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RESEARCH ARTICLE

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Analysis of tetramisole metabolites – Is "Aminorex" found in forensic samples of cocaine users actually 4-phenyl-2-imidazolidinone?

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Abstract

Phenyltetrahydroimidazothiazole (PTHIT, tetramisole) is a common adulterant in cocaine samples. Little is known about its human metabolism. *p*-hydroxy-PTHIT has long been the only proven phase-I-metabolite. Another putative metabolite is the stimulant aminorex. However, data on its analytical proof is rare and contradictory. Even less known is its constitutional isomer 4-phenyl-2-imidazolidinone which has only been proven in animal samples so far. The aim of the study was to get insight into the metabolism of PTHIT after controlled nasal uptake of PTHIT and in real forensic cocaine/benzoylecgonine-positive samples.

A liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was validated for quantification of 4-phenyl-2-imidazolidinone and *p*-hydroxy-PTHIT (LOQ 0.05 ng/ml each). Selectivity was ensured for 4-phenyl-2-imidazolidinone and aminorex (LOD 0.05 ng/ml). After controlled nasal uptake of tetramisole (10 mg, n = 3) a shorter half-life for *p*-hydroxy-PTHIT (3.4–5.8 h) was determined than for 4-phenyl-2-imidazolidinone (14.0–15.9 h). *p*-hydroxy-PTHIT (33%) and 4-phenyl-2-imidazolidinone (51%) were also detected in serum samples from cocaine users tested previously positive for PTHIT (n = 73). Aminorex was never detected.

The potential of misinterpreting 4-phenyl-2-imidazolidinone as aminorex was tested using a gas chromatography–mass spectrometry (GC–MS) method used in the literature and an in-house liquid chromatography-time-of-flight mass spectrometry (LC-QTOF) screening-method. Using GC–MS the analysed bis-trimethylsilyl-derivatives cannot be differentiated due to co-elution. Both substances were chromatographically separated using the LC-QTOF method, but library comparison workflows misinterpreted 4-phenyl-2-imidazolidinone as aminorex.

It seems likely that aminorex, which was allegedly identified as a metabolite of PTHIT in samples of cocaine users in previous studies, is in fact 4-phenyl-2-imidazolidinone.

KEYWORDS
aminorex, cocaine, isomers, metabolite, tetramisole

1 | INTRODUCTION

Phenyltetrahydroimidazothiazole (PTHIT, tetramisole) has recently gained attention as one of the most frequently used adulterants in cocaine.[1,2] Whereas PTHIT has been primarily known as a deworming (anthelmintic) agent in veterinary medicine, it has also been used as an immune modulator in human medicine. PTHIT is a chiral compound and exists in the two enantiomeric forms dexamisole (R) and levamisole (S) (Figure 1). Since levamisole had been identified as the pharmacologically active enantiomer (eutomer) soon after discovery, PTHIT was mainly used in formulations as a single enantiomer.[3] However, the racemate tetramisole is still commercially available and used for veterinary purposes.[4] Regarding the enantiomeric composition of PTHIT-tainted cocaine, PTHIT is currently predominantly added in its racemic form as tetramisole, while levamisole or non-racemic mixtures with an excess of levamisole are less frequently found.[5,6]

In general, little is known about the metabolism of PTHIT in humans. Although there is evidence suggesting an excessive hepatic metabolism, it has been studied only rudimentarily using levamisole.[7] In many cases, structural formulas are only suggested based on MS-data and it remains unclear to what extent these putative metabolites are formed.[8–10] During the pharmaceutical use of levamisole, *p*-hydroxy-levamisole (Figure 1) and its glucuronide were the only proven human metabolites.[8,11,12] Recently, this hydroxylated metabolite has been qualitatively detected in human urine of cocaine users.[10] In addition to such an aromatic hydroxylation, the thiazolidine ring (scission and aliphatic oxidation, hydrolysis and dehydration) is a further metabolic target.[8,13] A putative metabolite of PTHIT of forensic interest is the stimulant aminorex (Figure 1). Aminorex (2-amino-4,-5-dihydro-5-phenyl-2-oxazol) exerts amphetamine-like effects[14] and is an interesting substance itself with an unusual history: Being initially sold as an over-the-counter anorectic in Western Germany, Switzerland and Austria between 1965 and 1968 as Apiquel® and Menocil®, it has soon been withdrawn from the market after having caused a series of fatal pulmonary hypertension.[15,16] Its short availability was restricted to only a few European countries. Aminorex was found again in horse racing from 2004 on when it caused positive doping tests. In early 2007, a connection between a deworming treatment with levamisole and the metabolic formation of aminorex was concluded for the horse.[15] Soon afterwards, aminorex was found in human urine after controlled oral intake of levamisole.[17] From then on, it is often cited as a human metabolite of PTHIT[18–20] even though data relating to the analytical proof of aminorex in forensic samples is still quite rare and contradictory in the literature.[10,21] Moreover, there are further less known putative metabolites of PTHIT sharing the same sum formula ($C_9H_{10}N_2O$). Among these constitutional isomers are rexamino (4-Phenyl-4,5-dihydro-1,3-oxazol-2-amine) and 4-phenyl-2-imidazolidinone. Above all, 4-phenyl-2-imidazolidinone (Figure 1) has been found in higher concentrations in horse urine and plasma as aminorex after oral and subcutaneous administration of levamisole.[15] While 4-phenyl-2-imidazolidinone was included early in the analysis of doping-positive horse samples to ensure the finding of aminorex after levamisole administration,[15] this analyte has not been considered in studies examining forensic specimens. This entails the potential of mass-spectrometric misinterpretation and may be a reason for the contradictory findings in the detection of aminorex in forensic sample material.[10]

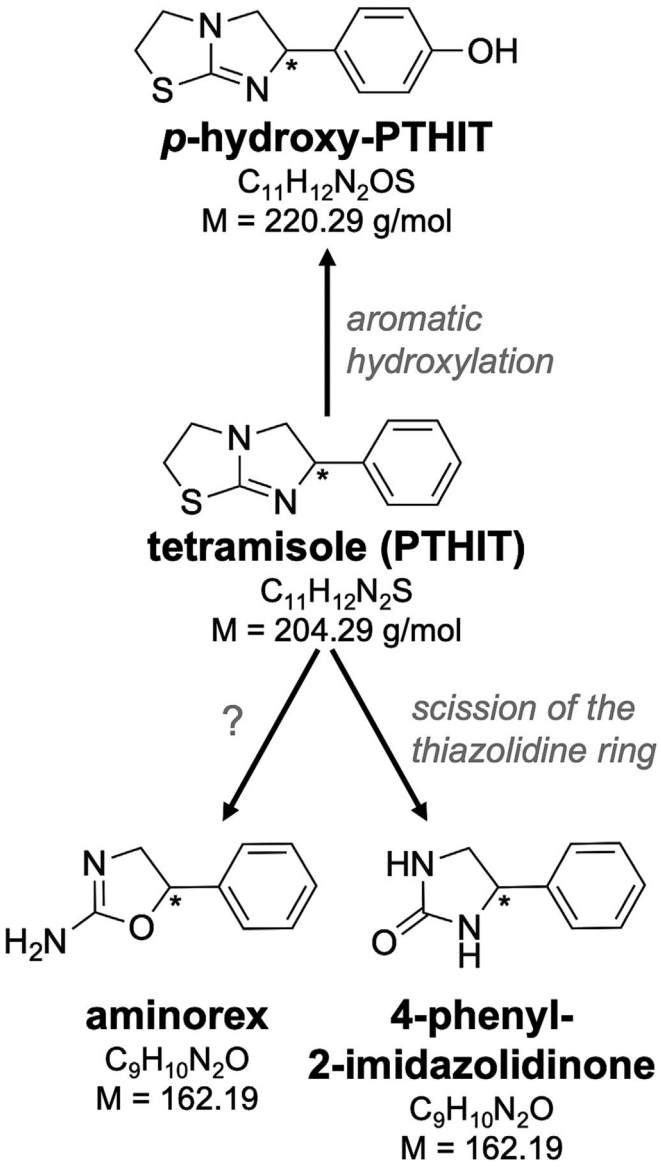


FIGURE 1 Structural formulas of putative tetramisole metabolites mentioned

After having quantified dexamisole and levamisole in serum samples of cocaine users and having elucidated the chiral pharmacokinetics of tetramisole enantiomers after a controlled nasal application,[6] the aim of this study was to detect PTHIT metabolites in human blood samples. An achiral LC–MS/MS method was developed and validated to quantify *p*-hydroxy-PTHIT and 4-phenyl-2-imidazolidinone as well as to detect aminorex. The previously examined sample collective, consisting of the serum samples after controlled nasal tetramisole administration and 74 blood samples that were tested positive for cocaine (> 1 ng/ml) and/or benzoylecgonine (> 25 ng/ml),[6] was therefore re-analysed for the presence of tetramisole metabolites.

