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DETERMINATION OF AMOXICILLIN AND COTRIMOXAZOLE CONCENTRATIONS IN SPUTUM OF PATIENTS WITH CYSTIC FIBROSIS

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Abstract:

In the management of cystic fibrosis, treatments against *Staphylococcus aureus* and *Haemophilus influenzae* such as amoxicillin or cotrimoxazole have to be prescribed and the antibiotherapy efficacy may be linked to the concentration that reaches the infected site. As cystic fibrosis patients present disturbed pharmacokinetics parameters, a drug monitoring would be relevant to assess lungs distribution of antibiotics and to optimize dosing regimens. In this context, the aim of the study was to develop and validate HPLC-based methods for the determination of both antibiotics in bronchial sputum from cystic fibrosis patients, in order to assess drugs distribution into lungs. Plasma proteins were precipitated by acetonitrile, amoxicillin concentrations in sputum were determined by HPLC coupled with tandem-mass spectrometry. Following a liquid extraction with ethyl acetate, cotrimoxazole was quantified by HPLC using ultraviolet detection. Both methods were found rapid, specific, accurate and reproducible. The method was applied to patient's samples. In three treated patients, concentration of amoxicillin in sputum were closed and below the lower limit of quantification (0.1 µg/g) and in six patients, sputum concentrations up to 11.1 µg/g and 6.4 µg/g were measured for sulfamethoxazole and trimethoprim respectively.

Keywords: Amoxicillin, cotrimoxazole, high performance liquid chromatography, sputum, cystic fibrosis, therapeutic drug monitoring

1. INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive disorder due to the mutation of a gene encoding the chloride channel cystic fibrosis transmembrane conductance regulator (CFTR). Dysfunction of CFTR in epithelial cells leads to secretion of a thick sticky mucus in the lungs and the digestive tract. This environment results in inflammation and infections development (Davies et al., 2007). Bacterial colonization of lungs occurs early in the natural history of the disease. The main bacteria responsible for this early colonization are *Haemophilus influenzae* and *Staphylococcus aureus*. Infections have been involved in morbidity and mortality of CF patients. Antibiotic treatment plays, therefore, a crucial role in the management of the disease (Davies et al., 2007; Thumerelle, 2003).

Among antibiotics used to treat respiratory tract infections caused by *Haemophilus influenzae* and *Staphylococcus aureus*, oral amoxicillin/clavulanic acid association or cotrimoxazole (sulfamethoxazole (SMX) / trimethoprim (TMP) synergistic association) are recommended by guidelines (Grim et al., 2005; H.A.S., 2017).

Therapeutic drug monitoring (TDM) of amoxicillin (AMOX) and SMX/TMP in plasma is proposed to optimize drug exposure and adjust dosage in infected patients (Jager et al., 2016; Roberts et al., 2010). In CF patients, important pharmacokinetic parameter variations, such as volume of distribution and clearance increases are expected, making TDM of antibiotics useful in this population (Lindsay & Bosso, 1993; Touw, 1998). In respiratory infections, there is a relationship between the efficacy of an antibiotic treatment and drug concentrations at the infection site (Bergogne-Bérézin, 1995). However, in CF, the drug diffusion from the vascular compartment to the bacterial inoculum might be reduced because of the abnormal mucus excretion. Thus, determining drug concentration in bronchial sputum in addition to plasma could be a relevant biomarker to predict and optimize antibiotics efficacy and to improve treatment outcomes. Moreover, sputum samples are relatively minimally invasive and can be relatively easily obtained from patients.

Data regarding AMOX and SMX/TMP quantification in sputum are scarce. Thus, it appears relevant to develop new and sensitive bioanalytical methods using liquid chromatography with either ultraviolet or mass spectrometry detection to measure antibiotics in this unconventional matrix.

Thus, the aim of this study was to validate analytical methods suitable for TDM of AMOX and SMX/TMP in bronchial sputum of cystic fibrosis patients.

2. MATERIALS AND METHODS

2.1. Reagents and chemicals

Amoxicillin, SMX, TMP, sulfadiazine and dichloromethane of HPLC grade were purchased from Sigma (Saint-Quentin, France). Cefotaxim was purchased from Panpharma (Luitré, France). HPLC grade acetonitrile, phosphoric acid and hydrochloric acid were provided by Carlo Erba Reagents (Val de Reuil, France). Formic acid and methanol of LC/MS grade were provided by Fisher Chemicals (Waltham, USA). Ethyl acetate of HPLC grade was obtained from Merck Millipore (Billerica, USA). The Dithiotreitol 10X (DTT) formulation used was the DigestEUR® solution purchased from Eurobio (Courtboeuf, France). Milli-Q-water (Saint-Quentin, France) was used throughout the experimentations.

