1. INTRODUCTION

Background and Objective:

The objective of this study was to independently validate analytical methods, one for the determination of Fluindapyr (F9990/ IR9792) and one for its metabolites (cis-1-carboxy-Fluindapyr, trans-1-carboxy-Fluindapyr and 3-hydroxy-Fluindapyr) in drinking water by direct injection LC-MS/MS.

Method Principles:

The methods to be validated independently consists of injecting aliquots of the water sample directly into the LC-MS/MS instrument.

2. MATERIALS AND METHODS

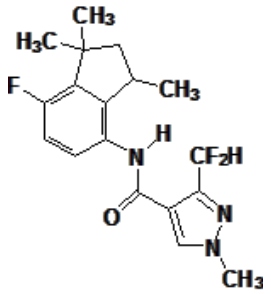
2.1 Test System

Drinking water e.g. tap water was taken in the rooms of EAG Laboratories GmbH. Typical physical parameter are controlled annually. The certificate of analysis is shown in the appendix.

2.2 Analytical Test / Reference Item

The following standard provided by the Sponsor was used as test / reference item:

Common name: **Fluindapyr (IR9792, F9990)**

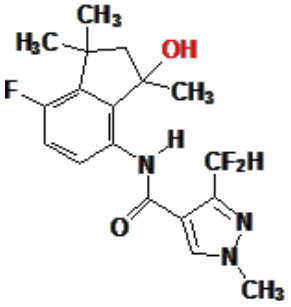


IUPAC Name: 3-(difluoromethyl)-N-(7-fluoro-2,3-dihydro-1,1,3-trimethyl-1H-inden-4-yl)-1-methyl-1H-Pyrazole-4-carboxamide

CAS no.: 1383809-87-7

Molecular Mass: 351.2 g/mol.

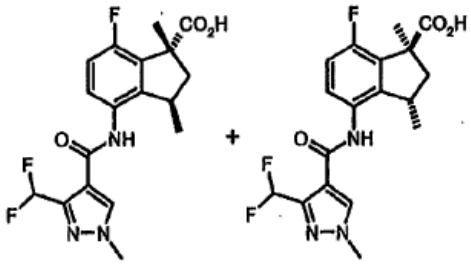
Common name: **3-Hydroxy-IR9792/F9990 (3-OH-Fluindapyr)**



Molecular formula: $C_{18}H_{20}F_3N_3O_2$

Molecular mass: 367.4 g/mol

Common name: **cis-1-Carboxy-IR9792/F9990 (cis-1-COOH-Fluindapyr)**

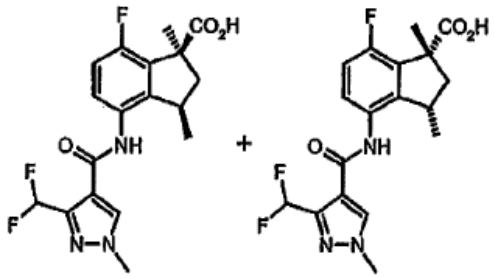


Molecular formula: $C_{18}H_{18}F_3N_3O_3$

Molecular mass: 381.4 g/mol

Racemic mixture of (1R,3R)-4-({[3(difluoromethyl)-1-methyl-1H-pyrazole-4-yl]carbonyl}amino)-7-fluoro-1,3-dimethylindane-1-carboxylic acid and (1S,3S)-4-({[3(difluoromethyl)-1-methyl-1H-pyrazole-4-yl]carbonyl}amino)-7-fluoro-1,3-dimethylindane-1-carboxylic acid

Common name: **trans-1-Carboxy-IR9792/F9990 (trans-1-COOH-Fluindapyr)**



Molecular formula: $C_{18}H_{18}F_3N_3O_3$

Molecular mass: 381.4 g/mol

Racemic mixture of (1R,3S)-4-({[3(difluoromethyl)-1-methyl-1H-pyrazole-4-yl]carbonyl}amino)-7-fluoro-1,3-dimethylindane-1-carboxylic acid and (1S,3R)-4-({[3(difluoromethyl)-1-methyl-1H-pyrazole-4-yl]carbonyl}amino)-7-fluoro-1,3-dimethylindane-1-carboxylic acid

See Appendix 2 for information on the analytical reference item used.

