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Identification and characterization of urinary prenylamine metabolites by means of liquid chromatography-tandem mass spectrometry

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Prenylamine is a vasodilator of phenylalkylamine structure and was used for the treatment of angina pectoris, until reports of undesirable effects including ventricular tachycardia led to a decreasing use of the drug in the 1980s. Metabolic *N*-dealkylation of orally ingested prenylamine can liberate amphetamine in humans and cause positive findings for amphetamine in doping and forensic analysis. In 2010, the World Anti-Doping Agency (WADA) classified prenylamine as a non-specified stimulant according to the 2010 Prohibited List, thus banning its use in sports in-competition. Supporting the development of a liquid chromatography-tandem mass spectrometry (LC-MS/MS) based detection method, a post-administration urine sample following a single oral prenylamine ingestion (Segontin® 60 mg) was analyzed for urinary metabolites. The LC-separated analytes were ionized in positive electrospray ionization (ESI) mode and detected as protonated ions using an AB Sciex TripleTOF 5600 quadrupole-time-of-flight hybrid mass spectrometer. Over 40 phase I metabolites were detected, including previously unknown mono- bis-, tris- and tetra-hydroxylated prenylamine, several hydroxylated and methoxylated prenylamine metabolites and (hydroxylated) diphenylpropylamine. Investigation of the collision-induced dissociation behaviours of the metabolites by high resolution/high accuracy mass spectrometry allowed for the assignment of the nature and the site of observed metabolic transformations. The most abundant phase I metabolite was confirmed as *p*-hydroxy-prenlyamine by chemical synthesis and stable isotope labelling of reference material. An existing routine screening assay based on direct injection and LC-MS/MS analysis of urine was modified and validated according to common guidelines, in order to allow for the detection of *p*-hydroxy-prenylamine in sports drug testing. The assay demonstrated the ability to detect the target metabolite at 0.1 ng/ml at intra- and inter-day imprecisions below 10%.

Keywords: prenylamine metabolism; doping; urine analysis; stimulants; TripleTOF

Introduction

Prenylamine ((RS)-(3,3-diphenylpropyl)(1-methyl-2-phenylethyl) amine, elemental composition $C_{24}H_{27}N$, M = 329.2143 g/mol) is a calcium antagonist of amphetamine-like structure and was formerly used as vasodilator for the treatment of angina pectoris.[1] It was marketed under several brand names such as Segontin (R), Sedolatan (R), and Seccidin (R). Numerous reports of adverse side effects including ventricular tachycardia associated with prenylamine intake and a strong inter-individual variability in physiologic response, however, led to a decrease in the use of the drug in the 1980s.[2,3]

In 2010, the World Anti-Doping Agency (WADA) classified prenylamine as a non-specified stimulant according to the 2010 Prohibited List (category S.6a), thus banning its in-competition use in sports.[4] While mentioned for the first time by WADA, prenylamine was already listed in the category 'drugs containing doping substances' by Manfred Donike, the pioneer of modern doping analysis, at least since 1984.[5] In fact, metabolic *N*-dealkylation of orally ingested prenylamine can produce amphetamine in humans to varying extents and cause positive findings for amphetamine in doping and forensic analysis.[6]

The pharmacokinetics and the metabolic pathways of prenylamine have been thoroughly investigated since the 1960s by various analytical techniques including thin layer chromatography (TLC), gas chromatography-flame ionization detection (GC-FID), TLC-mass spectrometry (TLC-MS), GC-MS, and liquid chromatography-ultraviolet detection (LC-UV).[7–11] The most extensive study on the identification and structural characterization of prenylamine metabolites was carried out by Remberg *et al*. using TLC and GC-MS in combination with radio-isotope labelling.[12] The active drug is extensively metabolized and hardly excreted unchanged into the urine, complicating its unequivocal identification in forensic and sports drug testing. Besides amphetamine, 3,3-diphenylpropylamine (DPPA) is the product of metabolic C-N bond cleavage at the longer alkyl chain of the secondary amine function. In doping analysis, however, detection of neither amphetamine, nor DPPA unambiguously diagnoses an illicit prenylamine intake: both analytes could also derive from other origins, such as the therapeutic use of structurally related phenylalkylamine derivatives like fendiline.[13–15]

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Presumably, the detection of metabolites with intact molecular backbone yields the most reliable results for the determination of illicit prenylamine intake. To date, however, a validated analytical assay for the detection of urinary prenylamine metabolites, based on modern chromatographic and mass spectrometric instrumentations, is not available.

Therefore, in order to comply with the recent ban of prenylamine in sports and the subsequent need for a validated determination method in sports drug testing, a post-administration urine sample of a male patient, collected after a single oral dose of prenylamine (Segontin® 60 mg) was analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Since the quantitative pharmacokinetics of the drug have been thoroughly investigated in the past, the emphasis of the study was put on the identification and qualitative LC-MS/MS-based characterization of prenylamine metabolites (phase I and II), utilizing high resolution/high accuracy quadrupole/time-of-flight hybrid mass spectrometry. The results were supported by chemical synthesis, stable isotope labelling and nuclear magnetic resonance spectroscopy of proposed mono- and dihydroxy-metabolites. The novel chromatographic and mass spectrometric data on prenylamine metabolites were included into an existing routine screening method based on direct injection and LC-MS/MS analysis of urine, resulting in the reliable determination of illicit prenylamine use in sports.

Materials and methods

Chemicals and reagents

4-hydroxy phenylacetone was obtained from TCI Europe (Eschborn, Germany), while 3-hydroxy phenylacetone and 3, 4-dihydroxy phenylacetone were from Chemos (Regenstauf, Germany). Sodium borohydride, sodium borodeuteride, phosphorous pentoxide, deuterium chloride solution (20% in D_2O), methanol-*d* and methanol-d_4 were purchased from Sigma-Aldrich (Deisendorf, Germany), β-glucuronidase (*E. coli*) and β-glucuronidase/arylsulfatase (*Helix Pomatia*) were from Roche Diagnostics (Mannheim, Germany). Methyl-tert-butylether (MTBE) was obtained from Applichem (Darmstadt, Germany). Acetonitrile and methanol for HPLC as well as potassium carbonate were of analytical grade and purchased from VWR International (Langenfeld, Germany). MiliQ water was used for all aqueous buffers and solutions.

Post-administration urine samples

A male patient (59 years) receiving a therapeutic dosage of Segontin® 60 mg (containing 76.4 mg of prenylamine lactate salt) collected spot urine samples 8.5 h and 29.5 h after oral ingestion of the drug. A written consent for the use of the samples for research purposes was provided.

Sample preparation

For the identification of total phase I metabolites, 2 ml of the post-administration urine sample (8.5 h) were mixed with 1 ml of 0.2 M sodium acetate buffer (pH 5.2), followed by addition of 50 µl of β-glucuronidase/arylsulfatase from *Helix Pomatia*. Enzymatic hydrolysis of conjugates was conducted for 3 h at 50 °C. After cooling of the samples, the solution was transferred to Oasis HLB cartridges 60 mg (Waters, Eschborn, Germany)) for solid-phase extraction (SPE). After loading of the samples, the SPE columns were washed with 2 ml of water and the analytes eluted with 2 x 0.5 ml of methanol. The eluate was concentrated in vacuum, reconstituted in 2 ml of 2% aqueous acetic acid containing 10% acetonitrile and subsequently analyzed by LC-MS/MS. For the detection of free phase I metabolites or the intact phase II conjugated metabolites, the enzymatic hydrolysis was omitted.

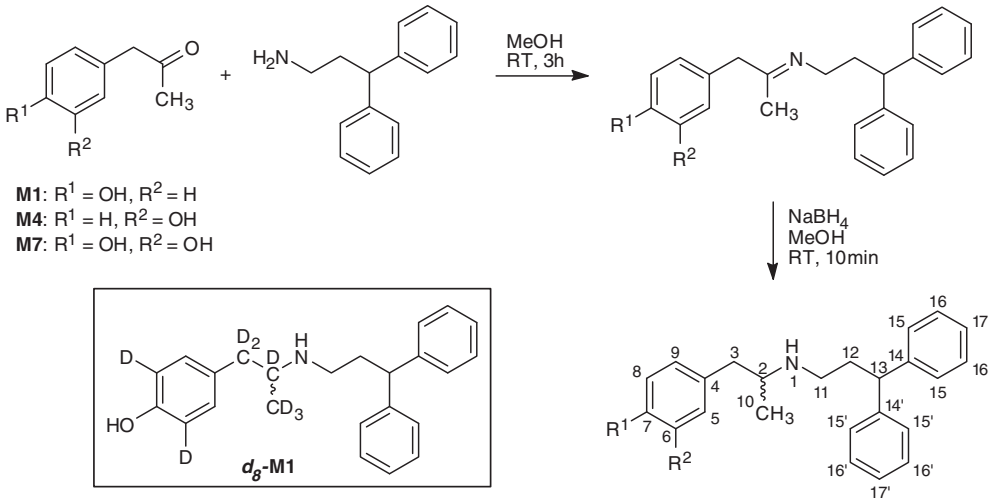
Liquid chromatography-tandem mass spectrometry

Urinary metabolites of prenylamine were detected by means of high-performance liquid chromatography (HPLC) coupled to high resolution tandem mass spectrometry via electrospray ionization (ESI). The instrumental LC set-up consisted of an Agilent Technologies 1260 liquid chromatograph (Waldbronn, Germany), equipped with a Phenomenex Gemini C6-Phenyl analytical column (2.1 x 100 mm, 3 µm particle size, Aschaffenburg, Germany). Using 0.2% formic acid (A) and acetonitrile (B) as eluents and a flow rate of 300 µl/min, the gradient elution was programmed as follows: 90% A -> 70% A (20 min), 70% A -> 30% A (5 min), 100% B (2 min), 90% A (5 min), run time 32 min.

