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Article

Simultaneous Determination of 11 Illicit Phenethylamines in Hair by LC–MS-MS: *In Vivo* Application

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Existing phenethylamines are a class of synthetic compounds that differ from each other only in small changes to a largely conserved chemical structure. The recreational and illicit use of phenethylamines is a widespread problem. A simple procedure for the simultaneous quantitative determination in hair of 11 phenethylamines that are officially recognized as illicit by Italian legislation (*p*-methoxyamphetamine; *p*-methoxymethamphetamine; 3,4,5-trimethoxyamphetamine; 2,5-dimethoxyamphetamine; 2,5-dimethoxy-4-methylamphetamine; 2,5-dimethoxy-4-ethylamphetamine; 2,5-dimethoxy-4-bromoamphetamine; 2,5-dimethoxy-4-bromophenethylamine; 2,5-dimethoxy-4-iodophenethylamine; 2,5-dimethoxy-4-ethylthiophenethylamine and 2,5-dimethoxy-4-*n*-propylthiophenethylamine) has been developed and validated. Extraction from the matrix was performed after incubation in methanolic HCl and filtered reconstituted extracts were injected into a liquid chromatography/tandem mass spectrometry system (LC–MS-MS) without any further purification steps. This validated LC–MS-MS method has been used to determine the *in vivo* accumulation/retention of the above target analytes in hair after repeat oral administration to rats. This experiment further permitted investigation of the effect of pigmentation on the uptake of these phenethylamines by hair and the effect of hair pigmentation. The developed method could potentially be used for forensic and toxicological purposes, in the detection and quantitation of these illicit substances in human hair in workplace drug testing; drug-facilitated crime investigation; driver re-licensing; determining drug abuse history and postmortem toxicology.

Introduction

Phenethylamines are a class of synthetic compounds, which stimulate activity in the central nervous system. The chemical structure of these molecules is similar to monoamines. Their mechanism of action involves an increase in monoaminergic transmissions (1–3). Amphetamine is the prototype drug of this class of compounds causing central and peripheral stimulation (4). Through structural modifications, it is possible to obtain a significant number of novel related products, with new drugs not considered illicit until they are legally defined as such by the Italian state. This explains the marked and growing interest in the illegal commercialization of these substances. Plasma, urine and blood are the most commonly used, conventional matrices for illicit drug testing. Hair is an interesting, alternative biological site for the measurement of drug exposure. Hair analysis is a powerful tool in the area of forensic and clinical toxicology and has been used to evaluate drug exposure in several fields of application, such as chronic exposure to environmental toxicants (5), but especially to detect therapeutic and illicit drug use (6–8) (workplace drug testing; drug-facilitated crimes; driver re-licensing; defining drug abuse history and postmortem toxicology).

Hair is a complex biological matrix (9) in which drugs and endogenous compounds circulating in the blood may ultimately become incorporated, depending on their chemical nature (10). Despite this complexity, hair has numerous advantages over traditional matrices (e.g., blood or urine) (11), and it is an important specimen for forensic drug analysis (12). First, it allows the investigation of a longer detection window (months to years), as substances remain in hair for a long time without significant loss/degradation. Furthermore, analysis of hair has been used to prove chronic drug use as it is less affected by adulteration or short-term abstinence than other matrices. Moreover, its sampling is not invasive, as collecting head hair is less intrusive and causes less embarrassment. Additionally, the sample is durable, stable and easy to store.

Literature data report that it is possible to use rat hair when it is not possible to undertake *in vivo* experiments in human subjects (13, 14). Even though the appearance and structure of human and rat hair are different, drugs and their metabolites are readily incorporated into both types of hair with higher concentrations usually found in pigmented hair (10).

Some methods have been described in the scientific literature for the determination of amphetamines alone or in combination with other illicit drugs in hair samples. In these methods, several techniques, such as gas chromatography/mass spectrometry (15–17) and liquid chromatography/mass spectrometry (LC–MS) (18–20), are used. To the best of our knowledge, there are no reported methods for the simultaneous determination of the specified 11 phenethylamines in this matrix.