2.3 Analytical Method

2.3.1 Method

The methods to be validated independently consist of injecting aliquots of the water samples directly into the LC-MS/MS instrument. Both methods achieve a limit of quantification (LOQ) of 0.1 µg/L for each of the four analytes.

2.3.2 Apparatus

Analytical balance:
Mettler-Toledo, XS205DU
Ultrasonic bath:
VWR international, USC 300 TH.
Various:

Typical lab ware (for example: volumetric flasks and pipettes).

Used for the determination of Fluindapyr:

LC-MS/MS System:

AB Sciex API 6500+ Triple quadrupole LC-MS/MS system, equipped with TurboIonspray ESI source, Agilent 1290 Series HPLC system (vacuum solvent degasser, binary HPLC pump), MayLab MistrSwitch column oven, MayLab EluSwitch and CTC Analytics HTC-xt PAL Autosampler, Analyst 1.6.3 Instrument control and data acquisition software.

LC Column:

Waters XTerra C_{18}, 50 mm length x 4.6 mm i.d., 3.5 µm particle size with pre-column Phenomenex C_{18}, 4 x 3 mm.

Used for the determination of the metabolites:

LC-MS/MS System:

AB Sciex API 5500 Triple quadrupole LC-MS/MS system, equipped with TurboIonspray ESI source, Agilent 1290 Series HPLC system (vacuum solvent degasser, binary HPLC pump), MayLab MistrSwitch column oven and CTC Analytics HTC-xt eksigent PAL Autosampler, Analyst 1.6.3 Instrument control and data acquisition software.

LC Column:

Phenomenex Kinetex C_{18}, 50 mm length x 2.1 mm i.d., 2.6 µm particle size with pre-column Phenomenex C_{18}, 4 x 3 mm.

2.3.3 Reagents and Chemicals

Methanol, Sigma Aldrich, HPLC grade.
Millipore water (supply at EAG Laboratories GmbH).
Water, Merck, LC-MS grade.
Methanol, Merck, LC-MS grade.
Formic acid, Promochem, 99%.
Ammonium Acetate, Sigma Aldrich, LC-MS grade.

2.3.4 Preparation of Standard Solutions

See Appendix 2 for complete information provided with the analytical reference item used.

2.3.4.1 Stock and Fortification Solutions

A 1.0 mg/mL stock solution of each reference substance was prepared in methanol as exemplified below:

Analyte	Weight [mg]	Volume Solvent [mL]	Obtained concentration* [mg/mL]
Fluindapyr	10.33	101.66	0.10
3-OH-Fluindapyr	10.32	101.48	0.10
cis-1-COOH-Fluindapyr	10.78	100.64	0.10
trans-1-COOH-Fluindapyr	10.80	100.94	0.10

*Purity taken into account.

The stock solution was used to prepare fortification solutions by appropriate dilution in drinking water as follows:

For the determination of Fluindapyr the stock solution was diluted as follows:

1.0 mL of the respective stock solution were dosed into a 100 mL volumetric flask and filled up to 100 mL with drinking water resulting in a 1.0 µg/mL solution used as intermediate dilution. This solution was further diluted by dosing 10 mL into a 100 mL volumetric flask and filling up to 100 mL with drinking water, resulting in a 0.10 µg/mL fortification solution, used for the 10xLOQ level. A further dilution of this solution was made by dosing 10 mL into a 100 mL volumetric flask and filling up to 100 mL with drinking water, resulting in a 0.010 µg/mL fortification solution, used for the LOQ level.

For the determination of 3-OH-Fluindapyr, cis- and trans-1-COOH-Fluindapyr mixed fortification solutions were prepared from the stock solutions as follows:

An intermediate solution containing all three analytes were prepared by dosing 1.0 mL of each stock solution into a 100 mL volumetric flask and filled up to 100 mL with drinking water resulting in a 1.0 µg/mL solution.

This intermediate solution was diluted by dosing 5.0 mL into a 100 mL volumetric flask and filled up to 100 mL with drinking water resulting in a 0.050 µg/mL solution used for the

fortification of the 10xLOQ level. This fortification solution was diluted further by dosing 10 mL into a 100 mL volumetric flask and filled up to 100 mL with drinking water resulting in a 0.0050 µg/mL solution used for the fortification of the LOQ level.

