

1 Summary

This report describes the independent laboratory validation of a method for the determination of DPX-MP062 and metabolites IN-MS775, IN-JT333, IN-MP819, IN-JU873 and IN-KG433 in groundwater, surface water and drinking water using LC-MS/MS. The method used was developed and previously validated by DuPont [1]. Water was extracted using solid phase extraction. After a clean up by rinsing with acetonitrile/water 70/30 V/V, the analytes were desorbed by acetonitrile. The extracts were then analyzed using high performance liquid chromatography with mass spectrometric/mass spectrometric detection (MS/MS). Positive ion atmospheric pressure ionisation was used, and one precursor \rightarrow product ion transition was monitored. All analytes except IN-MS775 and IN-MP819 were initially measured using both atmospheric pressure chemical ionisation (APCI) and electrospray ionisation (ESI). IN-MS775 did not produce sufficient signal using APCI, and IN-MP819 did not produce sufficient signal using ESI. The limit of quantification was 0.05 $\mu\text{g/L}$ for all six analytes. The analytical method generated acceptable recoveries for the three types of water fortified with DPX-MP062, IN-JT333, IN-MP819, IN-JU873, IN-KG433 (after 2nd attempt) and IN-MS775 (after 3rd attempt) at LOQ and 10 times LOQ level.

3 Introduction

3.1 Background

DPX-MP062 is a mixture of two optical isomers, 75% DPX-KN128 also known as indoxacarb and 25% IN-KN127. Indoxacarb is insecticidally active and IN-KN127 is not. DPX-KN128 and IN-KN127 cannot be separated without the use of chiral techniques.

IN-MS775, IN-JT333, IN-MP819, IN-JU873 and IN-KG433 are five metabolites of DPX-MP062.

3.2 Aim of the study

The aim of the study was to demonstrate that the proposed method [1] for water analysis can be reproduced, and that method performance characteristics meet the requirements as laid down in SANCO/825/00 [3]. The method was validated on representative drinking and surface waters.

3.3 Study parameters

The following parameters were determined during validation: recovery and repeatability at 0.05 µg/L (LOQ) and 0.5 µg/L level, and specificity. In addition, the limits of detection (LOD) were estimated.

The surface water sample was characterised by determination of total hardness, TOC, pH and silt content.

3.4 Study outline

Control and fortified control water samples were extracted using solid phase extraction with a Waters Oasis® HLB polymeric resin cartridge. After elution with acetonitrile the extracts were analysed by LC-MS/MS. From the data obtained, the validation parameters were determined and tested against the criteria.

3.5 Conduct of the study

The study was conducted:

- 1 In compliance with the study protocol, P5033/02, approved by the study director on 6 March 2003
- 2 In compliance with the OECD principles of Good Laboratory Practice (GLP) [2], except for the determination of the surface water characteristics.

3.6 Duration and timing

Experimental start date : 10 March 2003
Experimental completion date : 11 April 2003

3.7 Protocol deviations

1) the study title was changed, it now reads Independent laboratory validation instead of Inter laboratory validation.

Reason: Independent is the correct terminology as used in SANCO/825/00.

2) The name of the subcontractor for water characterization changed into Alcontrol.

3) Acetic acid was also added to (diluted) stock solutions in acetonitrile.

Reason: to prevent degradation and/or adsorption of analytes (IN-KG433).

4) LC-MS/MS: during the first validation attempt four analytes (DPX-MP062, IN-JT333, IN-JU873 and IN-KG433) were measured both by LC-APCI-MS/MS and LC-ESI-MS/MS.

Reason: the above mentioned analytes could be measured using both interfaces. Depending on the sample, the analyte, the instrument used and conditions applied, one interface may show favourable results over the other.

5) The LC conditions used with APCI were different than mentioned in the protocol.

Reason: the conditions used were as described in the sponsor's method. In the protocol the conditions were incorrect.

6) During the 3rd validation attempt for IN-MS775, acetic acid was added to eluent B (acetonitrile) at a concentration of 10 mM.

Reason: this way the concentration of acetic acid in the mobile phase was constant over the gradient applied.

7) Recoveries were not corrected for peaks detected in control samples.

Reason: in case peaks were detected the corresponding levels were inconsistent and low compared to the LOQ level ($\leq 12\%$). Subtraction of control peaks would not have resulted in average recoveries below 70%.