The ion source was operated in positive electrospray mode at 500 °C, using an ionization voltage of 5500 V. The protonated analytes were detected with an AB Sciex TripleTOF 5600 quadrupole-time-of-flight hybrid mass spectrometer (Darmstadt, Germany), using full ToF-MS as well as product ion MS/MS scans of theoretical metabolite ion traces (e.g. *m/z* 346 and 362 for mono- and di-hydroxylation, respectively). In order to ensure a mass error of <5 ppm throughout the analysis, automatic mass calibration was conducted every five injections by post-column T-split infusion of the AB Sciex APCI positive calibration solution (diluted 1:10 in methanol) using the external calibrant delivery system. Spectra were processed with the Analyst TF Software package (AB Sciex), including the PeakView software for calculation of elemental compositions and mass accuracies of precursor and product ions.

Synthesis of metabolites and stable isotope labelled analogues

The synthetic route for the preparation of prenylamine metabolites is depicted in Scheme 1. For the synthesis of **M1**, 4-hydroxy phenylacetone was dissolved in dry methanol and treated with 1.1 equivalents (eq.) of 3,3-diphenylpropylamine at room temperature for 3 h under argon atmosphere. The intermediate imine (which could be detected by ESI-MS/MS analysis through direct injection of the diluted sample) was subsequently reduced by the slow addition of 1.6 eq. of solid sodium borohydride to the reaction mixture and stirring at room temperature. After 10 min, the reaction was stopped by addition of saturated aqueous Na_2CO_3 solution and the product was extracted 3x with MTBE. The combined organic phases were concentrated under reduced pressure and the crude product taken up in methanol/2% aqueous acetic acid 1:1 (*v/v*, concentration approx. 20 mg/ml). Purification of the reaction product was achieved by preparative HPLC with UV-DAD detection, using a Phenomenex Gemini C_6-phenyl column (10x250 mm) with 5 mM ammonium acetate (pH 3.5) and methanol as eluents, applying a linear gradient run at a flow rate of 5 ml/min. The analyte-containing fractions were collected from 16 HPLC-runs using an injection volume of 100 µl per injection. The methanol contained in the fractions was removed under reduced pressure, the pH adjusted to



Scheme 1. Synthetic route for the preparation of prenylamine metabolites **M1**, **M2** and **M7**. The NMR signals are assigned according to the atom numbering. The synthesis of stable isotope labelled ***d*$_8$-M1** (insert) was achieved by stirring the starting material 4-OH-phenylacetone in DCl/MeOD at 150 °C for 24 h in a pressure flask, followed by imine formation in MeOD and subsequent reduction by $NaBD_4$.

approx. 10 with aqueous Na_2CO_3 solution and the purified analyte extracted with MTBE. The organic phases were washed with MiliQ water, concentrated, and the pale-white residue dried over P_2O_5.

The preparation of **M4** was conducted analogously but using 3-hydroxy phenylacetone as starting material. **M7** was synthesized in small scale according to the same procedure, using 1 mg of 3,4-dihydroxy phenylacetone as starting material. Due to the low yield, this product was only characterized by LC-MS/MS analysis. For the synthesis of the stable-isotope labelled analogue ***d*$_8$-M1**, 4-hydroxy phenylacetone was dissolved in methanol-*d*, concentrated under reduced pressure and dissolved in methanol-*d* again. The solution was diluted 1:1 (*v/v*) with a solution of 20% deuterium chloride in D_2O and heated to 150 °C in a pressure flask for 24 h. After cooling, the mixture was quenched by addition of dry Na_2CO_3 and the partly H/D-exchanged 4-OH-phenylacetone extracted with MTBE, concentrated, and dried over P_2O_5. The exchange-product was reacted with 3,3-diphenylpropylamine in methanol-*d* and the intermediate imine was directly reduced with sodium borodeuteride. The reaction was stopped by the addition of Na_2CO_3-solution in D_2O. All other reaction conditions as well as the work-up were the same as described above.

Nuclear magnetic resonance spectroscopy

The molecular structures of **M1**, **M4** and **d_8-M1** were confirmed by nuclear magnetic resonance spectroscopy (NMR) with ^{1}H, H, H-COSY, H,C-HMQC, H,C-HMBC and ^{13}C DEPT experiments employing a Bruker DRX 500 instrument (Bruker, Karlsruhe, Germany) equipped with a 5 mm inverse probe head (z-gradient coil). All spectra were recorded at room temperature from solutions of approximately 5 mg of analyte per ml of methanol-d_4. The spectra were calibrated using the methanol residual peak as reference signal.

Routine doping analysis

According to a recently published procedure for direct-injection urine analysis,[16] 90 µl of urine were fortified with 10 µl of a solution of ***d*$_8$-M1** as internal standard (final concentration of 20 ng/ml). The sample was mixed and 5 µl were injected for LC-ESI-MS/MS analysis. The analytical system consisted of an Agilent Technologies 1260 Series HPLC (Waldbronn, Germany) coupled to an API 5500 QTrap mass spectrometer (AB Sciex, Darmstadt, Germany). For the routine screening a Macherey Nagel (Düren, Germany) Nucleodur C18 Pyramid analytical column (2.1 x 50 mm, 3 µm particle size) with a Phenomenex Gemini C6-Phenyl precolumn (4 x 2 mm) was utilized for the separation of analytes. The gradient elution was carried out with 5 mM ammonium acetate buffer at pH 3.5 and acetonitrile as mobile phases A and B, respectively, and a flow rate of 350 µl/min. The gradient started at 100% A and was linearly decreased to 10% A in 4.1 min, followed by re-equilibration at 100% A for 6 min with a flow rate of 500 µl/min and 0.25 min with 350 µl/min. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode with ESI and scan-to-scan polarity switching. The MS parameters for MRM detection of **M1** and the internal standard were tuned by direct infusion of analyte solution (500 ng/ml in acetonitrile/formic acid 0.5%, 1:1), while the settings for the **M1**-glucuronide were optimized by repeated analysis of the post-administration urine sample (Table 1). All

Table 1. MRM parameters for the detection of **M1** and its glucuronide in routine screening

Analyte	Ion transition	Collision energy [V]	Declustering potential [V]
M1	346-107	45	100
	346-135	29	
	346-212	25	
	346-167	45	
M1-glucuronide	522-346	30	100
	522-107	40	
	522-135	35	
	522-212	40	
***d*$_8$-M1 (ISTD)**	354-212	25	100
	354-143	29	

additional MS settings were the same as previously described by Guddat *et al.*[16]

Method validation

Validation of the qualitative detection method was carried out for the criteria specificity, lower limit of detection (LLOD), linearity, intra- and inter-day precision and ion suppression/enhancement effects according to the guidelines of the International Conference on Harmonization and WADA.[17,18]

Specificity

Ten different blank urine samples from five healthy male and female volunteers, respectively, were prepared and analyzed as described above in order to check for interfering peaks at the respective retention times.

Lower limit of detection

Ten blank urine samples were spiked with the ISTD and the mean signal-to-noise ratio (S/N) at the retention time of **M1** was calculated. Then, the mean signal intensity for **M1** in ten fortified urine samples (10 ng/ml of **M1**) was assessed. The lower limit of detection (LLOD) was estimated by calculation of the concentration at an extrapolated S/N value of 3.

Linearity

The linearity of the method was determined by triplicate analysis of a 10-point calibration curve at concentrations between 10 and 100 ng/ml.

Intra-day and inter-day precision

Intra-day precisions were calculated as the coefficient of variation (CV) from batches of ten urine samples of low (10 ng/ml), medium (50 ng/ml) and high (100 ng/ml) concentrations, respectively (n = 10 + 10 + 10). The analysis was repeated on three consecutive days for the calculation of the inter-day precision (n = 30 + 30 + 30).

Ion suppression/enhancement effects

The influence of matrix effects on the ESI process was tested by analyzing three male and three female blank urine samples as well as solvent only with continuous co-infusion of **M1** (solution concentration 1 μg/ml, flow rate 7 μl/min) using a post column T-connector.[19,20] At the retention time of **M1**, the base-line-elevated chromatograms of the blank urine samples as well as the solvent injection were examined for suppression and/or enhancement effects.

Results and discussion

The data obtained from one and two-dimensional ^{1}H and ^{13}C NMR experiments with the synthesized prenylamine derivatives confirmed the molecular structures as depicted in Scheme 1. The purity of the synthesized standards was >90% according to NMR analysis.

M1: ^{1}H NMR (methanol-d_4, 500 MHz): δ (ppm) = 1.01 (d, J = 6.3 Hz, 3H, CH_3-10), 2.15-2.30 (m, 2H, CH_2-12) 2.43-2.67 (m, 4H, CH_2-3/11), 2.78 (sex, J = 6.6 Hz, 1H, CH-2), 3.83 (t, J = 8.0 Hz, 1H, CH-13), 6.76-7.81 (m, 2H, CH-6/8), 6.99-7.04 (m, 2H, CH-5/9), 7.15-7.25 (m, 6H, CH-15/15′, CH-17/17′), 7.28-7.31 (m, 4H, CH-16/16′). **^{13}C NMR** (methanol-d_4, 125 MHz): δ (ppm) = 19.69 (CH_3-10), 36.05 (CH_2-12), 43.44 (CH_2-3), 46.22 (CH_2-11), 50.39 (CH-13), 55.98 (CH-2), 116.72 (CH-6/8), 127.57/127.59 (CH-17/17′), 129.05/129.07 (CH-15/15′), 129.80 (CH-16/16′), 131.14 (C-4), 131.56 (CH-5/9), 146.15/146.22 (C-14/14′), 157.53 (C-7).

