

Climbazole – Determination of dihydroxy-climbazole and carboxyhydroxyclimbazole in urine by UPLC-MS/MS

Biomonitoring Method – Translation of the German version from 2026

Keywords

climbazole; biomonitoring; urine; UPLC-MS/MS

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Citation Note:

Küpper K, Schmidtkunz C, Schönraht I, Gries W, Leng G, Scherer G, Scherer M, Riedel K, Göen T, Hartwig A, MAK Commission.

Climbazole – Determination of dihydroxyclimbazole and carboxyhydroxyclimbazole in urine by UPLC-MS/MS. Biomonitoring Method – Translation of the German version from 2026. MAK Collect Occup Health Saf. 2026 Sep;11(3):Doc058. https://doi.org/10.34865/bi3808317e11_3or

Manuscript completed:
16 Nov 2023

Publication date:
30 Sep 2026

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Abstract

The working group “Analyses in Biological Materials” of the German Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area (MAK Commission) developed and verified this analytical method for the determination of two important urinary metabolites of climbazole – dihydroxy-climbazole ((OH)₂-climbazole) and carboxyhydroxyclimbazole (cx-OH-climbazole). For the quantitation of the analytes, enzymatic hydrolysis of the urine sample is followed by online SPE-UPLC-MS/MS. The two diastereomers of (OH)₂-climbazole are resolved chromatographically, and thus quantified separately. On the other hand, the diastereomers of cx-OH-climbazole coelute in one single peak. Calibration is performed using calibration standards prepared in pooled urine and processed analogously to the samples to be analysed. (OH)₂-climbazole-D₆ and cx-OH-climbazole-D₆ are applied as internal standards. The method provides reliable and accurate analytical results, as shown by the good precision data with standard deviations no greater than 9.2%. Good accuracy data were obtained with a mean relative recovery in the range of 96.9–102%. The method is both selective and sensitive, and provides a quantitation limit of 0.5 µg/l for (OH)₂-climbazole (per diastereomer) and cx-OH-climbazole (sum of diastereomers), respectively. The method enables the determination of occupational and environmental exposure to climbazole.

1 Characteristics of the method

Matrix and analytical principle

Matrix	Urine
Analytical principle	Ultra-high-performance liquid chromatography with tandem mass spectrometry (UPLC-MS/MS)

Parameters and corresponding hazardous substance

Hazardous substance	CAS No.	Parameter	CAS No.
1-(4-Chlorophenoxy)-1-(imidazol-1-yl)-3,3-dimethylbutan-2-one (climbazole)	38083-17-9	4-(4-Chlorophenoxy)-3-hydroxy-4-(1 <i>H</i> -imidazol-1-yl)-2,2-dimethylbutanoic acid (carboxyhydroxyclimbazole; cx-OH-climbazole)	3029805-89-5
		4-(4-Chlorophenoxy)-4-(1 <i>H</i> -imidazol-1-yl)-2,2-dimethylbutane-1,3-diol (dihydroxyclimbazole; (OH) ₂ -climbazole)	3029805-86-2

Reliability criteria

(OH)₂-Climbazole – Diastereomer 1

The exact configuration of Diastereomer 1 is unknown; see also [Section 4.2](#).

Within-day precision:	Standard deviation (rel.)	$s_w = 9.2\%, 7.2\%, \text{ or } 6.4\%$
	Prognostic range	$u = 21.8\%, 16.9\%, \text{ or } 15.1\%$
	at a spiked concentration of 1 µg, 10 µg, or 100 µg (OH) ₂ -climbazole (Diastereomer 1) per litre of urine and n = 8 determinations	
Day-to-day precision:	Standard deviation (rel.)	$s_w = 6.0\%, 3.6\%, \text{ or } 4.2\%$
	Prognostic range	$u = 15.5\%, 9.3\%, \text{ or } 10.8\%$
	at a spiked concentration of 1 µg, 10 µg, or 100 µg (OH) ₂ -climbazole (Diastereomer 1) per litre of urine and n = 6 determinations	
Accuracy:	Recovery (rel.)	$r = 101\%, 102\%, \text{ or } 98.2\%$
	at a spiked concentration of 1 µg, 10 µg, or 100 µg (OH) ₂ -climbazole (Diastereomer 1) per litre of urine and n = 48 determinations	
Limit of detection:	0.2 µg (OH) ₂ -climbazole (Diastereomer 1) per litre of urine	
Limit of quantitation:	0.5 µg (OH) ₂ -climbazole (Diastereomer 1) per litre of urine	

(OH)₂-Climbazole – Diastereomer 2

The exact configuration of Diastereomer 2 is unknown; see also [Section 4.2](#).

Within-day precision:	Standard deviation (rel.)	$s_w = 4.9\%, 3.6\%, \text{ or } 3.5\%$
	Prognostic range	$u = 11.6\%, 8.6\%, \text{ or } 8.3\%$
	at a spiked concentration of 1 µg, 10 µg, or 100 µg (OH) ₂ -climbazole (Diastereomer 2) per litre of urine and n = 8 determinations	

Day-to-day precision:	Standard deviation (rel.)	$s_w = 3.0\%$, 2.5%, or 2.9%
	Prognostic range	$u = 7.8\%$, 6.5%, or 7.5%
	at a spiked concentration of 1 µg, 10 µg, or 100 µg (OH) ₂ -climbazole (Diastereomer 2) per litre of urine and n = 6 determinations	
Accuracy:	Recovery (rel.)	$r = 99.0\%$, 102%, or 96.9%
	at a spiked concentration of 1 µg, 10 µg, or 100 µg (OH) ₂ -climbazole (Diastereomer 2) per litre of urine and n = 48 determinations	
Limit of detection:	0.2 µg (OH) ₂ -climbazole (Diastereomer 2) per litre of urine	
Limit of quantitation:	0.5 µg (OH) ₂ -climbazole (Diastereomer 2) per litre of urine	

cx-OH-Climbazole

Within-day precision:	Standard deviation (rel.)	$s_w = 7.2\%$, 7.6%, or 2.5%
	Prognostic range	$u = 17.0\%$, 18.0%, or 5.8%
	at a spiked concentration of 1 µg, 10 µg, or 100 µg cx-OH-climbazole per litre of urine and n = 8 determinations	
Day-to-day precision:	Standard deviation (rel.)	$s_w = 3.5\%$, 2.5%, or 3.5%
	Prognostic range	$u = 9.0\%$, 6.5%, or 8.9%
	at a spiked concentration of 1 µg, 10 µg, or 100 µg cx-OH-climbazole per litre of urine and n = 6 determinations	
Accuracy:	Recovery (rel.)	$r = 99.3\%$, 102%, or 99.9%
	at a spiked concentration of 1 µg, 10 µg, or 100 µg cx-OH-climbazole per litre of urine and n = 48 determinations	
Limit of detection:	0.2 µg cx-OH-climbazole per litre of urine	
Limit of quantitation:	0.5 µg cx-OH-climbazole per litre of urine	

2 General information on climbazole

Climbazole (1-(4-chlorophenoxy)-1-(imidazol-1-yl)-3,3-dimethylbutan-2-one) is a white to light-brown, crystalline solid with a melting point of 96.6 °C. At 25 °C, the water solubility of the substance is 58 mg/l (pH 7.4) and its octanol-water partition coefficient is 3.83 (pH 10), whereby both factors are largely dependent on the pH value (ECHA 2024). Due to its antifungal effects, climbazole is used as a preservative in cosmetics (European Parliament and European Council 2009), in veterinary products (Cavana et al. 2015 a, b), and in finger paints (DIN 2025). It is further applied as an active substance in anti-dandruff shampoos (European Parliament and European Council 2009) and as an antimycotic in human pharmaceuticals and veterinary products (Cavana et al. 2015 a, b; Difonzo et al. 2017).

Since climbazole acts as an endocrine disruptor in the environment, its use in cosmetic products is restricted within the EU (ECHA 2024; SCCP 2009; SCCS 2018). As a result, hair tonics, face creams, and foot-care products are only allowed to contain up to 0.2% climbazole, whereas a climbazole content of 0.5% is permitted in shampoos; in both cases, climbazole is applied as a preservative. In anti-dandruff shampoos, however, up to 2.0% climbazole may be added as an active substance (European Commission 2019; European Parliament and European Council 2009). Climbazole concentrations in veterinary products are based on values established by the Scientific Committee on Consumer Safety (Cavana et al. 2015 a, b; SCCS 2018). Climbazole has not yet been evaluated by the MAK Commission.

