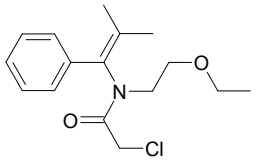
1. INTRODUCTION

Objective:

The objective of the present study was to validate analytical methods for the confirmation of pethoxamid in soil and in water at limits of quantification (LOQ) of 0.01 mg/kg (soil) and 0.1 µg/L (drinking and surface water), using LC/MS/MS for quantitation and confirmation.

Pethoxamid (TKC-94)



Principles of the Methods and Validation:

Analytical procedures as reported by Todd (17-Aug-2000, Huntingdon Life Sciences Project ID TON 025) were used in the present study:

20 g-soil specimens were extracted with 200 mL acetone/water (3/1, v/v) by shaking for two hours. After centrifugation, an aliquot of the supernatant was transferred in an autosampler vial, evaporated to dryness and re-dissolved in acetonitrile/water (1/1,v/v) for LC/MS/MS analysis.

100 mL-water specimens were extracted using solid phase extraction (SPE) using C_{18} cartridges with elution of the analyte using acetonitrile. An aliquot thereof was diluted for LC/MS/MS analysis.

LC/MS/MS was used to monitor two parent-daughter ion MRMs for quantitation and quantitative confirmation of pethoxamid. The soil extraction method achieves a limit of quantitation (LOQ) of 0.01 mg/kg and a limit of detection (LOD) of 0.002 mg/kg. The water extraction method achieves a limit of quantitation (LOQ) of 0.1 µg/L and a limit of detection (LOD) of 0.02 µg/L.

Method validations were accomplished by analyzing for soil and for surface and drinking water 2 blank control specimens, 5 replicate specimens fortified at LOQ, and 5 replicate specimens fortified at 10xLOQ.

2. EXPERIMENTAL

2.1 Test Systems

As European standard soil a sandy loam (according to USDA) soil (LUFA 5 M) was chosen.

Drinking (tap) water drawn at PTRL. The water was clear, had no smell, pH was 7.47, total hardness was 2.2 mmol/L corresponding to 12 °dH.

Surface (river) water collected from the Danube River on 02-Sep-08 near the Bundesstraße B10 "Ruderverein" in Ulm (Germany). The water was yellow, pH was 7.8, total hardness was 2.5 mmol/L corresponding to 14 °dH. The surface water was characterized by accredited Institute Alpha (Ulm, Germany following common DIN or EN guidelines and methods), resulting in the following (non-GLP):

TOC (total organic carbon, EN 1484:1997):	13 mg/L
DOC (dissolved organic carbon, EN 1484:1997):	4.0 mg/L
Silt content (filtered particles, DIN 38 409 H 2-3):	44 mg/L
Turbidity (EN 7027:1999)	58.0 NTU

2.2 Test and Reference Items

The analytical standard used was provided by the sponsor. See Appendix 1 for information provided.

2.3 Solvents, Chemicals, Equipment, and LC/MS/MS Instrumentation

Solvent, Chemicals, and Miscellaneous:

Acetone, acetonitrile, Promochem, HPLC or Pesticide grade. Millipore-H_2O, PTRL-Europe. Acetic acid 100 %, Merck. Ammonium acetate ≥ 98%, Fluka.

Bond Elut-C_{18} SPE cartridges, 500 mg, 6 mL, Varian.

pH meter, Denver Instr. Corp.

Total water hardness test, 0.1 – 3.6 mmol/L, Merck.

Equipment:

Analytical balance: Sartorius RC 210 D. Laboratory balance: Sartorius ED 2202S-CW. Horizontal shaker, JKA. Pierce Reacti-Vap. Ultrasonic bath: Elma Transsonic 460. Centrifuge: Rotixa 50 S, Hettich. SPE stations Baker SPE-100.

Typical glass and plastic ware and laboratory equipment.

LC/MS/MS Instrumentation:

Agilent 1100 Series HPLC system (vacuum solvent degasser, binary HPLC pump, column oven), and CTC Analytics HTC-Pal autosampler.

HPLC column: Thermo Aquasil (C_{18}), 150 mm length, 3.0 mm i.d., 3 µm particle size. Pre-column: Thermo Hypersil Gold, 10 x 3 mm, 5 µm particle size.

Applied Biosystems MDS Sciex API 3000 triple quadrupole LC/MS/MS system with TurboIonspray ESI source. Analyst 1.4.2 Instrument control and data acquisition software.

2.4 Standard Solutions and Stability

2.4.1 Stock Solutions

A stock solution of the analytical reference item was prepared in acetone by accurately weighing 10 mg (purity considered) into 10 mL volumetric flasks to obtain concentrations of 1.0 mg/mL.