***d_8*-M1: ^{1}H NMR** (methanol-d_4, 500 MHz): δ (ppm) = 2.16-2.31 (m, 2H, CH_2-12), 2.45-2.54 (m, 1H, CH_2-11α), 2.58-2.69 (m, 1H, CH_2-11ß), 3.83 (t, J = 8.0 Hz, 1H, CH-13), 7.01 (s, 2H, CH-5/9), 7.15-7.25 (m, 6H, CH-15/15′, CH-17/17′), 7.28-7.31 (m, 4H, CH-16/16′). **^{13}C NMR** (methanol-d_4, 125 MHz): δ (ppm) = 36.03 (CH_2-12), 46.45 (CH_2-11), 50.39 (CH-3), 127.58/127.60 (CH-17/17′), 129.05/129.07 (CH-15/15′), 129.81 (CH-16/16′), 131.02 (C-4), 131.41 (CH-5/9), 146.15/146.21 (C-14/14′), 157.43 (C-7).

M4: ^{1}H NMR (methanol-d_4, 500 MHz): δ (ppm) = 1.03 (d, J = 6.3 Hz, 3H, CH_3-10), 2.15-2.31 (m, 2H, CH_2-12) 2.45-2.59 (m, 2H, CH_2-11), 2.59-2.68 (m, 2H, CH_2-3), 2.84 (sex, J = 6.6 Hz, 1H, CH-2), 3.83 (t, J = 8.0 Hz, 1H, CH-13), 6.65-7.70 (m, 2H, CH-5/9), 6.71-6.75 (m, 1H, CH-7), 7.15-7.25 (m, 7H, CH-15/15′, CH-8, CH-17/17′), 7.25-7.31 (m, 4H, CH-16/16′). **^{13}C NMR** (methanol-d_4, 125 MHz): δ (ppm) = 19.75 (CH_3-10), 36.00 (CH_2-12), 44.31 (CH_2-3), 46.16 (CH_2-11), 50.38 (CH-13), 55.74 (CH-2), 114.82 (CH-7), 117.33 (CH-5/CH-9), 121.76 (CH-5/CH-9), 127.57/127.60 (CH-17/17′), 129.06/129.08 (CH-15/15′), 129.80/129/81 (CH-16/16′), 130.98 (C-8), 142.10 (CH-4), 146.14/146.19 (C-14/14′), 159.15 (C-6).

Previous TLC- and GC-MS-based studies of the metabolism of ^{14}C-prenylamine in man identified four different metabolites with unchanged molecular backbone, mainly formed by hydroxylation and/or methoxylation at different positions.[12]

Due to the relatively high molecular mass and the increasing number of polar functional groups introduced into the prenylamine molecule by metabolic transformation, LC-MS/MS is a powerful analytical alternative to GC-MS for the direct detection of metabolites with intact prenylamine backbone. Application of high resolution/high accuracy hybrid MS/MS allows for the determination of elemental compositions of analytes and their product ions, eventually offering an additional dimension for the separation of the complex urinary metabolite mixture. Therefore, using positive ESI, TOF product ion scans of protonated precursor ions with *m/z* of theoretical metabolites were conducted. Extraction of exact masses corresponding to potential metabolites (e.g. 346.2165 ± 4 mDa from the TOF product ion chromatogram of 346 for mono-oxygenation) facilitated metabolite identification. By interpretation of product ion mass spectra and extraction of product ion chromatograms diagnostic for either modified or unmodified molecular moieties, the site of metabolic transformation could be suggested. In the product ion scan of *m/z* 346, obtained from LC-MS/MS analysis of an enzymatically hydrolyzed post-administration urine sample, four protonated mono-oxygenated prenylamine metabolites can be identified (Figure 1a, **M1-M4**).

The product ion spectrum of the most abundant metabolite **M1** eluting at 15.52 min (Figure 1b) shows an abundant signal at *m/z* 212.1436, with the molecular composition $C_{15}H_{18}N$ (theoretical mass 212.1434, error 1.0 ppm, Table 2), corresponding to the unmodified 3,3-diphenylpropylamine (DPPA) residue. The two base peak product ions at *m/z* 135.0807 ($C_9H_{11}O$, 2.1 ppm) and 107.0494 (C_7H_7O, 2.4 ppm) are proposed to be formed by cleavage of the N-C2 bond and the C2-C3 bond of the amphetamine moiety, respectively (Figure 1b). The presence of the oxygen atom in these product ions together with the detection of unmodified DPPA delineates a hydroxylation at the phenylethyl moiety. Apart from differences in the relative ion intensities, all product ions observed after CID of protonated **M1** are

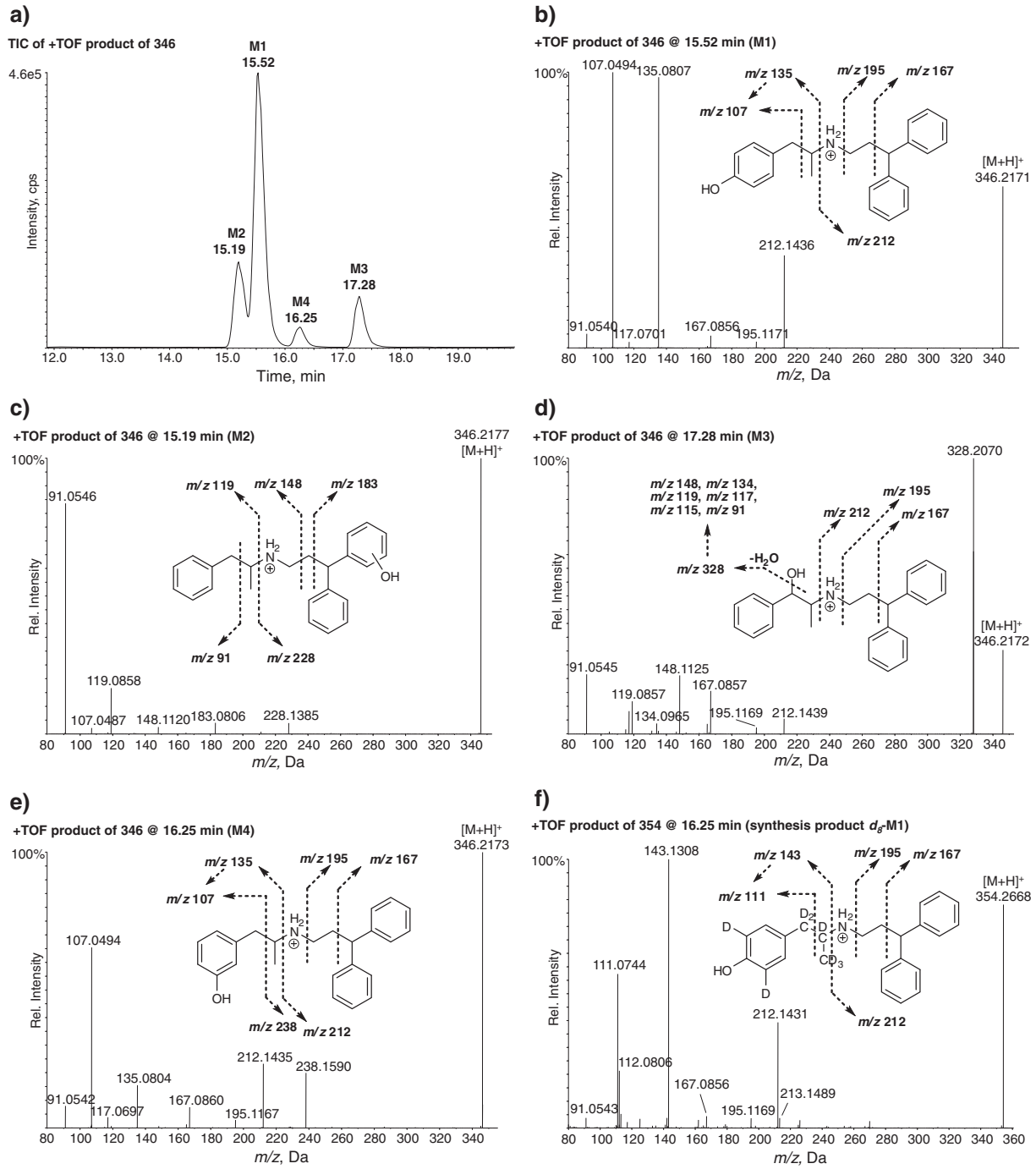


Figure 1. (a) +TOF product ion chromatogram of *m/z* 346 of a post-administration urine sample (8.5 h) after enzymatic hydrolysis with ß-glucuronidase/aryl sulfatase. (b-e) +TOF product ion mass spectra of protonated metabolites **M1-M4**, extracted from the LC-MS/MS product ion scan of *m/z* 346. (f) LC-MS/MS product ion mass spectrum of synthesized stable isotope labeled ***d*$_8$-M1**.

also visible in the MS/MS spectrum of the minor hydroxy-metabolite **M4** at 16.25 min (Figure 1e), indicating a close structural isomerism of both compounds. The additional product ion at *m/z* 238.1590 will be discussed later. *Para (p)*- and *meta (m)*-hydroxy-prenylamine have previously been identified as main metabolites in rat urine by TLC-MS,[7] while metabolic studies with ^{14}C-prenylamine in humans[12] did not achieve a definite assignment of the exact position of the hydroxylation. In order to unambiguously identify the regioisomerism of **M1** and **M4**, reference material of *p*- and *m*-hydroxy-prenylamine was synthesized in a facile one-pot procedure (Scheme 1). Both molecular structures were confirmed by ^{1}H and ^{13}C NMR spectroscopy. Comparison of retention times as well as the relative product ion intensities of the synthesized reference standards to the post-administration urine sample clearly substantiates the *p*-hydroxy-prenylamine structure for the most abundant phase I metabolite **M1**, while the minor hydroxy-metabolite **M4** was identified as *m*-hydroxy-prenylamine. Interestingly, the product ion at *m/z* 238.1590 ($C_{17}H_{20}N$, -0.2 ppm) is exclusively observed in the MS/MS spectrum of protonated

Table 2. High resolution/high accuracy MS/MS data of monohydroxy-prenylamine metabolites

