The MAK Collection for Occupational Health and Safety

Neonicotinoids with a 6-chloropyridinyl group (e.g. imidacloprid, acetamiprid, thiacloprid, nitenpyram, boscalid) – Determination of 6-chloronicotinic acid in urine by GC-MS

Biomonitoring Method - Translation of the German version from 2018

W. Gries[1], G. Leng[2], H.-W. Hoppe[3], T. Göen[4,*], A. Hartwig[5,*], MAK Commission[6,*]

1 *Method development, Currenta GmbH & Co. OHG, CUR-SEL-ANT-UWA, R800, 12, 47829 Uerdingen, Germany*

2 *Method development, Currenta GmbH & Co. OHG, CUR-SEL-SER-GS-BLM, Institute for Biomonitoring, Building L9, 51368 Leverkusen, Germany*

3 *External verification, Medical Laboratory Bremen, Haferwende 12, 28357 Bremen, Germany*

4 *Head of the working group "Analyses in Biological Materials" of the Permanent Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area, Deutsche Forschungsgemeinschaft, Friedrich-Alexander-Universität Erlangen-Nürnberg, Institute and Outpatient Clinic of Occupational, Social, and Environmental Medicine, Henkestraße 9–11, 91054 Erlangen, Germany*

5 *Chair of the Permanent Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area, Deutsche Forschungsgemeinschaft, Institute of Applied Biosciences, Department of Food Chemistry and Toxicology, Karlsruhe Institute of Technology (KIT), Adenauerring 20a, Building 50.41, 76131 Karlsruhe, Germany*

6 *Permanent Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area, Deutsche Forschungsgemeinschaft, Kennedyallee 40, 53175 Bonn, Germany*

***** *email: T. Göen (thomas.goeen@fau.de), A. Hartwig (andrea.hartwig@kit.edu), MAK Commission (arbeitsstoffkommission@dfg.de)*

Keywords: neonicotinoids; imidacloprid; acetamiprid; thiacloprid; nitenpyram; boscalid; 6-chloronicotinic acid; biomonitoring; urine; GC-MS

Citation Note: Gries W, Leng G, Hoppe H-W, Göen T, Hartwig A, MAK Commission. Neonicotinoids with a 6-chloropyridinyl group (e.g. imidacloprid, acetamiprid, thiacloprid, nitenpyram, boscalid) – Determination of 6-chloronicotinic acid in urine by GC-MS. Biomonitoring Method – Translation of the German version from 2018. MAK Collect Occup Health Saf [Original edition. Weinheim: Wiley-VCH; 2018 Jul;3(3):1687-1704]. Corrected republication without content-related editing. Düsseldorf: German Medical Science; 2026. https://doi.org/10.34865/bi532623e2218_w

Republished (online): 06 Mar 2026

Originally published by Wiley-VCH Verlag GmbH & Co. KGaA; https://doi.org/10.1002/3527600418.bi532623e2218

Manuscript completed: 12 Oct 2012

Published (online): 26 Jul 2018

The commission established rules and measures to avoid conflicts of interest.

Neonicotinoids with a 6-chloropyridinyl group (e.g. imidacloprid, acetamiprid, thiacloprid, nitenpyram, boscalid) – Determination of 6-chloronicotinic acid in urine by GC-MS

Biomonitoring Methods

W. Gries[1], G. Leng[2], H.-W. Hoppe[3], T. Göen[4,*], A. Hartwig[5,*], MAK Commission[6,*]

DOI: 10.1002/3527600418.bi532623e2218

Abstract

The working group „Analyses in Biological Materials" of the Permanent Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area verified the presented biomonitoring method. Neonicotinoids with a 6-chloropyridinyl structure are metabolised in warm-blooded organisms to 6-chloronicotinic acid that is excreted in urine. This analytical method permits the specific quantification of 6-chloronicotinic acid in urine. For determination, 2 mL of a urine sample are hydrolysed using 500 µL concentrated hydrochloric acid to cleave the conjugates, which are subsequently extracted with methyl tert-butyl ether. The solvent is evaporated to dryness under a stream of nitrogen and the residue is dissolved in acetonitrile. Then 6-chloronicotinic acid is derivatised with hexafluoroisopropanol (HFIP) in the presence of diisopropylcarbodiimide. In a subsequent washing and extraction step, the formed HFIP ester is extracted using isooctane and an aliquot is injected in the GC-MS system for quantitative analysis. Calibration is performed using calibration standards that are prepared in pooled urine and processed in the same way as the samples to be analysed. The method was extensively validated and the reliability data were confirmed by an independent laboratory, which has established and cross-checked the whole procedure.