2.2. Chromatographic system

Amoxicillin determination:

Amoxicillin was determined by high performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS). Analysis was carried out on a Finnigan Surveyor LC system (Thermo Fisher scientific, Waltham, USA), composed of a quaternary gradient pump, a degasser, an autosampler and a column oven. The column was a C-18 reversed phase HPLC column Atlantis® T3 (100 x 2.1 mm, 3 µm) coupled with a column guard Atlantis® T3 (10 x 2.1 mm, 3 µm) (Waters, Saint-Quentin, France). The column temperature was set at 30 °C and the autosampler was set at 10°C during analysis. The mobile phase was composed of formic acid 0.1% in water (eluent A) and methanol (eluent B). A linear gradient was used with a flow rate of 300 µL /min and was programmed as follow: equilibration at initial conditions with 10% of B, increase to 90% of B in 8 min, reversion to the starting conditions at 10% of B from 8.0 min to 8.1 min and re-equilibration with the initial composition from 8.1 min to 12 min. Data acquisition and system control were performed using LC Quan software (version 2.5. Thermo Fisher scientific, Waltham, USA). Following HPLC separation, compounds were detected with a triple quadrupole mass spectrometer Finnigan TSQ Quantum (Thermo Scientific, Waltham, USA), connected to the Thermo master tune software (version 2.0.5). The MS conditions were programmed as

follows: sheath and auxiliary nitrogen nebulizer gas pressure were set to 50 and 20 (arbitrary units) respectively. The collision gas pressure (argon) was 1.5 mTorr. Electrospray ionization in the positive mode was applied for all compounds. The MS run time was 12 min performed in the selective reaction monitoring (SRM) scanning mode. Capillary temperature was 270°C, and spray voltage was 3000 Volt. Transitions monitored were m/z 366.11→114.01 and m/z 456.05→166.70 for AMOX and cefotaxime, respectively. Collision energy and tube lens were respectively 20 volt and 120 volt for both analytes.

Cotrimoxazole determination:

SMX/TMP were determined by HPLC and ultraviolet detection (HPLC-UV). Analysis was carried out on a Dionex Ultimate 3000 system (Waltham, USA), composed of a quarterly gradient pump, a degasser, an autosampler, a column oven and an UV diode array detector. The column was a C-18 reversed phase HPLC column Atlantis T3 (150 x 4.6 mm, 5 µm) (Waters, Saint-Quentin, France) coupled with a column guard Atlantis® T3 (20 x 4.6 mm, 5 µm) (Waters, Saint-Quentin, France). The column temperature was set at 25 °C and the autosampler maintained samples at 10°C during analysis. The mobile phase was composed of a 10mM phosphoric acid solution adjusted to pH 2.0 with hydrochloric acid (eluent A) and acetonitrile (eluent B). Starting composition of the mobile phase was 93% of A and 7% of B. A linear gradient used with a flow rate of 2 mL/min was programmed as follow: increase to 31% of B in 12 min, reversion to the starting conditions at 7% of B from 12 min to 12.1 min and re-equilibration with the initial composition from 12.1 min to 14 min. The compounds were monitored and quantified at 270 nm. Data acquisition and process were performed with Chromeleon™ software (version 6.80, Dionex-Thermo Fisher scientific, Waltham, USA).

2.3. Preparation of standards and quality control samples

Stock solutions of AMOX and SMX/TMP were prepared in water at 240 mg/L and stored at -80°C. Working solutions of each calibration standard were daily prepared by dilution of the stock solution in water to reach the concentrations of 0.1-0.5-2.5-10-30-60 mg/L. Quality controls (QCs) samples were prepared by diluting working solutions of AMOX or cotrimoxazole 10-fold in “blank” drug free plasma (Provided by the French blood establishment). Three levels of concentrations were prepared for each antibiotic (0.3-15-45 mg/L for AMOX and 1-15-45 mg/L or 0.1-1.5-4.5 for SMX and TMP respectively). QCs

samples were stored at -80°C until analysis. Two batches of stock solutions per analyte were made to independently prepare QCs and calibration standard working solutions.

2.4. Sample preparation

Sputum pre-treatment

Bronchial sputum was collected in a clean container. Because of the high viscosity of sputum collected in CF patients, samples had to be dispersed and homogenized before analysis. Depending on the sample viscosity, ultrapure water or a diluted aqueous solution of DTT (10X) was added into the sample in equal volumetric proportion and the diluted sample was vortex mixed with a pulsing mixer (900 rounds per min (rpm), 50 Hz. (VWR international. Pessac. France) for 15 min. Samples was then centrifuged for 10 min at 4000 rpm and 4°C. Supernatant was kept and stored at -80°C until analysis.