Metabolite (ret. time/min)	Precurs. ion (*m/z*)	Elemental comp.	Error (ppm)	Product ions (*m/z*)	Elemental comp.	Error (ppm)
M1	346.2171	$C_{24}H_{28}NO$	1.5	212.1436	$C_{15}H_{18}N$	1.5
(15.52)				195.1171	$C_{15}H_{15}$	1.2
				167.0856	$C_{13}H_{11}$	0.1
				165.0963	$C_{13}H_{9}$	−3.3
				135.0807	$C_{9}H_{11}O$	2.1
				117.0701	$C_{9}H_{9}$	1.7
				107.0494	$C_{7}H_{7}O$	2.4
				91.0540	$C_{7}H_{7}$	−2.1
M2	346.2177	$C_{24}H_{28}NO$	3.5	228.1385	$C_{15}H_{18}NO$	0.8
(15.19)				183.0806	$C_{13}H_{11}O$	1.1
				148.1120	$C_{10}H_{14}N$	−0.4
				119.0858	$C_{9}H_{11}$	2.6
				107.0487	$C_{7}H_{7}O$	−3.7
				91.0546	$C_{7}H_{7}$	3.6
M3	346.2172	$C_{24}H_{28}NO$	1.9	328.2070	$C_{24}H_{26}N$	3.1
(17.28)				212.1439	$C_{15}H_{18}N$	2.3
				195.1169	$C_{15}H_{15}$	−0.1
				167.0857	$C_{13}H_{11}$	1.3
				165.0698	$C_{13}H_{9}$	−0.1
				148.1125	$C_{10}H_{14}N$	2.7
				134.0965	$C_{9}H_{12}N$	0.6
				119.0857	$C_{9}H_{11}$	1.0
				117.0701	$C_{9}H_{9}$	1.6
				115.0544	$C_{9}H_{7}$	1.9
				91.0545	$C_{7}H_{7}$	2.8
M4	346.2173		2.1	238.1590	$C_{17}H_{20}N$	−0.2
(16.25)				212.1435	$C_{15}H_{18}N$	0.4
				195.1167	$C_{15}H_{15}$	−0.4
				167.0860	$C_{13}H_{11}$	2.7
				165.0712	$C_{13}H_{9}$	2.8
				135.0804	$C_{9}H_{11}O$	−0.1
				117.0697	$C_{9}H_{9}$	−1.8
				107.0697	$C_{7}H_{7}O$	2.7
				91.0542	$C_{7}H_{7}$	0.0

m-hydroxy-prenylamine while it is completely absent in case of the *p*-hydroxy isomer. MS/MS analysis combined with stable isotope labelling and H/D-exchange experiments conducted with synthesized **M4** (not shown), substantiates the migration of a proton from the charged nitrogen to the benzylic methylene group, cleavage of the α-C-C bond and formation of a protonated N-ethylidene-3,3-diphenylpropylamonium product ion (CID scheme in Figure 1e). The neutral elimination product is *m*-cresol. In case of CID of protonated **M1**, α-cleavage leads to the favoured transfer of the positive charge to the resonance stabilized *p*-cresol product ion with *m/z* 107 (Figure 1b). The MS/MS spectrum of the protonated stable isotope labelled analogue ***d*$_8$-M1** (Figure 1f), which was synthesized for the support of analytical assay development, shows correspondent product ions and corroborates the dissociation pathways described above.

Interpretation of the CID pathways of the protonated metabolite **M2** eluting at 15.19 min, allows for the structural assignment depicted in Figure 1c. A mass increment of the DPPA product ion (*m/z* 212) by 16 u yields the product ion at *m/z* 228.1385 ($C_{15}H_{18}NO$, 0.8 ppm) and indicates a hydroxylation at this part of the molecule. The product ion at *m/z* 183.0806 ($C_{13}H_{11}O$, 1.1 ppm) corresponds to a hydroxylated diphenylmethane cation, which provides evidence for the oxygen atom to be bound either aromatically or to the disubstituted aliphatic carbon atom. In accordance to previous studies, however, an unambiguous differentiation of the exact position of the hydroxy substituent in diphenylmethyl part was not possible by means of LC-MS/MS.

Elucidation of the CID behaviour of protonated metabolite **M3** (RT = 17.28 min, Figure 1a and d) is not as straightforward as described above. While the presence of *m/z* 212.1439 corresponds to an unmodified DPPA moiety, the base peak product ion at *m/z* 328.2070 ($C_{20}H_{26}N$, 3.1 ppm), generated by the favoured loss of water from the protonated precursor, indicates an aliphatic hydroxylation. Unfortunately, all product ions that might provide mass spectral evidence for the exact position of the hydroxy function are generated from the already dehydrated product ion at *m/z* 328, which hampers the unambiguous localization of the hydroxylation site. Conceivable would be either the benzylic position at C-3 or the methyl group (C-10), while metabolic hydroxylation of C-2 would produce an unstable amino-hemiacetal, probably leading to cleavage of the C2-N

bond. Due to the fact, however, that norephedrine and cathine, which were detected in minor concentrations also in the present post-administration urine sample (not shown), are known prenylamine metabolites formed by aliphatic hydroxylation and *N*-dealkylation of the active drug, the benzylic position of the hydroxy group as shown in Figure 1d seems to be plausible. Remarkably, **M3** was found to be almost exclusively excreted into the urine as glucuronic acid conjugate and was hardly detectable as free metabolite if the enzymatic hydrolysis step was omitted. All other hydroxy-metabolites were also present in the urine in unconjugated form to varying extents (data not shown).

Analysis of the urine sample without previous enzymatic hydrolysis also allowed for the direct detection of glucuronic acid conjugates of **M1-M4**. Figure 2a-i shows the chromatogram of the corresponding product ion scan for *m/z* 522 (346 + 176), revealing four metabolite peaks. All glucuronides were amenable to complete enzymatic deconjugation by *ß*-glucuronidase/aryl sulfatase from *Helix Pomatia* (Figure 2a-ii) as well as by *ß*-glucuronidase from *E.coli* (iii). The identity of the glucuronides was unambiguously confirmed by high resolution MS/MS analysis and comparison with the previously discussed CID pathways of the free monohydroxy metabolites (Table 3). **M1** and **M2** were found to be additionally excreted into the urine as sulfo-conjugates, which was corroborated by the product ion scan for *m/z* 426, yielding two peaks corresponding to OH-prenylamine sulfate metabolites at 23.84 min and 24.15 min, respectively (Figure 2b). MS/MS data, supporting the structural identification of **M1** and **M2** sulfate, respectively, are given in Table 3. Enzymatic hydrolysis with *ß*-glucuronidase/aryl sulfatase fully deconjugated the **M2** sulfate, while the **M1** sulfoconjugate was only incompletely hydrolysed and still detectable after hydrolysis (Figure 2b).

Besides the abundant monohydroxy metabolites and its conjugates, a variety of dihydroxylated prenylamine metabolites were identified from the product ion scan for *m/z* 362 (Figure 3a). Using extracted ion chromatograms of diagnostic product ions, the hydroxylation sites of the partly coeluting analytes could be interpreted. The results of the structure identification are included in Table 4. The intense product ions at *m/z* 212.1436 ($C_{15}H_{18}N$, 1.4 ppm), *m/z* 151.0755 ($C_9H_{11}O_2$, 1.3 ppm) and *m/z* 123.0441 ($C_7H_7O_2$, 0.8 ppm) of the protonated precursor of **M7** (most abundant dihydroxy metabolite) indicate a dihydroxylation of the amphetamine phenyl ring. LC-MS/MS analysis of synthesized *m-,p*-dihydroxy-prenylamine, prepared in analogy to **M1** and **M4** from 3,4-dihydroxy-phenylacetone, and comparison of retention times as well as product ion spectra (Figures 3b and 3c, Table 4) substantiated the structural assignment as shown in Figure 3d. **M7** was found to be present in the native urine in the form of two different glucuronides and two sulfoconjugates, respectively (Table 3), while the free phase I metabolite was detected only in traces, when the urine samples were not subjected to enzymatic hydrolysis. As to be concluded from the product ion patterns, the 12 dihydroxy metabolites with *m/z* 362 comprise the amphetamine-phenyl-dihydroxy metabolite **M7**, 3 combinations of amphetamine-phenyl and -benzylic hydroxylations (**M5**, **M6**, **M9**), two metabolites with modification at the amphetamine-phenyl and the DPPA moiety (**M14**, **M15**), two combined benzyl-DPPA-hydroxy metabolites (**M8**, **M10**) and four metabolites with different hydroxylations at the DPPA part of the drug (**M11-M13**, **M16**; Table 4).

In accordance to previous reports,[12] combined methoxylation and hydroxylation constitutes another major metabolic pathway of prenylamine modification. Two abundant metabolites with *m/z* 376 and the elemental composition $C_{25}H_{30}NO_2$ could be identified at 15.74 min and 15.07 min, respectively (Table 4). As to be concluded from the product ion spectra, both the hydroxy- and the methoxy-modification are located either at the amphetamine phenyl ring (**M17**) or the DPPA moiety (**M18**). Using this approach of exact and accurate product ion chromatograms, a plethora of additional metabolites that are formed by variable combination of hydroxylations and methoxylations was identified (Table 4). In summary and in addition to the previously described metabolites, the enzymatically hydrolysed post-administration urine sample contained seven trishydroxy metabolites with *m/z* 378 (**M19-M25**), seven dihydroxy-methoxy metabolites with *m/z* 392 (**M26-M32**), five tetrahydroxy metabolites with