For a long time, only climbazole studies from the 1970s and 1980s were available (SCCP 2005). These studies investigated the toxicity of climbazole as well as both metabolism and excretion after dermal and oral climbazole exposure. The reliability and validity of these studies are, however, limited, as they were carried out before the introduction of GLP (good

laboratory practice) and were based on analytical methods now considered obsolete, such as thin-layer chromatography with densitometry.

The oral administration of climbazole was investigated *in vivo* in mice, rats, dogs, and humans (Bayer AG 1976 (Ref. 67), 1977 (Ref. 18), 1979 (Ref. 25), 1980 (Ref. 22) as cited in SCCP 2005; Schönrath et al. 2024 a, 2025). At several points in time after oral administration, climbazole and its metabolite hydroxycimbazole (1-(4-chlorophenoxy)-1-(1-imidazolyl)-3,3-dimethyl-2-butanol; OH-climbazole; see Figure 1) were determined in the plasma of the laboratory animals (Bayer AG 1976 (Ref. 67), 1977 (Ref. 18), 1980 (Ref. 22) as cited in SCCP 2005). The studies showed that orally ingested climbazole is rapidly absorbed via the gastrointestinal tract and is then metabolised to OH-climbazole. In the human study conducted by Bayer AG (n = 1, 2 mg climbazole/kg body weight; Bayer AG 1979 (Ref. 25) as cited in SCCP 2005), climbazole and OH-climbazole were quantified in both plasma and urine over a period of 24 hours after oral ingestion. The amount of climbazole and OH-climbazole excreted with the urine within 24 h was found to be only a fraction (0.05%) of the administered dose (Bayer AG 1979 (Ref. 25) as cited in SCCP 2005).

Climbazole metabolism after oral administration was once again investigated as part of the BMU-VCI initiative, whereby dihydroxycimbazole (4-(4-chlorophenoxy)-4-(1*H*-imidazol-1-yl)-2,2-dimethylbutane-1,3-diol; (OH)₂-climbazole) and carboxyhydroxycimbazole (4-(4-chlorophenoxy)-3-hydroxy-4-(1*H*-imidazol-1-yl)-2,2-dimethylbutanoic acid; cx-OH-climbazole) were used as biomarkers. These biomarkers, which had never before been described, were postulated based on the already established metabolic pathway of the structurally related triadimefon (Schmidt-kunz et al. 2021). Five subjects ingested climbazole as a single oral dose (49–77 µg climbazole/kg body weight); subsequently, (OH)₂-climbazole and cx-OH-climbazole were quantified in urine for a period of 48 hours following oral administration. This study found that, within 48 hours, 23.9% (18.0–33.4%) of the oral climbazole dose was excreted with the urine in the form of (OH)₂-climbazole and cx-OH-climbazole (Schönrath et al. 2024 a, 2025). The metabolite ratio of (OH)₂-climbazole to cx-OH-climbazole was found to be about 2 : 1, and the mean half-lives of biphasic elimination were 2.63 h and 10.69 h for (OH)₂-climbazole as well as 2.57 h and 12.17 h for cx-OH-climbazole (Schönrath et al. 2024 a).

With regard to dermal absorption, current *in-vitro* and *in-vivo* studies (Champmartin et al. 2025; Schönrath et al. 2024 a, 2025) are available in addition to *in-vivo* studies from the 1970s and 1980s (Bayer AG 1979 (Ref. 21), 1979 (Ref. 23), 1979 (Ref. 68), 1980 (Ref. 20) as cited in SCCP 2005). A comparative overview of results from diffusion-cell experiments conducted from 2006 to 2025 was published in Champmartin et al. (2025). Of the studies listed by Champmartin et al. (2025), the Proctor & Gamble study (2006 as cited in SCCP 2009) was consulted at the European level (ECHA 2024; SCCP 2009), such that the mean dermal absorption rates reported by the authors were used to calculate the margin relative to the NOEL for the use of climbazole-containing cosmetics. In this publication, based on *in-vitro* absorption studies (OECD 2004), it was calculated that 0.15% of climbazole is systemically available when using shampoos containing 2% climbazole under “rinse-off” conditions. In contrast, Champmartin et al. (2025), after conducting diffusion-cell experiments with freshly excised human skin, concluded that a sole quantification of non-metabolised climbazole in the receptor solution of the diffusion cell may lead to a significant underestimation of dermal resorption. Alongside climbazole, these authors detected the two diastereomers of the monohydroxylated climbazole metabolite in the receptor fluid, which accounted for 67% of the absorbed dose. In total (climbazole + diastereomers of OH-climbazole), 57% of the dose applied to the skin was absorbed, while a further 12.5% was present in the skin samples. On this basis, a potential systemic availability of approximately 70% of a dermally applied dose was derived (Champmartin et al. 2025).

In four *in-vivo* studies by Bayer AG (Bayer AG 1979 (Ref. 21), 1979 (Ref. 23), 1979 (Ref. 68), 1980 (Ref. 20) as cited in SCCP 2005), the use of climbazole-containing products was simulated and dermal absorption was measured. To this end, climbazole and OH-climbazole concentrations were quantified in plasma (Bayer AG 1980 (Ref. 20) as cited in SCCP 2005) or in plasma and urine (Bayer AG 1979 (Ref. 21), 1979 (Ref. 23), 1979 (Ref. 68) as cited in SCCP 2005) for up to 24 hours after application. The *in-vivo* studies specified above clearly show that climbazole is percutaneously absorbed. After washing hands forty times using a climbazole-containing shampoo (2%), maximum analyte concentrations of 20 µg/l in plasma and 5 µg/l in urine were reached (Bayer AG 1979 (Ref. 21) as cited in SCCP 2005). After washing hair with the same shampoo, the maximum analyte concentrations in plasma were found to be 4 µg climbazole/l and 5 µg OH-climbazole/l, whereas neither analyte could be detected in the urine (Bayer AG 1979 (Ref. 23) as cited in SCCP 2005).

The dermal application of a climbazole-containing shampoo as well as a climbazole-containing hair tonic was likewise investigated in a metabolism study on three subjects conducted by Schönrath et al. (2024 a, 2025). As part of the study, the excretion of the climbazole metabolites (OH)₂-climbazole and cx-OH-climbazole were quantified over a period of 72 hours after application. After using the shampoo, up to 0.4% of the applied climbazole was absorbed dermally, whereas up to 9.3% was absorbed after using the hair tonic. Systemic absorption and excretion progressed much more slowly with dermal application compared to oral ingestion, and the excretion was not yet complete at the last sampling timepoint (Schönrath et al. 2024 a, 2025). The ratios of the climbazole metabolites (OH)₂-climbazole to cx-OH-climbazole in the urine samples were found to be 4 : 1 (hair tonic) and 3 : 1 (shampoo), and the elimination half-lives were up to 28.9 h and 33.0 h for (OH)₂-climbazole and cx-OH-climbazole, respectively (Schönrath et al. 2024 a).

With respect to potential exposure, workers may be exposed to the substance during the synthesis, cleaning, or filling of climbazole as well as during the production of climbazole-containing end products. Moreover, exposure can be assumed in cases of occupational application of climbazole-containing products (hairdressers, veterinarians, farmers). The general population is primarily exposed via the use of climbazole-containing cosmetics and climbazole-containing products for household pets. Since climbazole is very stable and is not completely removed during water purification, exposure via drinking water and food products is likewise possible (Creusot et al. 2020; Tröger et al. 2018).

In marketed products, climbazole is usually employed as a 1 : 1 mixture (racemate) of its two enantiomers (European Commission 2019). Metabolism to (OH)₂- or cx-OH-climbazole therefore produces molecules with two chiral centres, such that a total of four stereoisomers may be present.

Figure 1 shows the postulated metabolism scheme for climbazole, including its urinary metabolites (OH)₂-climbazole and cx-OH-climbazole, according to Schmidtkunz et al. (2021) and Schönrath et al. (2024 a). The introduction of the second chiral centre through the reduction of the prochiral carbonyl group is clearly evident here.