8) LC-MS/MS, ions monitored: for each analyte one transition was monitored.

The deviations described above had no impact on the outcome of the study results.

4 Test system

4.1 Water samples

The validation was carried out using three types of water: drinking water (tap water, Zeist), groundwater (Netherlands, sample 699/04/1593), and surface water (local river/channel, Netherlands, sample 0699/05/0024). Water samples were stored at 2-10 °C until use.

The characteristics of the surface water were as follows:

pH	= 7.9	Silt	= 510 mg/l
Total organic carbon (TOC)	= 36 mg/l	Total hardness	= 14 °D

For each type of water the validation set consisted of:

2 control samples

5 samples fortified at LOQ (0.05 µg/L)

5 samples fortified at 10x LOQ (0.5 µg/L)

4.2 Test substances

DPX-MP062

Lot : 160
Purity : 99.4% Storage
: < 25 °C
Expiration date : 28 March 2003

IN-MS775

Lot : 002
Purity : 99.7 % Storage
: < 25 °C
Expiration date : 21 January 2006

IN-JT333

Lot : 017
Purity : 97.5%
Storage : < 25 °C
Expiration date : 14 November 2004

IN-MP819

Lot : 002
Purity : 98.6%
Storage : < 25 °C
Expiration date : 6 September 2004

IN-JU873

Lot : 005
Purity : 99.06%
Storage : < 25 °C
Expiration date : 2 May 2005

IN-KG433

Lot : 002
Purity : 96.5%
Storage : < -18°C
Expiration date : 27 November 2006

Test substances were supplied by the sponsor. The analyses for the identity and purity of the test substances were conducted by, or under responsibility of, the sponsor.

Structures and properties of the analytical test substances are given in appendix B01.

5 Procedures and methods

5.1 Safety requirements

The routine measures for laboratory work were applicable.

5.2 Preparation and storage of stock and fortification solutions

DPX-MP062, IN-MS775, IN-JT333, IN-MP819, IN-JU873 and IN-KG433

At least ca. 10 mg was accurately weight into 100 ml volumetric flasks and dissolved in acetonitrile (concentration approx. 100 µg/mL).

Standards were stored in the freezer (< -18 °C). Fresh standards were prepared for each validation attempt.

Method deviation: for the 2nd and 3rd validation attempts, acetic acid (0.1%) was added to the acetonitrile. This was considered to prevent degradation/adsorption.

5.3 Preparation and storage of multi-analyte standard solutions

A solution containing DPX-MP062 and the five metabolites at 1.0 µg/mL was prepared by pipetting all six individual stock solutions into one volumetric flask and dilution with acetonitrile. For the 2nd validation attempt, the mixture only contained IN-MS775 and IN-KG433. For the 3rd validation attempt the calibration solution only contained IN-MS775.

From the 1.0 µg/mL mixed solution further dilutions in acetonitrile were prepared to obtain 0.1 and 0.01 µg/mL solutions.

In case of re-validation, stock solutions were diluted in acetonitrile containing 0.1% acetic acid.

These solutions were used for fortification and preparation of calibration standards.

The 0.01-0.1 µg/L multi-analyte solutions were stored in the freezer (< -18°C). Calibration solutions were freshly prepared for each validation.

5.4 Preparation and storage of calibration solutions

Mixed calibration standards containing DPX-MP062 and the five metabolites were prepared by addition of 0.1 and 1 µg/mL standard solutions (according to table below) to a 10 mL volumetric flask. The standards were diluted to volume using a 50:50 acetonitrile:water mixture that contained 100 µl acetic acid/L.

Desired standard concentration (ng/mL)	Volume of 0.1 µg/mL multi-analyte standard required (µL)	Volume of 1.0 µg/mL multi-analyte standard required (µL)
0.60	60	-
1.0	100	-
5.0	-	50
10	-	100
15	-	150

Calibration standards were prepared freshly with each set of analysis

5.5 Sample fortification

Glass bottles were filled with aliquots of 40.0 mL of water. Fortification was done to each bottle individually using multi-analyte solutions.

To obtain a concentration in water of 0.05 µg/L, 200 µL of a 0.01 µg/mL solution was added to the bottles. To obtain a concentration of 0.5 µg/L, 200 µL of a 0.1 µg/mL solution was added to the bottles.