Keywords

Neonicotinoids; Imidacloprid; Acetamiprid; Thiacloprid; Nitenpyram; Boscalid; 6-Chloronicotinic acid; urine; biomonitoring; Analyses in Biological Materials; gas chromatography mass spectrometry; GC-MS

Author Information

[1] Method development, Currenta GmbH & Co. OHG, CUR-SEL-ANT-UWA, R800, 12, 47829 Uerdingen, Germany

[2] Method development, Currenta GmbH & Co. OHG, CUR-SEL-SER-GS-BLM-Institute for Biomonitoring, 51368 Leverkusen, Germany

[3] External verification, Medical Laboratory Bremen, Haferwende 12, 28357 Bremen, Germany

[4] Chair of the working group „Analyses in Biological Materials", Deutsche Forschungsgemeinschaft, Institute and Outpatient Clinic of Occupational, Social and Environmental Medicine, Friedrich-Alexander University Erlangen-Nürnberg (FAU), Henkestr. 9–11, 91054 Erlangen, Germany

[5] Chair of the Permanent Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area, Deutsche Forschungsgemeinschaft, Department of Food Chemistry and Toxicology, Institute of Applied Biosciences, Karlsruhe Institute of Technology (KIT), Adenauerring 20a, Building 50.41, 76131 Karlsruhe, Germany

[6] Permanent Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area, Deutsche Forschungsgemeinschaft, Kennedyallee 40, 53175 Bonn, Germany

[*] Email: T. Göen (thomas.goeen@fau.de), A. Hartwig (andrea.hartwig@kit.edu), MAK Commission (arbeitsstoffkommission@dfg.de)

Neonicotinoids with a 6-chloropyridinyl group (e.g. imidacloprid, acetamiprid, thiacloprid, nitenpyram, boscalid) – Determination of 6-chloronicotinic acid in urine by GC-MS

Matrix: Urine

Hazardous substances: Imidacloprid, acetamiprid, thiacloprid, nitenpyram, boscalid and other neonicotinoids with a 6-chloropyridinyl group

Analytical principle: Capillary gas chromatography with mass spectrometric detection (GC-MS)

Completed in: October 2012

Overview of the parameters that can be determined with this method and the corresponding hazardous substances:

Hazardous substance	CAS	Parameter	CAS
Imidacloprid	105827-78-9 138261-41-3	6-Chloronicotinic acid	5326-23-8
Acetamiprid	135410-20-7 160430-64-8		
Thiacloprid	111988-49-9		
Nitenpyram	150824-47-8		
Boscalid	188425-85-6		

Summary

Neonicotinoids with a 6-chloropyridinyl structure are metabolised in warm-blooded organisms to 6-chloronicotinic acid that is excreted in urine. This analytical method permits the specific quantification of 6-chloronicotinic acid in urine. For determination, 2 mL of a urine sample are hydrolysed using 500 µL concentrated hydrochloric acid to cleave the conjugates, which are subsequently extracted with methyl *tert*-butyl ether. The solvent is evaporated to dryness under a stream of nitrogen and the residue is dissolved in acetonitrile. Then 6-chloronicotinic acid is derivatised with hexafluoroisopropanol (HFIP) in the presence of diisopropylcarbodiimide. In a subsequent washing and extraction step, the formed HFIP ester is

extracted using isooctane and an aliquot is injected in the GC-MS system for quantitative analysis. Calibration is performed using calibration standards that are prepared in pooled urine and processed in the same way as the samples to be analysed.

Reliability data of the method

6-Chloronicotinic acid

Within-day precision:	Standard deviation (rel.)	s_w = 7.4% or 9.1%
	Prognostic range	u = 16.7% or 20.6%
	at a spiked concentration of 1 µg or 10 µg 6-chloronicotinic acid per litre urine and where n = 10 determinations	
Day-to-day precision:	Standard deviation (rel.)	s_w = 7.6% or 8.4%
	Prognostic range	u = 19.5% or 21.6%
	at a spiked concentration of 1 µg or 10 µg 6-chloronicotinic acid per litre urine and where n = 6 determinations	
Accuracy:	Recovery rate (rel.)	r = 112% or 102%
	at a spiked concentration of 1 µg or 10 µg 6-chloronicotinic acid per litre urine and where n = 10 determinations	
Detection limit:	0.2 µg 6-chloronicotinic acid per litre urine	
Quantitation limit:	0.5 µg 6-chloronicotinic acid per litre urine	

Neonicotinoids. Neonicotinoids are a class of highly effective insecticides. These are synthetic chemicals that bind to the neuronal nicotinic acetylcholine receptor and thus very selectively interfere with the transmission of impulses in the nervous system of insects [Tomizawa and Casida 2005]. Numerous neonicotinoids are approved for use as pesticides in the EU. Both industrial users and consumers are exposed to such pesticides. The latter exposure is due to the presence of pesticide residues in food. Therefore, dietary intake is considered to be the main route of exposure for consumers. The major routes of user exposure are especially dermal absorption and, to a lesser extent, inhalation. As there are various significant exposure pathways, an accurate assessment of the exposure level for all persons exposed to neonicotinoids is best enabled using biomonitoring [Kasiotis and Machera 2015; Göen 2016].

Animal experiments showed that neonicotinoids containing a 6-chloropyridinyl moiety are primarily metabolised to 6-chloronicotinic acid. Apart from other metabolites, urinary excretion of the unchanged insecticide is also of relevance [Wang et al. 2015; Göen 2016; Kasiotis and Machera 2015]. Figure 1 shows an example of a metabolism scheme of the neonicotinoid imidacloprid.