Dithiotreitol is used to homogenize viscous biological samples by splitting disulphide bridges. Nevertheless, the effect of DTT on AMOX and SMX/TMP was assessed to prevent any degradation of the drugs during the sample process and to avoid any misinterpretation in antibiotics quantification. Thus, in the method development step, the DTT effect was checked by preparing two groups of antibiotics solutions each one at four concentration levels (0.1-1-10-50 mg/L) either in water (group A) or in a 50:50 (v/v) mix of distilled water and DTT manufactured solution (group B). For each concentration levels, concentrations of the analytes analyzed in the aqueous solution or in the DTT solution were compared. To conclude that DTT has no effect on the molecule at the studied concentration range, differences between signal area in group A and in group B should be less than 15%.

Amoxicillin extraction

Aliquots of 100 µl of patient's sputum sample or QCs sample were added to 50 µl of water, 20 µl of cefotaxime at 1 mg/ml in water (use as internal standard) and 500 µl of acetonitrile. The calibration curve was prepared by mixing 100 µl of blank plasma with 50 µl of working standard solution, 20 µl of cefotaxime at 1 mg/mL and 500µl of acetonitrile. Samples were vortex mixed for 1 min and centrifuged 10 min at 4000 rpm and 4 °C. A volume of 400 µL of supernatant was diluted with 600 µl of water in vials and 20 µL were injected in the LC-MS/MS system.

Cotrimoxazole extraction

Aliquots of 200 μ l of patient's sputum or QCs were added to 50 μ l of water, 50 μ l of sulfadiazine at 1mg/ml (use as internal standard) and 1000 μ l of ethyl acetate. The calibration curve was prepared by mixing 200 μ l of blank plasma with 50 μ l of working standard solution, 50 μ l of sulfadiazine at 1 mg/mL and 1000 μ l of ethyl acetate. Samples were vortex mixed for 1 min (Vortex VWR Radnor, Pennsylvania, USA) and centrifuged 10 min at 3000 g and 4 °C (GR 412 Jouan, France). The supernatants were isolated and evaporated to dryness at 30°C under gentle nitrogen flow. Subsequently, dried extracts were reconstituted with 200 μ l of water and were vortex mixed for 10 min. A volume of 150 μ l of each extract was diluted with 1350 μ l of water in vials and 100 μ L were injected in the HPLC-UV system.

2.5. Method validation

The method was validated according to European medical agency guidelines for bioanalytical method (European medicines agency, 2011).

Matrix effect

For routinely analysis, blank plasma batches are more easily obtained than blank sputum batches. In order to realize calibration samples with blank plasma instead of blank sputum, matrix effect of sputum comparing to plasma was evaluated. For 3 batches of sputum (coming from three different patients), the matrix factor (MF) of each antibiotic was calculated by the ratio of the peak area in sputum over the peak area in plasma. Each blank matrix was spiked after extraction with the drug at a low and a high concentration levels (0.3 and 45 mg/L). The coefficient of variation (CV) of the MF for each level of concentration should not be greater than 15 % to exclude matrix effect.

Linearity

The linearity of the methods was assessed by analyzing calibration samples spiked with AMOX or SMX/TMP at 6 concentrations. Calibration standards analyses were performed on three different days. Calibration curves were established by the plotting peak area ratio of the target compounds to the internal standard (IS) versus the nominal concentrations. Linear regression was applied with weighting factor 1/x.

Precision and accuracy

The accuracy and precision of the methods were investigated by analyzing QC samples. To evaluate the intra-day accuracy and precision, 6 replicates of each of the three levels of concentrations of QC samples were extracted and assayed in a validation batch the same day. To evaluate the inter-day accuracy and precision, 3 batches of QC samples at three concentration levels were assayed with the same procedure on three different days. Each day, a freshly prepared calibration curve was constructed when the QC samples were extracted. Precision was expressed by the coefficient of variation (CV). Accuracy was reported as the relative error (RE). The acceptance criterion for precision and accuracy was $\pm 15\%$ at each QC level. The lower limit of quantification (LLQ) was defined as the lowest concentration on the calibration plot with a precision and accuracy within $\pm 20\%$. To be able to detect antibiotics in relevant concentrations, a LLQ of at least 0.1 mg/L was expected.

Recovery

Recoveries were determined by comparing the peak area of antibiotics and IS extracted from QC samples over those of antibiotics and IS spiked into blank plasma extracts at concentrations equivalent to a theoretical 100% recovery. Three replicates of the procedure were processed for each level of QC sample.