2 | MATERIAL AND METHODS

2.1 | Material

Aminorex was purchased from Cerilliant (Round Rock, USA). 4-phenyl-2-imidazolidinone and *p*-hydroxy-tetramisole were obtained from Toronto Research Chemicals (Toronto, Canada). Enantiopure *p*-hydroxy-levamisole was purchased from US Biological (Salem, USA). (RS)-tetramisole-d5 was obtained from Merck (Darmstadt, Germany). Methanol (LC–MS-grade) was from Honeywell (Seelze, Germany). Water (LC–MS-grade) and acetonitrile (LC–MS-grade) were purchased from Carl Roth (Karlsruhe, Germany). All reference substances had a purity of at least 96%, whereas all chemicals were at least of analytical grade. For the GC-derivatisation experiments, N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) and 1% trimethylsilyl iodide were purchased from Sigma-Aldrich (St. Louis, USA). Blank (drug-free) serum was provided by the blood bank of the University Medical Center of the Johannes Gutenberg University Mainz.

2.2 | Methods

2.2.1 | Quantification of 4-phenyl-2-imidazolidinone and *p*-hydroxy-PTHIT by LC–MS/MS

Method development and analytical parameters

A LC–MS/MS method was developed for the detection of aminorex, 4-phenyl-2-imidazolidione and *p*-hydroxy-PTHIT. Unfortunately, no further chiral examination as in our previous study was possible since the reference substance *p*-hydroxy-tetramisole appears not to be racemic (specific rotation −11.5° according to the manufacturer). Since *p*-hydroxy-tetramisole was of lower purity (> 96%), the enantiopure reference standard *p*-hydroxy-levamisole (> 98%) was used for quantification purposes. Chromatography was performed using a 1290 Infinity II UHPLC system, coupled via Jet Stream interface (ESI) to a 6495C triple quadrupole mass spectrometer. The system consisted of an Agilent Infinity Lab Poroshell 120 EC-C18 column (2.1 x 150 mm / 2.7 µm) guarded by an Infinity Lab Poroshell 120 EC-C18 column (2.1 x 5 mm / 2.7 µm). The mobile phase consisted of (A) 10mM ammonium acetate in water and (B) methanol. The column temperature was 50°C, injection volume was 1.5 µl and flow rate was 0.4 ml/min. The elution gradient started with 10% increasing to 45% B within 7 min. Subsequently, B was increased to 90% for column washing for 1 minute, followed by a 2 min post-run time for re-equilibration (total run time 10 min). For mass-selective detection, electrospray parameters were as follows: gas flow 11 L/min at 200°C; nebulizer 15 psi, sheath gas flow 12 L/min at 400°C; capillary voltage +3,500 V. Analytes were detected in multiple reaction monitoring (MRM) mode using the following transitions (*m/z*, collision energy in parentheses, target ion underlined): Aminorex and 4-phenyl-2-imidazolidinone 163 → 120 (13 eV),<u>103</u> (29 eV); *p*-hydroxy-PTHIT 221.1 → <u>107.0</u> (35 eV), 194.1 (25 eV); tetramisole-d5 210.1 → <u>183.1</u> (25 eV), 96.1 (45 eV).

Sample preparation

Briefly, 200 µl of serum were spiked with 10 µl internal standard (ISTD) solution (containing 200 ng/ml tetramisole-d5 in methanol, final concentration of 10 ng/ml in spiked samples). For protein precipitation, 200 µl acetonitrile were added. After mixing, the samples were centrifuged twice for 20 min at 21300 x g. Each time the supernatant was transferred into a new vessel. Finally, the extracts were evaporated to dryness under a gentle stream of nitrogen at 80°C. For analysis, residues were redissolved in 50 µl methanol.

Method validation

The method was validated for the analysis of serum samples according to a national guideline.[22,23] Validation parameters for 4-phenyl-2-imidazolidinone and *p*-hydroxy-PTHIT were selectivity, linearity of calibration, analytical limits, accuracy (bias), inter-day precision, recovery and matrix effects. Since aminorex was never detected in the sample collective, only selectivity and limit of detection (LOD) were tested.

Method selectivity was evaluated by measuring six drug-free samples (blank samples) and two samples after addition of ISTD-solution (zero samples). Linearity of the calibration was tested by threefold measurement of a calibration series from 0.05 to 50 ng/ml. The LOD was determined by evaluating the signal-to-noise ratio (both ion transitions S/N > 3), measuring a separate calibration series from 0.025 to 0.25 ng/ml. The lower limit of quantification (LLOQ) was determined by a sixfold measurement of the lowest calibrator (0.05 ng/ml), requiring <20% relative standard deviation (RSD) for precision and less than ±20% for bias. Accuracy (bias), inter-day precision, matrix effects and recovery were determined at low (0.75 ng/ml) and high concentrations (25 ng/ml) relative to the calibration range. Accuracy and inter-day precision were assessed by preparing and analysing spiked samples of each concentration level in duplicate on eight different days. Acceptance criterion for accuracy was a maximum bias of 15%. Inter-day precision was calculated as RSD (%). Matrix effects and recovery were determined according to Matuszewski et al. (2003).[24] Since the samples analysed in this study were measured right after preparation, the parameter stability in the extract was omitted. Validation data were statistically evaluated using Valistat 2.00.1 software (Arvecon; Walldorf, Germany).

2.2.2 | Investigated sample collective

Nasal application study

In our previous study, the chiral pharmacokinetics of dexamisole and levamisole were examined.[6] Therefore, an adulterated cocaine consumption was simulated, and a 10 mg dose of racemic tetramisole hydrochloride was administered nasally using a straw by three male healthy volunteers (27–38 years, weighting 69–83 kg, body mass index 22.3–23.9). In Germany, Ethics Committee approval is not required for scientific self-experiments. Blood samples were collected before the experiment (baseline) and approximately 10, 20, 40, 60, 90 minutes, 2, 3, 4, 6, 8, 12 and 24 hours after administration (exact

times were used for further evaluation). In deviation to that, for subject 1, samples were only drawn for a period of 8 hours after administration. These samples, taken in the scope of our previous study,[6] were re-analysed for metabolites approx. 15 months after the initial analysis. Blood samples were centrifuged immediately after collection. The resulting serum was stored at −18°C throughout the whole time. Basic pharmacokinetic parameters, including maximum serum concentrations observed (c_{max}) and corresponding times (t_{max}) as well as serum concentration at the last sampling point (c_{end}) were evaluated for each participant. Apparent elimination half-life ($t_{1/2}$) was calculated using exponential regression.