Due to their high selectivity for the insect nicotinic acetylcholine receptor, neonicotinoids generally exhibit a low acute toxicity potential to warm-blooded animals. Depending on the type of the specific neonicotinoid, the potential chronic toxicity of

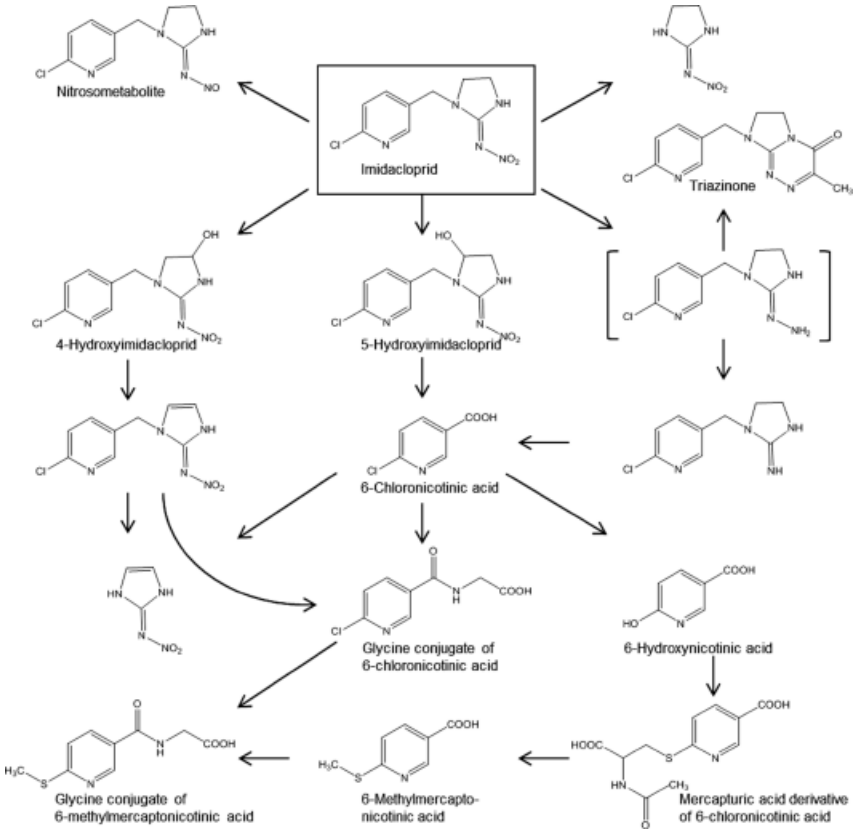

Figure 1 Postulated metabolic pathway of imidacloprid in rats according to Kavvalakis et al. [2013] and Klein [1987].

these insecticides varies considerably [Tomizawa and Casida 2005]. A toxicological evaluation of the individual neonicotinoids by the commission is not yet available.

So far, no studies concerning the specific assessment of occupational exposure to neonicotinoids using human biomonitoring and 6-chloronicotinic acid as parameter are available. However, a Chinese study did investigate 6-chloronicotinic acid levels in rural families that were partially exposed to neonicotinoids [Wang et al. 2015]. Here, 6-chloronicotinic acid was detected in many urine samples, but only in levels below 1 µg/L. In another study, 6-chloronicotinic acid was analysed in urine samples of European volunteers who changed their diet from conventional food to organic products. In that study 6-chloronicotinic acid was detectable in levels up to 7 µg/L. Thus, through biomonitoring it was possible to show the effect of the dietary change [Göen et al. 2017].

Contents

1 General principles

Neonicotinoids with a 6-chloropyridinyl structure are metabolised in warm-blooded organisms to 6-chloronicotinic acid that is excreted in urine. This analytical method permits the specific quantification of 6-chloronicotinic acid in urine. For determination, 2 mL of a urine sample are hydrolysed using 500 µL concentrated hydrochloric acid to cleave the conjugates, which are subsequently extracted with methyl *tert*-butyl ether. The solvent is evaporated to dryness under a stream of nitrogen and the residue is dissolved in acetonitrile. Then, 6-chloronicotinic acid is derivatised with hexafluoroisopropanol (HFIP) in the presence of diisopropylcarbodiimide. In a subsequent washing and extraction step, the formed HFIP ester is extracted using isooctane and an aliquot is injected in the GC-MS system for quantitative analysis. Calibration is performed using calibration standards that are prepared in pooled urine and processed in the same way as the samples to be analysed.

2 Equipment, chemicals and solutions

2.1 Equipment

GC-HRMS:

- Gas chromatograph with a split/splitless injector, high resolution sector field mass spectrometer, autosampler and data processing system (e.g. GC Agilent HP 5890 II+ with Waters MS AutoSpec)
- Capillary gas chromatographic column: stationary phase: 65%-phenyl-methyl-polysiloxane, length: 30 m; inner diameter: 0.25 mm; film thickness: 0.25 µm (e.g. Rtx 65, Restek No. 17023)

Alternatively: GC-Quadrupole-MS:

- Gas chromatograph with a split/splitless injector, quadrupole mass spectrometer, autosampler and data processing system (e.g. Agilent 7000 GC-MS/MS)
- Capillary gas chromatographic column: stationary phase: 5%-phenyl-methyl-polysiloxane, length: 30 m; inner diameter: 0.25 mm; film thickness: 0.25 µm (e.g. Rtx 5, Restek No. 102223)

- Pasteur pipettes (e.g. Sarstedt)
- Analytical balance (e.g. Sartorius)
- Laboratory centrifuge (e.g. Heraeus)
- Dry block heater (e.g. Diaglobal)
- Various volumetric flasks (e.g. VWR)
- 10-mL vials with screw caps and Teflon-coated septa (e.g. VWR)
- 10-µL and 100-µL transfer pipettes (e.g. Brand)
- 200-µL vials (e.g. Macherey-Nagel)
- Crimp caps with Teflon septa (e.g. CS Chromatographie-Service)
- Laboratory shaker (e.g. IKA Vibrax VXR)
- Nitrogen evaporator (e.g. Zymark)
- Urine containers (e.g. Sarstedt)

2.2 Chemicals

Unless otherwise specified, all chemicals must be at least p.a. grade.