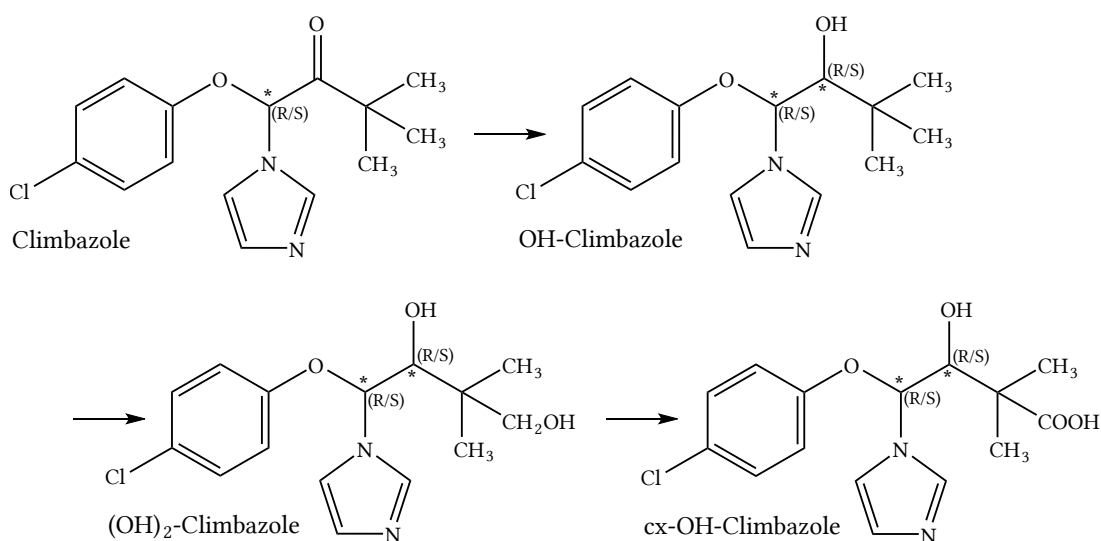


Fig. 1 Metabolism scheme of climbazole according to Schmidtkunz et al. (2021) and Schönrath et al. (2024 a)

3 General principles

The analytical procedure described herein enables the selective determination of the climbazole metabolites (OH)₂-climbazole and cx-OH-climbazole in urine. The determination of (OH)₂-climbazole is diastereoselective, whereas, for cx-OH-climbazole, only the sum of both diastereomers can be quantified. After adding the deuterated internal standards (ISTDs) (OH)₂-climbazole-D₆ and cx-OH-climbazole-D₆, enzymatic hydrolysis is performed with β -glucuronidase from *E. coli*. The analytes are purified by online SPE, separated by liquid chromatography, and quantified by down-

stream tandem mass spectrometry. Calibration is performed with standards prepared in pooled urine and processed analogously to the samples.

4 Equipment, chemicals, and solutions

4.1 Equipment

- UPLC-MS/MS system with two gradient pumps (e.g. Waters ACQUITY UPLC H-Class with 2D-LC option, coupled with a Waters Xevo-TQ-S tandem mass spectrometer, Waters GmbH, Eschborn, Germany)
- Analytical column (e.g. ACQUITY UPLC HSS C18, 1.8 μm , 2.1 mm $\times$ 150 mm, No. 1250977, Waters GmbH, Eschborn, Germany)
- Preconcentration column (e.g. EC 20/2 Chromabond HR-X, 15 μm , 2 mm $\times$ 20 mm, No. 732310.20, MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany)
- 96-well microtitre plates, 2 ml (e.g. No. 186002482, Waters GmbH, Eschborn, Germany) with sealing foil (e.g. No. 186002789, Waters GmbH, Eschborn, Germany) or sealing mats (No. 60180-M123, Thermo Fisher ScientificTM, Life Technologies GmbH, Darmstadt, Germany)
- Analytical balance (e.g. Sartorius AG, Göttingen, Germany)
- pH meter (e.g. SevenCompact S220, Mettler-Toledo GmbH, Gießen, Germany)
- Incubator (e.g. MMM Medcenter Einrichtungen GmbH, Planegg, Germany)
- 400-ml glass beakers (e.g. Schott AG, Mainz, Germany)
- 3.5-ml disposable pipettes (e.g. Sarstedt AG & Co. KG, Nümbrecht, Germany)
- Urine-collection cups (e.g. polypropylene) (e.g. Sarstedt AG & Co. KG, Nümbrecht, Germany)
- Various volumetric flasks (e.g. Schott AG, Mainz, Germany)
- Multipette[®] (e.g. Eppendorf AG, Hamburg, Germany)
- Variably adjustable pipettes (1–10 μl , 10–100 μl , and 100–1000 μl) with matching pipette tips (e.g. Eppendorf AG, Hamburg, Germany)

4.2 Chemicals

Unless otherwise specified, all chemicals must be a minimum of *pro analysi* grade. As reference substances, both diastereomers of (OH)₂-climbazole and cx-OH-climbazole were prepared by custom synthesis, each as a mixture of enantiomers. The absolute configurations of “Diastereomer 1” and “Diastereomer 2” were not determined. The following combinations are possible: (3*R*,4*S*-(OH)₂-climbazole + 3*S*,4*R*-(OH)₂-climbazole; 3*R*,4*R*-(OH)₂-climbazole + 3*S*,4*S*-(OH)₂-climbazole; 3*R*,4*S*-cx-OH-climbazole + 3*S*,4*R*-cx-OH-climbazole; 3*R*,4*R*-cx-OH-climbazole + 3*S*,4*S*-cx-OH-climbazole).

- (OH)₂-Climbazole, Diastereomer 1, 97.0% (HPLC area percentage, 210 nm) (e.g. custom synthesis, ChiroBlock GmbH, Wolfen, Germany)
- (OH)₂-Climbazole, Diastereomer 2, 95.0% (HPLC area percentage, 210 nm) (e.g. custom synthesis, ChiroBlock GmbH, Wolfen, Germany)
- cx-OH-Climbazole, Diastereomer 1, 97.4% (HPLC area percentage, 210 nm) (e.g. custom synthesis, ChiroBlock GmbH, Wolfen, Germany)
- cx-OH-Climbazole, Diastereomer 2, 96.7% (HPLC area percentage, 210 nm) (e.g. custom synthesis, ChiroBlock GmbH, Wolfen, Germany)

- (OH)₂-Climbazole-D₆, Diastereomer 1, 88.9% (qNMR, remaining content incl. 7.8% tetrahydrofuran, among other substances) or 95.5% (HPLC area percentage, 210 nm) (e.g. custom synthesis, ChiroBlock GmbH, Wolfen, Germany)
- (OH)₂-Climbazole-D₆, Diastereomer 2, 91.6% (qNMR, remaining content incl. 5.4% tetrahydrofuran, among other substances) or 96.3% (HPLC area percentage, 210 nm) (e.g. custom synthesis, ChiroBlock GmbH, Wolfen, Germany)
- cx-OH-Climbazole-D₆, Diastereomer 1, 89.4% (qNMR, remaining content incl. 7% dioxane + 1% acetic acid, among other substances) or 99.4% (HPLC area percentage, 210 nm) (e.g. custom synthesis, ChiroBlock GmbH, Wolfen, Germany)
- cx-OH-Climbazole-D₆, Diastereomer 2, 80.0% (qNMR, remaining content incl. 12% dioxane, among other substances) or 97.8% (HPLC area percentage, 210 nm) (e.g. custom synthesis, ChiroBlock GmbH, Wolfen, Germany)
- Acetonitrile, LiChrosolv® (e.g. No. 100030, Merck KGaA, Darmstadt, Germany)
- Ammonium acetate for mass spectrometry (e.g. No. 73 594, Fluka™, Honeywell Deutschland Holding GmbH, Offenbach, Germany)
- Formic acid (e.g. No. 56302, Fluka™, Honeywell Deutschland Holding GmbH, Offenbach, Germany)
- Glacial acetic acid, Suprapur® (e.g. No. 100066, Merck KGaA, Darmstadt, Germany)
- β-Glucuronidase from *E. coli* K 12, in 50% glycerine, ≥ 140 U/μl (e.g. No. 03707598001, Roche Life Science Products, Merck KGaA, Darmstadt, Germany)
- Ultra-pure water (e.g. Milli-Q® IQ 7000 Ultrapure Lab Water System, Merck KGaA, Darmstadt, Germany)

4.3 Solutions

- Ammonium acetate solution (1 mol/l, pH = 6.5)
In a 500-ml volumetric flask, 38.5 g ammonium acetate are weighed and dissolved in about 250 ml of ultra-pure water. The pH value is subsequently adjusted to pH = 6.5 using glacial acetic acid, and the volumetric flask is made up to the mark with ultra-pure water.

