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RESEARCH ARTICLE

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Chiral pharmacokinetics of tetramisole stereoisomers—Enantioselective quantification of levamisole and dexamisole in serum samples from users of adulterated cocaine

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Abstract

Phenyltetrahydroimidazothiazole (PTHIT, tetramisole) is the most frequently used adulterant of cocaine and exists in the two enantiomeric forms levamsiole (S) and dexamisole (R). Existing studies show diverse fractions of samples containing enantiopure levamsiole, levamisole-enriched mixtures, and racemic tetramisole as adulterant. However, blood samples have never been enantioselectively tested for PTHIT. Because enantiomers are usually metabolized stereoselectively, chiral analysis of blood samples can help estimate the time of drug use, provided that a racemic substance is ingested. Therefore, an enantioselective liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was developed using a chiral column. Validation of the method was carried out for methanolic substance samples as well as serum samples and showed satisfactory selectivity, sensitivity, linearity (0.05–100 ng/mL), precision, and accuracy; 151 cocaine samples seized in Germany between 2018 and 2021 were analyzed. Most (94%, $n = 48$) of the 51 PTHIT-positive samples contained racemic tetramsiole, whereas there were two samples containing levamisole-enriched mixtures and one sample containing nearly enantiopure levamisole. Furthermore, 157 cocaine and/or benzoylecgonine-positive forensic serum samples were tested with cocaine-positive samples showing the highest frequency of PTHIT detection (43%). All positive samples contained either dexamisole alone or (R)/(S)-concentration ratios >1 (1.05–70.6). Finally, a self-administration study was conducted with three subjects taking 10 mg of racemic tetramisole each. Although peak concentrations and corresponding times did not differ significantly between the enantiomers, dexamisole showed significantly longer apparent elimination half-lives (7.02–10.0 h) than levamisole (2.87–4.77 h). The resulting steadily increasing (R)/(S)-ratios can therefore be helpful in estimating the time of cocaine consumption.

KEYWORDS
chiral, enantiomers, forensic toxicology, pharmacokinetics, tetramisole

1 | INTRODUCTION

The anthelmintic drug phenyltetrahydroimidazothiazole (PTHIT, tetramisole) is the most frequently used adulterant of cocaine worldwide.[1,2] Since its first appearance in 2003,[3] its detection frequency increased rapidly over the next decade.[2,4–7] PTHIT possesses one stereocenter and therefore exists in two enantiomeric forms. The (S)-enantiomer is called levamisole, and the (R)-enantiomer is called dexamisole. While levamisole as well as the racemic mixture tetramisole are used for veterinary purposes, dexamisole represents the less potent distomer and is neither commercially available in isolated form nor has it ever been used medicinally.[4,8] Because most publications on these substances do not have a particular focus on stereochemistry, the terms levamisole and tetramisole are unfortunately often used as synonyms.[9]

Cocaine intoxications often play a role in criminal offenses, particularly traffic offenses (driving under the influence of drugs, DUID), and in the assessment of criminal responsibility.[10,11] In blood, cocaine exhibits a relatively short half-life, so that it is particularly difficult to assess the influence at the time of the offense on the basis of toxicological findings in the blood. In this regard, the in vitro degradation occurring between blood collection and analysis poses a further challenge.[12–15]

Existing chiral studies on PTHIT in hair[9] and urine[4] already suggested stereoselective pharmacokinetics. The interpretation of (R)/(S)-ratios has already been proposed for several other drugs to facilitate an assessment of the effect at the time of the offense.[16–18] Particularly when racemic tetramisole is taken, evaluation of the serum concentrations of the enantiomers and the resulting (R)/(S)-ratios could be helpful answering forensic questions. Therefore, it seems pertinent to examine the suitability of levamsiole and dexamisole to serve as surrogate markers for the forensic toxicological interpretation of cocaine intoxications. A few studies examining the PTHIT content of seized cocaine samples are available in the literature, but those that investigated the chiral composition of PTHIT yielded quite diverse results.[4,9,19] Furthermore, in the existing pharmacokinetic studies in human blood, enantiomerically pure levamisole was administered in each case but never tetramisole.[20–22] Forensic blood samples have only been investigated achirally to date.[20,23,24]

In the present study, a chiral liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the quantification of levamisole and dexamisole was developed and validated according to an international guideline. A total of 151 seized drug samples collected by the state police on the territory of Rhineland-Palatinate was tested for the presence of PTHIT enantiomers. Furthermore, a total of 157 forensic blood samples previously tested positive for cocaine or its metabolite benzoylecgonine were analyzed. Finally, chiral pharmacokinetics of tetramisole were investigated by means of a controlled nasal administration study involving 3 volunteers. Particular focus was placed on the forensic interpretation of the (R)/(S)-ratios.

2 | MATERIAL AND METHODS

2.1 | Material

(RS)-Tetramisole and (RS)-tetramisole-d_5 as well as ammonium bicarbonate and ammonia solution (25%, LC–MS-grade) were obtained from Merck (Darmstadt, Germany). Enantiopure levamisole was purchased from Cerilliant (Round Rock, USA). Methanol (LC–MS-grade) was from Honeywell (Seelze, Germay). Water (LC–MS-grade), acetonitrile (LC–MS-grade), isopropanol (LC–MS-grade), dichloromethane, acetone, glacial acetic acid, potassium dihydrogenphosphate, and potassium hydroxide were purchased from Carl Roth (Karlsruhe, Germany). All chemicals were at least of analytical grade. Mixed-mode cation-exchange solid phase extraction (SPE) columns (HF BE-CERTIFY, 300 mg, 3 mL) were purchased from Agilent (Waldbronn, Germany). Blank (drug-free) serum was provided by the blood bank of the University Medical Center of the Johannes Gutenberg University Mainz.

2.2 | Sample preparation

200 µL of serum were spiked with 10 µL internal standard (ISTD) solution (containing 200 ng/mL racemic tetramisole-d_5 in methanol). Samples with concentrations outside of the calibration range were diluted with blank matrix to reach appropriate concentrations and reanalyzed. An SPE method was used for sample extraction because the analytical column proved to be highly susceptible to sample impurities that occur with simpler methods. For protein precipitation, 600 µL acetonitrile were added. After mixing, samples were centrifuged for 10 min at 1200× g. The supernatants were decanted and mixed with 6 mL of phosphate buffer solution (0.1 M, pH 6). Equilibration of the SPE cartridges was performed by applying 2 × 3 mL methanol followed by 2 × 2 mL water. The entire samples were applied and washed with 2 × 2 mL water and 2 × 2 mL water/methanol (80/20, v/v). After addition of 1 mL acetic acid (0.1 M), cartridges were centrifuged for 10 min at 1000× g. Subsequent to flushing with 3 mL dichloromethane/acetone (50/50, v/v), elution was performed using 3 mL dichloromethane/isopropanol/ammonia solution (80/20/4, v/v/v). The extracts were then evaporated to dryness under a gentle stream of nitrogen at 40°C. For analysis, residues were redissolved in 50 µL of methanol.