Cocaine/benzoylecgonine-positive serum samples

Moreover, serum samples of cocaine users (cocaine ≥ 1 ng/ml and/or benzoylecgonine ≥ 25 ng/ml), were analysed for the presence of dexamisole and levamisole in our previous study.[6] The samples were taken between 11/2020 and 04/2021 and were sent to the Department of Forensic Toxicology at the Institute of Forensic Medicine in Mainz, Germany, from police stations in the federal state of Rhineland-Palatinate for toxicological analysis. Seventy-four samples previously tested positive for PTHIT (49 samples positive for both enantiomers, 25 samples positive for dexamisole) were re-analysed after approx. 15 months for PTHIT metabolites. The samples were constantly stored at −18°C.

2.2.3 | Examinations for misinterpretation of 4-phenyl-2-imidazolidinone as aminorex: GC–MS and untargeted LC-QTOF analyses

Since 4-phenyl-2-imidazolidinone and aminorex are constitutional isomers, the possibility of misinterpreting 4-phenyl-2-imidazolidinone as aminorex was further elucidated. Therefore, two methods, among them a GC–MS and a LC-QTOF method, were tested for their ability to differentiate between both substances. The GC–MS method was reconstructed according to a previously described method for the detection of aminorex.[17,25] In this analysis, aminorex has been previously identified in its form as a bis-trimethylsilyl-derivative (2-TMS). The LC-QTOF method is used in our laboratory for untargeted screening analysis.

GC–MS analysis as 2-TMS derivative

A total of 10 µl of a 1 mg/ml solution in methanol (aminorex or 4-phenyl-2-imidazolidinone) was evaporated to dryness under a gentle stream of nitrogen. After the addition of 50 µl of MSTFA, containing 1% iodotrimethylsilane, both substances were derivatised at 80°C for 30 min. After cooling, the samples were analysed by GC–MS in 1:100 dilution in ethyl acetate. Instrumental analysis was performed on an Agilent 8860 gas chromatograph equipped with an HP-5MS column (30 m x 0.25 mm x 0.25 µm) and a 5975B mass-selective detector. Helium was used as a carrier gas at a flow rate of 1 ml/min. The injection was performed in a splitless mode with an injection volume of 1 µl. The inlet temperature was set to 250°C. The oven temperature was programmed at 80°C for 1 min and then increased to 300°C at 20°C/min for 5 min. The mass-selective detector operated in scan mode.

LC-QTOF HRMS analysis

Chromatography was performed on an Agilent 1260 Infinity equipped with a 6590 Accurate Mass Q-TOF detector and connected with a Jet Stream Interface (ESI). The system was equipped with an Agilent Infinity Lab Poroshell 120 EC-C18 column (2.1 x 150 mm / 2.7 µm) guarded by an Infinity Lab Poroshell 120 EC-C18 (2.1 x 5 mm / 2.7 µm). The mobile phase consisted of (A) 10mM ammonium acetate in water and (B) methanol. Column temperature was 50°C, injection volume was 1.5 µl and flow rate was 0.4 ml/min. The elution gradient started with 10% increasing to 50% B in 8 min and further to 100% after 20 min. This was kept until 23.9 min had elapsed. The column was then re-equilibrated by a post-time of 5 min (total run time 29 min). The ESI-source parameters were as follows: gas flow 8 L/min at 320°C; nebulizer 35 psi, sheath gas flow 11 L/min at 380°C; capillary voltage +3,000 V. The first quadrupole was used for ion selection in a mass range from m/z 100 to 600. Further mass spectrometric parameters are described by Kleis et al. (2022).[26] Methanolic solutions of the reference substances at a concentration of 50 ng/ml were used for measurement and clarification of retention times. The resulting chromatograms were processed using in-house library comparison workflows.

3 | RESULTS

3.1 | Method validation of 4-phenyl-2-imidazolidinone and *p*-hydroxy-PTHIT

The method for quantification of *p*-hydroxy-PTHIT and 4-phenyl-2-imidazolidione was validated successfully for all tested parameters. Blank and zero samples showed no interfering signals. Separation of 4-phenyl-2-imidazolidione and aminorex was ensured (Figure 2). The LOD for aminorex was determined to be 0.05 ng/ml. Since it was known from previous data that nicotine could also disturb the examined mass transitions (*m/z* 163 → 120, 103), separation from 4-phenyl-2-imidazolidione was also confirmed. Nicotine elutes between aminorex and 4-phenyl-2-imidazolidinone at 3.7 min and differs in its target/qualifier-ratios (*m/z* 163 → 77, 120, 103 descending order) clearly from 4-phenyl-2-imidazolidinone and aminorex.

A linear calibration model without weighting could be used for quantification of all analytes. LOD was at 0.025 ng/ml for both analytes. The lowest calibration level (0.05 ng/ml) could be established as LLOQ with acceptable results for bias (RSD) and precision (less than −16.53% for p-hydroxy-PTHIT and 2.00% for 4-phenyl-2-imidazolidinone, respectively).

Also, the matrix effects (Table 1) for both analytes were within the allowed ranges for the low and high level. During method development, it was noted that 4-phenyl-2-imidazolidinone showed a weak and unreproducible response after the solid phase extraction (SPE) sample preparation technique used in our previous study.[6] Therefore,

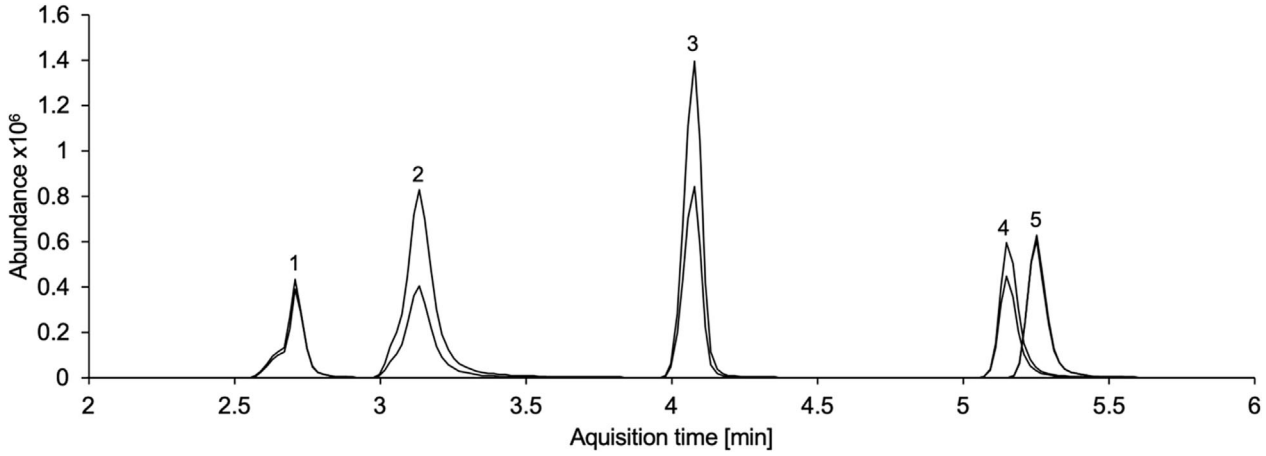


FIGURE 2 MRM chromatogram of a spiked blank serum for *p*-hydroxy-levamisole (1, 2.7 min), aminorex (2, 3.1 min), 4-phenyl-2-imidazolidinone (3, 4.1 min), tetramisole-d5 (4, 5.2 min) and tetramisole (5, 5.3 min) at a concentration level of 10 ng/ml by achiral LC-MS/MS analysis. The following ion transitions are displayed in decreasing order of intensity (*m/z*, target ion underlined): *p*-hydroxy-levamisole 221 → 107, 194, aminorex and 4-phenyl-2-imidazolidinone 163 → 120, 103, tetramisole-d5: 210.1 → 183.1, 96.1, tetramisole: 205.1 → 178, 91

TABLE 1 Validation results for the *p*-hydroxy-PTHIT and 4-phenyl-2-imidazolidinone.