The solution is stable for six months at room temperature.

- Eluent A (0.1% formic acid in ultra-pure water (water : formic acid; 99.9 : 0.1; v/v))
About 500 ml of ultra-pure water are placed in a 1000-ml volumetric flask; subsequently, 1 ml of formic acid is added and the volumetric flask is made up to the mark with ultra-pure water.

The solution is stable for at least one week at room temperature.

- Eluent B (0.1% formic acid in acetonitrile (acetonitrile : formic acid; 99.9 : 0.1; v/v))
About 500 ml of acetonitrile are placed in a 1000-ml volumetric flask; subsequently, 1 ml of formic acid is added and the volumetric flask is made up to the mark with acetonitrile.

The solution is stable for at least one week at room temperature.

4.4 Internal standards (ISTDs)

To serve as ISTDs, both diastereomers of (OH)₂-climbazole and cx-OH-climbazole were produced by custom synthesis; like the non-isotopically labelled reference substances, they were obtained as mixtures of enantiomers. Compounds which had been sixfold deuterated at their respective methyl groups were used.

- ISTD stock solution (1000 mg/l)
In separate 10-ml volumetric flasks, 10 mg each of (OH)₂-climbazole-D₆ (Diastereomer 1), (OH)₂-climbazole-D₆ (Diastereomer 2), cx-OH-climbazole-D₆ (Diastereomer 1), or cx-OH-climbazole-D₆ (Diastereomer 2) are weighed and dissolved in acetonitrile. Each flask is subsequently made up to the mark with acetonitrile.
- ISTD working solution (10 mg/l, each for the sum of the diastereomers)
Of each ISTD stock solution, 50 µl are pipetted into a 10-ml volumetric flask. The flask is subsequently made up to the mark with acetonitrile.
- ISTD spiking solution (1 mg/l, each for the sum of the diastereomers)
Of the ISTD working solution, 1 ml is pipetted into a 10-ml volumetric flask. The flask is subsequently made up to the mark with acetonitrile.

The ISTD stock, working, and spiking solutions are stored at 4 °C and are stable under these conditions for at least six months.

4.5 Calibration standards

- Stock solution (1000 mg/l)
In separate 10-ml volumetric flasks, 10 mg each of (OH)₂-climbazole (Diastereomer 1), (OH)₂-climbazole (Diastereomer 2), cx-OH-climbazole (Diastereomer 1), or cx-OH-climbazole (Diastereomer 2) are weighed and dissolved in acetonitrile. Each flask is subsequently made up to the mark with acetonitrile.
- Working solution (10 mg/l, each for the sum of the diastereomers)
Of each stock solution, 50 µl are pipetted into a 10-ml volumetric flask. The flask is subsequently made up to the mark with acetonitrile.
- Spiking solution I (1 mg/l, each for the sum of the diastereomers)
Of the working solution, 1 ml is pipetted into a 10-ml volumetric flask. The flask is subsequently made up to the mark with acetonitrile.
- Spiking solution II (0.1 mg/l, each for the sum of the diastereomers)
Of the working solution, 100 µl are pipetted into a 10-ml volumetric flask. The flask is subsequently made up to the mark with acetonitrile.
- Spiking solution III (0.01 mg/l, each for the sum of the diastereomers)
Of Spiking solution I, 100 µl are pipetted into a 10-ml volumetric flask. The flask is subsequently made up to the mark with acetonitrile.

The stock, working, and spiking solutions are stored at 4 °C and are stable under these conditions for at least six months.

For calibration, pooled urine from persons with no exposure to climbazole is used. To prepare the calibration standards in a concentration range of 0.1–500 µg/l, the urine is mixed with the spiking solutions in microtitre plates (or alternatively: in 2-ml polypropylene reaction vials) according to the pipetting scheme given in [Table 1](#). The calibration standards each contain both diastereomers of (OH)₂-climbazole as well as those of cx-OH-climbazole at a ratio of 1 : 1. Unspiked pooled urine is included to determine the blank value.

Tab. 1 Pipetting scheme for the preparation of calibration standards for the determination of (OH)₂-climbazole and cx-OH-climbazole in urine

Calibration standard	Spiking solution	Spiking solution [μl]	Urine [μl]	Analyte concentration [μg/l]	ISTD spiking solution [μl]
0	–	–	500	0.00	5
1	III	2.5	500	0.05	5
2	III	5	500	0.1	5
3	III	10	500	0.2	5
4	III	25	500	0.5	5
5	II	5	500	1	5
6	II	10	500	2	5
7	II	25	500	5	5
8	I	5	500	10	5
9	I	10	500	20	5
10	I	25	500	50	5

5 Specimen collection and sample preparation

5.1 Specimen collection

The urine sample is collected in a urine-collection cup (e.g. polypropylene). If the urine cannot be immediately processed, an aliquot is frozen and stored (at less than –10 °C) until analysis.

5.2 Sample preparation

Prior to analysis, the urine sample is thawed at room temperature and homogenised by shaking. An aliquot of 0.5 ml is pipetted into each cell of a 96-well plate (alternatively: 2-ml polypropylene reaction vials). After adding 5 μl of the ISTD spiking solution, 1 ml of the ammonium acetate solution, and 5 μl of the β-glucuronidase solution, the 96-well plate is sealed with sealing foil. The samples are then homogenised and incubated at 37 °C for three hours. Subsequently, 50 μl of each sample are directly injected into the UPLC-MS/MS system.

6 Operational parameters

Analytical determination was carried out using a device configuration comprised of a UPLC system with 2D-LC option and a tandem mass spectrometer (Waters Xevo TQ-S).

6.1 Liquid chromatography

Preconcentration column:	EC 20/2 Chromabond HR-X, 15 μm, 2 mm × 20 mm
Analytical column:	ACQUITY UPLC HSS C18, 1.8 μm, 2.1 mm × 150 mm
Mobile phase:	Eluent A: water : formic acid (99.9 : 0.1; v/v) Eluent B: acetonitrile : formic acid (99.9 : 0.1; v/v)
Runtime:	10 min
Column temperature:	50 °C

Sample temperature:	15 °C
Injection volume:	50 µl
Gradient programme of Pump P1:	see Table 2
Gradient programme of Pump P2:	see Table 3
Valve-switching times:	see Table 4

Schmidtkunz et al. (2021) depicts both the structure of the 2D-LC apparatus applied and an illustration of the switch positions of the six-port valve. Some of the parameters indicated above are instrument-specific and must be adjusted to the individual LC system used.

Tab. 2 Gradient programme of Pump P1 for the determination of (OH)₂-climbazole and cx-OH-climbazole in urine

Time [min]	Eluent A [%]	Eluent B [%]	Flow rate [ml/min]
0.0	95	5	0.8
1.0	95	5	0.8
1.5	0	100	0.8
3.0	0	100	0.8
3.5	95	5	0.8
10.0	95	5	0.8

Tab. 3 Gradient programme of Pump P2 for the determination of (OH)₂-climbazole and cx-OH-climbazole in urine

Time [min]	Eluent A [%]	Eluent B [%]	Flow rate [ml/min]
0.0	95	5	0.4
1.0	95	5	0.4
5.0	50	50	0.4
5.2	50	50	0.4
5.3	5	95	0.4
7.5	5	95	0.4
8.0	95	5	0.4
10.0	95	5	0.4

Tab. 4 Switch programme of the six-port valve

Time [min]	Valve position	Description
0–0.5	1	Enrichment of analytes, equilibration of analytical column
0.5–3.5	2	Transfer of analytes onto analytical column in back-flush mode, chromatographic separation of analytes
3.5–10.0	1	Reconditioning of preconcentration column via Pump P1, chromatographic separation of analytes via Pump P2

6.2 Tandem mass spectrometry

Ionisation:	Electrospray, positive (ESI+)
Capillary voltage:	2.0 kV
Cone voltage:	20 V
Source offset:	50 V
Source temperature:	150 °C
Desolvation temperature:	400 °C
Cone-gas flow:	150 l/h
Desolvation-gas flow:	800 l/h
Collision-gas flow:	0.15 ml/min
Nebuliser-gas flow:	5.0 bar
Collision gas:	Argon
Further parameters:	see Table 5