Specificity

The specificity of the method was determined by examining the susceptibility of the assay to interference from concomitant medications. It was evaluated by defining the retention time of potentially co-eluting compounds. Thus, retention times of ceftazidime, cefazolin, AMOX, SMX/TMP, sulfadiazine, ceftriaxone, piperacillin, cloxacillin, linezolid and DTT were compared. In addition, "blank" sputum from 5 patients with CF but not treated with AMOX or SMX/TMP were analyzed to check that no interfering compounds were eluted at the retention times of the analytes of interest.

Stability

Stability experiments were carried out to assess the stability of the target compounds in sputum samples and in post-extracted samples. Spiked samples of the antibiotics in sputum were prepared in triplicates from a freshly made stock solution and were investigated under different conditions. Short-term stability was assessed at room temperature after 6h of storage, and at 4°C after 2h of storage. Long-term stability was studied by assaying samples

that had been stored at -80°C for 1 month. The concentrations obtained were compared with blank sputum supplemented with AMOX or SMX/TMP at the same concentration and immediately assayed. The post-extraction stability was measured by testing the extract solution of QCs samples after 6h in autosampler at 10°C . The performance requirements for all stability measurements were a maximum relative error of $\pm 15\%$ between the immediately assayed sample (T_0) and tested samples.

2.6. Clinical application

In order to collect preliminary data regarding distribution of the studied antibiotics in sputum, induced bronchial expectorations were collected by respiratory physiotherapy from cystic fibrosis patients known to be treated with oral tablets of AMOX or SMX/TMP for at least 24 h. Sputum samples used in the study were excess amounts taken from samples usually collected for microbiological analysis during a periodic appointment at the regional cystic fibrosis Center of Rennes University Hospital. No extra expectorations were asked to the patients for this study. According to ethical standards, all patients were informed of the purpose of the study and agreed to participate. This part of the study was approved by the local ethical committee (Authorization n° 20.181). Because of the need to prevent antibiotics degradation in samples before analysis, pre-analytic conditions were controlled.

The way to express antibiotics concentration according to the volume or the weight of sputum is described in table S3.

For a few patients, when a therapeutic drug monitoring was clinically required, blood samples were also collected in a nearest time frame during the medical consultation, which enabled to monitor the antibiotics in plasma as well. Drugs were quantified in plasma according to a validated analytical method adapted from (Verdier et al., 2011). Drug dosage and time from the last administration was also collected.

3. RESULTS

3.1. Sputum pretreatment effect on analytes

Sputum samples collected has first to be dispersed and homogenized prior to be assayed. A method using ultrasound to disperse sputum was tested but was not satisfying. DTT solution was used because no denaturing effect on the analyte was found. The RE between distilled water solution and DTT solution of the antibiotic compared at the same concentration level

was lower than 15% for each drug studied in a large range of concentrations (0.1 to 50 µg/l) as presented in table 1.

3.2. Method validation

Matrix effect

The results of matrix effect assays (as shown in table 2) revealed that for low (0.3 or 1.5 mg/L) and high (45 mg/L) concentrations of antibiotics, there was no variation in the quantification of the analytes between plasma and sputum matrix. CV were lower than 15% for each drug. Consequently, the analytical procedure allows quantifying studied antibiotics in sputum using standard calibration curved samples prepared in plasma.

Linearity

The linearity study was carried out with concentrations ranging from 0.1 mg/L to 60 mg/L. Calibration curves shown in table S1, revealed excellent linearity through the regression coefficient $r^2 = 0.999, 0.999$ and 0.999 for AMOX, SMX and TMP, respectively.

Precision, accuracy

The precision and the accuracy of the developed methods at each level of concentration are given in table 3. The intra-day precision and accuracy ranged respectively from 2.5% to 8.7 % and from -2.4% to -14.1% for all drugs tested. The inter-day precision and accuracy ranged respectively from 2.6% to 14.3% and from -1.3% to 6.1% for all compounds tested. Highest variations are observed for low concentration levels. These results confirm that precision and accuracy of both methods developed for AMOX and SMX/TMP determination are good enough in the range of tested concentrations. The LLQ was validated at 0.1mg/L.

Recovery

Results are reported in table 4. Regarding the AMOX determination method, the mean percent of recovery ranged from 83.7% to 100%. Regarding SMX quantification, the mean recovery ranged from 71.2% to 73.2%.

Specificity

Blank plasma and sputum showed no interfering endogenous substances in the analysis of studied antibiotics. Potentially co-administered drugs had retention times that were different from the targeted analytes (table S2) or were not extracted or detected. Retention times for AMOX and cefotaxime were respectively 3.68 min and 5.53 min with the LC/MS/MS method. With the HPLC/UV method, retention times were 11.1 min for SMX, 6.8 min for TMP and 4.1 min for sulfadiazine. Drugs of interest and internal standards were well resolved.