2.3 | Chiral LC–MS/MS instrumentation and analytical parameters

Analysis was conducted using an LC–MS/MS system from Agilent (Waldbronn, Germany). Chromatography was performed using a 1290 Infinity II LC system, coupled via Jet Stream interface (ESI) to a 6495C triple quadrupole mass spectrometer. Enantiomeric separation was achieved using a polysaccharide based chiral Lux® 3 µm AMP

150 × 3.0 mm analytical column, guarded with a Lux® AMP 4 × 2.0 mm security guard cartridge (both Phenomenex; Aschaffenburg, Germany). The mobile phase consisted of (A) 5 mM ammonium bicarbonate solution adjusted to pH 11 by addition of ammonia solution and (B) methanol/acetonitrile/isopropanol (40/50/10, v/v/v). Column temperature was 30°C, injection volume was 5 µL, and flow rate was 0.4 mL/min. The following elution gradient was used: Starting with 30%, B was increased to 59% within 13.5 min. Subsequently, B was increased to 95% for 2 min for column washing. The column was then re-equilibrated at 30% B for 1 min, followed by a post-time of 2 min (total run time 18.5 min). 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 +3500 V. Analytes were detected in multiple reaction monitoring (MRM) mode using the following transitions (*m/z*, collision energy in parentheses, target ion underlined): tetramisole (both enantiomers) 205.1 → 178.0 (25 eV), 91.0 (45 eV); tetramisole-d_5 (both enantiomers) 210.1 → 183.1 (25 eV), 96.1 (45 eV). For quantification of dexamisole and levamisole, corresponding enantiomers of the internal standard were used. Identification of the elution order was conducted by injection of (nearly) enantiopure levamisole standard solution. For all other experiments, racemic solutions were used as analytical standard. Data evaluation was done using Agilent Mass Hunter Workstation Software (Version B.09.00).

2.4 | Method validation

The method was validated for the analysis of serum samples according to an international guideline.[25] Validation parameters were selectivity, linearity of calibration, analytical limits, accuracy (bias), inter-day precision, recovery, matrix effects, and processed sample stability. Data were statistically evaluated using Valistat 2.00.1 software (Arvecon; Walldorf, Germany). Method selectivity was evaluated by measurement of six drug-free samples (blank samples) and two samples after addition of ISTD-solution (zero samples). Linearity of the calibration was tested by fourfold measurement of a calibration series from 0.05–100 ng/mL per enantiomer. The limit of detection (LOD) was determined by means of signal to noise ratio (both ion transitions S/N > 3), measuring a separate calibration series from 0.025 to 0.25 ng/mL per enantiomer. The lower limit of quantification (LLOQ) was determined by 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 (0.15 ng/mL), medium (1.5 ng/mL), and high concentrations (15 ng/mL, each per enantiomer) relative to the calibration range. Accuracy and inter-day precision were assessed by preparing and analyzing spiked samples of each concentration level in duplicate on eight different days. Acceptance criteria 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.[26] Dilution integrity was validated according to the guideline of the European Medicines Agency (EMA).[27] For this purpose, five samples, each containing 500 ng/mL per enantiomer, were diluted with blank matrix by factor 10 (20 + 180 µL) and measured again. Processed sample stability was evaluated at low, medium, and high concentrations by pooling and subsequently splitting six samples of each concentration, followed by repeated analysis over 68 h. For quantification of seized drug samples, linearity and precision of the method were validated for the assessment of methanolic solutions. Linearity was assessed by measuring a methanolic calibration series from 0.05 to 125 ng/mL per enantiomer. For determination of the methods' precision, two solutions of racemic tetramisole (0.4 and 40 ng/mL per enantiomer) were prepared, processed, and measured tenfold.

2.5 | Investigated collectives

2.5.1 | Seized drug samples

A total of 151 cocaine samples from a total of 99 seizures were investigated for the presence of PTHIT enantiomers. All samples were seized by the state police on the territory of Rhineland-Palatinate, Germany in the years 2018–2021. Determination of the initial sample weight as well as of the relative cocaine content (via HPLC-DAD, method not published) was conducted in the State Office of Criminal Investigation Rhineland-Palatinate, Mainz. The original solutions obtained had cocaine concentrations from 1–100 µg/mL. For preparation of the seized drug samples, 5 µL of the original sample and 5 µL of ISTD solution (containing 10 µg/mL racemic tetramisole-d_5 in methanol) were diluted with 990 µL methanol. Samples outside the calibration range were further diluted and again measured. Solutions were stored at −18°C at all times. Quantification of levamisole and dexamisole contents was carried out using the calibration curve obtained by measuring the methanolic calibration series. The percentage PTHIT content was calculated in relation to the total mass as well as in relation to the cocaine content. (R)/(S)-concentration ratios were calculated as quotient of the calculated dexamisole and levamisole concentrations (c.f. Equation 1).

$$(\mathrm{R})/(\mathrm{S}) - \mathrm{ratio} = \frac{c_{\mathrm{dexamisole}}}{c_{\mathrm{levamisole}}} \quad (1)$$

Equation 1: Calculation of the (R)/(S)-ratios.

2.5.2 | Forensic serum samples

A total of 157 serum samples, that were tested positive for cocaine (>1 ng/mL) and/or benzoylecgonine (>25 ng/mL), were analyzed for the presence of dexamisole and levamisole. Concentrations of cocaine and benzoylecgonine were determined with a fully validated LC–MS/MS method. The samples originated from the years 2020 and 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. All blood samples were collected with fluoride-containing tubes to prevent hydrolysis of cocaine.[13,28] After centrifugation, serum was transferred into NaF-pretreated vessels to reach an NaF fraction of 0.5% and subsequently stored at −20°C. For statistical analysis, the collective was split into groups of samples, that contained cocaine (>1 ng/mL, $n = 111$, group A), samples that contained no cocaine but benzoylecgonine concentratios >75 ng/mL ($n = 21$, group B), and samples that contained benzoylecgonine concentrations of 25–75 ng/mL ($n = 25$, group C). According to the common jurisprudence in Germany, a benzoylecgonine serum concentration of >75 ng/mL may indicate a cocaine consumption that is not necessarily recent but less than 2 days ago.[11]

2.5.3 | Nasal application study

A 10 mg dose of racemic tetramisole hydrochloride was administered nasally using a straw by three male healthy volunteers (27–38 years old, weighting 69–83 kg, body-mass-index 22.3–23.9). In Germany, Ethics Committee approval is not required for scientific self-experiments. For each of the three participants, there was no dependency, neither to the authors nor to the affiliated institutes. Participation had neither positive nor negative consequences for each of the participants. Therefore, the experiment was regarded to be entirely voluntary. Blood samples were collected before the experiment (baseline) and approximately 10, 20, 40, 60, 90 min, 2, 3, 4, 6, 8, 12, and 24 h after administration (exact times were used for further evaluation). Samples were centrifuged immediately and stored at −18°C. Quantitative data were evaluated in terms of peak serum concentrations (c_{max}) and their corresponding time after administration (t_{max}). The apparent elimination half-life ($t_{1/2}$) was estimated from exponential regression of the concentrations from t_{max} to the last sampling time. Furthermore, the (R)/(S)-concentration ratio (quotient of dexamisole and levamisole concentrations) was calculated for every sample according to Equation 1. For all three subjects, linear and exponential regression of the time course of (R)/(S)-ratios was performed.