Tab. 5 Mass-spectrometric parameters for the determination of (OH)₂-climbazole and cx-OH-Climbazol in urine

Analyte or ISTD	Precursor ion (<i>m/z</i>)	Product ion (<i>m/z</i>)	Dwell time [s]	Cone voltage [V]	Collision energy [eV]	Retention time [min]
(OH) ₂ -Climbazole (Diastereomer 1)	311.4	69.1 ^{a)}	0.017	17.0	8.0	4.50
		111.0	0.017	17.0	58.0	
(OH) ₂ -Climbazole-D ₆ (Diastereomer 1)	317.4	69.1 ^{a)}	0.017	15.0	10.0	4.49
		121.2	0.017	15.0	14.0	
(OH) ₂ -Climbazole (Diastereomer 2)	311.4	69.1 ^{a)}	0.017	17.0	8.0	4.56
		111.0	0.017	17.0	58.0	
(OH) ₂ -Climbazole-D ₆ (Diastereomer 2)	317.4	69.1 ^{a)}	0.017	15.0	10.0	4.55
		121.2	0.017	15.0	14.0	
cx-OH-Climbazole	325.4	69.1 ^{a)}	0.017	31.0	10.0	4.41
		129.2	0.017	31.0	14.0	
cx-OH-Climbazole-D ₆	331.4	69.1 ^{a)}	0.017	25.0	8.0	4.40
		135.1	0.017	25.0	16.0	

^{a)} quantifier

The retention times given in [Table 5](#) can only serve as a point of reference. The user of this method must ensure the separation performance of the analytical column used as well as the resulting retention behaviour of the substances. In the representative chromatograms, the diastereomers of (OH)₂-climbazole are chromatographically separated ([Figure 2](#)), whereas those of cx-OH-climbazole are not ([Figure 3](#)).

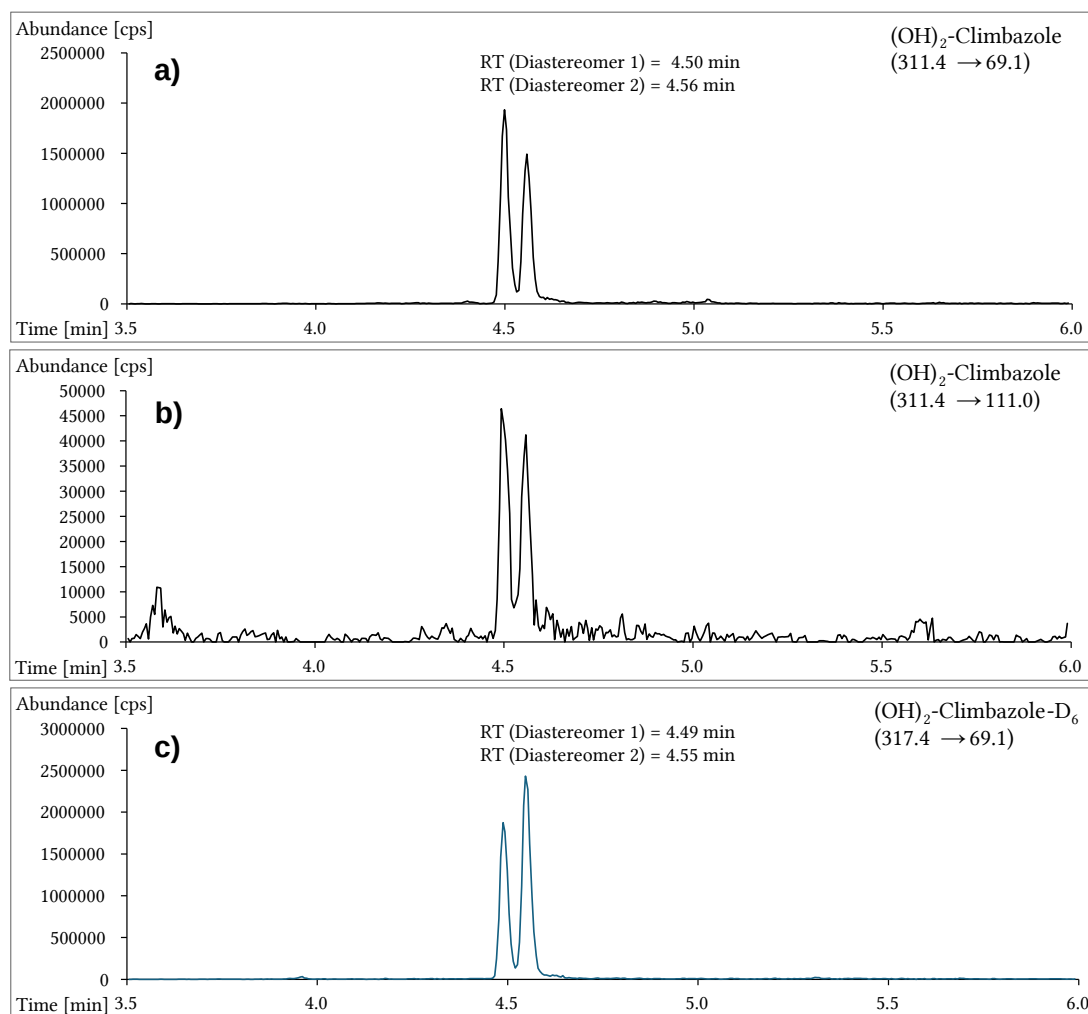


Fig. 2 Chromatogram of pooled urine spiked with $(\text{OH})_2$ -climbazole at a concentration of 10 $\mu\text{g/l}$; Part a) shows the quantifier transition of $(\text{OH})_2$ -climbazole, part b) shows the qualifier transition of $(\text{OH})_2$ -climbazole, and part c) shows the qualifier transition of the ISTD $(\text{OH})_2$ -climbazole- D_6 .