Concentration (ng/mL)	Analyte	Accuracy (bias) n = 8 (%)	Inter-day precision n = 8 (%)	Recovery n = 6 (%)	Matrix effects n = 6 (%)
0.75	*p*-hydroxy-PTHIT	−10.6	4.97	41.0 ± 18.1	106 ± 8.8
0.75	4-phenyl-2-imidazolidinone	5.00	7.40	44.7 ± 16.3	94.0 ± 9.4
25.0	*p*-hydroxy-PTHIT	−3.71	6.01	68.5 ± 18.2	91.8 ± 6.4
25.0	4-phenyl-2-imidazolidinone	−3.09	5.18	71.1 ± 17.9	92.4 ± 5.7

different SPE techniques routinely used in our laboratory, including C18-phases as well as mixed-mode-phases consisting of a non-polar C8 sorbent and a strong cation exchanger, were tested for their extraction efficiency for the analytes *p*-hydroxy-PTHIT and 4-phenyl-2-imidazolidinone. Moreover, a simple protein precipitation omitting an SPE step and the effect of evaporation under a steam of nitrogen were evaluated. Finally, a protein precipitation and a concentration step were chosen since this led to a satisfying recovery in combination with an increased sensitivity. At the low level, recovery for both analytes was decreased compared to the high level (Table 1). However, fluctuations in the recovery were compensated by the ISTD (data set low level 43.0 ± 20.9; data set high level 73.0 ± 14.0).

3.2 | Quantification of *p*-hydroxy-PTHIT and 4-phenyl-2-imidazolidinone in samples after a controlled nasal self-administration

Whereas aminorex was never detected, 4-phenyl-2-imidazolidinone and *p*-hydroxy-PTHIT could be found in the serum samples of all three volunteers. Time courses of *p*-hydroxy-PTHIT and 4-phenyl-2-imidazolidinone serum concentrations for all three subjects are given in Figure 3. Calculated pharmacokinetic properties for each participant are given in Table 2. Unfortunately, after finishing the initial study,[6] the remaining amounts in the 8 hours sample of subject 1 and the 24 hours sample of subject 3 were not sufficient for further analysis. Since the observed c_{max} was the last sampling point for 4-phenyl-2-imidazolidinone in case of subject 1, no elimination half-life could be determined. The calculated half-lives for *p*-hydroxy-PTHIT (3.4–5.8 h) were considerably shorter than that of 4-phenyl-2-imidazolidinone (14.1–15.8 h). For subject 2, two samples (65 and 87 min) were presumably diluted inadvertently, which may have affected the determination of the pharmacokinetic parameters. Serum concentrations at the last measurable sampling point (after 6 h for subject 1, after 12 h for subject 2 and after 24 h for subject 3) are also given in Table 2 (c_{end}). Whereas initially *p*-hydroxy-PTHIT showed higher concentrations than 4-phenyl-2-imidazolidinone, later (after 6, 12 and 24 hours for subjects 1, 2 and 3, respectively), the concentrations of 4-phenyl-2-imidazolidinone exceeded those of *p*-hydroxy-PTHIT.

3.3 | Quantification of *p*-hydroxy-PTHIT and 4-phenyl-2-imidazolidinone in blood samples of cocaine users tested positive for PTHIT

Seventy-four serum samples of cocaine users were previously tested positive for PTHIT.[6] In one case, the sample amount was not

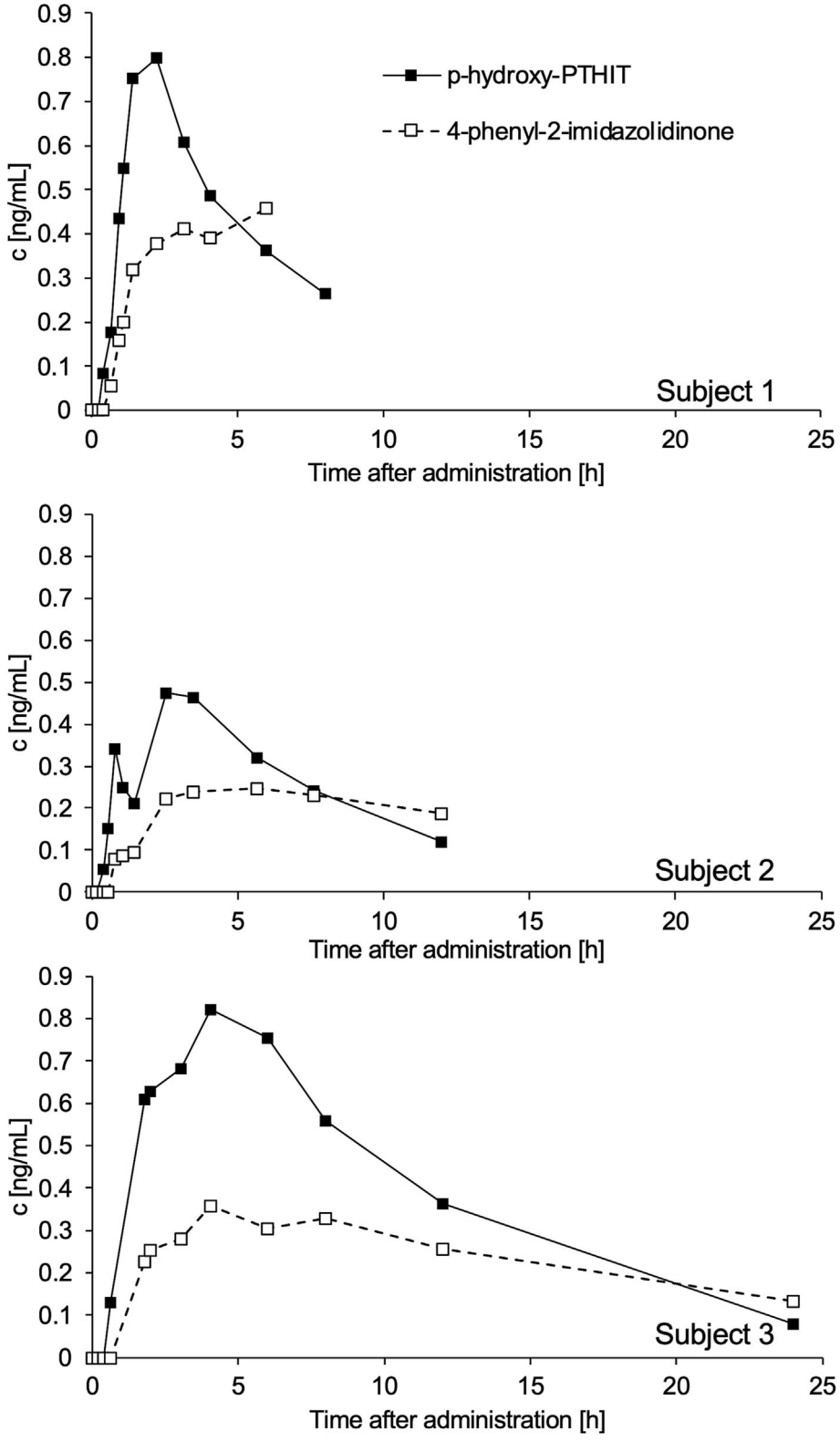


FIGURE 3 *p*-Hydroxy-PTHIT and 4-phenyl-2-imidazolidinone serum concentration-time curves after nasal administration of 10 mg racemic tetramisole hydrochloride for subject 1 (170 cm, 69 kg), subject 2 (189 cm, 83 kg) and subject 3 (187 cm, 78 kg)

sufficient for further analysis, so 73 samples were finally examined for the presence of aminorex, 4-phenyl-2-imidazolidinone and *p*-hydroxy-PTHIT. Briefly, *p*-hydroxy-PTHIT could be detected in 24 cases (33%) of which it was below the LOQ in 7 cases. 4-Phenyl-2-imidazolidinone was detectable in 37 cases (51%), of which it was below the LOQ in 4 cases. Also in these samples, aminorex was never detected. Figure 4 further summarises the frequency distribution of the detection of 4-phenyl-2-imidazolidinone and *p*-hydroxy-PTHIT in the sample collective.