2.3.4.2 Calibration Solutions and Matrix-Matched Standards

For the determination of Fluindapyr:

Using the 0.10 mg/mL stock solution, LC-MS/MS calibration solutions were prepared by accurate volumetric dilution in Millipore water to obtain the following intermediate concentrations: 1000, 100, 12, 6.0, 3.0, 1.0 and 0.20 all in ng/mL.

These intermediate solutions were diluted further by a factor of 10 in Millipore water (1.0 mL of the respective solution diluted with 9.0 mL Millipore water in a volumetric flask) to obtain the following concentrations: 1.20, 0.60, 0.30, 0.10 and 0.020 all in ng/mL.

For the preparation of matrix-matched standard solutions the same intermediate solutions were diluted in drinking water by a factor of 10, as described above.

For the determination of 3-OH-Fluindapyr, cis- and trans-1-COOH-Fluindapyr:

Using the intermediate solution, containing all three analytes at a concentration of 1.0 µg/mL. matrix-matched standard solutions were prepared by accurate volumetric dilution in drinking water to obtain following concentrations: 50, 10, 5.0, 1.0, 0.50, 0.10, 0.050 and 0.030 all in ng/mL.

All standard solutions were stored refrigerated in amber glass bottles when not in use.

2.3.4 Preparation of Samples and Fortifications

For the determination of Fluindapyr:

Recovery of Fluindapyr was assessed throughout the analytical procedure by fortifying five aliquots of drinking water at the limit of quantitation (LOQ: 0.10 µg/L) and five aliquots of drinking water at 1.0 mg/L (10 x LOQ).

Fortified samples were prepared by pipetting accurate volumes (1.0 mL) of the corresponding fortification solutions into 100 mL of drinking water.

In addition, two control samples and one reagent blank sample per matrix were analysed in order to verify that no significant interferences (> 20% of LOQ) were present at the retention time of the analyte in the test system.

For the determination of 3-OH-Fluindapyr, cis- and trans-1-COOH-Fluindapyr:

Recovery of 3-OH-Fluindapyr, cis- and trans-1-COOH-Fluindapyr were assessed throughout the analytical procedure by fortifying five aliquots of drinking water at the limit of quantitation (LOQ: 0.10 µg/L) and five aliquots of drinking water at 1.0 µg/L (10 x LOQ).

Fortified samples were prepared by pipetting accurate volumes (1.0 mL) of the corresponding fortification solutions into 50 mL of drinking water.

In addition, two control samples and one reagent blank sample per matrix were analysed in order to verify that no significant interferences (> 20% of LOQ) were present at the retention time of the analyte in the test system.

2.4 Instrumental Analysis

2.4.1 LC-MS/MS Method for the Determination of Fluindapyr

The water samples were analyzed without further clean-up by liquid chromatography with tandem mass spectrometric detection (LC-MS/MS).

LC System	Agilent 1290 HPLC system (vacuum solvent degasser, binary HPLC pump) MayLab MistraSwitch column oven, MayLab EluSwitch and CTC Analytics HTC-xt Pal Autosampler.
LC Column	Waters XTerra C_{18} column: Length: 50 mm, i.d.: 4.6 mm, particle size: 3.5 µm. Column temperature: 40 °C.
LC Injection Volume	10 µL.
LC Method	Solvent A: Water + 10 mM ammonium acetate + 0.2% formic acid Solvent B: Methanol + 0.2% formic acid Mobile Phase Composition: Time (min) / Flow rate (mL/min) / % A / % B 0.0 / 0.70 / 40 / 60 3.0 / 0.70 / 20 / 80 3.5 / 0.70 / 40 / 60 5.0 / 0.70 / 40 / 60
Retention time	≈ 3.1 min
MS/MS System	AB Sciex API 6500+ Triplequadrupole LC-MS/MS system with Turbo IonSpray (ESI) source, detection of positive ions.
Ion Source Conditions ESI Positive Polarity	Source temperature: 550 °C Gas supply GS 1: 60 (arbitrary units) Gas supply GS 2: 60 (arbitrary units) Curtain gas: 35 (arbitrary units) Entrance potential: 10 V IonSpray voltage: 4500 V Declustering potential: 91 V
MS/MS Conditions	Collision activated dissociation (CAD): 10 V Resolution Q1 unit Resolution Q3: unit Collision energy (CE): 25 V Cell exit potential (CXP): 55 V Dwell time: 100 ms 1st MS/MS transition: 352 m/z > 256 m/z 2nd MS/MS transition: 352 m/z > 312 m/z

The parent ion for detection of Fluindapyr (IR9792, F9990) (352 m/z) is related to the pseudo-molecular ion $[M+H]^+$of the compound cation. A product mass spectrum is shown in the original method validation report.