- Isooctane (e.g. Merck, No. 115440)
- Methyl *tert*-butyl ether (e.g. Sigma-Aldrich, No. 306975)
- Acetonitrile (e.g. Merck, No. 100017)
- Hydrochloric acid, 37% (e.g. Merck, No. 100317)
- Sodium hydrogen carbonate (e.g. Merck, No. 106329)
- Purified water (e.g. Milli-Q water)
- 1,1,1,3,3,3-Hexafluoroisopropanol (e.g. Sigma-Aldrich, No. 52517)
- N,N′-Diisopropylcarbodiimide (e.g. Sigma-Aldrich, No. 38370)
- 6-Chloronicotinic acid (e.g. Alfa Aesar, No. A15620)
- 2-Phenoxybenzoic acid (e.g. Merck, No. 841344)
- Urine from persons with no known exposure to neonicotinoids

2.3 Solutions

- 1 M sodium hydrogen carbonate solution
  42 g sodium hydrogen carbonate are weighed into a 500-mL volumetric flask and are dissolved in purified water. The flask is filled to the mark with purified water.

The solution is stable for a maximum of 6 months when stored in the refrigerator at +4 °C.

2.4 Internal standard

- IS stock solution (1 g/L)
  10 mg of 2-phenoxybenzoic acid (2-PBA) are weighed into a 10-mL volumetric flask and dissolved in acetonitrile. The flask is filled to the mark with acetonitrile.
- IS spiking solution (1 mg/L)
  0.1 mL of the IS starting solution are pipetted into a 100-mL volumetric flask. The flask is filled to the mark with acetonitrile.

The solutions of the internal standard are stable for at least 6 months when stored in the refrigerator at +4 °C.

2.5 Calibration standards

- Stock solution (1 g/L)
  10 mg 6-chloronicotinic acid are weighed into a 10-mL volumetric flask and dissolved in acetonitrile. The flask is filled to the mark with acetonitrile.
- Spiking solution I (10 mg/L)
  0.1 mL of the starting solution are pipetted into a 10-mL volumetric flask. The flask is filled to the mark with acetonitrile.
- Spiking solution II (1 mg/L)
  1 mL of spiking solution I is pipetted into a 10-mL volumetric flask. The flask is filled to the mark with acetonitrile.
- Spiking solution III (0.1 mg/L)
  0.1 mL of spiking solution I are pipetted into a 10-mL volumetric flask. The flask is filled to the mark with acetonitrile.
- Spiking solution IV (0.01 mg/L)
  0.1 mL of spiking solution II are pipetted into a 10-mL volumetric flask. The flask is filled to the mark with acetonitrile.

The solutions used to prepare the calibration standards are stable for at least 4 weeks when stored in the refrigerator at +4 °C.

Table 1 Pipetting scheme for the preparation of calibration standards to determine 6-chloronicotinic acid in urine.

Calibration standard	Spiking solution	Volume of the spiking solution [µL]	Concentration of 6-chloronicotinic acid [µg/L]
0	–	–	–
1	IV	20	0.1
2	IV	40	0.2
3	IV	100	0.5
4	III	20	1.0
5	III	40	2.0
6	III	100	5.0
7	II	20	10
8	II	40	20
9	II	100	50
10	I	20	100

Calibration standards are prepared by pipetting 2 mL of each pooled urine sample into 10-mL screw cap vials. The urine samples are then spiked with the standard solutions according to the pipetting scheme given in Table 1. The calibration standards are processed in the same way as the samples to be analysed (see Section 3.2).

3 Specimen collection and sample preparation

3.1 Specimen collection

The urine is collected in pre-cleaned plastic containers (rinsed off with methanol) and should be immediately analysed. If necessary, the urine samples can be stored at < 10 °C. Under these conditions, the analyte in the urine matrix is stable for at least 6 months. The effects of longer-term storage on the analyte levels were not investigated.

3.2 Sample preparation

For sample preparation, 2 mL of urine are pipetted into a 10-mL screw cap vial. Then 500 µL of concentrated hydrochloric acid and 20 µL of the internal standard spiking solution are added. The screw cap vial is closed, the sample is thoroughly mixed and then incubated for hydrolysis at 100 °C for 2 h in a dry block heater or drying oven. After the sample has cooled down to room temperature, 4 mL of methyl *tert*-butyl ether are added. After the screw cap vial has been closed again, the sample is mixed vigorously on a laboratory shaker for 10 min. Afterwards the sample is centrifuged

at 2200 × *g* for 5 min. The organic phase is transferred as completely as possible into a new 10-mL screw cap vial using a Pasteur pipette, while the aqueous phase is discarded. After the organic phase has been evaporated to dryness under a stream of nitrogen, the residue is dissolved in 250 µL of acetonitrile. Then 30 µL of HFIP and 15 µL of N,N′-diisopropylcarbodiimide are added for derivatisation. After the sample has been agitated for 10 min on the laboratory shaker, 1 mL of the sodium hydrogen carbonate solution is added and the sample is mixed thoroughly. Afterwards 250 µL of isooctane are added using a pipette and the sample is again shaken vigorously on the laboratory shaker for 10 min and centrifuged at 2200 × *g* for 5 min. Finally, the isooctane phase is transferred into a 200-µL microvial. A 1-µL aliquot of this phase is injected into the GC-MS system for analysis.