Stability

The results of stability tests are presented in table 5. Short-term stability study revealed that AMOX remains stable in sputum stored for 2h at 4°C. However, a significant loss is observed in sputum kept 6h at room temperature with a decrease of the drug amount of 63.6%. Otherwise, SMX/TMP remains stable in sputum for all the tested conditions since no decrease exceeding 15% of the initial concentrations was observed neither at room temperature nor at 4°C.

Long-term stability study indicated that both drugs are stable in frozen sputum when stored at -80°C for at least 1 month.

The stability tests performed on extracts maintained at 10°C in autosampler for 6h demonstrated that for low and high concentration levels, the amounts of antibiotics remained stable in extracts under these conditions.

3.3. Clinical Application in therapeutic drug monitoring

The analytical methods developed were applied to quantify AMOX and SMX/TMP in sputum from patients. Representative chromatograms of a blank samples and patients sample are reported in figure 1.

Amoxicillin concentrations were measured in the sputum of 3 patients. For one of them, concentration of the betalactam in plasma (C_p) was determined as well. SMX/TMP concentrations were measured in the sputum of 6 patients. Plasma concentration was also determined for one of them. Some sputum samples were not included in the analysis because of inappropriate pre-analytic treatment (e.g. lack of refrigerated storage, imprecise volume of diluent added). For 3 patients treated with SMX/TMP, information about the last intake of the drug was unavailable. Results of concentrations quantified in studied patients are presented in table 6. AMOX level was quantifiable in sputum for only one patient 3h after drug intake at

the very low value of 0.1 mg/L (or 0.3 µg/g). AMOX concentrations in other sputum or plasma samples were below the LLQ.

SMX/TMP concentrations in patients' sputum exhibited substantial inter-patient variability. Concentrations expressed in amount over volume of sputum sample ranged from 1.1 mg/L to 6.5 mg/L for SMX and from 0.5 mg/L to 3.7 mg/L for TMP. Similar and proportional values were observed for concentrations expressed in amount over weight of sputum sample. Mean level of concentrations observed was 3.0 mg/L for SMX and 1.9 mg/L for TMP. In the single plasma sample collected, the concentration of SMX was 35.0 mg/L and the concentration of TMP was 2.4 mg/L. The ratio sputum concentration over plasma concentration was calculated for the patient and was 0.05 for SMX and 0.52 for TMP. Besides, the SMX/TMP ratio (5/1) present in the pharmaceutical formulation was not conserved in sputum concentration.

4. DISCUSSION

Two analytical methods were validated for the determination of antibiotics in sputum from bronchial excretions. It is of particular interest in CF patients since plasma concentrations might not reflect the actual amount of drug distributed into the site of infection (i.e. respiratory tract). Noteworthy, the viscosity of the infectious micro-environment could be a mechanic hurdle to the drug diffusion.

To accurately determine drugs concentrations in sputum samples, some technical aspects have to be controlled. As explained by Hector *et al.*, due to the complex matrix of the sample material, precise and reliable analysis of the sputum critically rely on pre-analytical issues (Hector *et al.*, 2010). Indeed, sputum collections are viscous and need to be properly homogenized to avoid retention of the targeted drug in an aggregated part of the sample. Several homogenization and fluidizing methods have been reported. In their work, Wilms *et al.* homogenized sputum from cystic fibrosis patients by vortex mixing with glass pearls, an efficient but expensive method (Wilms *et al.*, 2008). Andrasi *et al.* developed a method to determine antibiotics in sputum by capillary electrophoresis and used lyophilizing as an effective pretreatment of viscous and thick samples (Andrási *et al.*, 2007). Some authors reported only a simple mechanic homogenization and volume measurement as pretreatment of sputum sample (Ehrlich *et al.*, 2001). Hector *et al.* also described a mechanical method where samples were processed in very small aliquots (Hector *et al.*, 2010). This method is not adapted to drug monitoring because it reduces the amount of drug available for

quantification. Eventually, we chose a method using DTT since it is efficient, cheap and easy. However, its denaturing effect can threaten some drugs stability. Besides, a consequent volume of product has to be added to the sputum sample, which dilutes it and can lead to decrease the analytical method sensitivity. These two challenges were overcome in our work.

Chromatographic procedures reported in the present study were adapted from previous validated methods. Some tests have not been conducted, such as standards stability study, or stability study after freeze-thaw cycles but existing data demonstrated that these aspects satisfy usual requirements (Amini & Ahmadiani, 2007; Verdier et al., 2011).