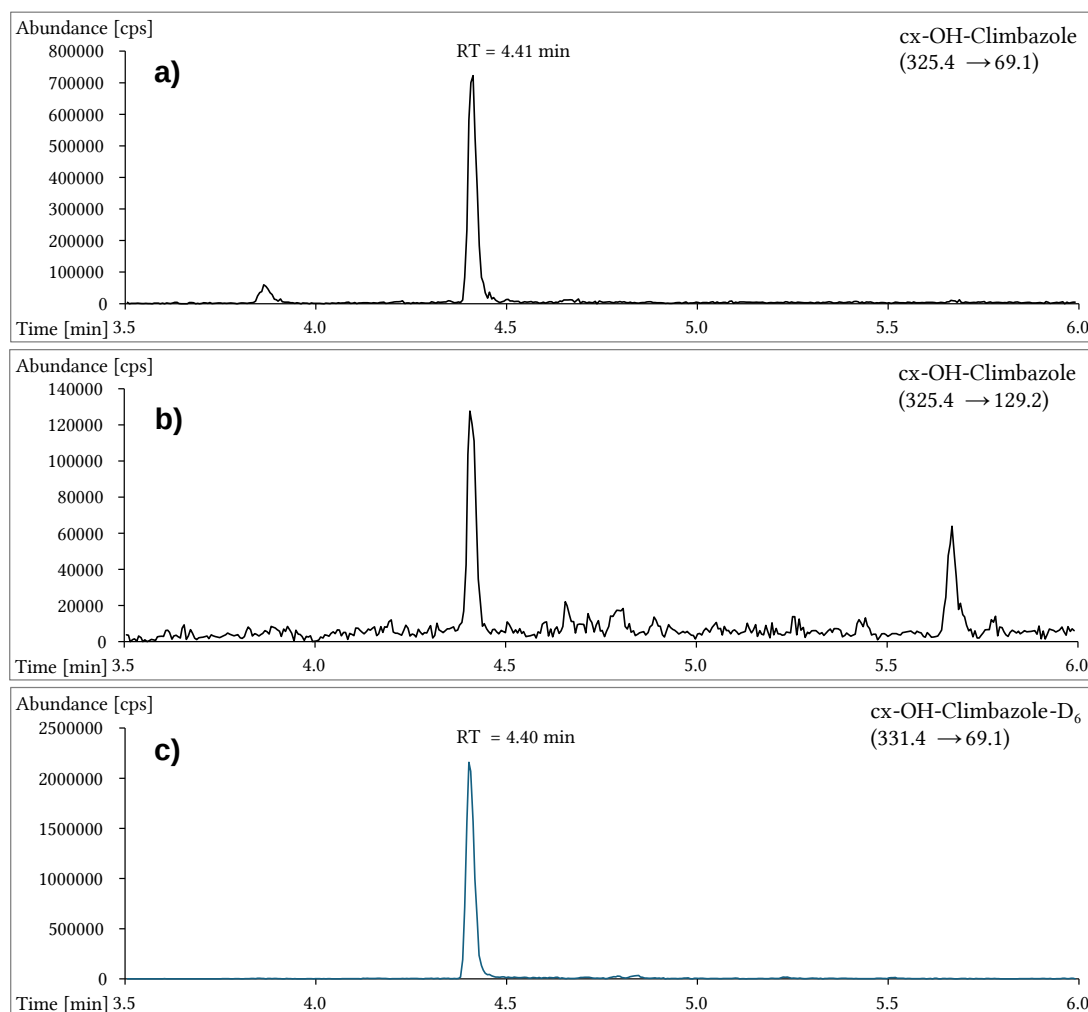


Fig. 3 Chromatogram of pooled urine spiked with cx-OH-climbazole at a concentration of 10 µg/l; Part a) shows the quantifier transition of cx-OH-climbazole, part b) shows the qualifier transition of cx-OH-climbazole, and part c) shows the qualifier transition of the ISTD cx-OH-climbazole-D₆.

7 Analytical determination

For analytical determination, the urine samples are processed as described in [Section 5](#), then 50 µl of each processed sample are injected into the UPLC-MS/MS system. The applied sample is preconcentrated and purified by online SPE. After elution in back-flush mode, chromatographic separation takes place. Whereas both diastereomers of (OH)₂-climbazole are separated, those of cx-OH-climbazole co-elute. The analytes are identified by their retention times as well as by their characteristic mass transitions (see [Table 5](#)).

8 Calibration

The calibration standards prepared as described in [Section 4.5](#) are processed analogously to the samples – without the further addition of ISTD (see [Section 5](#)) – and are analysed according to [Sections 6](#) and [7](#). The calibration curve is generated by plotting the peak-area ratio of the analyte and its corresponding ISTD against the spiked analyte concentration. To this end, the diastereomers of (OH)₂-climbazole are integrated individually, while those of cx-OH-climbazole can

only be integrated as a sum (co-elution). Representative calibration curves for the determination of (OH)₂-climbazole and cx-OH-climbazole in urine are depicted in Figure 4.

With the analytical instrumentation used during method development, a linear measurement range of 0.5–500 µg/l was ascertained. During external verification of the method, analyte carryover was observed after the highest calibration standards, which corresponded to an analyte concentration of up to 0.7 µg/l. To prevent this carryover, it is recommended – depending on the samples to be measured – to limit the upper end of the calibration range, for example to 20 or 50 µg/l.

Blank values from the tested sample material must be subtracted from all measurement points. During method development, however, no blank values of a relevant magnitude were observed. For longer sequences, it proved expedient to include a calibration series at the beginning and end of each measurement sequence in order to evaluate the samples – as long as no significant differences in peak-area ratios arose – using an averaged calibration curve.

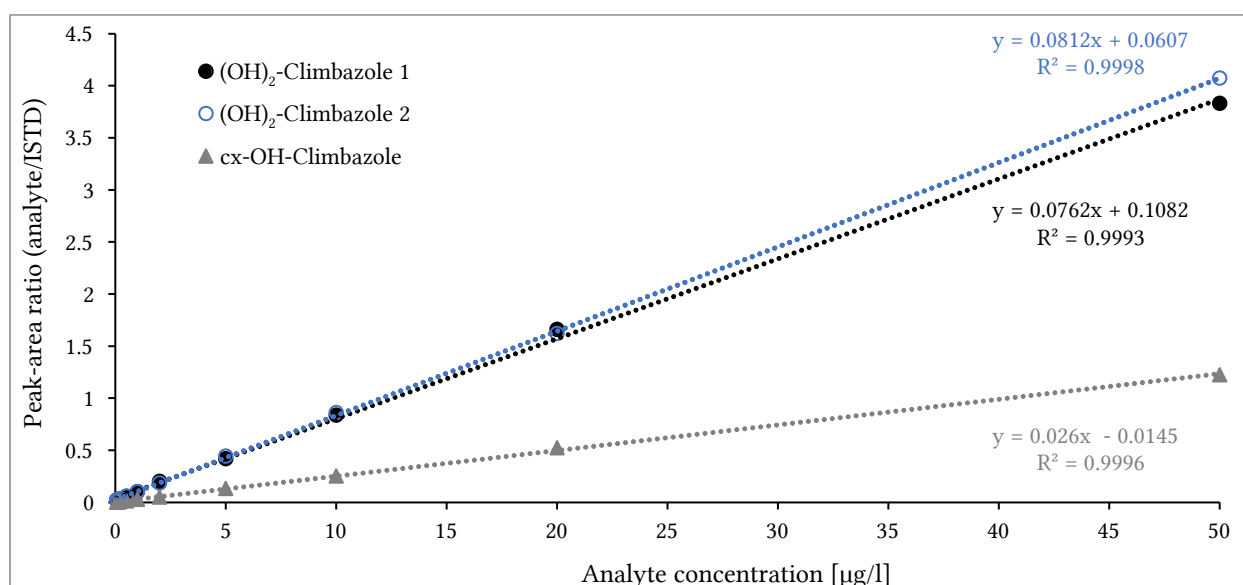


Fig. 4 Calibration curves for the determination of (OH)₂-climbazole and cx-OH-climbazole in urine

9 Calculation of analytical results

The analyte concentrations of the urine samples are calculated using the calibration functions specific to that particular analytical sequence (see Section 8). The peak area of the analyte is divided by the peak area of its corresponding ISTD. The quotient thus obtained is entered into the aforementioned calibration function, yielding the analyte concentration in µg/l. During method development, the analyte concentrations were calculated using the device software MassLynx 4.1 from Waters GmbH, Eschborn, Germany.

10 Standardisation and quality control

Quality assurance of the analytical results is carried out as stipulated in the guidelines of the *Bundesärztekammer* (German Medical Association) and in a general chapter published by the Commission (Bader et al. 2010; Bundesärztekammer 2023).

For the quality assurance of the individual analytical sequences, at least two quality-control samples with known analyte concentrations are determined alongside the samples to be analysed. Since commercial material is not available, this

quality-control material must be prepared in the in-house laboratory. To this end, pooled urine from persons with no known exposure to climbazole is spiked with standard solutions of the analytes, such that the analyte concentrations lie within the concentration range relevant for assessment. Furthermore, reagent blanks are included as part of each analytical sequence to enable the recognition of potential interferences caused by the reagents. In this step, ultra-pure water is used as the matrix instead of urine.

To prepare quality-control materials, the method developers spiked pooled urine with the analytes at concentrations of 1.0 µg/l, 10 µg/l, or 100 µg/l. Moreover, at least three reagent blank values were included in each analytical sequence during method development.

11 Evaluation of the method

The reliability of this method was confirmed by comprehensive validation as well as by replication and verification of the method in a second, independent laboratory.

11.1 Precision

Within-day precision was determined by processing and analysing the quality-control materials (see [Section 10](#)) eight times in parallel. The within-day precision data are given in [Table 6](#).

Tab. 6 Within-day precision for the determination of (OH)₂-climbazole and cx-OH-climbazole (n = 8)

Analyte	Spiked concentration [µg/l]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
(OH) ₂ -Climbazole (Diastereomer 1)	1.0	9.2	21.8
	10	7.2	16.9
	100	6.4	15.1
(OH) ₂ -Climbazole (Diastereomer 2)	1.0	4.9	11.6
	10	3.6	8.6
	100	3.5	8.3
cx-OH-Climbazole	1.0	7.2	17.0
	10	7.6	18.0
	100	2.5	5.8

Day-to-day precision was determined from the mean values of the eightfold measurements on each of six days. The precision data thus obtained are given in [Table 7](#).

