

1. During development and validation of the method the below equipment have been used:

- LC-MS/MS system consist of triple quadrupole MS-8050 detector coupled with Nexera X2 UHPLC system (Shimadzu, Kyoto, Japan),
- additional auxiliary LC-MS/MS equipment (Shimadzu, Kyoto, Japan): binary chromatographic pump (LC-30AD), degasser unit (DGU-20A5R), thermostatic column device (CTO-20AC), autosampler with thermostat compartment (SIL-30AC),
- centrifuge MPW- 375 (MPW Med Instruments, Warsaw, Poland),
- laboratory vortex (IKA, Warsaw, Poland),
- Vibrax shaker (IKA, Warsaw, Poland),
- shaking heater block (Thermo Scientific, Waltham, MA, USA),
- Simplicity 185 Millipore system for water deionizing (Merck Millipore, Burlington, MA, USA),

2. Lab materials:

- HyPurity C₁₈ chromatographic column:
- Pipettes, tips, Eppendorf tubes and other classic plastic materials,

3. Chemical and reagents:

- acetonitrile hyper grade for liquid chromatography (Merck, Darmstadt, Germany),
- super gradient methanol for liquid chromatography (Avantor Performance Materials, Gliwice, Poland),
- Zinc sulfate heptahydrate [ZnSO₄ • 7H₂O] (Merck, Darmstadt, Germany),
- Ammonium fluoride >99,99% of purity (Sigma-Aldrich, Saint Louis, MO, US),
- Formic acid for LC-MS >99,99% of purity (Sigma-Aldrich, Saint Louis, MO, US),
- Deionized water produced by Simplicity 185 Millipore system (Merck Millipore, Burlington, MA, USA)
- Reference standard: tacrolimus (Toronto Research Chemicals Inc., Toronto, ON, Canada, >99,10% of purity),
- Internal Standard: ascomycin (Sigma-Aldrich, Saint Louis, MO, US),

4. Biological material:

Whole blood from healthy volunteers not treated with tacrolimus from Regional Centre of Blood Donation and Haemotherapy (Warsaw, Poland).

5. Calibrators and working standard solutions:

- working solutions of TAC: 1000 ng/mL; 100 ng/mL
- working solution of ASC: 100 ng/mL; 25 ng/mL
- TAC calibrators: 0.5 ng/mL, 1.0 ng/mL, 2.5 ng/mL, 5.0 ng/mL, 10 ng/mL, 30 ng/mL, 60 ng/mL

6. Sample preparation procedure:

Stage 1a – calibration points preparation: to 50 µL of whole blood, add 10 µL of calibrator solution, 10 µL ASC (4.5 ng/mL),

Stage 1b – patients samples preparation after collection → go to Stage 3.

Stage 2- 1h-drying of VAMS

Stage 3 – VAMS tip extraction in 150 µL of pure water

Stage 4- Put on a shaking mixer at 1000 rpm at room temperature for 1h

Stage 5- Sample purification by protein precipitation - add 150 µL of 0.1 mol/L zinc sulfate mixture in acetonitrile (1:1; v/v);

Stage 6 - Centrifuge at 3500 rpm at 4°C for 10 min

Stage 7- Supernatant after purification and centrifugation should be moved into vial (~250-300 µL)

Stage 8- LC-MS/MS assay performing

4. Development of LC and MS conditions:

a) Mobile phases consist of:

(A) deionized water with ammonium fluoride (2 mmol/L) and 0.05% formic acid

(B) methanol: acetonitrile (50:50; v/v) with ammonium fluoride (2 mmol/L) and 0.05% formic acid.

b) Gradient was set as:

Time	Value of percentage phase concentration
0.50	A: 90% B: 10%
1.00	A: 5% B: 95%
3.00	A: 5% B: 95%
3.01	A: 90% B: 10%
5.00	CONTROLLER STOP

c) Additional LC- parameters:

- Total binary phase flow – 0.75 mL/min.
- The cooler of autosampler temperature set at 5°C.
- Injection volume – 10 µL.

5. MS/MS detector conditions

a) Measurement of TAC concentration was performed based on Multiple Reaction Monitoring (MRM) in positive electrospray ionization mode of ammonium adducts of analytes monitoring using following MRM pairs (m/z):

(1) TAC: 821,20 → 768,40 and 821,20 → 786,40 (as control pair),

(2) ASC: 809,20 → 756,55 and 809,20 → 564,35 (as control pair).

b) Proposed MS instrument parameters

parameter	value
electrospray voltage	0.70 kV
detector voltage	1.82 kV
dwel time	13.0 msec
single event time	0.048 sec
collision energy	22 V
temperature of interface	250 °C
desolvation temperature	526 °C
temperature of desolvation line	200 °C
temperature of heat block	250 °C
drying gas (nitrogen) flow	5.0 L/min
heating gas (air) flow	10.0 L/min
nebulizing gas (nitrogen) flow	1.0 L/min
CID gas (argon) pressure	270 kPa
thermostatic temperature	40 °C