The processed samples are stable for at least 14 days when stored at temperatures below +4 °C. The effects of longer-term storage were not investigated.

4 Operational parameters

GC-HRMS analysis was performed on a system consisting of GC HP 5890 II+ (Agilent), Autosampler CTC A200S (CTC Analytics) and AutoSpec Ultima sector field mass spectrometer (Waters). GC-Quadrupole-MS analysis was performed on an Agilent 7000 GC-MS/MS.

4.1 Gas chromatography

4.1.1 GC-HRMS

Capillary column:	Stationary phase:	Rtx 65 (65% phenyl-methylpolysiloxane)
	Length:	30 m
	Inner diameter:	0.25 mm
	Film thickness:	0.25 µm
	Detector:	MSD
Temperatures:	Column:	Initial temperature 60 °C, 1 min hold time, increase at a rate of 8 °C/min to 120 °C, then increase at a rate of 30 °C/min to 230 °C, then increase at a rate of 50 °C/min to 300 °C, then 5 min at the final temperature
	Injector:	280 °C
	Transfer line:	280 °C
Carrier gas:	Helium 5.0	
	Flow rate:	1.0 mL/min
Injection volume:	1 µL (splitless)	

4.1.2 GC-Quadrupole-MS

Capillary column:	Stationary phase:	Rtx 5 (5% phenyl-methylpolysiloxane)
	Length:	30 m
	Inner diameter:	0.25 mm
	Film thickness:	0.25 µm
	Detector:	MSD
Temperatures:	Column:	Initial temperature 80 °C, 1 min hold time, increase at a rate of 3 °C/min to 100 °C, then increase at a rate of 40 °C/min to 280 °C, then 3 min at the final temperature
	Injector:	280 °C
	Transfer line:	280 °C
Carrier gas:	Helium 5.0	
	Flow rate:	1.0 mL/min
Injection volume:	1 µL (splitless)	

4.2 Mass spectrometry

4.2.1 High resolution sector field mass spectrometer

Ionisation mode:	Electron impact ionisation (EI)
Ionisation energy:	70 eV
Source temperature:	230 °C
Interface temperature:	250 °C
Calibration gas:	Perfluorokerosene
Filament:	0.3 mA
Resolution:	5000
Detection mode:	Single Ion Resolution (SIR)

4.2.2 Quadrupole mass spectrometer

Ionisation mode:	Negative chemical ionisation (NCI)
Source temperature:	150 °C
Quadrupole temperature:	150 °C
Ionisation gas:	Methane
Collision gas:	Argon
Detection mode:	Single Ion Mode (SIM) Multi Reaction Mode (MRM)

All parameters serve as rough guidelines only and may have to be optimized in accordance with the manufacturer's specifications.

5 Analytical determination

For analytical determination of the urine samples prepared as described in Section 3, 1 µL of each sample solution is injected into the GC-MS system. The temporal profiles of the ion traces shown in Table 2 (GC-HRMS) and Table 3 (GC-Quadrupole-MS) are recorded.

The retention times given in Table 2 and Table 3 are intended to be a rough guide only. Users of the method must ensure proper separation performance of the column used influencing the resulting retention behaviour of the analytes. Figure 2 and 3 (in the Appendix) show GC-MS chromatograms of a spiked and processed urine sample for both detection techniques.

6 Calibration

The calibration standards (see Section 2.5) are processed in the same way as the urine samples and are then analysed as described in Sections 4 and 5. Calibration curves are obtained by plotting the peak area ratios of 6-chloronicotinic acid and of the internal standard against the spiked concentrations. The linearity of the analytical method was tested and validated over the concentration range from 0.1 µg/L to 100 µg/L. The calibration curve was proven to be linear in the range between 0.1 µg/L and 20 µg/L. Any blank values have to be subtracted from the analytical results. Principially, a set of processed calibration standards can be re-used for calibration (see Section 9.4). It should, however, be noted that the calibration stan-

Table 2 Retention times and detected fragment ions analysed by high resolution sector field mass spectrometry (GC-HRMS).

Analyte	Retention time [min]	Ion trace [*m/z*]
6-Chloronicotinic acid	6.36	139.9903
2-Phenoxybenzoic acid (IS)	12.56	364.0534

Table 3 Retention times and detected fragment ions analysed by quadrupole mass spectrometry.

Analyte	Retention time [min]	Ion trace [*m/z*][a]	
		SIM	MRM
6-Chloronicotinic acid	6.9	**156**	**156 → 112**
		158	156 → 35
		307	307 → 156
2-Phenoxybenzoic acid (IS)	10.9	**364**	**364 → 213**
		213	

[a] Quantifier ion traces are printed in bold

dards have to be analysed for each analytical series. Figure 4 (in the Appendix) shows an example of a calibration curve.