Tab. 7 Day-to-day precision for the determination of (OH)₂-climbazole and cx-OH-climbazole in urine (n = 6)

Analyte	Spiked concentration [µg/l]	Standard deviation (rel.) <i>s_w</i> [%]	Prognostic range <i>u</i> [%]
(OH) ₂ -Climbazole (Diastereomer 1)	1.0	6.0	15.5
	10	3.6	9.3
	100	4.2	10.8
(OH) ₂ -Climbazole (Diastereomer 2)	1.0	3.0	7.8
	10	2.5	6.5
	100	2.9	7.5
cx-OH-Climbazole	1.0	3.5	9.0
	10	2.5	6.5
	100	3.5	8.9

11.2 Accuracy

The accuracy of the method was ascertained from the total measurement data collected for determining precision. The relative recoveries thus obtained are listed in [Table 8](#).

Tab. 8 Mean relative recovery for the determination of (OH)₂-climbazole and cx-OH-climbazole in urine (n = 48)

Analyte	Spiked concentration [µg/l]	Mean recovery (rel.) <i>r</i> [%]	Range [%]
(OH) ₂ -Climbazole (Diastereomer 1)	1.0	101	79.0–114
	10	102	87.0–113
	100	98.2	87.5–111
(OH) ₂ -Climbazole (Diastereomer 2)	1.0	99.0	85.0–114
	10	102	88.9–125
	100	96.9	86.7–106
cx-OH-Climbazole	1.0	99.3	84.0–112
	10	102	88.9–125
	100	99.9	88.6–111

11.3 Selectivity

In order to determine the influence of different urine matrices on the analytical results, nine native urine samples were spiked with the analytes at concentrations of 1 µg/l, 10 µg/l, and 100 µg/l, then processed and analysed. The creatinine contents of the urines were determined by photometry following the Jaffé reaction (Taussky and Kurzmann 1954), and were found to lie in the range of 0.21–1.69 g/l. The relative standard deviations and mean relative recoveries for the determination of (OH)₂-climbazole and cx-OH-climbazole in the individual urines are presented in [Table 9](#).

Tab. 9 Selectivity for the determination of (OH)₂-climbazole and cx-OH-climbazole in individual urines (n = 9)

Analyte	Spiked concentration [µg/l]	Mean recovery (rel.) <i>r</i> [%]	Standard deviation (rel.) <i>s_w</i> [%]
(OH) ₂ -Climbazole (Diastereomer 1)	1.0	95.6	12.7
	10	95.3	7.6
	100	93.2	9.2
(OH) ₂ -Climbazole (Diastereomer 2)	1.0	91.8	18.0
	10	84.4	16.9
	100	93.7	9.9
cx-OH-Climbazole	1.0	103	33.1
	10	106	6.1
	100	108	5.9

The mean relative recovery was correct at a spiked analyte concentration of 1.0 µg/l, whereas the relative standard deviation at this concentration level exhibited a certain degree of matrix-related uncertainty in the determination.

The method verifiers also investigated the influence of individual urine matrices on the analytical results. During method verification, six different, unspiked pooled urines were analysed. Without spiking samples with the analytes, fulfilment of selectivity criteria would mean a signal of less than 20% of that obtained from spiking the samples at the limit of quantitation (limit of quantitation from method verification: 0.05 µg/l, see [Appendix](#)). For (OH)₂-climbazole, a range of 0.0–40.9% (mean: 23.4%) and for cx-OH-climbazole, a range of 12.8–26.1% (mean: 20.1%) was found. Without ISTD spiking, no signal was detected for (OH)₂-climbazole-D₆; for cx-OH-climbazole-D₆, a signal of 0.03–0.07% of the usual ISTD signal was measured. It can be concluded that the selectivity criteria were only marginally fulfilled during the determination of the analytes, although it remained unclear whether the urine samples were initially analyte-free.

11.4 Matrix effects

To measure potential matrix effects, the method verifiers followed the guidelines of the FDA (2018) and the EMA (2022). As part of external method verification, ultra-pure water and pooled urine were spiked with the analytes at concentrations of 0.1 µg/l and 400 µg/l. The same approach was taken with respect to the ISTDs (spiking ultra-pure water and pooled urine at concentrations of 20 µg/l and 400 µg/l). Comparison of the absolute areas of the analyte peaks showed a clear matrix effect in the form of a signal suppression of an average 20–30% caused by the urine matrix. This matrix effect appears to be somewhat greater for cx-OH-climbazole than for (OH)₂-climbazole ([Table 10](#)). These results highlight the importance of calibration in the urine matrix (rather than in water) and of using isotope-labelled ISTDs to achieve precise and accurate quantitation.

Tab. 10 Matrix effects in the determination of (OH)₂-climbazole and cx-OH-climbazole in pooled urines (n = 3)

Analyte or ISTD	Spiked concentration [µg/l]	Matrix effect [%]
(OH) ₂ -Climbazole (Diastereomer 1 + 2)	0.1	64–86
	400	79–82
cx-OH-Climbazole	0.1	55–82
	400	66–73
(OH) ₂ -Climbazole-D ₆ (Diastereomer 1 + 2)	20	76–87
	400	74–78
cx-OH-Climbazole-D ₆	20	59–69
	400	63–69

11.5 Limits of detection and quantitation

The limits of detection and quantitation (Table 11) were ascertained using the calibration-curve method based on DIN 32 645 (DIN 2008). To this end, the lowest calibration points of the calibration curves were used.

Tab. 11 Limits of detection and quantitation for the determination of (OH)₂-climbazole and cx-OH-climbazole in urine

Analyte	Detection limit [µg/l]	Quantitation limit [µg/l]
(OH) ₂ -Climbazole (Diastereomer 1)	0.2	0.5
(OH) ₂ -Climbazole (Diastereomer 2)	0.2	0.5
cx-OH-Climbazole (Diastereomer 1 + 2)	0.2	0.5

11.6 Sources of error

The analysis of climbazole metabolites herein described is generally very robust. Blank values from reagents or background levels from native urine samples do not appear to play any relevant role. Moreover, analytical sequences with up to 100 injections can be performed without any measurable decline in analytical quality. The method developers as well as the verifiers, however, consistently found matrix interferences in the form of interfering peaks in urines with high creatinine levels; these interferences were most problematic at very low analyte concentrations. Furthermore, in samples with analyte concentrations at the limit of quantitation, a distinct peak in the qualifier transition was frequently not observed. Although, based on the sensitivity of the analytical instruments (e.g. signal-to-noise ratio), a limit of quantitation well below 0.5 µg/l would be conceivable, depending on the urine matrix; in real samples, the described matrix interferences can complicate the reliable quantitation of analyte levels below 0.5 µg/l. The influence of interfering matrix components plays a much less significant role at higher analyte concentrations.

The use of isotope-labelled ISTDs is absolutely essential to adequately compensate for matrix-related fluctuations during workup, chromatographic separation, or ionisation.

To sensitively measure potential climbazole exposure in the general population, it is necessary to adjust the calibration range accordingly. Further optimisation of chromatographic conditions may increase the selectivity of the analysis, enabling a precise, accurate, and robust – with respect to various urine matrices – quantification into the sub-µg/l-range. Possibilities include adjusting valve-switch times or elution gradients in order to achieve not only satisfactory preconcentration but also improved purification via online SPE.

12 Discussion of the method

The method allows for a selective, sensitive, and robust analysis of climbazole metabolites (OH)₂-climbazole and cx-OH-climbazole, whereby the two diastereomers of (OH)₂-climbazole are separated. The results of an exposure study (Schönrath et al. 2024 a) show that the analytes detected with this method are suitable biomarkers for the measurement of both oral and dermal climbazole exposure. Schönrath et al. (2024 a) further describes the time-dependent and, where applicable, individual-dependent changes in the diastereomer ratios of both (OH)₂-climbazole diastereomers after oral administration and dermal application.

The method allows for the determination of (OH)₂-climbazole and cx-OH-climbazole with good precision and accuracy. However, the selectivity of the determination may be affected by matrix components at analyte concentrations close to the limit of quantitation (see Section 11.3).

Since only the non-glucuronidated analytes were synthesised as standards for method development, the efficacy of enzymatic hydrolysis could not be tested directly. To validate enzymatic hydrolysis, Schmidt-kunz et al. (2021) subjected two samples with relatively high analyte concentrations to tenfold enzymatic hydrolysis in parallel, then proceeded with

sample workup and analysis. The results showed precision data comparable to those of the simultaneously processed and measured samples to which only the free analytes had been added (Schmidtkunz et al. 2021). Furthermore, aliquots of the same samples were subject to either acidic hydrolysis or enzymatic hydrolysis, whereby the results showed excellent consistency (Schmidtkunz et al. 2021). In the samples investigated by Schmidtkunz et al. (2021) as part of a kinetics study, an average of 0.9% of (OH)₂-climbazole and 55% of cx-OH-climbazole were present in their unconjugated forms.