5.6 Extraction and purification procedures

DPX-MP062 and IN-JT333 have the tendency to adhere to glass and plastic surfaces. For this reason acetonitrile is added to the bottle containing the water sample. Sample handling accessories such as SPE solvent reservoirs and adapters should be avoided to minimize surface area.

1. Add 10.0 mL (\pm 1%) of acetonitrile and 40 µl acetic acid to the 40 mL water sample in the bottle. Cap and shake the samples vigorously.
2. Attach a 12 mL, 0.5-g Oasis HLB cartridge [Waters] to an SPE manifold. Precondition the cartridge with 5 mL of methanol and discard the conditioning solution. Do not let the cartridge go to dryness. Next condition the cartridge with 10 mL of HPLC grade water. Do not let the cartridge go to dryness.
3. Load the sample into the SPE. Using gravity flow, allow the sample to pass through the Oasis cartridges at a flow rate of 2-5 mL/min. Rinse the sample bottle with 10 mL of 70:30 (V/V) water:acetonitrile and load the rinse into the reservoir just before all of the sample passes through. Use vacuum to dry the cartridge for 5 minutes.
4. Elution of the analyte.
Rinse the sample bottle with 25 mL of acetonitrile and load the acetonitrile onto the cartridge, vacuum may be required to start the flow but should be turned off once the flow has started. Once the acetonitrile has passed through the cartridge, use a small amount of vacuum to empty the remaining liquid in the cartridge into the collection tube.
5. Evaporate the extract to a volume of approximately 100 µL using a flow of nitrogen at approximately 35°C*. Dilute the extract to 1 mL with acetonitrile, vortex the extract for 30 seconds and sonicate it for 5 minutes. Add 1.0 mL of water (final volume 2.0 mL). Add 10 µl of acetic acid. Vortex the tube and

transfer an aliquot of the extract using a disposable pipette into an HPLC vial. Analyse the solution by LC/MS/MS as described in the following section.

* Except for validation attempt 3, partially concentrated acetonitrile extracts were stored overnight in the freezer and further concentrated the next day. The acetic acid was added before storage in the freezer instead of after reconstitution.

Extracts were stored at 2-10 °C and analysed within 1 day, except for LC-APCI-MS/MS measurements (stored for 4 days), see appendix B02.

5.7 Analysis

Method deviations:

- During the first validation attempt DPX-MP062, IN-JT333, IN-JU873 and IN-KG433 were measured with both ACPI and ESI. In the original method all analytes except IN-MP819 were measured using the ESI interface.
- Only one transition for each analyte was measured.

5.7.1 LC-APCI-MS/MS

Instrumentation:

HPLC : Shimadzu
 Injection : 200 µl
 Column : 3.0 mm i.d. x 150 mm, Phenomenex C18, 3 µm
 Column temp. : 30 °C
 Sample temp. : 2-10 °C
 Flow rate : 0.5 mL/min

Eluent A : 10 mM aqueous formic acid

Eluent B : Acetonitrile

Gradient	time	%A	%B
	0	50	50
	0.5	50	50
	9	2	98
	12	2	98
	12.5	50	50
	16	50	50

Divert valve : not used

MS/MS : PE Sciex API3000

Interface : APCI (heated nebulizer)

Ion mode : positive

Acquisition : Multiple reaction monitoring (MRM)

Ion monitored : precursor → product ion:
 DPX-MP062 528 → 218
 IN-JT333 267 → 150
 IN-MP819 238 → 131
 IN-JU873 458 → 255
 IN-KG433 516 → 281
 Dwell time: 200 ms

Nebulizer temperature : 550 °C
 Nebulizer gas : 14
 Curtain gas : 12
 Collision gas (nitrogen) : 3
 Resolution : Q1 and Q3: low

5.7.2 LC-ESI-MS/MS

Instrumentation:

HPLC : Shimadzu
 Injection : 200 µl
 Column : 3.0 mm i.d. x 150 mm, Phenomenex C18, 3 µm
 Column temp. : 30 °C
 Sample temp. : 2-10 °C
 Flow rate : 0.4 mL/min

Eluent A : 10 mM aqueous acetic acid
 Eluent B : Acetonitrile (1st and 2nd attempt)

Method deviation: 10 mM of acetic acid was added to the acetonitrile during the 3rd attempt. This was considered to improve stability of response for IN-MS775