Both validated methods in this study were applied to assess the distribution of AMOX and SMX/TMP in sputum from cystic fibrosis patients. Although the results have to be confirmed in a larger cohort, it provides some interesting preliminary data about the behavior of the molecules and their diffusion in sputum.

Amoxicillin levels measured in patients' sputum were low or under the LLQ which reflects a low penetration of the antibiotic into sputum. These results are consistent with previous reports while most of them quantified AMOX in bronchial expectorations of patients suffering of other bronchial pathologies than CF. Frashini *et al.* found a peak of concentration of AMOX in sputum around 1 mg/L in patients with chronic bronchitis (Fraschini et al., 1990). Hill *et al.* used a bioassay and an enzyme-linked immunosorbent assay to determine AMOX in sputum as well (Hill et al., 1992). The mean concentration levels obtained over the 4h following oral administration of 500 mg of AMOX was about 0.4 mg/L.

Although several studies demonstrated the low penetration of AMOX in sputum, these conclusions may be discussed in the light of results obtained by Honeybourne *et al.* on bronchial mucosa biopsy collected in patients with respiratory symptoms (Honeybourne et al., 1988). Indeed, concentrations observed in patients' bronchial mucosa 3.5h after a 500mg oral dose of AMOX was about 2.7 µg/g. In addition, by comparison with plasma concentration, the percentage of penetration of the antibiotic bronchial mucosa was 75%. This highlights that the ability of a drug to largely diffuse into lungs is not a guarantee of appropriated drug exposition in pulmonary infections where bacterial inoculum could be trapped in viscous sputum.

Such data suggest that AMOX concentrations in bronchial secretion are not likely to be above the minimal inhibitory concentration (MIC of the antibiotic defined by the CA-SFM (antibiotic committee of the French microbiology society) for *S. aureus* and *H. influenzae* (2 mg/L for both germs) (CASFM / EUCAST : Société Française de Microbiologie Ed ; 2019,

s. d.). As betalactams are time-dependant antimicrobial agents, significant amount of antibiotic must be present in the infected site in order to efficiently treat the infection.

A significant amount of SMX/TMP was found in sputum samples. Nevertheless, results interpretation might be limited by the lack of information about times from the last intake of the drug for some patients. Concentrations observed (mean level around 3 mg/L) are close to MIC defined by the CA-SFM for *S. aureus* and *H. influenzae* (respectively of 2 and 0.5 mg/L) (CASFM / EUCAST: Société Française de Microbiologie Ed; 2019, s. d.). The penetration of TMP in sputum appears to be proportionally higher than SMZ. Then, the concentration ratio of both drugs should be enough to maintain the synergic bactericidal activity of the drug.

All these findings support the use of higher dosages or alternative administration modalities to increase *in situ* antibiotic concentrations in sputum of CF patients. Antibiotics active against *Pseudomonas aeruginosa* are already available under nebulization formulations (e.g. tobramycin, aztreonam, colistin) but antibiotics active against *Staphylococcus aureus* or *Haemophilus influenzae* still presented as oral or intravenous formulations. Concia *et al.* reported a significant increase in the AMOX level in bronchial secretions with an association of the antibiotic to a bronchial fluidizer (Concia *et al.*, 1982). This suggests that sputum aspect is a limitation to the drug distribution.

Further studies including more patients are warranted to assess the relationship between pharmacokinetics and the therapy outcome in CF patients treated with AMOX or SMX/TMP. The next step would be to compare concentrations of antibiotics and MIC values for bacteria isolated from a same sputum sample. In addition, a study of plasma/sputum concentrations relationship has to be conducted to better describe the distribution of the antibiotic to the infection site.

5. CONCLUSION

Two HPLC-based analytical methods were developed and validated. They were precise, accurate, specific, sensitive, convenient, cheap and routinely usable for the determination of AMOX and SMX/TMP in sputum of patients with CF. The clinical application of the analytical procedures suggests that AMOX penetration from plasma to bronchial sputum might be insufficient to ensure an effective anti-bacterial action. Concentrations of SMX/TMP assessed in the infected site were closed to the MIC of the drug for targeted

bacteria. Moreover, a low ratio of sputum/plasma concentration has been reported. Further studies have to be performed to confirm these preliminary observations. Pharmacokinetics analysis and drug monitoring must be considered and associated with microbiological analysis in the management of the patient treatment. It would lead to optimize dosing regimens and to improve clinical outcomes.