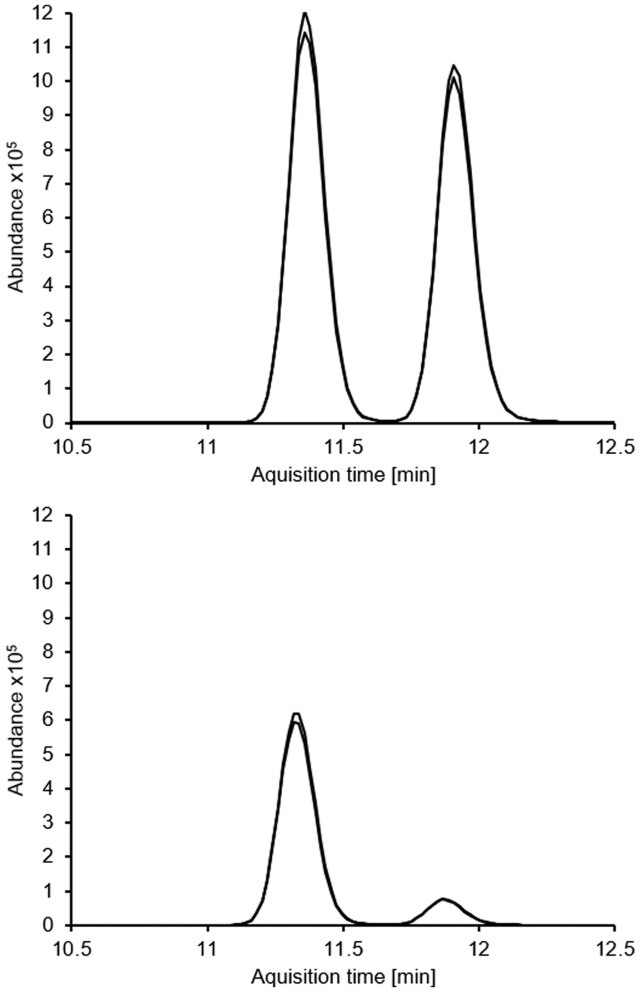

FIGURE 1 Chiral chromatographic separation of dexamisole (left) and levamisole (right). The figure shows multiple reaction monitoring (MRM) chromatograms of samples after nasal application of 10 mg racemic tetramisole hydrochloride (Subject 3) after 37 min (16.2 and 14.3 ng/mL) and after 24 h (7.4 and 0.89 ng/mL). The calculated (R)/(S)-concentration ratios were 1.13 and 8.27, respectively. The following ion transitions are displayed in decreasing order of intensity: 205.1 → 178.0 (target), 91.0 (qualifier)

3 | RESULTS

3.1 | Method validation

Baseline separation was achieved for levamisole (eluting second) and dexamisole, as well as for the respective ISTDs (c.f. Figure 1). The commercially acquired levamisole standard was nearly pure and showed a (R)/(S)-ratio of 0.005. Blank and zero samples showed no interfering signals. A linear calibration model without weighting could be used for quantification. LOD was at 0.025 ng/mL for both enantiomers. The lowest calibration level (0.05 ng/mL) could be established as LLOQ with acceptable results for bias (RSD) and precision (less than −12.3% and 5.2%, respectively). Further results for method validation were also within acceptable limits and are shown in Table 1. Whereas at low and medium concentration levels matrix effects were almost absent, slight ion enhancement was observed at the high level. For both enantiomers, recovery was between 60.7% and 68.4% at low and medium concentration levels, whereas recovery was lower at the high level (47.5%). Recovery for the enantiomers of the ISTD was in the same range (58.1 ± 5.6%). For diluted samples, accuracy (less than 9.7%) and precision (less than 3.0%) were within the EMA guidelines. Processed samples were stable for the observed time period of 68 h, because the decrease in absolute peak areas was less than 20% of the initial value. The calibration series in methanolic solution for quantification of the seized samples showed good linearity for both enantiomers ($R^2 > 0.999$). Calculated (R)/(S)-ratios of the tenfold measured control solutions of racemic tetramisole were between 0.98 and 1.02 (mean and median each 1.00).

TABLE 1 Validation results for the levamisole and dexamisole in serum

Concentration (ng/mL)	Analyte	Accuracy (bias) $n = 8$ (%)	Inter-day precision $n = 8$ (%)	Recovery $n = 6$ (%)	Matrix effects $n = 6$ (%)
0.15	Levamisole	−0.8	4.6	68.4 ± 7.1	103 ± 6.5
0.15	Dexamisole	−0.4	3.7	68.4 ± 7.2	101 ± 6.2
1.5	Levamisole	−0.8	4.8	60.7 ± 11.3	97.8 ± 13.9
1.5	Dexamisole	0.5	2.9	61.0 ± 11.3	96.1 ± 13.6
15	Levamisole	5.1	5.7	47.4 ± 5.5	115 ± 6.8
15	Dexamisole	8.9	3.0	47.5 ± 5.8	112 ± 7.2

3.2 | Seized drug samples

Fifty-one (34%) of the total 151 seized cocaine samples contained at least one of the PTHIT enantiomers. Of the total of 99 seizures, 37 (37%) contained at least one PTHIT-positive sample. In 2019 and 2020, the proportion of seizures containing at least one PTHIT-positive sample was nearly identical at 38% and 37%, respectively. Due to the small sample size, no trend can be derived from the 2018 ($n = 5$, 60% positive) and 2021 ($n = 11$, 27% positive) data. The mass fraction of PTHIT hydrochloride relative to the total mass of the samples ranged from 0.58% to 50.0% (mean 16.5%, median 10.9%). Whereas one sample contained more PTHIT base than cocaine base (152%), the PTHIT/cocaine ratios of the other samples ranged from 1.7 to 96.5%. Mean and median PTHIT/cocaine ratios for the whole collective were 29.4% and 18.0%, respectively. Comparing the 2019 and 2020 samples, there were no significant differences for either the proportion of PTHIT to the total mass (Mann–Whitney *U*-test $p = 0.86$) or the PTHIT/cocaine ratio (Mann–Whitney *U*-test $p = 0.53$). Calculated (R)/(S)-ratios for the precision control samples were between 0.98 and 1.02; 48 of the 51 PTHIT-positive samples showed comparable results with (R)/(S)-ratios close to 1.00 (0.97–1.01). (R)/(S)-Ratios of the other PTHIT-positive samples were 0.39, 0.19, and 0.02. The first two of these samples originated from the same seizure. Figure 2 shows boxplots of the calculated (R)/(S)-ratios for seized drug samples and control samples. The mass fraction of PTHIT hydrochloride relative to the total mass was in the medium range for all three outliers with 19.1%, 11.8%, and 10.4%.