Gradient	time	%A	%B
	0	70	30
	0.5	70	30
	4	35	65
	12	5	95
	15	5	95
	15.1	70	30

Divert valve : the flow was diverted before 7 and after 15 min

MS/MS : PE Sciex API3000
 Interface : ESI (Turbo ion spray)
 Ion mode : positive
 Acquisition : Multiple reaction monitoring (MRM)
 Ions monitored :

DPX-MP062	528 → 293
IN-MS775	412 → 209
IN-JT333	470 → 267
IN-JU873	458 → 149
IN-KG433	516 → 281

temperature : 550 °C
 gas : 8
 Curtain gas : 8
 Collision gas (nitrogen) : 4
 Ion spray voltage : 5000
 Entrance potential : 10
 Resolution : Q1 low, Q3 unit

5.8 Quantification

Quantification was performed using the average response factor for the three standards closest in response to the sample analysed. The standards selected included a minimum of one standard above and one standard below the response of the sample.

Average Response Factor (RF_{Avg}) were calculated as follows:

$$RF_{Avg} = \frac{(\text{Conc. A} \div \text{Area A}) + (\text{Conc. B} \div \text{Area B}) + (\text{Conc. C} \div \text{Area C})}{3}$$

$\mu\text{g/L}$ found were calculated as follows:

$$\mu\text{g/L Found} = \frac{(\text{Peak Area}) \times (RF_{Avg}) \times (\text{Final Volume})}{(\text{Sample Volume})}$$

The percent recovery found was calculated as follows:

$$\% \text{ Recovery} = \frac{(\mu\text{g/L Found})}{(\text{Fortification level})} \times 100$$

6.4 Limit of quantification and limit of detection

The limit of quantification (LOQ) is defined as the lowest fortification level evaluated at which acceptable average recoveries (70-110%, RSD < 20%) were achieved. In this study the LOQ was 0.05 µg/L for all six analytes in all three types of water.

The limit of detection (LOD) is defined as the concentration of analyte determined by extrapolation of the peak height obtained for low fortification samples at three times the chromatographic base line noise observed in unfortified control samples. The least sensitive analyte that determined the overall LOD for the method was IN-MP819. The LOD for this analyte was 0.02 µg/L. For the other analytes LODs were lower (< 0.001 – 0.005 µg/L). No differences for the three types of waters were observed.

6.5 Critical aspects and remarks on the proposed method

In general the proposed method could successfully be reproduced for the analysis of all six analytes involved. There were, however, some observations that resulted in a few minor method adjustments that may further improve the robustness of the method:

- choice of APCI vs ESI: four analytes (DPX-MP062, IN-JT333, IN-JU873, IN-KG433) out of the set of six can be measured with both APCI and ESI. In practise it will depend on the instrument used and the conditions applied which interface will be preferable (if any). In this study, APCI was preferable over ESI for two of the four analytes (DPX-MP062, IN-JT333).
- the hexane rinse step in the original SPE method [1] was omitted here because in an earlier study [4] it was found that IN-JT333 was partially (ca. 10%) eluted during this step.
- there were indications for degradation or adsorption to glass of IN-KG433 in some of the standard solutions in acetonitrile. Use of freshly prepared solutions and addition of acetic acid to the acetonitrile solvent improves stability of analyte concentration.
- stability of LC-MS/MS response for IN-MS775 was poor in some of the sample sets. Addition of acetic acid to the acetonitrile component of the LC gradient (thereby keeping the concentration of acetic acid constant over the gradient), and injecting the calibration standards in between the sample extracts (i.e. calibration standards after every 2-3 sample extracts) improves accuracy of the measurements.
- With addition of acetic acid to the final extract, DPX-MP062, IN-JT333, IN-MP819, and IN-JU873 were demonstrated to be stable for at least 7 days.