7 Calculation of the analytical results

The analyte concentration in the urine samples is calculated using the calibration function of Section 6. The peak area of the analyte 6-chloronicotinic acid is divided by the peak area of the internal standard. The quotient thus obtained is inserted into the calibration curve according to Section 6 in order to obtain the analyte concentration in µg/L. Any reagent blank values have to be subtracted. If the analytical result exceeds the calibration range, the respective sample is diluted appropriately and reanalysed.

8 Standardisation and quality control

Quality control of the analytical results is carried out as stipulated in the guidelines of the Bundesärztekammer (German Medical Association) and in a general chapter of the MAK-Collection for Occupational Health and Safety Part IV: Biomonitoring Methods [Bundesärztekammer 2014; Bader et al. 2010]. To check precision, at least two quality control samples with a known and constant analyte concentration are analysed in each analytical series. As material for quality control is not commercially available, it must be self-prepared in the laboratory. To this end, pooled urine is spiked with a standard solution. The analyte concentration of this control material should lie within the relevant concentration range (Q_{low}: 1 µg/L, Q_{high}: 10 µg/L). After thorough mixing, the quality control material thus obtained is aliquoted into vials (2 mL each) and stored at –18 °C. The nominal value and the tolerance ranges of the quality control material are determined in a pre-analytical period [Bader et al. 2010]. The concentration level of the quality control samples should also lie within the tolerance ranges obtained.

9 Evaluation of the method

The reliability of the method was proven by comprehensive validation and by implementation and validation of the procedure in a second independent laboratory.

9.1 Precision

Within-day precision was determined using urine samples that were spiked with 6-chloronicotinic acid at two different concentration levels. The urine samples were then processed and analysed repeatedly. The obtained within-day precision data are presented in Table 4.

Day-to-day precision was determined by processing and analysing the spiked urine samples on six consecutive days. The obtained day-to-day precision data are presented in Table 5.

9.2 Accuracy

Recovery tests were performed to determine the accuracy of the method. To this end, the spiked urine samples were analysed and the recovery rates were calculated. The results are presented in Table 6.

Table 4 Within-day precision data for the determination of 6-chloronicotinic acid in urine using GC-Quadrupole-MS (GC-MS) or high-resolution GC-MS (GC-HRMS).

Analytical method	Spiked concentration [µg/L]	Number of determinations	Rel. standard deviation s_w [%]	Prognostic range u [%]
GC-MS	1	10	7.4	16.7
	10	10	9.1	20.6
GC-HRMS	1	6	5.7	14.7
	10	6	11.8	30.3

Table 5 Day-to-day precision data for the determination of 6-chloronicotinic acid in urine using high-resolution GC-MS (GC-HRMS).

Spiked concentration [µg/L]	Number of determinations	Rel. standard deviation s_w [%]	Prognostic range u [%]
1	6	7.6	19.5
10	6	8.4	21.6

Table 6 Relative recovery rates for the determination of 6-chloronicotinic acid in urine using GC-Quadrupole-MS (GC-MS) or high-resolution GC-MS (GC-HRMS).

Analytical method	Spiked concentration [µg/L]	Number of determinations	Mean rel. recovery rate [%]	Range [%]
GC-MS	1	10	112	102–125
	10	10	102	90–115
GC-HRMS	1	6	101	92–106
	10	6	95	81–105

9.3 Limit of detection and limit of quantitation

The limit of detection and the limit of quantitation were determined for both procedures using the calibration curve method according to ISO 84661-1. The results are presented in Table 7.

9.4 Sources of error

During the method development, interferences for the most intense fragment ion *m/z* 307 were observed in both method variants. For this reason, less intense fragment ions were selected for quantification. In unspiked pooled urine, the interference at *m/z* 307 was approx. 0.5 µg 6-chloronicotinic acid per litre.

Derivatisation reactions with HFIP are taking place only under anhydrous conditions. It is therefore of particular importance that the separation of the methyl *tert*-butyl ether phase after extraction is performed as complete as possible but preferably without traces of water. In that case an optional further drying of the organic phase (e.g. using sodium sulphate) is not required. The external method validation showed a decrease in peak intensity of 6-chloronicotinic acid in the case of multiple injections from a vial that was sealed with Teflon-coated butyl rubber septa. In all probability, this decrease in intensity may be attributed to entrained septum particles and cannot be compensated for by the use of the internal standard 2-phenoxybenzoic acid. Therefore, rubber septa allow single injections only. This phenomenon was not observed when using Teflon septa. In vials with intact septa, however, the solutions are stable for at least two weeks.

10 Discussion of the method

This method allows the determination of 6-chloronictotinic acid as a metabolite of various neonicotinoids. In addition, the method can easily be extended to include the determination of the metabolites of natural pyrethrum and synthetic pyrethroids [Leng et al. 2006; Leng et al. 1997 a ; Leng et al. 1997 b]. However, as the two substance groups usually do not have to be determined at the same time, the 6-chloronicotinic acid method was developed as an independent method. 6-Chloronicotinic acid can be determined using either high resolution sector field mass spectrometry (GC-HRMS) in EI mode or by quadrupole MS in NCI mode. Sample preparation is identical for both procedures. A limit of detection of 0.2 µg/L

Table 7 Limits of detection and quantitation for the determination of 6-chloronicotinic acid in urine using GC-Quadrupole-MS (GC-MS) or high-resolution GC-MS (GC-HRMS).