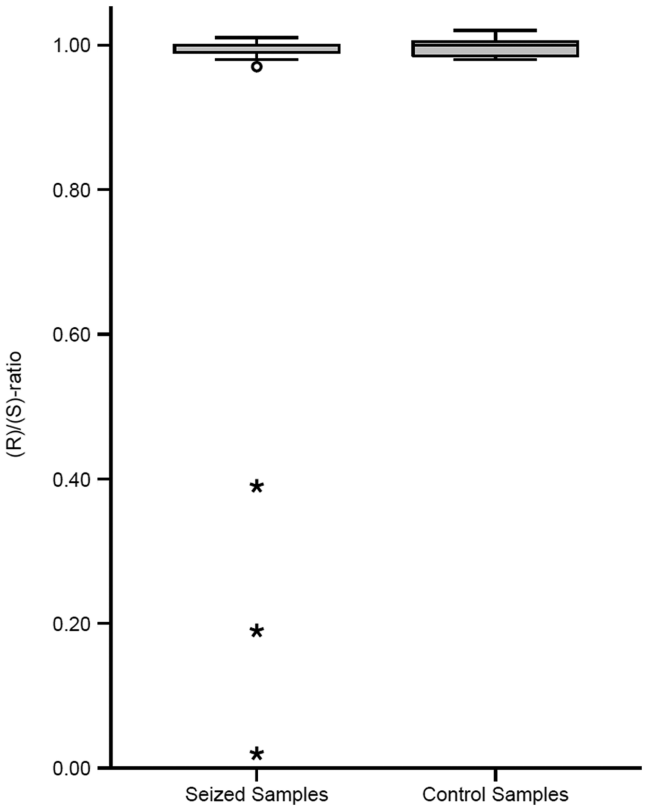

FIGURE 2 Boxplots of the calculated (R)/(S)-ratios for PTHIT-positive seized drug samples ($n = 51$) in comparison with racemic control samples ($n = 20$). Horizontal lines represent the median, and boxes represent the range between lower and upper quartile (interquartile range). Whiskers represent all samples within ±1.5 times the interquartile range. Outliers > median ±1.5 times the interquartile range are shown as circles. Extreme values with > median ±3 times the interquartile range are shown as asterisk

3.3 | Forensic serum samples

Although the LOD of the method was lower, only quantifiable results (>LLOQ) were considered for further evaluation. PTHIT was detected least frequently in group C (64% negative). The proportion of samples containing both dexamisole and levamisole was highest in group A (36%). There were no samples that exclusively contained levamisole. Figure 3 shows the frequency distribution of the detection of dexamisole and levamisole within the respective groups.

Median total PTHIT concentrations were highest in group A (3.90 ng/mL). Lower median concentrations were found in group B (2.60 ng/mL) and group C (1.10 ng/mL). In samples in which only dexamisole was found ($n = 25$, 16%), its concentrations were comparatively low (maximum 1.17 ng/mL, mean 0.16 ng/mL, median 0.10 ng/mL). Among the cases in which both enantiomers were found, the median concentrations for dexamisole and levamisole were highest in group A (3.47 and 0.75 ng/mL, respectively). In this group, also the overall highest concentrations were found with 115 ng/mL for dexamisole and 110 ng/mL for levamisole (same case, cocaine 230 ng/mL, benzoylecgonine 1000 ng/mL). However, comparatively

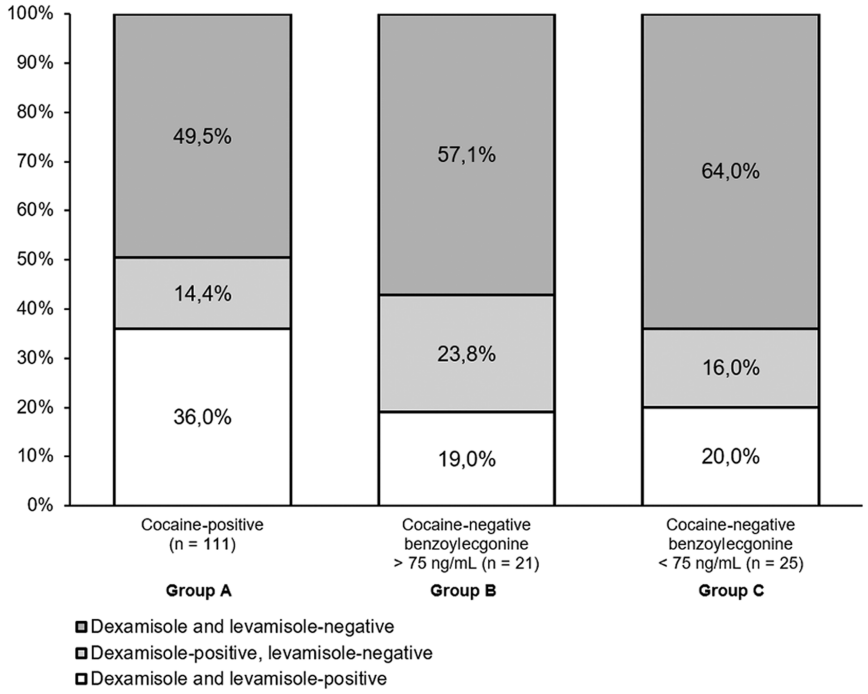

FIGURE 3 Frequency distribution of the detection of dexamisole and levamisole in NaF-serum samples. The collective was split into groups of samples that contained cocaine (>1 ng/mL, group A), samples that contained no cocaine but benzoylecgonine concentrations >75 ng/mL (group B), and samples that only contained benzoylecgonine concentrations of 25–75 ng/mL (group C)