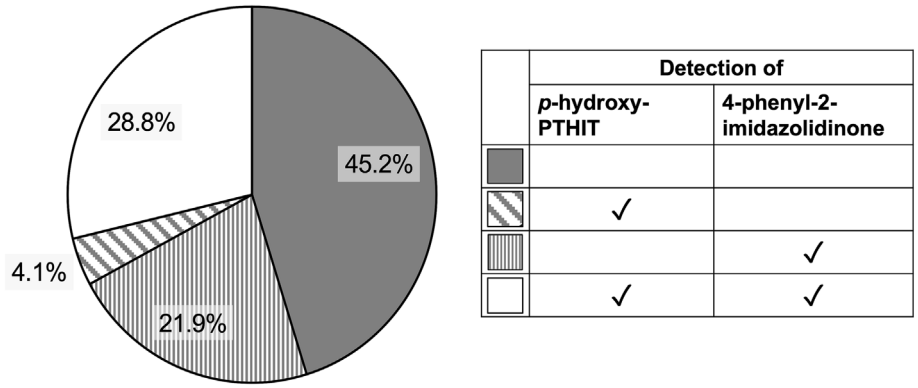
In the quantifiable cases, the median concentration of *p*-hydroxy-PTHIT was 0.411 ng/ml (range, 0.056–2.66 ng/ml) whereas the median 4-phenyl-2-imidazolidinone concentration was lower with

TABLE 2 Pharmacokinetic properties of *p*-hydroxy-PTHIT and 4-phenyl-2-imidazolidinone in serum after nasal administration of 10 mg tetramisole hydrochloride.

Subject	Analyte	c_{max} (ng/mL)	t_{max} (h)	$t_{1/2}$ (h)	c_{end} (ng/mL)
1	*p*-hydroxy-PTHIT	0.798	2.2	3.4	0.632 (6 h)
2		0.473	2.6	4.6	0.120 (12 h)
3		0.820	4.0	5.8	0.080 (24 h)
1	4-phenyl-2-imidazolidinone	0.457	6.0	-	0.457 (6 h)
2		0.248	5.7	15.9	0.189 (12 h)
3		0.357	4.0	14.1	0.133 (24 h)

Note: Summing up the concentrations determined for dexamisole and levamisole[6] lead to a concentration maximum of PTHIT of 49.4 ng/ml after 2.2 h, 32.0 ng/ml after 0.8 h and 55.0 ng/ml after 3.1 h for subjects 1, 2 and 3, respectively.

FIGURE 4 Frequency distribution of the detection of 4-phenyl-2-imidazolidinone and *p*-hydroxy-PTHIT in PTHIT-positive NaF-serum samples (n = 73)



0.191 ng/ml (range, 0.052–4.01 ng/ml). While for *p*-hydroxy-PTHIT a concentration above 1 ng/ml was found in three cases, there was only one case exceeding a 4-phenyl-2-imidazolidinone concentration of 1 ng/ml.

3.4 | Examinations for misinterpretation of 4-phenyl-2-imidazolidinone as aminorex: GC–MS and untargeted LC-QTOF

By means of the adopted GC–MS method by Bertol et al. (2011),[17] both substances, aminorex and 4-phenyl-2-imidazolidinone, were measured as 2TMS-derivatives. The resulting TIC-chromatograms are shown in Figure 5a,b. Since aminorex has been analysed before as 2TMS-derivative[17,20,25] exhibiting a base peak at *m/z* 291, extracted ion chromatograms were generated for this mass. By doing so, it can be recognised that both substances co-elute at a retention time of 8.6 min. Both derivatives also show the same fragmentation pattern (Figure 5c,d).

By means of the LC-QTOF untargeted screening analysis, both constitutional isomers were chromatographically separated. Whereas aminorex eluted at 4.4 min, 4-phenyl-2-imidazolidinone eluted at 6.1 min (Figure 6a). The mass spectra obtained for these peaks are nearly identical (Figure 6b,c) leading to 4-phenyl-2-imidazolidinone being misidentified as aminorex by running a library comparison workflow with a high score (93%). For further information on the fragments formed compare Hess et al. (2013).[9]

4 | DISCUSSION

4.1 | Quantification of *p*-hydroxy-PTHIT and 4-phenyl-2-imidazolidinone

In the present study, the two tetramisole metabolites *p*-hydroxy-PTHIT and 4-phenyl-2-imidazolidinone were detected after controlled nasal application of tetramisole and in serum samples of cocaine users. Aminorex was absent in the whole sample examined.

4.1.1 | Self-administration samples

Referring to human kinetic data on *p*-hydroxy-levamisole, there are two studies in which the single enantiomer levamisole was administered orally either once[7] or repeatedly for 5 or 3 days, respectively.[7,12] Contrastingly, in this study, the racemate tetramisole was administered nasally. Since only achiral data were collected in this study and the enantiomeric composition of the metabolite is unknown, the analyte is herein deliberately referred to as '*p*-hydroxy-PTHIT'. The half-life of *p*-hydroxy-levamisole is reported to be significantly shorter than the half-life of levamisole,[12] about 6.25 ± 2.40 hours after single administration and up to 8.60 ± 0.98 hours after repeated dosage.[7] In our study, the half-lives determined (3.4–5.8 h) are in the lower range of the literature values. After oral ingestion of levamisole, t_{max} of *p*-hydroxy-levamisole was reached after about 2.6 hours[7] which is similar to the t_{max} (2.2–4.0 hours) of *p*-