Analytical method	Limit of detection [µg/L]	Limit of quantitation [µg/L]
GC-MS	0.2	0.5
GC-HRMS	0.2	0.6

can easily be achieved with both analytical methods (GC-MS and GC-HRMS). 2-Phenoxybenzoic acid was used as the internal standard, which compensates sufficiently well for the analysis-related analyte losses. This is confirmed by the validation data obtained. However, meanwhile, an isotope labelled internal standard is commercially available ($^{13}C_6$-6-chloronicotinic acid, e.g. Cambridge Isotope Laboratories, No. CLM-9598) that may even be more suitable to enable a valid determination of 6-chloronicotinic acid in urine.

Instruments used:

- GC-Quadrupole-MS: Gas chromatograph Agilent 7000 GC-MS/MS (Agilent, USA)
- GC with high resolution sector field MS: GC 5890 II+ (Agilent, USA)
- Autosampler CTC A 200 S (CTC Analytics, USA)
- AutoSpec Sector Field MS (Waters, USA)

11 References

Bader M, Barr D, Göen Th, Schaller KH, Scherer G, Angerer J, Working group Analyses in Biological Materials (2010) Reliability criteria for analytical methods. In: Angerer J and Hartwig A (eds): The MAK-Collection for Occupational Health and Safety Part IV: Biomonitoring Methods, Vol. 12, Wiley-VCH, Weinheim
https://onlinelibrary.wiley.com/doi/abs/10.1002/3527600418.bireliabe0012

Bundesärztekammer (2014) Richtlinie der Bundesärztekammer zur Qualitätssicherung laboratoriumsmedizinischer Untersuchungen. Dt Ärztebl 111: A1583-1618

Göen Th (2016) Biomonitoring von beruflichen und außerberuflichen Pestizidbelastungen. Zbl Arbeitsmed 66: 276-285

Göen Th, Schmidt L, Lichtensteiger W, Schlumpf M (2017) Efficiency control of dietary pesticide intake reduction by human biomonitoring. Int J Hyg Environ Health 220: 254-260

Kasiotis KM, Machera K (2015) Neonicotinoids and their metabolites in human biomonitoring: a review. Hellenic Plant Protection Journal 8: 33-45

Kavvalakis MP, Tzatzarakis MN, Theodoropoulou EP, Barbounis EG, Tsakalof AK, Tsatsakis AM (2013) Development and application of LC-APCI-MS method for biomonitoring of animal and human exposure to imidacloprid. Chemosphere 93: 2612-2620

Klein O (1987) (14C)-NTN 33893: Biokinetic part of the 'General metabolism study' in the rat. Unpublished report from Bayer AG, report No. PF2889, dated 9 November 1987, GLP. Submitted to WHO by Bayer AG, Monheim, Germany

Leng G, Gries W, Selim S (2006) Biomarker of pyrethrum exposure. Toxicol Lett 162: 195-201

Leng G, Kühn KH, Idel H (1997a) Biological monitoring of pyrethroids in blood and pyrethroid metabolites in urine: applications and limitations. Sci Total Environ 199: 173-181

Leng G, Kühn KH, Leng A, Gries W, Lewalter J, Idel H (1997b) Determination of trace levels of pyrethroid metabolites in human urine by capillary gas chromatography-high resolution mass spectrometry with negative chemical ionization. Chromatographia 46: 265-274

Tomizawa M and Casida JE (2005) Neonicotinoid insecticide toxicology: mechanism of selective action. Annu Rev Pharmacol Toxicol 45: 247-268

Wang L, Liu T, Liu F, Zhang J, Wu Y, Sun H (2015) Occurence and profile characteristics of the pesticide imidacloprid, preservative parabens, and their metabolites in human urine from rural and urban china. Environ Sci Technol 49: 14633-14640

Method development: W. Gries, G. Leng
External verification: H.-W. Hoppe
Chair of the working group "Analyses in Biological Materials", Deutsche Forschungsgemeinschaft: Th. Göen
Chair of the Permanent Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area, Deutsche Forschungsgemeinschaft: A. Hartwig
Permanent Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area of the Deutsche Forschungsgemeinschaft: MAK Commission

12 Appendix

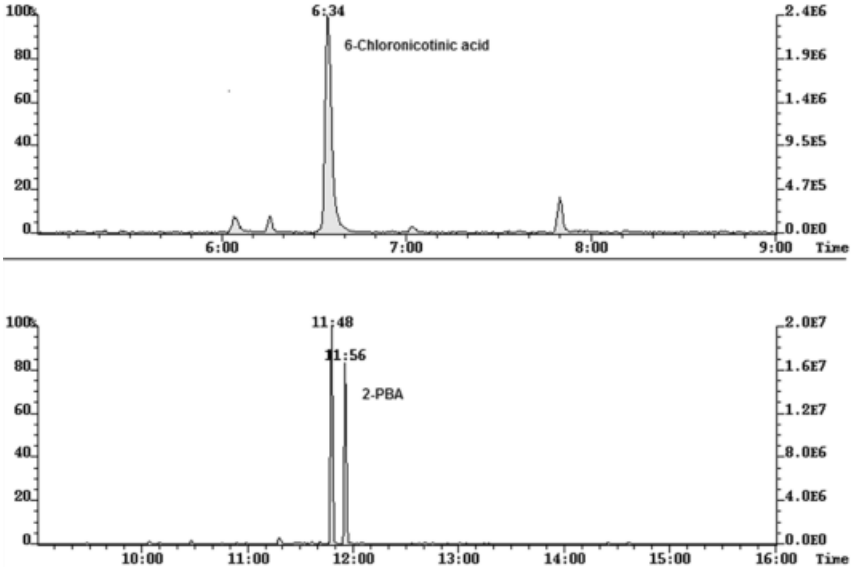

Figure 2 GC-HRMS chromatogram of a urine sample spiked with 10 µg 6-chloronicotinic acid/L.