Repeatability of LC-MS/MS determination was demonstrated by duplicate injection of selected fortified specimen extracts.

LC-MS/MS, monitoring two structurally characteristic parent – daughter ion transitions, is considered to be highly specific, thus not requiring further confirmation of detected residues.

2.4.2 Calibration and Evaluation for the Determination of Fluindapyr

Linear LC-MS/MS calibration functions were established by injecting standard solutions in solvent and using both the 256 m/z and the 312 m/z daughter ion peak areas for separate quantification/confirmation. Calibration levels ranged from 0.020 ng/mL to 1.2 ng/mL.

The concentration of Fluindapyr (IR9792, F9990) in the water samples from the control and recovery specimens was evaluated by external calibration, employing the LC-MS/MS software.

2.4.3 LC-MS/MS Method for the Determination of 3-OH-Fluindapyr, cis- and trans-1-COOH-Fluindapyr

The water samples were analyzed without further clean-up by liquid chromatography with tandem mass spectrometric detection (LC-MS/MS).

LC System	Agilent 1290 HPLC system (vacuum solvent degasser, binary HPLC pump) MayLab MistraSwitch column oven, MayLab EluSwitch and CTC Analytics HTC-xt Pal Autosampler.
LC Column	Phenomenex Kinetex C_{18} column: Length: 50 mm, i.d.: 2.1 mm, particle size: 2.6 µm. Column temperature: 25 °C.
LC Injection Volume	40 µL.
LC Method	Solvent A: Water + 0.1% formic acid Solvent B: Methanol + 0.1% formic acid Mobile Phase Composition: Time (min) / Flow rate (mL/min) / % A / % B 0.0 / 0.60 / 90 / 10 3.5 / 0.60 / 90 / 10 6.0 / 0.60 / 60 / 40 15.0 / 0.60 / 37.5 / 62.5 15.1 / 0.60 / 5.0 / 95 18.0 / 0.60 / 5.0 / 95 18.1 / 0.60 / 90 / 10 22.0 / 0.60 / 90 / 10
Retention times	trans-1-COOH-Fluindapyr ≈ 7.7 min cis-1-COOH-Fluindapyr ≈ 8.3 min 3-OH-Fluindapyr ≈ 12.0 min
MS/MS System	AB Sciex API 5500 Triplequadrupole LC-MS/MS system with Turbo IonSpray (ESI) source, detection of positive ions.
Ion Source Conditions ESI Positive Polarity (cis- and trans-1-COOH-Fluindapyr)	Source temperature: 550 °C Gas supply GS 1: 40 (arbitrary units) Gas supply GS 2: 60 (arbitrary units) Curtain gas: 20 (arbitrary units) Entrance potential: 10 V IonSpray voltage: 4500 V

MS/MS Conditions for cis- and trans-1-COOH-Fluindapyr	Collision activated dissociation (CAD): Resolution Q1 Resolution Q3: 1st MS/MS transition: Collision energy (CE): Cell exit potential (CXP): Dwell time: Declustering potential: 2nd MS/MS transition: Collision energy (CE): Cell exit potential (CXP): Dwell time: Declustering potential:	9 unit unit 382 m/z > 296 m/z 35 V 20 V 100 ms 76 V 382 m/z > 336 m/z 21 V 10 V 100 ms 50 V
Ion Source Conditions ESI Negative Polarity (3-OH-Fluindapyr)	Source temperature: Gas supply GS 1: Gas supply GS 2: Curtain gas: Entrance potential: IonSpray voltage:	550 °C 40 (arbitrary units) 60 (arbitrary units) 20 (arbitrary units) -10 V -4500 V
MS/MS Conditions for 3-OH-Fluindapyr	Collision activated dissociation (CAD): Resolution Q1 Resolution Q3: Declustering potential: 1st MS/MS transition: Collision energy (CE): Cell exit potential (CXP): Dwell time: 2nd MS/MS transition: Collision energy (CE): Cell exit potential (CXP): Dwell time:	9 unit unit -110 V 366 m/z > 131 m/z -40 V -7 V 100 ms 366 m/z > 175 m/z -30 V -11 V 100 ms

The parent ion for detection of cis- and trans-1-COOH-Fluindapyr (382 m/z) is related to the pseudo-molecular ion $[M+H]^+$of the compound cation. The parent ion for detection of 3-OH-Fluindapyr (366 m/z) is related to the pseudo-molecular ion $[M-H]^-$of the compound cation . A product mass spectrum is shown in the original method validation report.