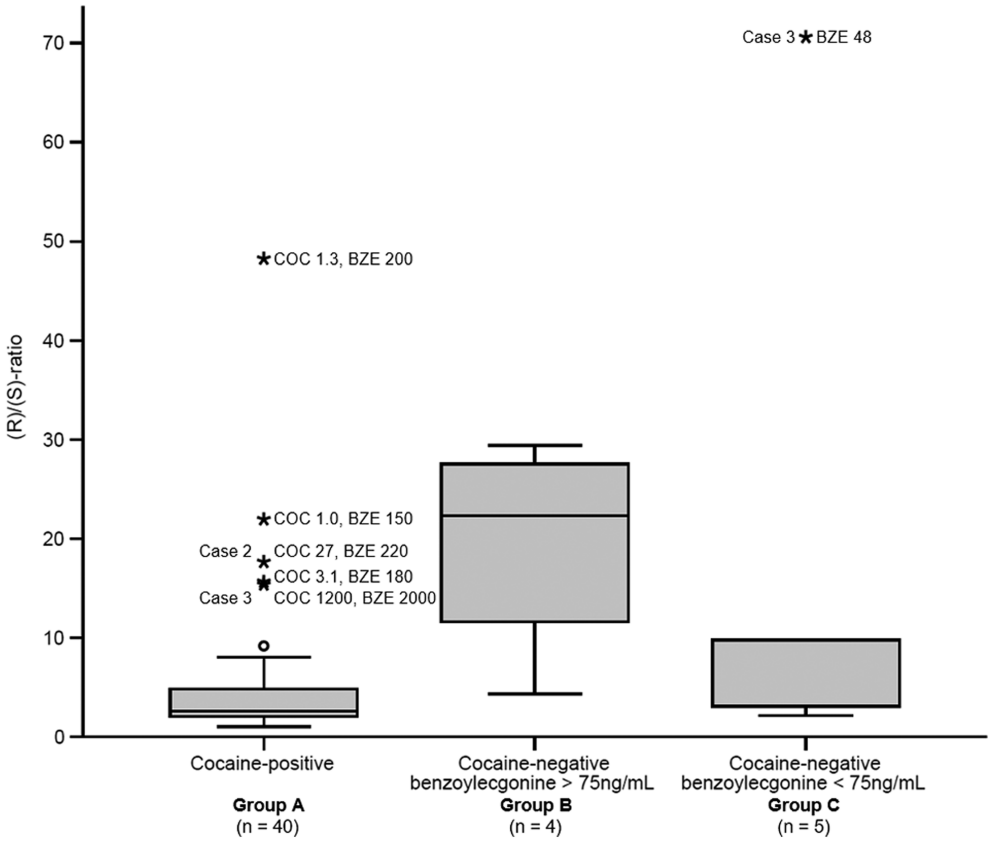

FIGURE 4 Boxplots of the observed (R)/(S)-concentration ratios in NaF-serum samples that contained both dexamisole and levamisole. The collective was split into groups of samples that contained cocaine (>1 ng/mL, group A), samples that contained no cocaine but benzoylecgonine concentrations >75 ng/mL (group B), and samples that only contained benzoylecgonine concentrations of 25–75 ng/mL (group C). Horizontal lines represent the median, and boxes represent the range between lower and upper quartile (interquartile range). Whiskers represent all samples within ±1.5 times the interquartile range. Outliers > median ±1.5 times the interquartile range are shown as circles. Extreme values with >median ±3 times the interquartile range are shown as asterisk. For extreme values, serum concentrations (ng/mL) of cocaine (COC) and benzoylecgonine (BZE) are given

high concentrations of PTHIT were also found in groups B (9.5 and 0.37 ng/mL) and C (41 and 4.2 ng/mL).

Figure 4 shows boxplots of the calculated (R)/(S)-concentration ratios for all three groups. For better comparison, statistical data are additionally presented in Table 2. All calculated ratios were between 1.05 and 70.6 (mean 8.18, median 3.09). The highest (R)/(S)-ratio (dexamisole 5.16 ng/mL vs. levamisole 0.07 ng/mL) was found in a case with only minor concentrations of benzoylecgonine (48 ng/mL,

TABLE 2 Statistical data for the observed (R)/(S)-concentration ratios in NaF-serum samples that contained both dexamisole and levamisole

	Group A Cocaine-positive $n = 40$	Group B Cocaine-negative benzoylecgonine > 75 ng/mL $n = 4$	Group C Cocaine-negative benzoylecgonine 25–75 ng/mL $n = 5$
Median	2.62	18.2	9.76
Mean	5.68	16.6	16.1
Minimum	1.05	4.36	2.16
Maximum	48.3	25.7	70.6

Note: The collective was split into groups of samples that contained cocaine (>1 ng/mL, group A), samples that contained no cocaine but benzoylecgonine concentrations >75 ng/mL (group B), and samples that only contained benzoylecgonine concentrations of 25–75 ng/mL (group C).

case 1). However high (R)/(S)-ratios (17.7 and 15.4, respectively) were also found in combination with higher (27 ng/mL) and very high (1200 ng/mL) cocaine concentrations (cases 2 and 3, respectively). Nevertheless, median (R)/(S)-ratios were lowest within group A (2.26). The difference was significant compared with group B (median 18.2, Mann–Whitney *U*-test $p < 0.005$) but not to group C (median 9.76, $p = 0.11$). Also, no significant differences were found comparing the (R)/(S)-ratios of groups B and C ($p = 0.41$).

3.4 | Nasal application study

After consumption of tetramisole, all subjects experienced a burning sensation in the nose and a bitter taste in the throat. Apart from that, no physical effects were noticeable. Dexamisole and levamisole serum concentration-time curves for all three subjects are shown in Figure 5. Calculated pharmacokinetic parameters are presented in Table 3. Baseline samples were negative for all subjects. Two samples from Subject 2 (65 min and 87 min) showed markedly lower concentrations for both dexamisole and levamisole than the samples before and after. Therefore, the pharmacokinetic parameters for Subject 2 should be considered with caution. While t_{max} of dexamisole and levamisole did not differ for Subjects 1 and 2, the time to reach maximum serum concentration of dexamisole was about twice as long (1.78 vs. 4.03 h) for Subject 3. Whereas serum concentrations of the enantiomers increased at approximately the same rate in the first hour after administration, c_{max} of dexamisole was higher for all subjects (mean 28.2 vs. 18.3 ng/mL, 139–174%). Dexamisole and levamisole concentrations in the last sample drawn (after 24 h) were 2.57 and 0.23 ng/mL for Subject 2 and 7.39 and 0.89 ng/mL for Subject 3. Unfortunately, for Subject 1, sampling was not possible on the next day, so that the last sample was collected after 8 h. For all three subjects, apparent elimination half-lives for dexamisole were about twice as long (mean 8.2 vs. 3.9 h, 180–245%) as for levamisole.