The analytes that are determined with this method are valid biomarkers for the measurement of internal burden after exposure to climbazole. The method hereby presented is sufficiently sensitive to quantify climbazole absorption and excretion after a single dermal application of hair-care products (shampoo or hair tonic; Schönrrath et al. 2024 a, 2025). The climbazole metabolites (OH)₂-climbazole and cx-OH-climbazole could be quantified up to at least 48 h after shampoo application and up to at least 72 h after application of hair tonic (Schönrrath et al. 2024 a, 2025).

The method verifiers used devices from different manufacturers (LC-20AB; Shimadzu Deutschland GmbH, Neufahrn, Germany and QTrap 6500+; AB Sciex Germany GmbH, Darmstadt, Germany) as well as a different analytical column (Acquity UPLC BEH C8, 1.7 µm, 2.1 × 150 mm; Waters GmbH, Eschborn, Germany). Moreover, β-glucuronidase from a different supplier (Megazyme Ltd., Bray, Ireland) was used, and the samples were centrifuged for 10 min at 37 500 × g after the hydrolysis step.

With these instrumental and equipment-related modifications (for further details, see Appendix), the diastereomers of (OH)₂-climbazole could also be separated, while this was not possible for those of cx-OH-climbazole. During external method verification, calibration was performed in a range of 0.05–500 µg/l, and lower limits of quantitation of 0.05 µg/l were achieved for the sum of the (OH)₂-climbazole diastereomers as well as for the sum of the cx-OH-climbazole diastereomers. Despite these improved quantitation limits, the analytes were not detectable in ten pooled-urine samples from the general population.

The method developers investigated 40 individual urine samples from individuals without occupational exposure to climbazole, whereby none of the samples exhibited analyte concentrations >1.5 µg/l. At a later point in time, the method developers investigated 300 urine samples from the general population, provided by the German Environmental Specimen Bank (samples taken between 2002 and 2022), whereby 16 samples exhibited concentrations of at least one analyte over the detection limit of 0.5 µg/l; maximum concentrations of 10.23 µg/l and 2.53 µg/l were measured for (OH)₂-climbazole and cx-OH-climbazole, respectively. There was a tendency for climbazole levels to decrease over time (Schönrrath et al. 2024 b).

Instruments used UPLC-MS/MS system (Waters ACQUITY UPLC H-Class with 2D-LC option, coupled with Waters Xevo-TQ-S tandem mass spectrometer, Waters GmbH, Eschborn, Germany); analytical column (ACQUITY UPLC HSS C18, 1.8 µm, 2.1 mm × 150 mm, Waters GmbH, Eschborn, Germany); preconcentration column (EC 20/2 Chromabond HR-X, 15 µm, 2 mm × 20 mm, MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany)

Notes

Competing interests

The established rules and measures of the Commission to avoid conflicts of interest (https://www.dfg.de/mak/conflicts_interest) ensure that the content and conclusions of the publication are strictly science-based.

The views expressed in these publications are those of the individual authors acting in their personal capacity as experts and do not represent the positions of their respective institutions or employers.

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Appendix

Equipment, chemicals, and solutions during external verification

During the method verification, lower limits of quantitation of 0.05 µg/l urine were achieved. The general procedure followed the method described by the developers; therefore, only the UPLC-MS/MS system used for external verification and any changes to chemicals and solutions are described here to provide supplemental information.

Equipment

- UPLC-MS/MS system with a gradient pump LC-20AB (Shimadzu Deutschland GmbH, Neufahrn, Germany) coupled with a QTrap 6500+ tandem mass spectrometer (AB Sciex Germany GmbH, Darmstadt, Germany)
- Analytical column (e.g. Acquity UPLC BEH C8, 1.7 µm, 2.1 × 150 mm, No. 186003377, Waters GmbH, Eschborn, Germany)

Chemicals

- β-Glucuronidase from *E. coli*, in solution (Tris-HCl/NaCl/EDTA), ~250 U/µl (e.g. E-BGLAEC, No. 700004191 Megazyme Ltd., Bray, Ireland)

Solutions

- Eluent B (0.1% formic acid in water/acetonitrile; water : acetonitrile : formic acid (94.95 : 4.95 : 0.1; v/v/v))
About 500 ml of ultra-pure water are placed in a 1000-ml volumetric flask, then 49.5 ml of acetonitrile and 1 ml of formic acid are added, and the volumetric flask is subsequently made up to the mark with ultra-pure water.

The solution is stable for least three months at room temperature.

Liquid chromatography

As the pump for the preconcentration column was not a gradient pump, corresponding modifications were made to the eluent as well as to the programme for the pump.

Preconcentration column:	EC 20/2 Chromabond HR-X, 15 µm, 2 mm × 20 mm
Analytical column:	Acquity UPLC BEH C8, 1.7 µm, 2.1 × 150 mm
Mobile phase:	Eluent A: water : acetonitrile : formic acid (94.95 : 4.95 : 0.1; v/v/v) Eluent B: acetonitrile : formic acid (99.9 : 0.1; v/v)
Runtime:	10 min
Column temperature:	50 °C
Sample temperature:	15 °C

Injection volume: 50 µl
 Programme of Pump P1: see [Table 12](#)
 Gradient programme of Pump P2: see [Table 3](#)
 Valve-switch times: see [Table 13](#)

Tab. 12 Programme of Pump P1 for the preconcentration column

Time [min]	Eluent A [%]	Eluent B [%]	Flow rate [ml/min]
0.0	100	0	0.8
1.25	0	100	0.8
3.5	100	0	0.8

Tab. 13 Valve-switch times

Time [min]	Valve position	Description
0–0.5	1	Enrichment of analytes, equilibration of analytical column
0.5–3.5	2	Transfer of analytes onto analytical column in back-flush mode, chromatographic separation of analytes
3.5–10.0	1	Reconditioning of preconcentration column via Pump P1, chromatographic separation of analytes via Pump P2

Tandem mass spectrometry

Ionisation: Electrospray, positive (ESI+, unit/unit)
 Curtain gas (CUR): 20
 Collision gas (CAD): medium
 Ion-spray voltage: 4500 V
 Temperature: 400 °C
 Ion Source Gas 1 (GS1): 7
 Ion Source Gas 2 (GS2): 0
 Entrance Potential (EP): 10
 Temperature: 400 °C
 Collision-gas flow: medium
 Further settings: see [Table 14](#)

Tab. 14 Mass-spectrometric parameters for the determination of (OH)₂-climbazole and cx-OH-climbazole in urine

Analyte or ISTD	Precursor ion (<i>m/z</i>)	Product ion (<i>m/z</i>)	Dwell time [s]	Cone voltage [V]	Collision energy [eV]	Retention time [min]
(OH) ₂ -Climbazole (Diastereomer 1)	311	69 ^{a)}	0.01	20	13	6.0
		115	0.01	20	21	
		111	0.01	20	46	
(OH) ₂ -Climbazole-D ₆ (Diastereomer 1)	317	69 ^{a)}	0.01	20	13	6.1
		121	0.01	20	21	
(OH) ₂ -Climbazole (Diastereomer 2)	311	69 ^{a)}	0.01	20	13	6.2
		111	0.01	20	46	
(OH) ₂ -Climbazole-D ₆ (Diastereomer 2)	317	69 ^{a)}	0.01	20	13	6.2
		121	0.01	20	21	
cx-OH-Climbazole	325	69 ^{a)}	0.01	20	13	6.0
		129	0.01	20	21	
cx-OH-Climbazole-D ₆	331	69 ^{a)}	0.01	20	13	6.0
		135	0.01	20	21	

^{a)} quantifier

Limits of detection and quantitation

The limits of quantitation were ascertained in accordance with FDA (2018) and ICH M10 guidelines (EMA 2022) using three different, analyte-free urines. The urine samples were each spiked five times at the limit of quantitation (0.05 µg/l) with (OH)₂-climbazole as well as cx-OH-climbazole, processed and analysed. The accuracy and precision of the determinations served as criteria. For both climbazole metabolites, the individual accuracy values were between 80% and 120% at the limit of quantitation; with respect to precision, a coefficient of variation of 20% was not exceeded.