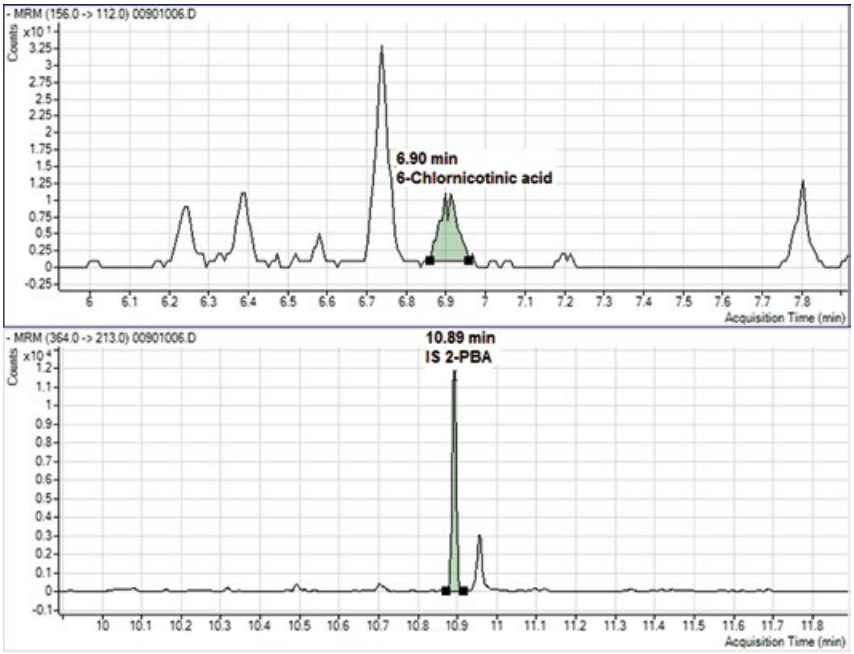

Figure 3 GC-Quadrupole-NCI-MS chromatogram of a processed native urine sample. Determined concentration of 6-chloronicotinic acid: 0.06 µg/L.

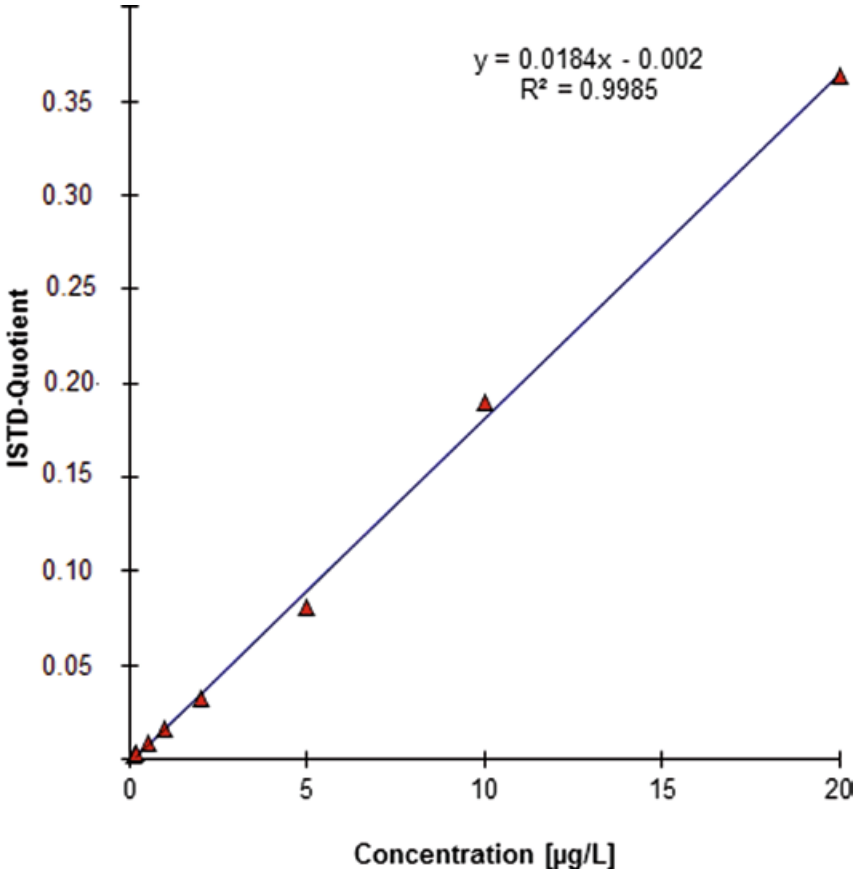

Figure 4 Calibration curve established in pooled urine for the determination of 6-chloronicotinic acid in urine.