Calculated (R)/(S)-concentration ratios were continuously increasing with time for each subject. In the first sample drawn (10–14 min), concentrations of both enantiomers were close to equal (ratios 1.03–1.16). Within the first 3 h, (R)/(S)-ratios for all three subjects increased

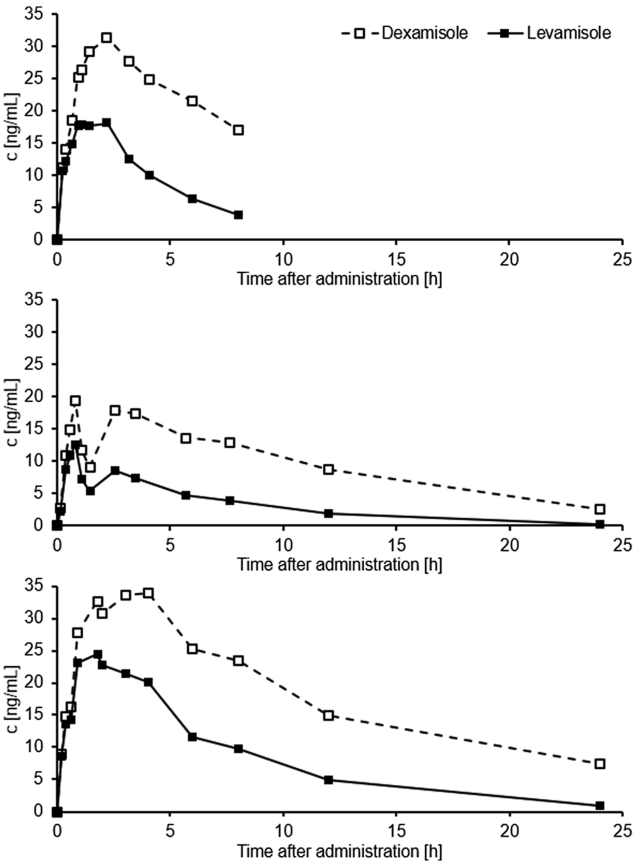

FIGURE 5 Levamisole (■) and dexamisole (□) serum concentration-time curves after nasal administration of 10 mg racemic tetramisole hydrochloride for Subject 1 (170 cm, 69 kg, top), Subject 2 (189 cm, 83 kg, middle), and subject 3 (187 cm, 78 kg, bottom). Two early samples from Subject 2 may be diluted inadvertently with NaCl solution of the attached infusion during blood collection. For Subject 1, the last sampling time was 8 h after administration

almost linearly ($R^2 > 0.93$ for each subject), reaching ratios of 1.57–2.07 after approximately 3 h. Calculated slopes were 0.18, 0.36, and 0.37 h (c.f. Figure 6). For sampling times > 3 h, an exponential fit showed better correlation ($R^2 > 0.95$ for each subject, c.f. Figure 7). After 24 h, (R)/(S)-ratios were 11.0 for Subject 2 and 8.3 for Subject

3. Ratios increased most rapidly for Subject 1, reaching 4.4 in the last sample collected (8 h after administration).

4 | DISCUSSION

In the present study, the chiral composition of PTHIT in seized cocaine samples as well as the chiral pharmacokinetics of PTHIT in serum samples was observed. The developed sensitive and selective chiral LC–MS/MS method fulfilled all validation criteria and was successfully applied to all sample collectives. To the best of our knowledge, this is the first study to enantioselectively detect levamisole and dexamisole in human serum samples.

TABLE 3 Pharmacokinetic properties of levamisole and dexamisole 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	Levamisole	18.1	2.22	2.87	3.87
2		12.6*	0.80*	4.15*	0.23
3		24.4	1.78	4.77	0.89
1	Dexamisole	31.3	2.22	7.02	17.0
2		19.4*	0.80*	7.47*	2.58
3		33.9	4.03	10.0	7.39

Note: Maximum serum concentrations observed (c_{max}) and corresponding times (t_{max}) are given. Apparent elimination half-life ($t_{1/2}$) was calculated using exponential regression. Serum concentrations at the last sampling point (after 8 h for Subject 1 and after 24 h for Subjects 2 and 3) are also given (c_{end}). For Subject 2, two samples (65 and 87 min) were presumably diluted inadvertently, which may have affected the determination of the pharmacokinetic parameters (marked with asterisk).

4.1 | Seized drug samples

In this study, 34% of the seized cocaine samples contained PTHIT. This is in line with studies from other European countries from the years 2012 to 2017 that found proportions in the range of 30–40%.[29–31] However, there are other European studies reporting higher detection frequencies of 64%[32] and 85%.[6] High fractions of PTHIT-adulterated cocaine were also found in Brazil (56%)[33] and the United States (78%[34] and 79%[4]). While PTHIT fractions in seized cocaine were increasing in the early 2000s,[4–7] the presented results suggest a stagnant trend. Other studies confirm both the variable PTHIT/cocaine-ratios[4,29,33] as well as the variable total mass fraction of PTHIT hydrochloride[33,34] found in this study. Also, the presence of a sample containing more PTHIT than cocaine is consistent with some individual findings in the literature.[4,32] Regarding the mean mass fraction of PTHIT in the total sample of 16.5% found in this study, partially similar (17.8%)[34] but also lower (1.5–7.5%)[6,33] results can be found in the literature. The mean PTHIT/cocaine ratio of 29.4% found here is in the same range as observed by Casale et al. (23%)[4] but lower than observed by Hofmaier et al. (59%)[32] and higher than observed by Lapachinske et al. (13%).[33] However, the comparison of mean values is only of limited significance considering the asymmetrical distribution of the results.

The calculated (R)/(S)-ratios for 48 of the 51 PTHIT-positive samples were between 0.97 and 1.01. Because this is nearly the same range as obtained for the control samples (0.98–1.02), these samples can be considered racemic. The proportion of dexamisole in the

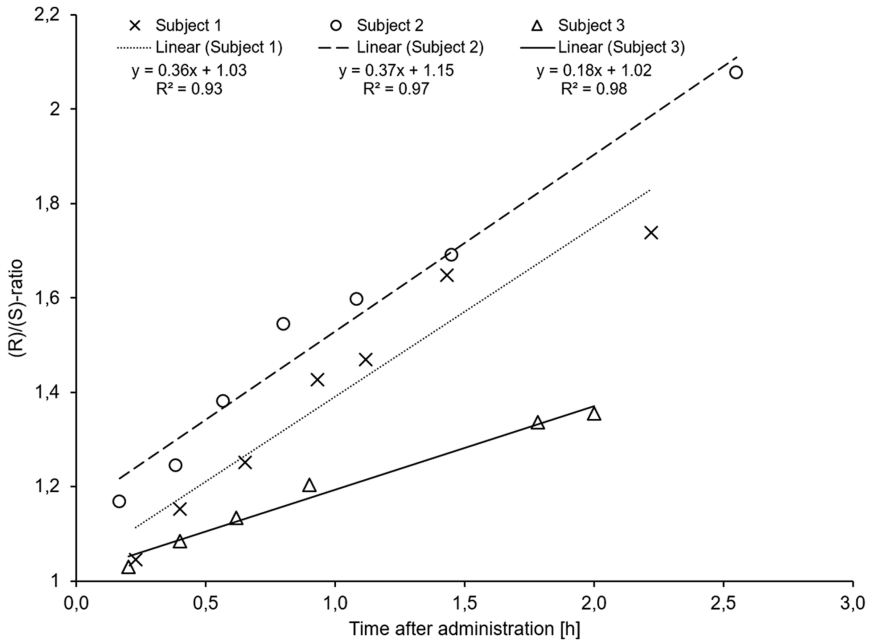

FIGURE 6 Time course of the calculated PTHIT (R)/(S)-ratios within the first 3 h after nasal administration of racemic tetramisole (6–7 time points). Linear regression was carried out for all three subjects and showed good correlation (R^2 > 0.93 for each subject)