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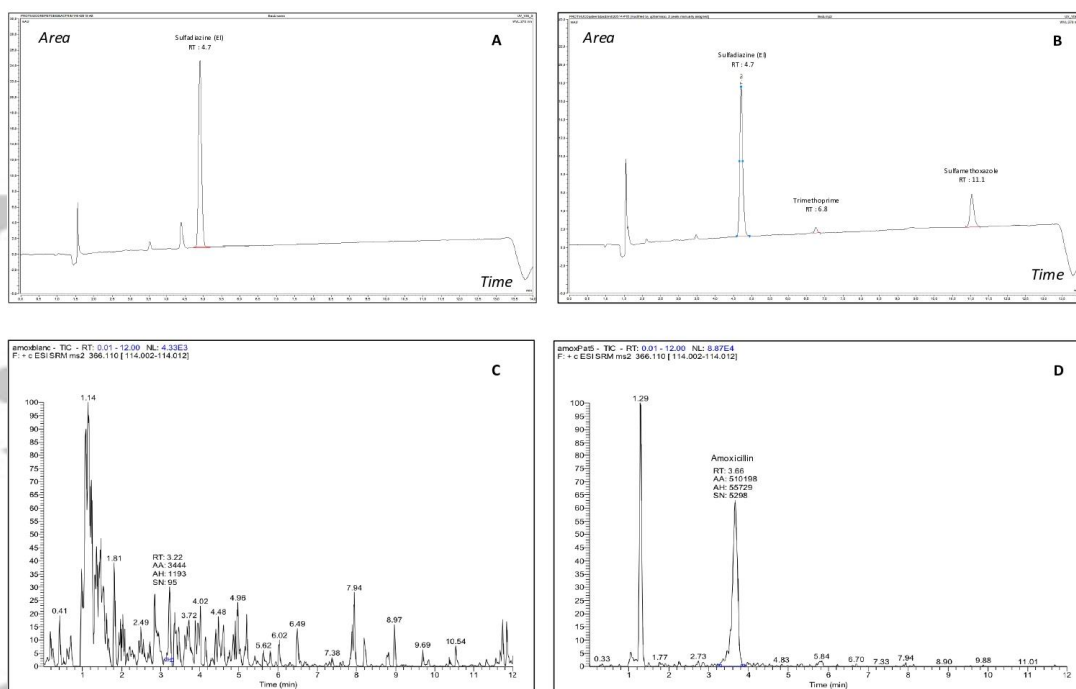


Figure 1: Representative chromatograms. (A) blank sample analyzed by the HPLC-UV method, (B) chromatogram of samples from a patient treated by cotrimoxazole and analyzed by the HPLC-UV method. (C) blank sample analyzed by the HPLC-MS/MS method, (D) chromatogram of samples from a patient treated by amoxicillin and analyzed by the HPLC-MS/MS method.

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Table 1: Effect of DTT on amoxicillin and cotrimoxazole

	Theoretical concentration (mg/L)	Concentration measured in distilled water solution		Concentration measured in DTT solution		RE (%)
		(Mean)	CV	(Mean)	CV	
		(mg/L) (n=3)	(%)	(mg/L) (n=3)	(%)	
AMOX	0.1	0.1	0.5	0.1	4.6	-4.2
	1.0	0.9	1.0	0.9	1.0	1.5
	10.0	9.3	0.3	9.2	0.6	-1.5
	50.0	46.0	0.4	45.8	0.4	-0.4
SMX	0.1	0.3	3.4	0.3	6.5	9.0
	1.0	2.9	0.6	2.9	1.9	0.1
	10.0	30.4	1.2	29.9	3.8	-1.7
	50.0	147.3	0.5	145.6	2.1	-1.2
TMP	0.1	0.1	1.5	0.1	8.0	5.9
	1.0	1.5	7.2	1.5	4.3	0.9
	10.0	14.7	0.9	14.7	4.8	-0.5
	50.0	77.3	3.0	79.8	2.7	3.2

No denaturation if relative error (RE) < ±15%, DTT: dithiotreitol, AMOX: amoxicillin, SMX: sulfamethoxazole, TMP : trimethoprim, CV : coefficient of variation

Table 2: Matrix effect assay

Antibiotic	Theoretical	MF	CV MF (%)
	concentration (mg/L)	(Mean +/- SD n=3)	
AMOX	0.3	0.97+/- 0.05	5.9
	45	0.91+/- 0.05	5.5
SMX	1	1.00+/- 0.01	1.3
	45	1.03+/- 0.04	3.9
TMP	0.1	0.88+/- 0.12	14.1
	1.5	0.96+/- 0.07	7.2

No relative matrix effect if MF sputum/plasma is <15%

MF: matrix factor, AMOX: amoxicillin, SMX: sulfamethoxazole, TMP: trimethoprim, CV: coefficient of variation, SD: standard deviation