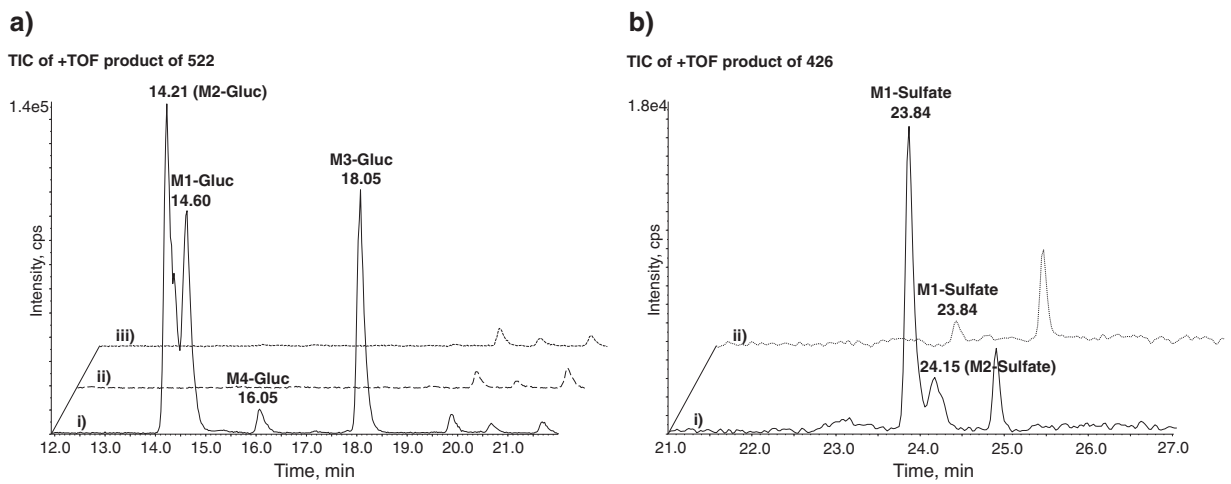


Figure 2. (a-i) +TOF product ion chromatogram of *m/z* 552 of the post-administration urine sample without previous enzymatic hydrolysis. The chromatogram shows signals for glucuronic acid conjugates of the monohydroxy-prenylamine metabolites **M1-M4**. (a-ii) Post-administration urine sample after enzymatic hydrolysis with *ß*-glucuronidase/aryl sulfatase from *Helix Pomatia*, and (a-iii) with *ß*-glucuronidase from *E.coli* (60 min, 50 °C, pH7.0). (b) Chromatograms of +TOF product ion scan of *m/z* 426 diagnosing sulfate conjugates of **M1** and **M2** in urine samples without previous hydrolysis (i) and with enzymatic hydrolysis (ii).

Table 3. High resolution/high accuracy MS/MS data of selected phase II prenylamine metabolites

Metabolite (ret. time/min)	Precurs. ion (*m/z*)	Elemental comp.	Error (ppm)	Product ions (*m/z*)	Elemental comp.	Error (ppm)
M1-Gluc	522.2495	$C_{30}H_{36}NO_7$	1.6	346.2166	$C_{24}H_{28}NO$	0.8
(14.60)				212.1435	$C_{15}H_{18}N$	0.3
				135.0804	$C_9H_{11}O$	−0.7
				107.0491	C_7H_7O	0.1
M2-Gluc	522.2506	$C_{30}H_{36}NO_7$	3.7	346.2178	$C_{24}H_{28}NO$	3.6
(14.21)				228.1380	$C_{15}H_{18}NO$	−1.3
				148.1122	$C_{10}H_{14}N$	0.7
				119.0855	C_9H_{11}	0.0
M3-Gluc	522.2499	$C_{30}H_{36}NO_7$	2.4	346.2170	$C_{24}H_{28}NO$	1.3
(18.05)				228.2067	$C_{24}H_{26}N$	2.1
				148.1122	$C_{10}H_{14}N$	0.7
				119.0854	C_9H_{11}	0.8
M4-Gluc	522.2502	$C_{30}H_{36}NO_7$	3.0	346.2167	$C_{24}H_{28}NO$	0.6
(16.05)				238.1592	$C_{17}H_{20}N$	0.9
				212.1422	$C_{15}H_{18}N$	>5
				107.0475	C_7H_7O	>5
M1-Sulf	426.1733	$C_{24}H_{28}NSO_4$	0.1	346.2173	$C_{24}H_{28}NO$	2.2
(23.84)				215.0378	$C_9H_{11}SO_4$	2.7
				212.1434	$C_{15}H_{18}N$	0.3
				187.0055	$C_7H_7SO_4$	−2.4
				167.0856	$C_{13}H_{11}$	0.4
				135.0804	$C_9H_{11}O$	−0.1
				107.0485	C_7H_7O	>5
M2-Sulf	426.1734	$C_{24}H_{28}NSO_4$	0.1	346.2164	$C_{24}H_{28}NO$	−0.5
(24.15)				308.0949	$C_{15}H_{18}NSO_4$	−0.8
				228.1390	$C_{15}H_{18}NO$	3.2
				119.0842	C_9H_{11}	>5
M7-Gluc1	538.2450	$C_{30}H_{36}NO_8$	2.9	362.2121	$C_{24}H_{28}NO_2$	2.1
(16.27)				238.1607	$C_{17}H_{20}N$	−2.5
				212.1442	$C_{15}H_{18}N$	3.9
				151.0753	$C_9H_{11}O_2$	0.5
				123.0442	$C_7H_7O_2$	1.4
M7-Gluc2	538.2439	$C_{30}H_{36}NO_8$	0.7	362.2120	$C_{24}H_{28}NO_2$	1.5
(14.55)				238.1604	$C_{17}H_{20}N$	−1.9
				212.1411		>5
				151.0748	$C_9H_{11}O_2$	−4.0
				123.0415	$C_7H_7O_2$	−4.2
M7-Sulf1	442.1694	$C_{24}H_{28}NSO_5$	2.7	362.2117	$C_{24}H_{28}NO_2$	0.7
(24.26)				212.1440	$C_{15}H_{18}N$	2.8
				151.0760	$C_9H_{11}O_2$	4.4
				123.0432		>5
M7-Sulf2	442.1688	$C_{24}H_{28}NSO_5$	1.2	362.2122	$C_{24}H_{28}NO_2$	2.0
(23.91)				212.1425	$C_{15}H_{18}N$	−4.1
				151.0754	$C_9H_{11}O_2$	0.6
				123.0443	$C_7H_7O_2$	2.2
				107.0490	C_7H_7O	−1.5
				105.0697	C_8H_9	−1.6
				91.0539	C_7H_7	−3.4

m/z 394 (**M33-M37**), three trihydroxy-methoxy metabolites with *m/z* 408 (**M38-M40**) and two dihydroxy-dimethoxy metabolites with *m/z* 422 (**M41**, **M42**). Most of these phase I metabolites were present in the native urine sample in the form of glucuronic acid and/or sulfo-conjugates (data not shown).

Finally, *N*-dealkylation of the intact drug as well as of various metabolites on both sides of the amine function, liberating either free or modified amphetamine or DPPA, respectively, adds ultimate complexity to the metabolic profile of prenylamine. Amphetamine was also detected in the post-administration urine samples (data not shown) but since the chromatographic and mass spectral properties of this stimulant and its metabolites/analogues are well described elsewhere, only the data of three exemplary DPPA metabolites are included in Table 4, for example, unmodified DPPA (*m/z* 212, **M43**), hydroxy-DPPA (*m/z* 228, **M44**) and dihydroxy-DPPA (*m/z* 244, **M45**).

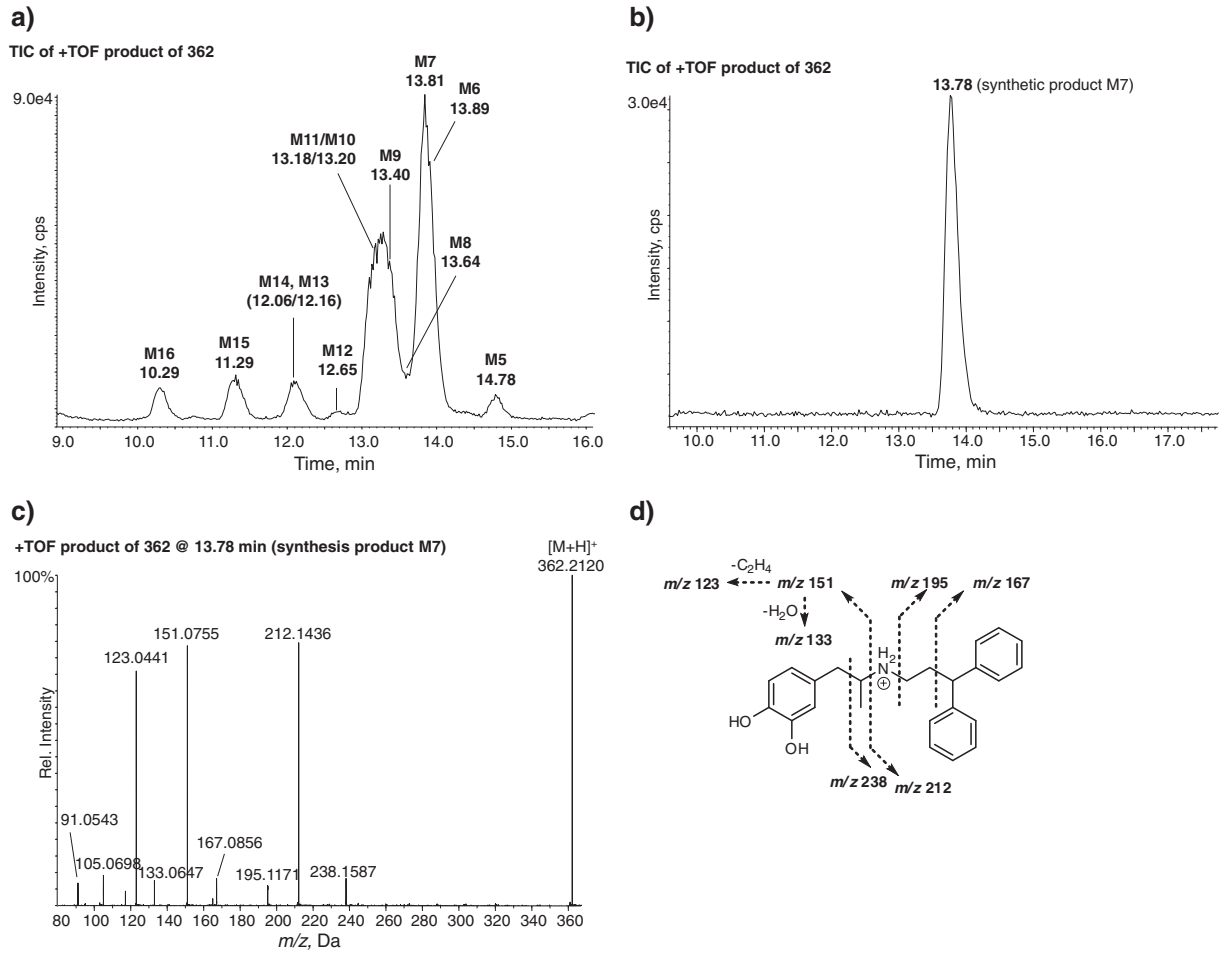


Figure 3. (a) +TOF product ion chromatogram of *m/z* 362, corresponding to the mass of protonated dihydroxy-prenylamine, of a post-administration urine sample after enzymatic hydrolysis with ß-glucuronidase/aryl sulfatase. (b) +TOF product ion chromatogram and (c) product ion spectrum of the synthesis product **M7**, respectively. (d) Structure of **M7** and proposed CID pathways.