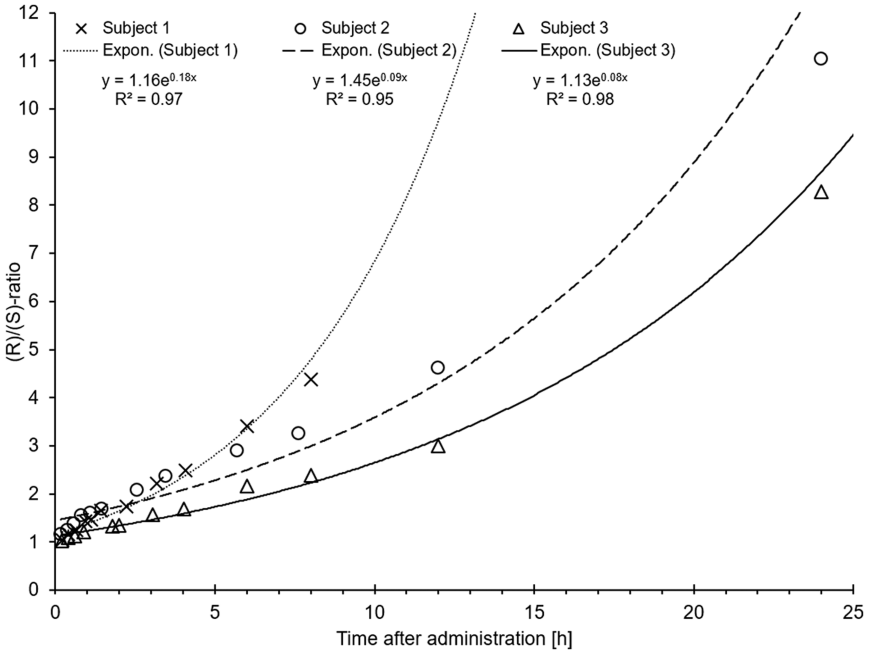

FIGURE 7 Time course of the calculated PTHIT (R)/(S)-ratios over the whole time period observed. For Subject 1, the last sampling time was 8 h after nasal administration of racemic tetramisole. Exponential regression showed good correlation (R^2 > 0.95 for each subject) for all three subjects

sample that contained almost exclusively levamisole ((R)/(S)-ratio 0.02) was greater than the dexamisole contamination in the analytical levamisole standard ((R)/(S)-ratio 0.005). Thus, either the levamisole used for adulteration was of lower purity, as proposed by Casale et al.[4] or the sample was cross-contaminated with other cocaine samples during manufacture or trafficking. Moreover, the contamination of the levamisole analytical standard demonstrates once again that for chiral analysis, quantification should always be done using racemic standards. The other two non-racemic samples ((R)/(S)-ratios 0.19 and 0.39) were likely originally adulterated with one compound only and later further adulterated with the other adulterant or blended with other cocaine batches.

In only a few studies, the enantiomeric composition of PTHIT in seized cocaine samples was determined.[4,9,19] As in this study, no samples containing pure dexamisole or enantiomeric mixtures with an excess of dexamisole were found in any of these studies.[4,9,19] This would also not be expected, because PTHIT is commercially available either as racemic tetramisole or as enantiomerically pure levamisole.[4] The relative proportion of samples adulterated with racemic tetramisole observed here (94%) is close to the proportion observed in Switzerland by Madry et al. (88%, 2013–2016, $n = 24$)[9] but higher than observed in the United States by Casale et al. (19%, 2010, $n = 752$).[4] In a total of four cocaine samples seized in Italy, Bertucci et al. found levamisole and tetramisole two times each.[19] As seen in this study, Madry et al. also found one sample containing pure levamisole and two non-racemic samples with excess of levamisole. Contrastingly, in the United States, Casale et al. found mainly enantiopure levamisole as adulterant (66%) and more samples containing non-racemic mixtures with excess of levamisole (15%). Accordingly, there may have been a shift towards racemic adulteration in the last 10 years, or the differences may be explained by varying supply chains.

4.2 | Forensic serum samples

Approximately half of the samples of group A were positive for PTHIT. The detection frequency in blood samples is therefore slightly higher than in the seized drug samples. Because PTHIT was not present in all samples, its absence does not allow any conclusions to be drawn about the presence, amount, or frequency of cocaine use.

The small sample size of groups B and C makes comparison difficult. The lower frequency of PTHIT detection and lower median concentrations could be explained by advanced excretion, which is to be assumed in the absence of cocaine. In another study from Germany (2011–2012), detection frequency in cocaine-positive serum samples was higher, with 45 of 106 (43%) containing PTHIT.[20] With 224 ng/mL, the maximum concentration detected there was nearly identical with that of the present study (225 ng/mL). Maximum concentrations detected in cocaine-positive serum samples by Eiden et al. (112 ng/mL)[23] and benzoylecgonine-positive plasma samples by Handley et al. (64 ng/mL)[24] were significantly lower, although only eight cases were observed each. Due to the different LOQs in the other studies, a comparison of the mean and median values is not feasible. In general, the wide variation of PTHIT concentrations in blood can be explained by the different proportions of PTHIT in cocaine. Very high serum concentrations of PTHIT were probably caused by accumulation from multiple use (binge), which is common for cocaine.[35,36]

In all PTHIT-positive serum samples, either dexamisole alone or higher concentrations of dexamisole were found ((R)/(S)-ratio >1). Considering the fact that the cocaine seizures either contained racemic tetramisole, or a higher proportion of levamisole, it can be assumed that levamisole is eliminated more rapidly. An inversion of the configuration in vivo (as observed, e.g., for ibuprofen[37]) is not reported for PTHIT. Although there was one seized cocaine specimen

that contained almost enantiopure levamisole, none of the serum specimens tested contained levamisole alone.

The detection of cocaine in serum samples indicates a recent consumption.[38] It is therefore consistent that the lowest median (R)/(S)-ratios were found group A. Also, the presence of the maximum (R)/(S)-ratio (70.6) in group C can be explained in this way. Furthermore, samples containing only dexamisole exhibited low concentrations (<1.17 ng/mL), which also indicates advanced excretion. Individual findings of high (R)/(S)-ratios along with high cocaine concentrations (cases 2 and 3) can probably be explained by an accumulation of dexamisole upon binge consumption.