Our aim was to develop a liquid chromatography/tandem mass spectrometry (LC–MS-MS) multiresidual method to identify and quantify in hair the 11 phenethylamines officially recognized as illicit by Italian legislation (21, 22). The method validated has been applied to an *in vivo* experiment in Lister Hooded outbred rats. Both dark gray and white hair were analyzed to investigate the effect of pigmentation on the distribution of the target analytes, between pigmented and non-pigmented hair.

Experimental

Chemicals and reagents

The target analytes (*p*-methoxyamphetamine, PMA; *p*-methoxymethamphetamine, PMMA; 3,4,5-trimethoxyamphetamine, TMA;

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2,5-dimethoxyamphetamine, DMA; 2,5-dimethoxy-4-methylamphetamine, DOM; 2,5-dimethoxy-4-ethylamphetamine, DOET; 2,5-dimethoxy-4-bromoamphetamine, DOB; 2,5-dimethoxy-4-bromophenethylamine, 2C-B; 2,5-dimethoxy-4-iodophenethylamine, 2C-I; 2,5-dimethoxy-4-ethylthiophenethylamine, 2C-T-2 and 2,5-dimethoxy-4-*n*-propylthiophenethylamine, 2C-T-7) were purchased from Lipomed AG (Arlesheim, Switzerland). The internal standard (2,3-dimethoxyphenethylamine-d_3, IS) was synthesized in our laboratory. Deionized and distilled water was filtered through a Milli-Q water system (Millipore, Billerica, MA, USA). Other reagents and solvents used were of the highest commercial quality and were obtained from Sigma-Aldrich (Milan, Italy). Individual methanolic stock solutions (1.0 mg/mL of each analyte and the IS) were used to prepare the working solutions by appropriate dilution. All solutions were stored at −20°C in the dark.

Sample preparation

Human hair samples were provided voluntarily by the laboratory staff and were obtained from the vertex posterior, as close as possible to the scalp. Rat hair samples were obtained from 7-week-old male, Lister Hooded outbred rats (see the section '*In vivo* application').

The samples were submitted to an initial decontamination, by washing them twice; first with acetonitrile (5 mL, 2 min, at room temperature) and then with Milli-Q water (5 mL, 2 min, at room temperature). After complete removal of the washing solvent, the hair was dried at room temperature in a gentle nitrogen stream and subsequently cut into small pieces of ~2 mm with scissors. Hair samples (100 mg of each) were incubated with 1% HCl in methanol (10 mL) at 45°C for 24 h with continuous stirring. Thereafter, the samples were filtered, and each filtrate evaporated to dryness under a stream of nitrogen. The residues were reconstituted in 100 μL of IS solution (100 ng/mL) and 5 μL were directly injected into the LC–MS-MS system.

LC–MS-MS analysis

LC was performed using a Shimadzu LC-20AD$_{XR}$ (Shimadzu Italia, Milano, Italy). Chromatographic separation was carried out on a Kinetex PFP column (75 × 2.1 mm i.d., particle size 2.6 μm, Phenomenex, Torrance, CA, USA) fitted with a 2.6-μm security guard cartridge (4 × 2.1 mm i.d., Phenomenex). Two solvents were used for gradient elution: solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). Chromatography was undertaken using a linear gradient: from 10% B to 70% B in 6.5 min at a flow rate of 0.3 mL/min. A 5-min re-equilibration period at the initial conditions (10% B) was performed before each injection. Each sample was injected three times.

Tandem mass spectrometric analysis was performed on an Applied Biosystem MDS Sciex (Concord, ON, Canada) API 2000 triple quadrupole, equipped with an electrospray ionization (ESI) interface. Infusion experiments by the syringe pump method were carried out for each analyte and the IS (10 μg/mL) in both negative and positive ESI modes. A multiple reaction monitoring (MRM) method was developed in positive mode, as the signal intensities were greater. For each phenethylamine, two transitions were selected; one transition was taken for confirmation of the identity/the presence of the analyte and the other for its quantitation. For the IS, only one transition was selected. The precursor/product ion transitions monitored are reported in Table I. The ion source temperature was set at 400°C. The ion spray voltage was set at 5,000 V, the curtain gas was set at 6 arbitrary units and the collision gas at 6 arbitrary units. The optimized MS parameters are reported in Table I. Qualitative analysis was performed according to the selected MRM transitions and retention times. Positive analyte identification in samples required retention times to be within ± 0.20 min of the average retention time of reference standards. Since two product ions were selected for each analyte, a further criterion of identification was considered the ratio of the qualifier transition to the quantifier transition, which was required to be within ± 30% of the average, measured ratio from the calibration curve samples (23). Data acquisition was accomplished using the Applied Biosystem Analyst version 1.6 software. Quantitation was performed using the Applied Biosystem MultiQuant version 2.1 software, using the IS method.