Repeatability of LC-MS/MS determination was demonstrated by duplicate injection of selected fortified specimen extracts.

LC-MS/MS, monitoring two structurally characteristic parent – daughter ion transitions, is considered to be highly specific, thus not requiring further confirmation of detected residues.

2.4.4 Calibration and Evaluation for the Determination of 3-OH-Fluindapyr, cis- and trans-1-COOH-Fluindapyr

Linear LC-MS/MS calibration functions were established by injecting matrix-matched standard solutions in drinking water and using the 296 m/z and the 336 m/z (for cis- and trans-1-COOH-Fluindapyr), respectively 131 m/z and 175 m/z (for 3-OH-Fluindapyr) daughter ion peak areas for separate quantification/confirmation. Calibration levels ranged from 0.030 ng/mL to 5.0 ng/mL.

The concentrations in the water samples from the control and recovery specimens were evaluated by external calibration, employing the LC-MS/MS software.

2.5 Calculation

Results derived from LC-MS/MS determination and subsequent calculations are presented in the table section.
No calculation of the residue is necessary for the determination after direct injection without dilution of the water sample.
Recoveries (Rec.) were calculated for the fortified specimens as follows:

$$\text{Rec.} = (\text{Residue}_{\text{found}} / \text{Residue}_{\text{fortified}}) \times 100\%$$

2.6 Example for Calculation

The calculation of the recovery of the analyte is exemplified below for the sample P4865-45 fortified with Fluindapyr at 0.10 µg/L (LOQ). The final extract was examined by LC-MS/MS in run file P4865API6#015 (see Figure 5). The final concentration (C_{End}) was determined to be 0.106 ng/mL for the primary transition (*m/z* 352 → 256).

$$\text{Rec.} = (R_{found} / R_{fortified}) \times 100\%$$
$$= (0.106\ \mu g/L / 0.10\ \mu g/L) \times 100\%$$
$$= 106\%$$

All calculations in the Excel sheets were performed with full precision, but were reported with rounding.

3.5 Minor Adaptions of the Method

The following minor adaptions were made due to different instrumental equipment available in the laboratory.

For the determination of Fluindapyr:

- A Waters Xterra C_{18} LC column with dimensions of 50x4.6 mm and 3.5 µm particles was used which is similar to the Phenomenex Kinetex C_{18} LC column (50x4.6 mm, 2.6 µm particles) used in the original validation.
- LC flow rate was adapted from 1200 µL/min to 700 µL/min

Using these conditions similar retention of the analyte was achieved. Thus, these adaptions to the equipment available were considered to be only minor.

For the determination of 3-OH-Fluindapyr, cis- and trans-1-COOH-Fluindapyr:

- A Phenomenex Kinetex C_{18} LC column with dimensions of 50x2.1 mm and 2.6 µm particles was used which is similar to the Phenomenex Kinetex C_{18} LC column (50x4.6 mm, 2.6 µm particles) used in the original validation.
- Methanol containing 0.1% formic acid water containing 0.1% formic acid were used as the mobile phases. The original validation employed methanol containing 0.2% formic acid and water containing 10 mM ammonium acetate and 0.2% formic acid.

The elution order of the analytes remained unchanged and a good retention and a good resolution of cis- and trans-1-COOH-Fluindapyr was achieved.

Thus, these adaptions to the equipment available were considered to be only minor.

There is no significant impact on the validity of the ILV by these minor adaptions, however they demonstrate the robustness of the methods.

4. CONCLUSION

Two analytical methods based on Direct-Injection-LC-MS/MS were successfully validated independently for the determination of Fluindapyr respectively 3-OH-Fluindapyr, cis- and trans-1-COOH-Fluindapyr in drinking water with a limit of quantification of 0.1 µg/L. The method fulfills the requirements of EC Guidance document of pesticide residue analytical methods SANCO/825/00 rev. 8.1, 16/11/2010, and OECD guidance document (ENV/JM/MONO (2007)17).