Determination of prenylamine in routine doping analysis

Current doping control test methods are developing towards the reduction of sample preparation workloads in order to deal with the increasing complexity and number of analytical targets. Constant instrumental improvements including optimized chromatographic systems as well as more sensitive, robust, and rapid mass detectors allow for more economic analytical approaches like the direct injection of urine samples without previous purification procedures.

In compliance with these developments, the most abundant phase I metabolite **M1**, *p*-hydroxy-prenylamine, was included into an existing direct injection-LC-MS/MS-based routine urinary doping detection method. For optimal compensation of potential matrix effects, the stable isotope labelled ***d*$_8$-M1** was used as internal standard. The assay was fully validated regarding the criteria specificity, intra- and inter-day precision, LLOD, linearity, and ion suppression/enhancement effects. The specificity of the method is illustrated in Figure 4. While there is significant matrix background signal for the two most abundant ion transitions 346–107 and 346–135 (Figure 4a), the ion chromatogram for 346–212 shows high specificity for **M1** at the respective retention time and is therefore recommended as main screening tool. Under the rapid chromatographic conditions applied, the expected co-elution of **M1** and **M4** combined with their very similar CID behaviour does not allow for a differentiation of these isobaric isomers. This would require a significantly extended gradient run or the chromatographic set-up described for the metabolite identification studies (*vide supra*). The specificity of the method for the internal standard is demonstrated in Figures 4a and 4b. The method proved to be linear in the concentration range from 10 to 100 ng/ml (with linear regression coefficients of > 0.99) and ion suppression/ enhancement effects accounted for less than 10% of signal intensity at the respective retention times of the analyte (not shown). The LLOD is estimated with 0.1 ng/ml and the deviations calculated for the intra-day as well as inter-day precisions are better than 10% CV (Table 5).

Figure 4c shows the selected screening ion transitions of a directly injected post-administration urine sample (29.5 h after drug administration). Since the amount of free urinary phase I metabolite may be subject to considerable inter-individual as well as intra-individual variation from sample to sample (as previously reported for the general prenylamine metabolism) and no enzymatic hydrolysis is conducted in the screening procedure, the glucuronic acid conjugate of **M1** at *m/z* 522 can serve as an alternative or additional screening target (Figure 4c, transition 522-212). The specificity for the respective ion transition was also demonstrated by the analysis of ten different blank urine

Table 4. High resolution/high accuracy MS/MS data of multiply modified phase I metabolites

Metabolite (ret. time/min)	Precurs. ion (*m/z*)	Elemental comp.	Error (ppm)	Product ions (*m/z*)	Elemental comp.	Error (ppm)	Metabolic transformation		
							amph. phen.	amph. Benz.	DPPA
M5	362.2124	$C_{24}H_{28}NO_2$	2.7	344.2011	$C_{24}H_{26}NO$	0.6	*m*-OH	OH	-
(14.78)				238.1595	$C_{17}H_{20}N$	2.1			
				212.1442	$C_{15}H_{18}N$	4.1			
				195.1157	$C_{15}H_{15}$	>5			
				167.0861	$C_{13}H_{11}$				
				164.1068	$C_{10}H_{14}NO$	−0.9			
				135.0809	$C_9H_{11}O$				
				117.0700	C_9H_9	1.3			
				107.0490	C_7H_7O	−1.5			
				105.0697	C_8H_9	−1.6			
				91.0539	C_7H_7	−3.4			
M6	362.2116	$C_{24}H_{28}NO_2$	0.3	344.2017	$C_{24}H_{26}NO$	2.3	*p*-OH	OH	-
(13.89)				195.1169	$C_{15}H_{15}$	0.2			
				167.0853	$C_{13}H_{11}$	−1.1			
				164.1064	$C_{10}H_{14}NO$	−3.7			
				135.0803	$C_9H_{11}O$	−0.9			
				117.0699	C_9H_9	0.4			
				107.0490	C_7H_7O	−1.5			
				105.0696	C_8H_9	−2.4			
				91.0542	C_7H_7	−0.3			
M7	362.2121	$C_{24}H_{28}NO_2$	1.8	238.1592	$C_{17}H_{20}N$	0.9	*m*-OH	-	-
(13.81)				212.1437	$C_{15}H_{18}N$	1.4	+		
				195.1171	$C_{15}H_{15}$	1.1	*p*-OH		
				151.0756	$C_9H_{11}O_2$	1.3			
				133.0647	C_9H_9O	−0.5			
				123.0442	$C_7H_7O_2$	0.8			
				105.0695	C_8H_9	−3.6			
				91.0542	C_7H_7	−0.4			
M8	362.2119	$C_{24}H_{28}NO_2$	1.2	344.2013	$C_{24}H_{26}NO$	1.3	-	OH	OH
(13.64)				183.0806	$C_{13}H_{11}O$	1.0			
				148.1120	$C_{10}H_{14}N$	−0.6			
				146.0966	$C_{10}H_{12}N$	1.3			
				119.0856	C_9H_{11}	1.0			
				91.0540	C_7H_7	−2.2			
M9	362.2121	$C_{24}H_{28}NO_2$	1.8	212.1436	$C_{15}H_{18}N$	1.2	*p*-OH	OH	-
(13.40)				195.1169	$C_{15}H_{15}$	0.1			
				167.0858	$C_{13}H_{11}$	1.4			
				107.0492	C_7H_7O	0.8			
				91.0542	C_7H_7	0.0			
M10	362.2120	$C_{24}H_{28}NO_2$	1.6	344.2015	$C_{24}H_{26}NO$	1.7	-	OH	OH
(13.20)				211.1121	$C_{15}H_{15}O$	1.5			
				183.0811	$C_{13}H_{11}O$	3.6			
				148.1122	$C_{10}H_{14}N$	0.8			
				146.0964	$C_{10}H_{12}N$	0.1			
				119.0856	C_9H_{11}	0.9			
M11	362.2120	$C_{24}H_{28}NO_2$	1.6	244.1333	$C_{15}H_{18}NO_2$		-	-	$(OH)_2$
(13.18)				199.0759	$C_{13}H_{11}O_2$	2.9			
				123.0441	$C_7H_7O_2$	0.4			
				119.0856	C_9H_{11}	0.9			
M12	362.2107	$C_{24}H_{28}NO_2$	−2.0	148.1123	$C_{10}H_{14}N$	1.5	-	-	$(OH)_2$
(12.65)				119.0855	C_9H_{11}	0.0			
				91.0542	C_7H_7	−0.3			
M13	362.2128	$C_{24}H_{28}NO_2$	3.7	148.1121	$C_{10}H_{14}N$	0.5	-	-	$(OH)_2$
(12.16)				119.0854	C_9H_{11}	−0.6			
				91.0542	C_7H_7	−0.1			

