a bovine liver blank run (top) and of a standard solution of three antibiotics spiked in bovine liver (bottom) separated on Chromolith® FastGradient® RP-18 endcapped 50-2 mm analytical monolithic silica column and Chromolith® RP-18 endcapped 5-2 mm monolithic silica guard column. For conditions and peak annotiations see Figure 2.

The third example simulates excreted human urine after antibiotic medication. Urine pretreatment followed by spiking of the resulting liquid with an antibiotic syrup formulation. This sample was analysed via LC-MS utilising a combination of monolithic silica analytical and guard columns and a short two-step gradient *(Figure 4)*. For the blank syrup no guard column was applied. The chromatogram of the formulation reveals only four signals, of which the first can be attributed to sugar eluting in the void volume. The intensive and narrow peak at approximately 4.3 minutes is caused by phenoxymethylpenicillin, while two additional signals at 3.1 minutes remain unassigned. The spiked urine sample shows several peaks equally distributed throughout the whole chromatographic runtime. Phenoxymethylpenicillin is eluting as a narrow and almost baseline separated peak at 4.9 minutes and was identified by its spectrum pattern (m/z 160.0 and 351.1, see inset in *Figure 4* bottom). A visible shift in retention of this signal (in comparison to the blank syrup sample) is caused by the use of a guard column.

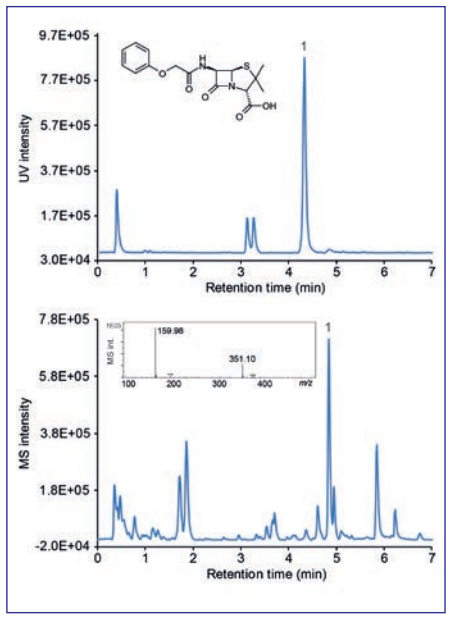

Figure 4. LC-MS chromatogram of an antibiotic drug formulation (top) and of the same formulation spiked in human urine (bottom). Insert: MS spectrum of 1 (phenoxymethylpenicillin) at 4.91 min. Sample analysis was performed on Chromolith® FastGradient® RP-18 endcapped 50-2 mm analytical monolithic silica column coupled with a Chromolith® RP-18 endcapped 5-2 mm monolithic silica guard column. Detection: Positive ESI-MS (m/z 100 – 500), base peak chromatogram (BPC). Mobile phase A: acetonitrile + 0.1% formic acid, mobile phase B: water + 0.1% formic acid; flow rate 0.4 mL/min; gradient: 0' 5% A, 3.5' 70% A, 5' 95% A, 7' 95% A.

Conclusion

This work shows that monolithic silica technology combined with fast sample preparation methods is a robust system ideally suited for the analysis of antibiotics in complex food and urine matrices. Particulate matter and highly unpolar matrix components were removed via the sample preparation process, and most of the more polar compounds were separated from the antibiotics applying fast gradient runs. The target analytes were then easily identified via UV or MS detection. Due to the low backpressure characteristics of the monolithic silica columns it was possible to perform all chromatographic runs on standard HPLC systems. In order to protect the analytical column and to increase it's lifetime, a guard column was utilised.