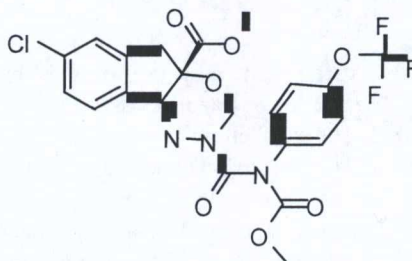
6.6 Communications with sponsor during study

During the study intermediate results were communicated with the sponsor. In case of unacceptable results, several suggestions were made by the sponsor with respect to minor method adjustments:

Structures and properties of test substances

Common Name Indoxacarb

Structure



DPX Number DPX-KN128

CAS Chemical Name (S)-methyl 7-chloro-2,5-dihydro-2-[[[(methoxycarbonyl)[4-(trifluoromethoxy)phenyl]amino]carbonyl]=indeno[1,2-e][1,3,4]oxadiazine-4a(3H)-carboxylate

CAS Number 173584-44-6

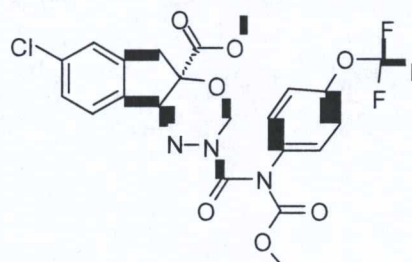
Formula $C_{22}H_{17}ClF_3N_3O_7$

Molecular Weight 527.8409

Monoisotopic Weight 527.0707

Common Name None

Structure



DPX Number DPX-KN127

CAS Chemical Name (R)-methyl 7-chloro-2,5-dihydro-2-[[[(methoxycarbonyl)[4-(trifluoromethoxy)phenyl]amino]carbonyl]=indeno[1,2-e][1,3,4]oxadiazine-4a(3H)-carboxylate

CAS Number 173584-44-6

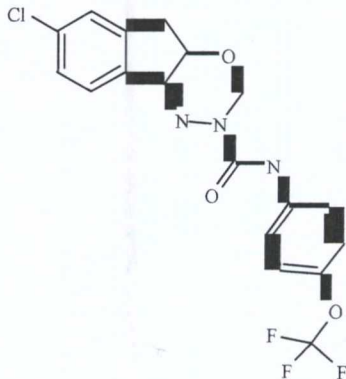
Formula $C_{22}H_{17}ClF_3N_3O_7$

Molecular Weight 527.8409

Monoisotopic Weight 527.0707

Common Name None

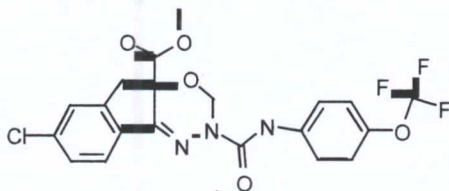
Structure



DPX Number IN-MS775
Formula C₁₈H₁₃ClF₃N₃O₃
Molecular Weight 411.7675
Monoisotopic Weight 411.0598

Common Name None

Structure

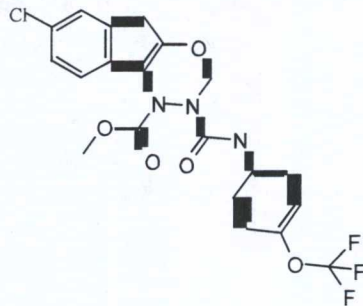


DPX Number IN-JT333
Formula C₂₀H₁₅ClF₃N₃O₅
Molecular Weight 469.8042
Monoisotopic Weight 469.0652

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Common Name None

Structure



DPX Number IN-MP819

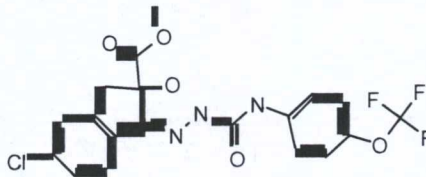
Formula $C_{20}H_{15}ClF_3N_3O_5$

Molecular Weight 469.8042

Monoisotopic Weight 469.0652

Common Name None

Structure



DPX Number IN-JU873

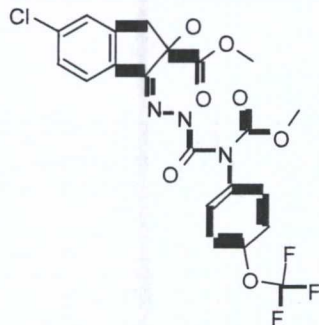
Formula $C_{19}H_{15}ClF_3N_3O_5$

Molecular Weight 457.7932

Monoisotopic Weight 457.0652

Common Name None

Structure



DPX Number IN-KG433
Formula $C_{21}H_{17}ClF_3N_3O_7$
Molecular Weight 515.8299
Monoisotopic Weight 515.0707