Table 4. *(Continued)*

Metabolite (ret. time/min)	Precurs. ion (*m/z*)	Elemental comp.	Error (ppm)	Product ions (*m/z*)	Elemental comp.	Error (ppm)	Metabolic transformation		
							amph. phen.	amph. Benz.	DPPA
M14 (12.06)	362.2118	$C_{24}H_{28}NO_2$	0.8	228.1385	$C_{15}H_{18}NO$	1.1	OH	-	OH
				164.1068	$C_{10}H_{14}NO$	−1.2			
				135.0801	$C_9H_{11}O$	−2.3			
				107.0486	C_7H_7O	−5.0			
M15 (11.29)	362.2117	$C_{24}H_{28}NO_2$	0.7	228.1386	$C_{15}H_{18}NO$	1.4	*p*-OH	-	OH
				183.0776	$C_{13}H_{11}O$	>5			
				135.0800	$C_9H_{11}O$	−2.9			
				107.0489	C_7H_7O	−2.6			
M16 (10.29)	362.2121	$C_{24}H_{28}NO_2$	1.8	199.0748	$C_{13}H_{11}O_2$	−2.7	-	-	$(OH)_2$
				148.1120	$C_{10}H_{14}N$	−0.5			
				119.0854	C_9H_{11}	−0.6			
				107.0491	C_7H_7O	−2.6			
				91.0540	C_7H_7O	−2.6			
M17 *(15.74)*	376.2284	$C_{25}H_{30}NO_2$	2.7	238.1591	$C_{17}H_{20}N$	0.4	OMe + OH	-	-
				212.1436	$C_{15}H_{18}N$	1.5			
				165.0913	$C_{10}H_{13}O_2$	3.2			
				137.0597	$C_8H_9O_2$	2.1			
				133.0647	C_9H_9O	2.5			
				105.0696	C_8H_9	1.0			
				91.0540	C_7H_7	−2.1			
M18 *(15.07)*	376.2283	$C_{25}H_{30}NO_2$	3.1	213.0914	$C_{14}H_{13}O_2$	2.0	-	-	OMe + OH
				148.1125	$C_{10}H_{14}N$	2.5			
				137.0599	$C_8H_9O_2$	1.3			
				119.0858	C_9H_{11}	2.6			
				91.0546	C_7H_7	4.6			
M19 (12.41)	378.2067	$C_{24}H_{28}NO_3$	1.1	360.1962	$C_{24}H_{26}NO_2$	0.9	$(OH)_2$	OH	-
				212.1441	$C_{15}H_{18}N$	3.3			
				180.1018	$C_{10}H_{14}NO_2$	0.0			
				167.0848		>5			
				151.0752	$C_9H_{11}O_2$	−0.2			
				123.0448		>5			
				117.0708		>5			
				91.0543	C_7H_7	0.4			
M20 (11.61)	378.2068	$C_{24}H_{28}NO_3$	1.2	360.1962	$C_{24}H_{26}NO_2$	1.0	-	OH	$(OH)_2$
				199.0759	$C_{13}H_{11}O_2$	2.7			
				148.1123	$C_{10}H_{14}N$	1.6			
				146.0965	$C_{10}H_{12}N$	0.2			
				134.0969	$C_9H_{12}N$	3.4			
				123.0441	$C_7H_7O_2$	0.7			
				119.0857	C_9H_{11}	1.5			
				105.0700	C_8H_9	0.8			
				91.0545	C_7H_7	3.0			
M21 (11.28)	378.2068	$C_{24}H_{28}NO_3$	1.0	360.1964	$C_{24}H_{26}NO_2$	1.7	-	OH	$(OH)_2$
				244.1334	$C_{15}H_{18}NO_2$	0.9			
				227.1064	$C_{15}H_{15}O_2$	−1.3			
				199.0755	$C_{13}H_{11}O_2$	0.7			
				148.1121	$C_{10}H_{14}N$	0.5			
				146.0961	$C_{10}H_{12}N$	−2.4			
				134.0965	$C_9H_{12}N$	0.3			
				123.0443	$C_7H_7O_2$	2.3			
				119.0854	C_9H_{11}	−1.5			
				117.0693	C_9H_9	−4.6			
				91.0542	C_7H_7	−0.7			
M22	378.2063	$C_{24}H_{28}NO_3$	−0.3	360.1961	$C_{24}H_{26}NO_2$	0.8	-	OH	$(OH)_2$

Table 4. *(Continued)*

Metabolite (ret. time/min)	Precurs. ion (*m/z*)	Elemental comp.	Error (ppm)	Product ions (*m/z*)	Elemental comp.	Error (ppm)	Metabolic transformation		
							amph. phen.	amph. Benz.	DPPA
(11.01)				244.1339	$C_{15}H_{18}NO_2$	2.8			
				227.1072	$C_{15}H_{15}O_2$	2.3			
				199.0758	$C_{13}H_{11}O_2$	2.2			
				148.1123	$C_{10}H_{14}N$	1.8			
				146.0962	$C_{10}H_{12}N$	−1.3			
				134.0968	$C_9H_{12}N$	3.2			
				123.0443	$C_7H_7O_2$	2.3			
				119.0854	C_9H_{11}	−1.1			
				117.0696	C_9H_9	−2.7			
				105.0700	C_8H_9	1.2			
				91.0542	C_7H_7	−0.7			
M23 (9.47)	378.2068	$C_{24}H_{28}NO_3$	1.2	360.1961	$C_{24}H_{26}NO_2$	0.7	$(OH)_2$	-	OH
				254.1541	$C_{17}H_{20}NO$	0.7			
				228.1384	$C_{15}H_{18}NO$	0.3			
				183.0819		>5			
				151.0755	$C_9H_{11}O_2$	1.2			
				123.0441	$C_7H_7O_2$	0.4			
				119.0855	C_9H_{11}	−0.2			
				105.0700	C_8H_9	2.1			
				91.0543	C_7H_7	0.6			
M24 (9.21)	378.2067	$C_{24}H_{28}NO_3$	1.0	244.1338	$C_{15}H_{18}NO_2$	2.4	*p*-OH	-	$(OH)_2$
				199.0754	$C_{13}H_{11}O_2$	0.0			
				135.0804	$C_9H_{11}O$	−0.2			
				107.0491	C_7H_7O	−0.1			
M25 (8.23)	378.2070	$C_{24}H_{28}NO_3$	1.7	360.1971	$C_{24}H_{26}NO_2$	3.7	-	-	$(OH)_3$
				260.1297		>5			
				215.0699	$C_{13}H_{11}O_3$	−1.9			
				148.1120	$C_{10}H_{14}N$	−0.6			
				146.0969	$C_{10}H_{12}N$	3.0			
				119.0855	C_9H_{11}	−0.6			
				91.0543	C_7H_7	0.3			
M26 *(14.13)*	392.2228	$C_{25}H_{30}NO_3$	1.9	374.2123	$C_{25}H_{28}NO_2$	2.4	OMe + OH	OH	-
				359.1892	$C_{24}H_{25}NO_2$	3.4			
				212.1430	$C_{15}H_{18}N$	1.7			
				194.1176	$C_{11}H_{16}NO_2$	0.2			
				167.0859	$C_{13}H_{11}$	2.3			
				165.0913	$C_{10}H_{13}O_2$	1.8			
				117.0698	C_9H_9	−0.9			
				91.0541	C_7H_7	−1.2			
M27 (13.20)	392.2229	$C_{25}H_{30}NO_3$	2.3	374.2126	$C_{25}H_{28}NO_2$	3.1	-	OH	OMe + OH
				250.1591	*$C_{18}H_{20}N$*	*0.2*			
				241.1227	$C_{16}H_{17}O_2$	1.6			
				213.0912	$C_{14}H_{13}O_2$	1.0			
				181.0644	$C_{13}H_9O$	−2.0			
				148.1123	$C_{10}H_{14}N$	1.3			
				146.0967	$C_{10}H_{12}N$	1.6			
				137.0599	$C_8H_9O_2$	1.3			
				119.0855	C_9H_{11}	0.0			
				105.0698	C_8H_9	−0.8			
				91.0543	C_7H_7	1.0			
M28 (12.97)	392.2229	$C_{25}H_{30}NO_3$	2.3	374.2126	$C_{25}H_{28}NO_2$	3.0	-	OH	OMe + OH
				250.1593	*$C_{18}H_{20}N$*	*1.1*			
				241.1229	$C_{16}H_{17}O_2$	2.5			
				213.0915	$C_{14}H_{13}O_2$	2.4			

Table 4. *(Continued)*

Metabolite (ret. time/min)	Precurs. ion (*m/z*)	Elemental comp.	Error (ppm)	Product ions (*m/z*)	Elemental comp.	Error (ppm)	Metabolic transformation		
							amph. phen.	amph. Benz.	DPPA
				181.0655	$C_{13}H_9O$	−4.1			
				148.1123	$C_{10}H_{14}N$	1.6			
				146.0966	$C_{10}H_{12}N$	1.1			
				137.0598	$C_8H_9O_2$	0.5			
				119.0855	C_9H_{11}	0.1			
				105.0698	C_8H_9	−0.8			
				91.0544	C_7H_7	2.0			
M29 (12.04)	392.2226	$C_{25}H_{30}NO_3$	1.4	194.1180	$C_{11}H_{16}NO_2$	2.1	*m*-OMe + *p*-OH	-	OH
				165.0911	$C_{10}H_{13}O_2$	0.6			
				151.0751	$C_9H_{11}O_2$	−1.8			
				137.0596	$C_8H_9O_2$	−1.1			
				133.0649	C_9H_9O	1.0			
				105.0699	C_8H_9	−0.3			
M30 (11.40)	392.2230	$C_{25}H_{30}NO_3$	2.4	254.1539	$C_{17}H_{20}NO$	−0.2	*p*-OMe + *m*-OH	-	OH
				228.1388	$C_{15}H_{18}NO$	2.3			
				165.0908	$C_{10}H_{13}O_2$	−1.4			
				137.0602	$C_8H_9O_2$	3.4			
				133.0652	C_9H_9O	2.8			
				105.0697	C_8H_9	−2.2			
				91.05444	C_7H_7	2.3			
M31 (11.23)	392.2228	$C_{25}H_{30}NO_3$	2.1	258.1486	$C_{16}H_{20}NO_2$	−1.0	*p*-OH	-	OMe + OH
				164.1078	$C_{10}H_{14}NO$	4.8			
				135.0807	$C_9H_{11}O$	1.8			
				107.0491	C_7H_7O	−0.3			
				91.0542	C_7H_7	0.3			
M32 (10.27)	392.2224	$C_{25}H_{30}NO_3$	1.0	229.0868	$C_{14}H_{13}O_3$	4.0	-	-	OMe + $(OH)_2$
				148.1126	$C_{10}H_{14}N$	3.7			
				119.0857	C_9H_{11}	1.5			
				91.0540	C_7H_7	−2.2			
M33 *(13.81)*	*394.2021*	*$C_{24}H_{28}NO_4$*	*2.0*	*238.1597*	$C_{17}H_{20}N$	*3.0*	---------- *$(OH)_4$*----------		
				195.1160	*$C_{15}H_{15}$*	*−4.4*			
				172.0972	*$C_8H_{14}NO_3$*	*2.3*			
				167.0854	*$C_{13}H_{11}$*	*−0.7*			
				117.0693	C_9H_9	*−4.8*			
				91.0539	C_7H_7	*−3.6*			
M34 *(12.32)*	*394.2012*	*$C_{24}H_{28}NO_4$*	*−0.2*	*238.1593*	$C_{17}H_{20}N$	*1.3*	---------- *$(OH)_4$*----------		
				212.1432	$C_{15}H_{18}N$	*−0.7*			
				167.0853	*$C_{13}H_{11}$*	*−1.3*			
				123.0443	$C_7H_7O_2$	*1.7*			
				117.0701	C_9H_9	*1.8*			
				91.0542	C_7H_7	*−0.7*			
M35 *(10.68)*	*394.2017*	*$C_{24}H_{28}NO_4$*	*1.0*	*276.1229*	$C_{15}H_{18}NO_4$	*−0.3*	-	-	*$(OH)_4$*
				216.1025	$C_{13}H_{14}NO_2$	3.0			
				201.0910	$C_{13}H_{13}O_2$	0.0			
				148.1223	$C_{10}H_{14}N$	1.3			
				119.0855	C_9H_{11}	0.0			
				91.0543	C_7H_7	0.5			
M36 (10.14)	394.2017	*$C_{24}H_{28}NO_4$*	0.9	*276.1222*	*$C_{15}H_{18}NO_4$*	−3.2	-	-	*$(OH)_4$*
				216.1020	?	0.5			
				201.0905	?	−2.4			
				119.0853	C_9H_{11}	−2.2			
				91.0542	C_7H_7	−0.1			
M37 (6.15)	394.2015		0.5	215.0692	$C_{13}H_{11}O_3$	−4.9	-	OH	$(OH)_3$
				148.1120	$C_{10}H_{14}N$	−1.0			