Table 3: Intra-day and inter-day precision and accuracy

Antibiotic	Theoretical concentration (µg/mL)	Intra-day (n = 6)		Inter-day (n = 6)	
		Precision (CV %)	Accuracy (RE %)	Precision (CV %)	Accuracy (RE %)
AMOX	0.3	7.0	6.7	14.3	5.1
	15	6.5	-2.4	6.3	-3.6
	45	8.6	6.5	5.5	6.0
SMX	1	3.6	-4.0	7.2	-4.8
	15	5.1	-6.9	5.1	-7.9
	45	2.5	-5.2	2.5	-7.0
TMP	1.5	7.2	-10.8	4.1	-1.3
	4.5	4.9	-14.1	7.3	-7.5

AMOX: amoxicillin, SMX: sulfamethoxazole, TMP: trimethoprim, CV: coefficient of variation, SD: standard deviation, RE: relative error

Table 4: Recovery of amoxicillin extraction by protein precipitation and cotrimoxazole extraction by ethyl acetate liquid extraction (n=3)

Antibiotic	Theoretical concentration (µg/mL)	Recovery (%)
AMOX	0.3	100.9
	15	83.7
	45	88.7
SMX	1	71.2
	15	73.2
	45	72.5
TMP	1.5	/
	4.5	/

AMOX: amoxicillin, SMX: sulfamethoxazole, TMP: trimethoprim

Table 5: Stability of amoxicillin and cotrimoxazole in sputum samples and in extracts

	Stability in sputum samples (n=3)					Stability post extraction in autosampler (n=3)
	Nominal concentration (mg/L)	Initial concentration (mg/L)	-80C° 1month (bias %)	Room temperature 6h (bias %)	4°C 2h (bias %)	10 C° 6h (bias %)
AMOX	25	23.1	1.6	-63.7	-10.3	
AMOX	0.1	0.1				-6.6
AMOX	60	59.3				4.2
SMX	1	1.0	2.5	11.4	1.2	
SMX	30	32.9	-2.9	-5.0	-3.0	
SMX	0.5	0.5				4.6
SMX	60					-1.1
TMP	3	3.1	-2.9	-2.1	4.1	
TMP	6	6.1				-1.1

Results are expressed as relative bias compared to initial concentration

AMOX: amoxicillin, SMX: sulfamethoxazole, TMP: trimethoprim

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Table 6: Clinical application of the methods in cystic fibrosis patients

	Patie nts	Ws (g)	Vs (mL)	Vd (mL)	Dosage (per os)	Time from last admini- stration	Ca (mg/L)	Cvs (mg/L)	Cws (µg/g)	Cp (mg/L)	Sputum/ plasma Ratio (Cvs/Cp)
AMOX	1	1.12	1.87	1	<i>1g thrice a day</i>	15h	<0.1	<0.1	<0.1	<0.1	NC
	2	1.47	3.5	0.5		3h	0.1	0.1	0.3	NC	NC
	3	0.18	0.45	0.15		3h	<0.1	<0.1	<0.1	NC	NC
	4	0.77	1.29	2	<i>800 mg thrice a day</i>	3h	1.74	4.44	7.40	NK	NC
SMX	5	0.47	0.78	2	<i>1200 mg twice a day</i>	NK	0.66	2.35	3.92	NK	NC
	6	0.31	0.52	2	<i>800 mg twice a day</i>	NK	0.55	2.68	4.47	NK	NC
	7	2.37	3.95	1.5		NK	0.77	1.06	1.77	NK	NC
	8	1.52	2.54	2		3h	3.61	5.94	12.11	NK	NC
	9	0.81	1.35	1		12h	1.02	1.78	2.97	35.02	0.05
4	0.77	1.29	2	<i>160mg thrice a day</i>		3h	0.68	1.73	2.88	NK	NC
TMT	5	0.47	0.78	2	<i>240mg twice a day</i>	NK	0.24	0.86	1.43	NK	NC
	6	0.31	0.52	2	<i>160mg twice a day</i>	NK	0.76	3.72	6.20	NK	NC
	7	2.37	3.95	1.5		NK	0.37	0.51	0.85	NK	NC
	8	1.52	2.54	2		3h	1.91	3.14	6.39	NK	NC
	9	0.81	1.35	1		12h	0.70	1.22	2.03	2.36	0.52

Cws = Concentration of antibiotic in sputum (µg/mg); Cvs = Concentration of antibiotic in sputum (mg/L);
Ca = Concentration measured in sputum (mg/L); Cp: concentration measured in plasma; Vs = Volume of the
sputum sample (mL); Vd = volume of diluent added (mL); Ws = weight of the sputum sample (mg); NK: not
known, NC: not calculable