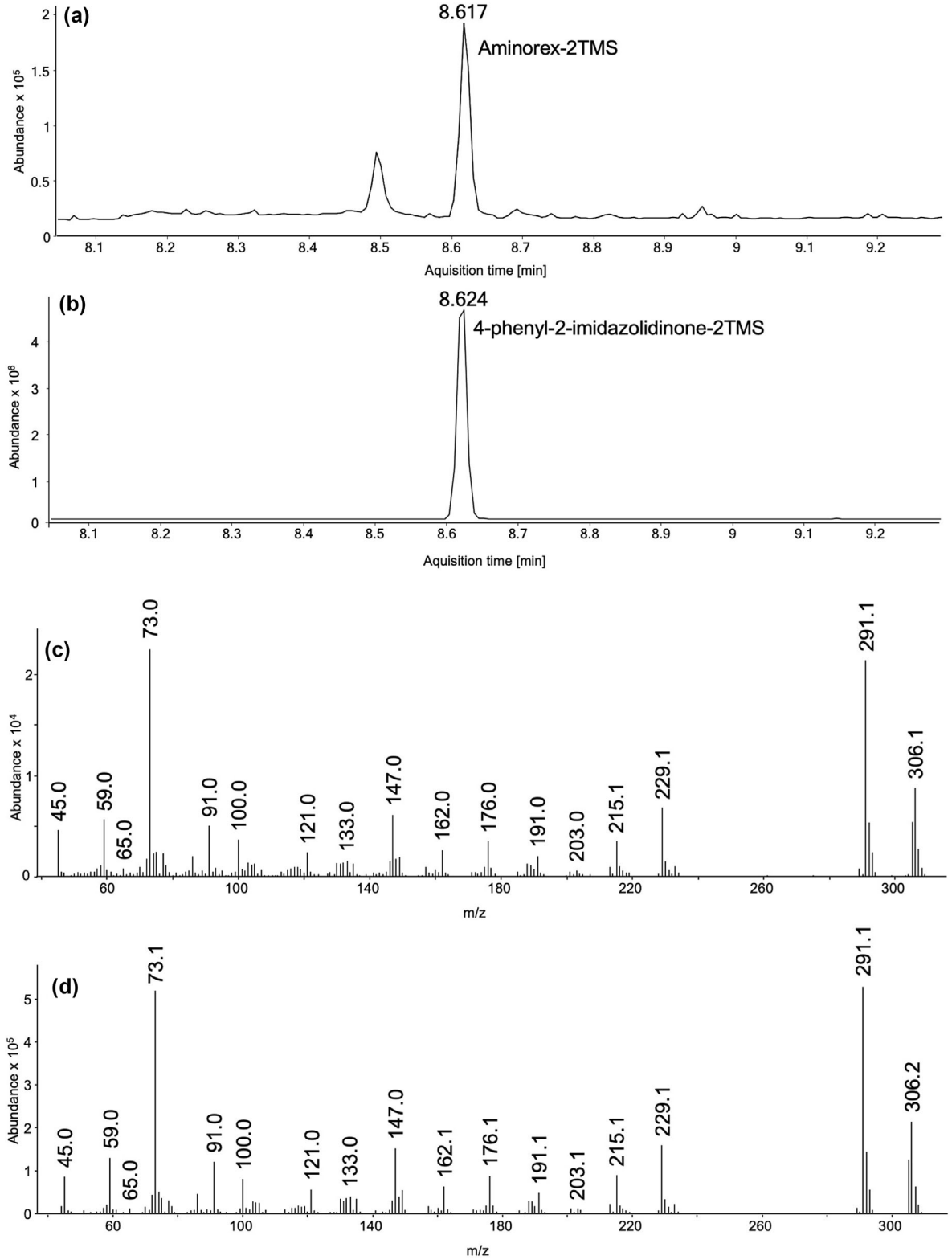


FIGURE 5 Total ion chromatogram of trimethylsilyl-derivatives of aminorex (a) resp. 4-phenyl-2-imidazolidinone (b) reference substance. Mass spectra of the aminorex-2TMS (c) resp. 4-phenyl-2-imidazolidinone-2TMS derivatives (d) eluting at 8.6 min

hydroxy-PTHIT determined in the present study after nasal administration. Moreover, c_{max} of *p*-hydroxy-levamisole after oral ingestion is reported to be approximately 5% of c_{max} of levamisole.[7] After nasal ingestion of PTHIT, c_{max} of *p*-hydroxy-PTHIT was only 1.5–1.6% of c_{max} of PTHIT for the three subjects. The proportion of *p*-hydroxy-PTHIT formed after oral ingestion might be higher than after nasal administration due to a stronger first pass effect. Previously, the first pass effect of levamisole after oral intake was estimated to be about

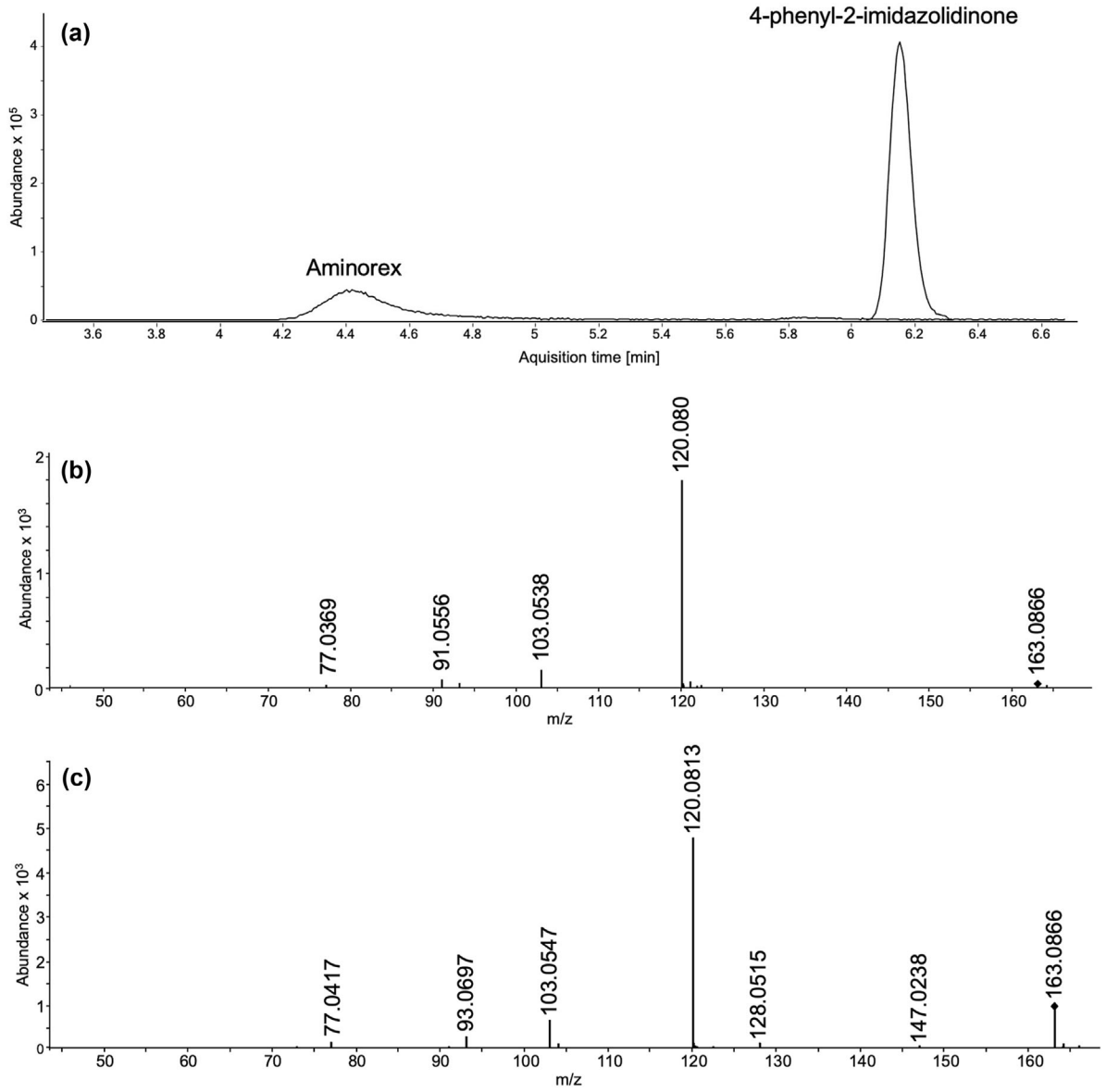


FIGURE 6 Overlay of total compound chromatogram of aminorex and 4-phenyl-2-imidazolidinone in a methanolic reference substance solution at a concentration of 50 ng/ml by LC-QTOF analysis (a). HRMS/MS spectra of aminorex (b) and 4-phenyl-2-imidazolidinone (c) with precursor ions of $m/z = 163.0866$ $[M + H^+]$ using a collision energy of 13.9 eV

30%.[12] A more pronounced para-hydroxylation for levamisole than for dexamisole could also be a possible explanation. Our own study (*data not shown*) showed that *p*-hydroxy-PTHIT is formed hepatically via CYP enzymes, which are known to be stereoselective.[27] Hess et al. (2013)[9] reported the detection of seven metabolites with the mass of *p*-hydroxy-PTHIT in urine samples from a subject after ingestion of 100 mg levamisole. In at least 3 of the mass spectra given, the mass fragments *m/z* 107 and 194 appear, which were also measured here. Therefore, it should be considered that there are far more metabolites as those studied here. Although fragment 107 is indicative of hydroxylation on the aromatic ring,[10] a limitation of this study is that the formation of *m*- and *o*-hydroxy-PTHIT cannot be ruled out, as co-elution must be taken into account.

Unlike *p*-hydroxy-PTHIT, 4-phenyl-2-imidazolidinone is a rarely studied compound. In fact, to the best of the authors' knowledge, this is the first in vivo detection of 4-phenyl-2-imidazolidinone in human material. Previously, 4-phenyl-2-imidazolidinone has been described as an in vitro metabolite of levamisole in dogs, pigs, sheep, cattle and human[8,15] and as an in vivo metabolite of dogs, monkeys, rats and horses.[15] In veterinary levamisole formulations and also in a 4-year-old methanolic levamisole solution, 4-phenyl-2-imidazolidinone has been confirmed as a degradation product to a small extent. Most likely, it is formed by slow auto hydrolysis with a subsequent loss of the labile mercaptoethyl group.[15] In the case of the tetramisole reference substance consumed in the self-administrations experiments here, initial contamination by 4-phenyl-2-imidazolidinone was

TABLE 3 Summary on the detection of aminorex in forensic samples reported in the literature.