Table 4. *(Continued)*

Metabolite (ret. time/min)	Precurs. ion (*m/z*)	Elemental comp.	Error (ppm)	Product ions (*m/z*)	Elemental comp.	Error (ppm)	Metabolic transformation		
							amph. phen.	amph. Benz.	DPPA
				146.0963	$C_{10}H_{12}N$	−0.2			
				119.0860	C_9H_{11}	2.0			
				105.0696	C_8H_9	2.2			
				91.0543	C_7H_7	1.0			
M38 (9.48)	408.2175	$C_{25}H_{30}NO_4$	1.4	390.2074	$C_{25}H_{28}NO_3$	2.7	----------$(OMe)_2(OH)_2$----------		
				244.1335	$C_{15}H_{18}NO_2$	1.1			
				165.0910	$C_{10}H_{13}O_2$	−0.1			
				137.0603	$C_8H_9O_2$	4.6			
				133.0969		>5			
				105.0701	C_8H_9	2.4			
M39 (8.31)	408.2171	$C_{25}H_{30}NO_4$	1.2	390.2069	$C_{25}H_{28}NO_3$	1.3	-	OH	OMe + $(OH)_2$
				229.0857	$C_{14}H_{13}O_3$	−1.1			
				148.1126	$C_{10}H_{14}N$	3.3			
				146.0965	$C_{10}H_{12}N$	0.7			
				119.0859	C_9H_{11}	3.5			
				105.0691	C_8H_9	>5			
				91.0539	C_7H_7	−3.3			
M40 (6.48)	408.2169	$C_{25}H_{30}NO_4$	0.0	165.0918	$C_{10}H_{13}O_2$	4.7	----------$(OMe)_2(OH)_2$----------		
				135.0798	$C_9H_{11}O$	−4.7			
				107.0492	C_7H_7O	0.2			
M41 (11.43)	422.2338	$C_{26}H_{32}NO_4$	2.8	258.1487	$C_{16}H_{20}NO_2$		OMe + OH	-	OMe + OH
				165.0910	$C_{10}H_{13}O_2$	−0.1			
				137.0597	$C_8H_9O_2$	0.0			
				133.0645	C_9H_9O	−2.2			
				105.0702	C_8H_9	3.3			
M42 (10.36)	422.2338	$C_{26}H_{32}NO_4$	2.8	148.1124	$C_{10}H_{14}N$	2.0	-	-	$(OMe)_2$ + $(OH)_2$
				119.0865		>5			
				91.0541	C_7H_7	−1.4			
M43 (6.83) (DPPA)	212.1440	$C_{15}H_{18}N$	3.2	167.0860	$C_{13}H_{11}$	3.1	-	-	-
				165.0702	$C_{13}H_9$	2.0			
				152.0625	$C_{12}H_8$	3.0			
				117.0702	C_9H_9	2.6			
				115.0543	C_9H_7	0.9			
				91.0545	C_7H_7	3.3			
M44 (2.83)	228.1378	$C_{15}H_{18}NO$	−2.2	211.1114	$C_{15}H_{15}O$	−1.7	-	-	OH
				183.0809	$C_{13}H_{11}O$	2.5			
				165.0699	$C_{13}H_9$	0.1			
				107.0492	C_7H_7O	0.7			
				91.0545	C_7H_7	2.9			
M45 (1.73)	244.1342	$C_{15}H_{18}NO_2$	3.9	227.1071	$C_{15}H_{15}O_2$	2.2	-	-	$(OH)_2$
				199.0760	$C_{13}H_{11}O_2$	3.0			
				181.0654	$C_{13}H_9O$	3.3			
				153.0697	$C_{12}H_9$	−1.2			
				123.0443	$C_7H_7O_2$	1.7			
				117.0698	C_9H_9	−0.7			
				91.0543	C_7H_7	1.1			

specimens (not shown). A convenient procedure for the potential confirmation of suspicious screening results for the **M1**-glucuronide, for which synthetic reference material is not available, is based on direct enzymatic hydrolysis. In this approach, which has previously been successfully applied for the determination of urinary phthalate concentrations,[21] ß-glucuronidase from *E.coli* is added straight to the unprocessed urine sample (25 µl/ml urine) followed by direct confirmatory analysis of free urinary **M1** using the synthesized standard as a reference compound. Determination of the hydrolysis kinetics of the **M1**-glucuronide showed complete cleavage of the conjugate instantly after addition of the enzyme, no matter if the pH of the urine was buffered to 7.0 or not. Analysis of ten different blank urine samples with this direct hydrolysis method showed no additional interfering signals at the retention time of **M1**, indicating the specificity of this approach (data not shown).

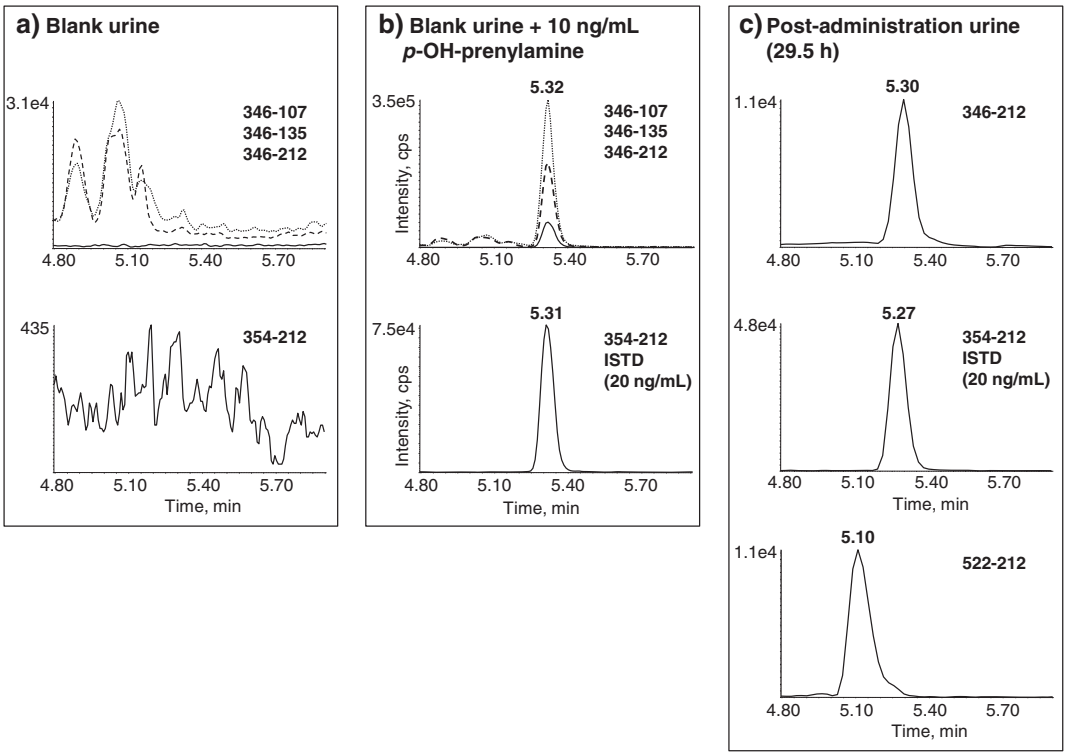


Figure 4. Illustration of the specificity of the adapted routine screening method showing: (a) the extracted MRM ion chromatograms of *p*-hydroxyprenylamine (**M1**) and the internal standard (ISTD) d_8-**M1** in a blank urine sample. No influence of the ISTD on the signal of M1 was observed (data not shown). (b) A urine specimen fortified with 10 ng/mL of **M1** and 20 ng/mL of the ISTD and (c) the selected screening ion transitions of a post-administration urine sample (29.5 h after drug administration) including the transition 522-212 for the **M1**-glucuronide.

Table 5. Validation results for routine detection of **M1**

Concentration [ng/ml]	Intraday precision (n = 10 + 10 + 10)	Interday precision (n = 30 + 30 + 30)	LLOD [ng/ml]
10	6.48%	6.31%	0.1
50	7.43%	8.90%	
100	4.66%	5.83%	

Conclusion

Numerous studies of the metabolism of prenylamine have been conducted in the past but so far, the knowledge of the metabolic fate of prenylamine was neither complete, nor was it based on modern chromatographic and mass spectrometric instrumentation. For the support of analytical assay development in sports drug testing, the urinary metabolic profile of prenylamine administration by LC-MS/MS analysis was studied. Using sensitive HPLC-high resolution/high accuracy hybrid tandem mass spectrometry, a detailed picture of the metabolic fate can be drawn, including the direct detection of intact phase II conjugates. It is to be said that due to the known inter-individual variability in physiologic response and metabolism of prenylamine, this study should be understood as a case study only. Nevertheless, the metabolic pathways reported herein are in good accordance with previous investigations and allowed for the development of a sensitive and reliable screening method for urinary doping analysis. The results are also of relevance for forensic drug testing, where the differentiation between illicit intake of amphetamine-like stimulants and therapeutic – non-prohibited – prenylamine use is of importance for law enforcement processes.

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