Validation of an LC-MS/MS method for the assay of the free fraction of an antiobiotic in human feces and assessment of its stability in the matrix using incurred samples

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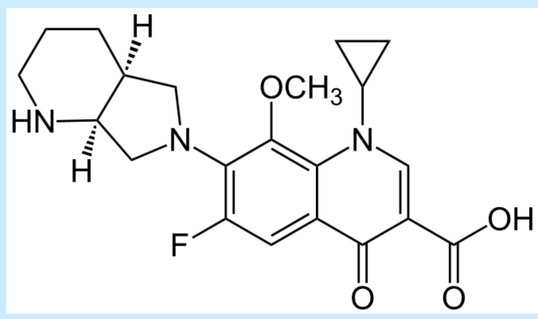
INTRODUCTION

In the current concern of increasing antibiotic resistance as a public-health issue, Da Volterra has developed a medical device to decrease the antibiotic free concentration in the gut.

In order to support a clinical study, DAV132-CL-1002, AmatsiAvogadro developed an analytical method allowing the assay of the free fraction of a widely used antibiotic, moxifloxacin, in human feces. Moxifloxacin is a fourth-generation synthetic fluoroquinolone, with a wide antibacterial spectrum. Among antibiotics, moxifloxacin was selected, as it is very frequently involved in the occurrence of *C. difficile* infections. The feces free fraction of the drug is supposed to lead to side effects like emergence of bacterial resistance as well as the occurrence of *C. difficile* associated colitis.

The moxifloxacin not bound to feces obtained by dissolution in buffer was assayed by an LC-MS/MS method.



Moxifloxacin

METHOD

Preparation of moxifloxacin free fraction Supernatant after gentle homogenization performed by mixing feces in pH 7 phosphate buffer

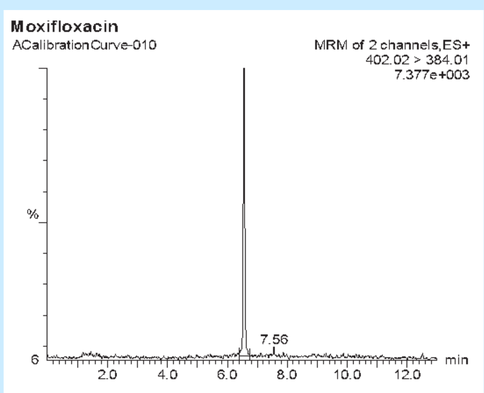
Range of Quantification 40 to 4000 ng/g of feces

Spiking procedure
Calibration standard and QC samples > Spiking of the feces free fraction
Stability samples > Spiking of feces

IS Moxifloxacin-D4

Extraction procedure
Protein precipitation with acetonitrile
Purification by SPE on Molecularly Imprinted Polymer (MIP) Fluoroquinolone (Supelco)

LC-MS/MS analysis
Column: Kinetex Phenylhexyl 2.6 µm, 100 x 3.0 mm (Phenomenex)
Gradient elution with pH 2.7 20 mM ammonium formate buffer/0.1% formic acid in acetonitrile, 0.4 mL/min
MRM acquisition in positive ion mode



Chromatogram at LLOQ

VALIDATION RESULTS

Selectivity:	Verified
Carry-over:	No significant remaining peaks
Regression parameter:	Simple linear regression with 1/X weighting factor
Matrix factor:	Moxifloxacin: from 87.8 % to 109.7 % Moxifloxacin-D4: from 81.1 % to 105.9 %
Extraction recovery:	Moxifloxacin 75.1 % / Moxifloxacin-D4 (IS): 73.4 %

Within and between -run accuracy:	From -1.1 % to 13.0 %
Within and between-run precision:	From 2.5 % to 8.0 %
Dilution:	Up to 100-fold dilution
Stability in solvent:	6 h at room temperature and 20 days at *ca.* +5°C
Stability in free fraction:	19 h at room temperature / 70 h at *ca.* +5°C
Post preparative stability:	36 h at +5°C

The method was successfully validated according to FDA and EMA guidelines.

STABILITY IN FECES

1) Spiked samples

Parameters	Mean concentration found (ng/g of feces)	% Diff *vs.* T0	CV % (n=4)
T0	204.8	-	13.3 %
21.5 h at room temperature	63.6	-68.9 %	8.4 %
42.5 h at *ca.* +5°C	103.6	-49.4 %	7.9 %
2 freeze/thaw cycles	106.6	-47.9 %	11.5 %
3 freeze/thaw cycles	96.6	-52.8 %	9.0 %

- One level of concentration
- Aberrant % difference with several sets of spiked stability samples
- *In-vitro* equilibrium of the moxifloxacin free fraction not reproducible from day to day
- No analytical parameter could be identified to solve the issue, the stability investigation was discontinued

➡ **The stability parameters were then evaluated using incurred samples**

2) Incurred samples (Low concentration level)

Parameters	Mean concentration found (ng/g of feces)	% Diff *vs.* T0	CV % (n=5)
T0	583.5	-	9.3 %
4 h at room temperature	659.4	13.0 %	12.9 %
53 h at *ca.* +5°C	655.9	12.4 %	6.6 %
3 freeze/thaw cycles	633.1	11.4 %	7.5 %
138 days at *ca.* -80°C	627.7	7.6 %	19.7 %

- 2 batches of incurred samples to evaluate stability at 2 concentration levels
- No difference observed for T0 value before and after one freeze/thaw cycle
- All stability parameters successfully evaluated and stability data determined for each storage condition

➡ **The procedure to obtain spiked stability sample is not representative of the incurred sample and thus of the free fraction**

CONCLUSION

The LC-MS/MS method for the assay of the free fraction of moxifloxacin in human feces was successfully validated, and relevant stability data were determined to cover the storage of human feces samples, to support clinical studies.