Study	Sample collective and type of administration	Matrix	Results	
			PTHIT	Aminorex
GC–MS-based analyses: Aminorex was analysed as 2TMS-derivative.				
Bertol et al. (2011)[17]	Controlled oral administration, ingestion of 47–57 mg levamisole (n = 8)	urine	40.63 ng/ml (range 30.05–53.22 ng/ml, after 3 hours) 20.63 ng/ml (range 12.45–28.34 ng/ml, after 6 hours)	30.63 ng/ml (range 22.52–38.12 ng/ml, after 3 hours) 28.32 ng/ml (range 20.08–43.6 ng/ml, after 6 hours)
	Cocaine users	cocaine and/or benzoylecgonine-positive urine (n = 12)	Qualitative detection of aminorex and PTHIT in 4 samples	
Karch et al. (2014)[25]	Case report of a fatal cocaine overdose (cocaine powder found by the deceased contained 6.9% PTHIT)	urine	75.05 ng/ml	38.62 ng/ml
		blood	15.05 ng/ml	8.92 ng/ml
		hair	12.15 ng/mg	7.35 ng/mg
Serinelli et al. (2021)[20]	Case report of two fatal oral cocaine overdoses (qualitative, without reference substances)	urine	Qualitative detection of aminorex and PTHIT	
		blood	No detection of aminorex, qualitative detection of PTHIT	
LC–MS/MS-based analyses				
Hess et al. (2014)[29]	Controlled oral administration, ingestion of 100 mg levamisole (n = 1)	serum	c_{max} 214 ng/ml after 1.98 hours; detected for 36 hours (LOQ 0.85 ng/ml).	> LOD (0.09 ng/ml) for 30 hours, but always < LOQ (0.34 ng/ml), detection of an aminorex isomer for up to 11 hours (c_{max} 8 ng/ml, quantified by aminorex reference standard)
	Cocaine users	cocaine-positive serum (n = 106)	45 (43%) PTHIT-positive, range 2.2 to 224 ng/ml (mean 45.8 ng/ml)	Qualitative detection in 12 (11%) samples (< LOQ), detection of an aminorex isomer in 13 (12%) samples (0.95 ng/ml–22.1 ng/ml, mean 8.1 ng/ml, quantified by aminorex reference standard).
		urine (n = 12, positive cocaine blood finding)	11 PTHIT-positive, ranging from 3.7 - about 2,920 ng/ml	Negative
		post-mortem urine (n = 3, positive cocaine blood finding)	2 PTHIT-positive, about 1,270 ng/ml about 3,790 ng/ml	6.1 ng/ml 1.0 ng/ml
		cocaine-positive post-mortem blood (n = 2)	171 ng/ml 15.8 ng/ml	Positive (< LOQ) 0.37 ng/ml

TABLE 3 (Continued)

Study	Sample collective and type of administration	Matrix	Results	
			PTHIT	Aminorex
Eiden et al. (2015)[21]	Cocaine users	cocaine-positive urine (n = 42)	32 (76%) PTHIT-positive, range 30–258,000 ng/ml (median 390 ng/ml [?], mean 19,016 ng/ml)	negative (LOQ 30 ng/ml)
		cocaine-positive serum (n = 8)	7 PTHIT-positive, range 2.6–112 ng/ml (mean 58.5 ng/ml)	Detection in 3 samples (2 cases < LOQ 1.0 ng/ml, one case 2.4 ng/ml)
		cocaine-positive post-mortem blood (n = 7)	6 PTHIT-positive, range 1–125 ng/ml (mean 38.4 ng/ml)	Detection in 1 sample (< LOQ 1.0 ng/ml)
LC-HRMS-based analyses				
Hess et al. (2013)[9]	Controlled oral administration, ingestion of 100 mg levamisole-hydrochloride (n = 1)	urine	c_{max} 1,250 ng/ml after 3 hours, concentration stayed > 1,000 ng/ml for 20 hours (detectable for 39 hours; LOD 0.51 ng/ml).	Concentration peaked after 4–8 hours at about 45 ng/ml and stayed > 30 ng/ml for 30 hours (detectable for 54 hours; LOD 0.65 ng/ml). Additionally, five isomers were detected. One isomer could be detected longer (up to 61 h) than PTHIT and aminorex.
Handley et al. (2019)[10]	Cocaine users	benzoylecgonine-positive urine samples (n = 100)	72 (72%) PTHIT-positive, range 4–72,970 ng/ml (Median 565 ng/ml, LOQ 1 ng/ml).	No detection of aminorex (LOQ 1 ng/ml), isomers or aminorex-derivatives in 5 urine samples with high PTHIT concentrations.
		blood samples (n = 8, corresponding to 8 urine samples)	8 PTHIT-positive, range 0.9–64.1 ng/ml (median 10.6 ng/ml, LOQ 0.1 ng/ml)	No detection (LOQ 0.1 ng/ml)

excluded. Only for the horse rudimentary pharmacokinetic data after subcutaneous injection or oral administration of levamisole is published.[15] After 6–8 hours c_{max} of 4-phenyl-2-imidazolidinone was reached at 40–60 ng/ml (c_{max} of levamisole was 380–590 ng/ml, reached after 1 hour) corresponding to approximately 10% of the concentration of the parent compound.[15] Similarly, in this study, the maximum serum concentration of 4-phenyl-2-imidazolidinone was reached between 4.0 and 5.7 hours after nasal administration of the racemate. However, the c_{max} of 4-phenyl-2-imidazolidinone compared to c_{max} of PTHIT was clearly below 1% in all 3 subjects, showing significant deviations from the data from horses. The half-life of 4-phenyl-2-imidazolidinone calculated here (14.1–15.8 hours) is about twice as long as that of its constitutional isomer aminorex (7.7 hours in humans[28]).

4.1.2 | NaF-serum samples of cocaine users tested previously positive for PTHIT

Neither for *p*-hydroxy-PTHIT nor for 4-phenyl-2-imidazolidinone quantitative data have previously been collected in biological samples from cocaine users. Only *p*-hydroxy-PTHIT was previously detected qualitatively by Handley et al. (2019)[10] in 48% of a collective of urine samples tested previously positive for benzoylecgonine and PTHIT (n = 72). In this study, *p*-hydroxy-PTHIT was found in slightly lower frequency with 33% of the PTHIT- and benzoylecgonine/cocaine-positive serum samples examined (n = 73). 4-phenyl-2-imidazolidinone was detected more frequently (51%) in serum samples from cocaine users. Due to its longer half-life, 4-phenyl-2-imidazolidinone is still detectable in the late phase of elimination and is therefore more likely to exhibit accumulation effects in case of binge consumption.

4.2 | Absence of aminorex

Despite a comparatively low limit of detection (LOD 0.05 ng/ml), aminorex could not be detected in any of the samples examined in this study. To demonstrate that the detection of aminorex in human material is quite limited and contradictory, the literature is summarised in greater detail in Table 3. On the one hand, there are two experiments in which aminorex was detected in serum[29] or urine[9,17] after controlled oral levamisole ingestion. On the other hand, there are studies examining the presence of aminorex in samples of cocaine users including two case reports of fatal cocaine intoxications[20,25] as well as four studies examining forensic urine or serum sample collectives.[10,17,21,29] In the following comparison of studies examining forensic sample collectives (Table 3), it should be considered as a limiting factor that no information on the cocaine consumed (purity, adulterants) is available. While some of these studies only report qualitative data anyway,[17,20] the few aminorex detections in quantitative studies are mostly below the LOQ. The highest reported aminorex concentration is 45 ng/ml in urine (controlled intake of 100 mg levamisole[9]) or 8.92 ng/ml in blood (post-mortem sample[25]). However, especially in the latter case, the reported aminorex concentration is remarkably high in relation to the PTHIT concentration (approx. 60%).