2.4.2 Fortification Solutions

Fortification solutions were prepared in acetone by volumetric dilution of the stock solution. 0.10 mL of the stock solution were diluted into 10 mL acetone to obtain a 10 µg/mL fortification solution used for the 10xLOQ level of soil specimens, dosing 0.20 mL to 20 g soil dry mass.

This fortification solution was diluted (1.0 mL into 10 mL of acetone) to obtain a 1.0 µg/mL solution used for LOQ soil fortifications, dosing 0.20 mL to 20 g soil dry mass. The 1.0 µg/mL solution was used to fortify water specimens at the 10xLOQ level by dosing 0.10 mL to 100 mL of water volume.

The latter fortification solution was further diluted (0.10 mL into 10 mL of acetone) to obtain a fortification solution with a concentration of 10 ng/mL. This fortification solution was used to fortify water specimens at the LOQ level by dosing e.g. 1.0 mL to 100 mL of water volume.

2.4.3 Calibration Solutions

For preparation of calibration solutions, an intermediate solution was prepared in 10 mL of acetonitrile / water (1/1, v/v) by volumetric dilution of the stock solution (100 µL) to obtain a concentration of 10 µg/mL.

Calibration solutions were prepared by volumetric dilutions of the intermediate solution into acetonitrile / water (1/1, v/v) with the following concentrations:
100, 25, 10, 5.0, 2.5, 1.0, 0.50, 0.25, 0.10 and 0.05, all ng/mL.

All standard solutions were stored refrigerated when not in use. Stability of standard solutions was demonstrated by consistent LC/MS/MS results throughout the duration of the experimental phase of the study.

2.5 Soil Extraction Method

1. 20 g soil dry mass (W) were weighed into centrifuge bottles.
2. Fortifications are performed at this stage by dosing 0.20 mL of the fortification solutions: 1.0 µg/mL for LOQ (0.010 mg/kg), respectively 10 µg/mL for 10xLOQ (0.10 mg/kg).
3. 200 mL (V_{Ex}) of acetone/water (3/1, v/v) were added and specimens were shaken mechanically for at about 300 rpm for two hours.
4. Specimens were centrifuged for 5 minutes at about 3000 rpm.

5. A 0.5 mL aliquot (V_1) of the supernatant was transferred into an autosampler vial, evaporated to dryness using a gentle stream of nitrogen and re-constituted in a final volume of 1.0 mL (V_{End}) acetonitrile/water (1/1, v/v) for subsequent LC/MS/MS analysis.

2.6 Solid Phase Extraction (SPE) of Water

1. 100 mL aliquots (V_W) of the water specimen are measured.
2. Fortifications are performed at this stage by dosing 1.0 mL of the 10 ng/mL fortification solution for LOQ (0.10 µg/L) respectively 0.10 mL of the 1000 ng/mL fortification solution for 10xLOQ (1.0 µg/L).
3. Varian C_{18} SPE cartridges (500 mg adsorbens, 6 mL) are placed on SPE station(s) equipped with Teflon stop cocks, vacuum manifold(s) attached to water suction pump(s) or vacuum pumps.
   75-mL SPE reservoirs are fitted with adapters onto cartridges.
4. SPE cartridges are pre-conditioned with 3 mL of acetonitrile and 3 mL of water.
5. The 100-mL water specimens are added into the reservoirs and the water is drawn through the cartridge drop wise, the extracted water discarded.
6. SPE cartridges are washed with 20 mL of de-ionized water, followed by 3 mL acetonitrile/water (1/1, v/v).
7. 2.5 mL (V_{Ex}) of acetonitrile are added to the cartridge and drawn through the cartridges to elute the analyte from the C_{18} material.
8. The acetonitrile eluate is collected and an aliquot of 0.1 mL (V_1) is diluted with acetonitrile/water (1/1, v/v) to a final volume of 1.0 mL (V_{End}) for subsequent LC/MS/MS analysis.

2.7 LC/MS/MS Analysis

2.7.1 RP-HPLC Method

HPLC System	Agilent 1100 HPLC system (vacuum solvent degasser, binary HPLC pump, column oven), and CTC Analytics HTC-Pal Autosampler.
HPLC Column	Thermo Aquasil C_{18}, 150 mm length, 3.0 mm i.d., 3 µm particle size. Pre-column: Thermo Hypersil Gold, 10 x 3 mm, 5 µm particle size. Column oven 35 °C.
Injection Volume	20 µL.