4.3 | Nasal application study

The administered quantity was based on the assumption of the intake of a rather high cocaine dose with an absolute quantity of 50 mg,[39] which was adulterated with tetramisole by 20%. Nasal application is by far the most frequently used route of administration.[40] Significant differences between the enantiomers were already evident in the absorption phase. In contrast to the other subjects, Subject 3 showed almost constant serum concentrations for dexamisole over a period of 1.8–4.0 h. This also explains the significantly longer t_{max} of dexamisole observed for this subject. For all subjects, c_{max} of dexamisole was higher than that of levamisole. Although the concentrations of dexamisole and levamisole were approximately equal in the first sample drawn, the effects of the relatively short half-lives are thus already apparent during the absorption phase. A similar effect could be observed for MDMA (3,4-methylendioxy-*N*-methylamphetamine) in several studies.[16,41,42] After oral intake of racemic MDMA, both t_{max} and c_{max} of the enantiomers showed significant differences. However, in studies with oral intake of amphetamine and 4-fluoroamphetamine (4-FA), c_{max} and t_{max} of the enantiomers were identical.[17,43] Observed c_{max} after controlled nasal administration was far below the maximum concentrations observed in the forensic serum samples. Because an already relatively high dosage with a relatively high percentage of adulteration was assumed for this study, this suggests that the high concentrations found in the forensic samples were probably caused by binge consumption. Intake of either a very high single dose or a highly adulterated dose could also explain this observation. However, mean and median concentrations of PTHIT detected in group A were far below that observed in this experiment. This suggests that many blood samples were taken at later time points after administration or that either the cocaine dosage or the proportion of adulteration was mainly lower than assumed for our experiment. As already derived from the forensic serum collective, levamisole exhibited a shorter apparent elimination half-life than dexamisole. Because differences in the elimination of enantiomers are the rule rather than the exception,[44] this was to be expected. As observed for the (S)-configurated levamisole, also (S)-enantiomers of MDMA,[16,41] amphetamine,[43] 4-FA,[17] and methamphetamine[45] show significantly shorter elimination half-lives than their corresponding (R)-enantiomers. The observed apparent elimination half-lives for levamisole (2.87–4.77 h) are in line with other studies with controlled (oral) intake of the single enantiomer. Luyckx et al. found mean plasma half-lives between 3.3 and 5.1 h in healthy subjects and cancer patients depending on the dose (2.5 and 5 mg/kg).[21] In healthy subjects, mean half-lives ranged from 2.7 to 6.0 h. Kouassi et al. found plasma half-lives of 5.6 ± 2.5 h in healthy subjects (150 mg oral).[22] Hess et al. found a half-live of 2.0 h for one subject after oral ingestion of 100 mg levamisole.[20] Pharmacokinetic data after human intake of tetramisole or dexamisole are not available in the literature.

As already observed with the forensic serum samples, also in this experiment, higher concentrations of dexamisole than of levamisole are present at all time points. This is in contrast to observations for amphetamine[43,46] and 4-FA,[17] where (R)/(S)-ratios <1 were observed in the absorption phase. On the other hand, for MDMA, (R)/(S)-ratios were >1 at any time as well.[16,41,42] Due to the longer half-life of dexamisole compared with levamisole (180–245%), calculated (R)/(S)-ratios increase steadily. The two samples from Subject 2, which showed significantly lower absolute concentrations than the samples before and after, however, fitted into the continuously increasing (R)/(S)-ratios. Because (R)/(S)-ratios are inherently not affected by dilution, the samples may be diluted inadvertently with NaCl solution of the attached infusion during blood collection. Therefore, the pharmacokinetic parameters of Subject 2 must be considered with caution. In particular, the observed early t_{max} was probably caused by this. Due to the considerable differences between the half-lives of the enantiomers, very high (R)/(S)-ratios were obtained in individual forensic cases (maximum 70.6). The maximum detectable ratio is naturally highly dependent on the LLOQ of the method.

For modeling the course of the (R)/(S)-ratios in the first 2 h, a linear fit can be applied (c.f. Figure 6). Under the assumption of first-order elimination kinetics, an exponential fit must be applied for longer observation periods (c.f. Figure 7). While Subject 2 shows higher (R)/(S)-ratios than the other two subjects within the first 4 h, Subject 1 shows a more rapid increase in (R)/(S)-ratios due to the greater relative differences in half-lives. Time course of (R)/(S)-ratios was suggested for estimating the time of drug consumption.[16–18] In contrast to the previous approaches, in which the drug itself was used to estimate the time of consumption, the idea here is to use PTHIT as a surrogate for the evaluation of acute stimulant effects after a simultaneous cocaine consumption. Therefore, the suitability of PTHIT as a surrogate marker for the evaluation of acute stimulant effects after cocaine consumption is examined. Due to the predominance of samples containing racemic tetramisole, it is reasonable to conclude on the time of cocaine consumption using (R)/(S)-ratios of PTHIT. The acute stimulant effect after cocaine use usually lasts no longer than 2 h.[47–49] Subject 2, which exhibited the higher (R)/(S)-ratios than the other subjects during the first hours, has a calculated (R)/(S)-ratio of 1.90 after 2 h. Accordingly, (R)/(S)-ratios >2.0 were not detected within the first 2 h after intake within the three participants. It could be suggested that an (R)/(S)-ratio above this value indicates the absence of acute effects. However, to use a cutoff like this in court decisions, more data with a higher number of participants using different doses should be recorded. Due to interindividual differences, this

approach can only be used to exclude but not to prove an acute effect. In the present study, for example, Subject 2 exhibited an (R)/(S)-ratio of 1.90 after 2 h, whereas Subject 3 exceeds such an (R)/(S)-ratio only after 5 h. Based on the little data presented here, it cannot be excluded that there may well be even greater interindividual variations in time course of (R)/(S)-ratios. Obviously, this kind of argumentation is only possible if PTHIT is present in the blood. Because this was not the case in about two-thirds of the samples, this option becomes redundant in the most cases. In one of the seized drug samples, almost pure levamisole was found as adulterant. In the case of consumption of cocaine adulterated with pure levamisole, an assessment is also not possible. On the other hand, consumption of a quantities adulterated with an excess levamisole, as found in two of the examined cocaine samples, would probably lead to an underestimation of the time interval to consumption. In contrast, binge use would result in overestimation of the last consumption interval due to accumulation of dexamisole. Furthermore, it is possible that in the context of binge use, first adulterated and afterwards pure cocaine is consumed. This would also explain case 3 in which extremely high cocaine and benzoylecgonine concentrations were found (1200 and 2000 ng/mL) despite relatively low total concentrations (1.42 and 0.09 for dexamisole and levamisole, respectively) and a high (R)/(S)-ratio of 15.4 (c.f. Figure 4).

5 | CONCLUSION

The present study showed the presence of PTHIT in about one-third of the seized drug samples and in approximately the same amount of cocaine-positive serum samples. With three exceptions, the substance samples all contained racemic tetramisole. All forensic serum samples contained higher concentrations of dexamisole than of levamisole. Also, after controlled nasal administration of racemic tetramisole, an excess of dexamisole was found in all serum samples. There, calculated (R)/(S)-concentration ratios were steadily increasing for all participants. Under certain conditions, (R)/(S)-ratios can be used as surrogate markers for the assessment of an influence by cocaine.

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