Like in our study, Handley et al. (2019)[10] detected no aminorex at all. A few theories were suggested to explain the inconsistencies in aminorex detection. Firstly, enzyme polymorphism was proposed.[17] A multi-step proceeding reaction after protonation of PTHIT – and therefore preferably taking place in the acidic milieu – was suggested as metabolic pathway.[15,30] Since aminorex was mainly found in urine samples after controlled oral consumption of levamisole,[9,17] it was proposed that aminorex was only formed after oral intake and protonation in the stomach.[21] Therefore, the absence of aminorex in most samples from cocaine users may be explained due to nasal, inhalative or intravenous consumption.[10] A recently published case report describes an unusual double-suicide after oral ingestion of PTHIT-tainted cocaine. Putatively, aminorex had been qualitatively detected in post-mortem urine but not in blood.[20] Another explanation for the frequent detection of aminorex in studies after controlled oral intake[9,17,29] compared to samples from cocaine users is the significantly higher administered dose. Also in horses, the dose given that resulted in a detectable amount of aminorex was much higher.[15] However, if aminorex is only a minor metabolite in humans, the dose of PTHIT (usually <20% PTHIT relative to total mass of seized powder[6]) taken by cocaine users may not be sufficient to produce a detectable amount of aminorex in most cases. Moreover, at least with regard to urine as a sample matrix, acidification may increase excretion of aminorex due to its weakly basic nature[31] and lead to inconsistencies in detection. Degradation of aminorex in the sample matrix is unlikely since it was stable in urine at room temperature for 2 weeks.[10] Although no data is available on long-term stability in serum,[10] structurally similar stimulants such as amphetamine are stable for up to 10 months at −20°C.[32]

4.3 | Misinterpretation of 4-phenyl-2-imidazolidinone as aminorex?

Another explanation for the inconsistencies of aminorex detection in the literature may be the misinterpretation of mass spectrometric data.[10] This theory is supported by the identified aminorex isomer 4-phenyl-2-imidazolidinone in human sample material in our study. Since levamisole was known to produce a metabolite of same molecular weight than aminorex,[31] 4-phenyl-2-imidazolidinone was included in the horse doping analysis very early on. For the horse, it was concluded that both substances, 4-phenyl-2-imidazolidinone and aminorex, are indeed metabolites of levamisole.[15] Contrastingly, 4-phenyl-2-imidazolidinone has not been involved in forensic analysis so far since it was not commercially available as reference substance[9] and could only be obtained by self-synthesis.[15]

For the analysis of aminorex in forensic samples, either GC–MS, LC–MS/MS or LC techniques coupled to high resolution mass spectrometry (HRMS) were used. All studies utilising GC–MS have in

common that 2TMS-derivatives were detected.[17,25,33] Therefore, the first published GC–MS method[17] was reconstructed. Like aminorex, 4-phenyl-2-imidazolidinone forms a 2TMS-derivative, which co-eluted using this method. Even the spectra of both derivatives cannot be distinguished from each other and correspond to the spectrum of aminorex-2TMS previously reported.[17] Also, by decreasing the slope of the temperature gradient (80°C 1 min; 10°C/min to 150°C, hold 3 min; 20°C/min to 300°C, hold 3 min) the derivatives could not be separated *(data not shown)*. The detection of aminorex by Bertol et al. (2011),[17] Karch et al. (2014)[25] and Serinelli et al. (2021)[20,33] are therefore questionable. Despite the co-elution of both compounds, it is even more likely that the peak observed was produced exclusively by 4-phenyl-2-imidazolidinone. Because the signal produced by 4-phenyl-2-imidazolidinone-2TMS is even more intensive than that of aminorex-2TMS this may lead to high putative concentrations of aminorex. This may be an explanation for the remarkably high aminorex concentration reported by Karch et al. (2014).[25]

Aminorex has also been detected in forensic samples by means of LC–MS/MS methods. Eiden et al. (2015)[21] used a pentafluorophenyl propyl column and a mobile phase consisting of a mixture of acetonitrile and formate buffer (pH 3.2). Hess et al. (2013)[9] used a phenyl hexyl column and a gradient elution consisting of a mixture of 0.1% formic acid in water resp. 0.1% formic acid in acetonitrile. Like in our LC–MS/MS analysis, in both studies the same mass transitions for aminorex (*m/z* 163 → 120, 103) were analysed. Hess et al. (2013)[9] also analysed a third mass transition (*m/z* 163 → 77) which was also monitored in our analyses. However, as this transition was more prone to interferences it was not included in the final method. Since 4-phenyl-2-imidazolidinone can be detected with similar target/qualifier-ratios, a misinterpretation of 4-phenyl-2-imidazolidinone may be explanation for the detection of aminorex in a blood sample by Eiden et al. (2015).[21] Hess et al. (2014)[29] on the other hand reported the detection of an aminorex isomer with higher signal intensities compared to aminorex. Unfortunately, no target/qualifier-ratios were given. Additionally, during our method development, it was observed that nicotine shared the same mass transitions. Nicotine differs in its sum formula but exerts the same molecular weight and can therefore not be distinguished by means of low-resolution mass spectrometry (unit mass resolution). Separation was ensured in our study, and different target/qualifier-ratios were observed for nicotine.

Finally, there are two studies using LC-HRMS techniques.[9,10] Hess et al. (2013)[9] used a C18 column and a mobile phase consisting of a mixture of 10mM ammonium formate in water and methanol. Handley et al. (2019)[10] performed chromatography based on a phenyl hexyl column and a mobile phase consisting of a mixture of 10mM aqueous ammonium formate containing 0.1% formic acid and 0.1% formic acid in methanol/acetonitrile (1/1, v/v). Whereas Hess et al. (2013)[9] found aminorex as well as five isomers in urine of a subject who ingested 100 mg levamisole, Handley et al. (2019)[10] neither found aminorex nor any isomers of identical mass in benzoylecgonine-positive urine samples despite of high PTHIT concentrations. We observed in our laboratory that within the LC-QTOF screening analysis 4-phenyl-2-imidazolidinone was falsely identified as aminorex in some cocaine/benzoylecgonine-positive samples by library comparison.

5 | CONCLUSION

4-Phenyl-2-imidazolidinone as well as *p*-hydroxy-PTHIT were found as metabolites after a nasal administration of 10 mg tetramisole. Moreover, both metabolites were quantified for the first time in cocaine/benzoylecgonine-positive serum samples whereas aminorex could never be detected. 4-phenyl-2-imidazolidinones shows a longer half-life than *p*-hydroxy-PTHIT and is more likely to be detected in the late elimination phase. An aminorex detection after consuming cocaine tainted with tetramisole appears to be very unlikely. If it is formed in humans at all, PTHIT-concentrations after cocaine consumption usually not seem to be sufficient to result in detectable amounts of aminorex, since even 4-phenyl-2-imidazolidinone and *p*-hydroxy-PTHIT reach concentrations > 1 ng/ml only in isolated cases. A review of the studies available in literature revealed considerable evidence that 4-phenyl-2-imidazolidinone may be misinterpreted as aminorex. In general, GC-based techniques with trimethylsilylation lack selectivity, whereas LC–MS/MS-based techniques showed a better performance in separating both constitutional isomers. However, a detection of aminorex should never be claimed without ensuring selectivity, given that a reference substance for 4-phenyl-2-imidazolidinone is now available. In addition, without an analytically reliable aminorex detection, the cause of death after the consumption of PTHIT-tainted cocaine should not be explained by the effects of aminorex (e.g. pulmonary hypertension).

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