HPLC Method	Solvent A: 0.1 % acetic acid and 0.01 M ammonium acetate in water/acetonitrile (80/20, v/v) Solvent B: 0.1 % acetic acid and 0.01 M ammonium acetate in acetonitrile/water (80/20, v/v) Mobile Phase Composition: Time (min) Flow rate (µL/min) % A % B 0.00 250 30 70 3.00 250 0 100 9.00 250 0 100 9.10 250 30 70 12.00 250 30 70

2.7.2 MS/MS Method

MS System	Applied Biosystems MDS Sciex API 3000 triple quadrupole LC/MS/MS system with TurboIonspray (ESI) source
Electrospray Ion Source Conditions Polarity: Positive	Source temperature: 450 °C Nebulizer gas (NEB): 14 Curtain gas (CUR): 12 Ion spray voltage (IS): 4500 V Entrance potential (EP): 10 V Collision gas (CAD): 4 Resolution Q1 and Q3: Unit
MS/MS Conditions	Declustering potential (DP): 36 Focussing potential (FP): 220 *296.1 m/z →131.1 m/z (Dwell Time : 500msec) :* CE: 30 CXP: 10 *296.1 m/z →*250.1 m/z (Dwell Time *: 500msec):* CE: 15 CXP: 22
Retention Time	approx. 8.5 min

External calibration was used for quantification and confirmation of the analyte by LC/MS/MS. Calibrations were established with standard solutions prepared in acetonitrile / water (1/1, v/v), which were injected interspersed with specimen extracts. The calibration usually ranged from 0.10 ng/mL or 0.05 ng/mL to 10 ng/mL or 25 ng/mL with ≥ 5 concentration levels.

Linear regression equations were generated with 1/x weighting, resulting in calibration functions with excellent correlation ($r \geq 0.997$), as exemplified in Figure 1 showing representative calibration functions and diagrams for both ion transitions.

LC/MS/MS chromatograms of standard solutions are exemplarily shown in Figure 2.

Figure 3 gives examples of LC/MS/MS chromatograms of fortified soil (10xLOQ: 0.10 mg/kg, LOQ: 0.010 mg/kg) and blank control soil specimens. Figure 4 gives examples of LC/MS/MS chromatograms of fortified drinking water (10xLOQ: 1.0 µg/L, LOQ: 0.10 µg/L) and blank control water specimens and Figure 5 gives examples of LC/MS/MS

chromatograms of fortified surface water (10xLOQ: 1.0 µg/L, LOQ: 0.10 µg/L) and blank control water specimens.

2.8 Calculation of Results

2.8.1 Calculation of Concentrations

Concentrations in the final extracts (ng/mL) were determined by substituting the peak area responses into the regression equation, using the LC/MS/MS Analyst 1.4.2 Instrument control and data acquisition software.

2.8.2 Calculation of Residues

Calculations were performed by Excel with full precision; discrepancies may arise when recalculated with pocket calculator.

For the calculation of residues the following formulas were used:

R *Soil* = c_{End} x (V_{Ex} x V_{End} / V_1 x W) / 1000 ng/µg

R *Water* = c_{End} x (V_{Ex} x V_{End} / V_1 x V_w)

= c_{End} x Multiplier M

Where:

R: Analyte residue in mg/kg or µg/L.

c_{End}: Final concentration of analyte in extract in ng/mL.
(where multiple injections were evaluated: mean).

V_{Ex}: Extraction volume: *Soil*: 200 mL; *Water*: 2.5 mL.

V_1: Aliquot of V_{Ex}: *Soil*: 0.50 mL; *Water*: 0.10 mL.

W: Weight of soil dry mass: 20 g

V_W: Water volume extracted by SPE: 100 mL.

V_{End}: Volume of final extract used for LC/MS/MS: 1.0 mL.

M: Multiplier: *Soil*: 0.020; *Water*: 0.25.

Recoveries (Rec.) were calculated for the fortified specimens as follows:

Rec. = (R / $R_{fortified}$) x 100 %

2.8.3 Example

The calculation is exemplified with a drinking water specimen fortified with pethoxamid at 1.0 µg/L or 10xLOQ (P1278-70, see Table 2).

The whole 100 mL (V_W) were enriched on a C_{18} SPE cartridge, eluted, and an aliquot of 0.10 mL (V_1) was diluted in a final volume (V_{End}) of 1.0 mL for LC/MS/MS determination.

The final extract was examined by LC/MS/MS (ESI) in run file P1578API#195 (Figure 4), resulting in a final concentration c_{End} for pethoxamid (m/z 296.1-> 131.1) calculated with 3.67 ng/mL.

Thus:

R = c_{End} x (V_{Ex} x V_{End} / V_1 x V_w)

= c_{End} x Multiplier M

= 3.67 ng/mL x (2.5 mL x 1.0 mL / 0.10 mL x 100 mL)

= 3.67 ng/mL x 0.25

= 0.92 µg/L

The result gave a recovery of 92 %.