Method validation

The method was validated according to the Society of Hair Testing guidelines for drug testing in hair (11), using drug-free rat hair samples obtained from different sources. The parameters determined were: selectivity, linearity, limit of detection (LOD), limit of quantification (LOQ), matrix effect, precision, accuracy, extraction efficiency and stability.

Selectivity was assessed by analyzing 10 blank matrix samples from different sources, extracted as described above. The presence of possible interference from endogenous substances was verified by monitoring the MRM chromatograms specific for each investigated compound and the IS at their expected

Table I
Tandem Mass Spectrometric Conditions

Compound	Parent ion (*m*/*z*)	Fragment ions (*m*/*z*)	DP (V)	FP (V)	EP (V)	CE (V)	CXP (V)
PMA	166.1	149.1[a]	10	200	6.0	13	2.2
		121.1[b]				27	2.0
PMMA	180.1	121.1[a]	15	200	6.0	18	2.2
		149.1[b]				25	4.6
TMA	226.2	209.2[a]	15	200	4.0	16	4.6
		181.2[b]				29	3.0
DMA	196.2	151.2[a]	15	200	8.0	16	3.2
		179.2[b]				24	6.0
DOM	210.2	193.2[a]	10	200	6.0	18	4.3
		178.2[b]				27	3.5
DOET	224.2	207.2[a]	11	300	4.0	20	4.7
		179.2[b]				27	7.4
DOB	274.0	257.0[a]	15	300	5.0	22	5.9
		229.0[b]				33	4.3
2C-B	260.0	243.0[a]	15	300	4.0	18	10
		228.0[b]				30	4.6
2C-I	308.1	291.1[a]	15	300	5.0	21	6.5
		276.1[b]				35	5.4
2C-T-2	242.2	225.2[a]	15	200	5.0	19	4.8
		134.2[b]				37	5.0
2C-T-7	256.2	239.2[a]	15	300	5.0	19	5.4
		197.2[b]				30	8.0

DP, declustering potential; FP, focusing potential; EP, entrance potential; CE, collision energy; CXP, collision cell exit potential.
[a]Fragment ion used for quantitation.
[b]Fragment ion used for confirmation.

retention times. The absence of interference from the IS and from the analytes has been determined by analyzing three blank hair samples fortified only with IS, and three blank samples fortified only with the analytes, respectively.

For calibration curves, blank hair samples fortified with appropriate amounts of each analyte at five concentration levels ranging from LOQ to 5.0 ng/mg (LOQ, 0.5, 1.5, 3.0 and 5.0 ng/mg) were analyzed as described above. Quantitative data resulting from area counts were corrected using the IS signal areas. The linearity of the compound-to-IS peak ratios versus the theoretical concentrations in hair was verified by using a $1/\times$ weighted linear regression. The correlation coefficients (r) were tested on a set of five calibration curves. LODs and LOQs were calculated using the signal-to-noise criteria (S/N) of 3 and 10, respectively (24).

The matrix effect on ion suppression or enhancement (25–27) was also determined using the post extraction addition method (28). Nine drug-free hair samples were extracted and subsequently fortified with analytes at three different concentrations (0.2, 2.0 and 4.0 ng/mg). For each analyte, the chromatographic peak areas obtained from the extracts were compared with the corresponding peak areas produced by the reference solutions prepared in the initial mobile phase at the same concentration. Each sample was analyzed three times. The matrix effect percentage was calculated according to the following equation:

$$\text{Matrix effect (\%)} = [(\text{Mean peak area of 'reconstituted hair extract'} - \text{mean peak area of 'reference solution'})/(\text{Mean peak area of 'reference solution'})] \times 100.$$

The precision of the method was evaluated at three concentrations over the linear dynamic range (0.2, 2.0 and 4.0 ng/mg) and was expressed as the percent relative, standard deviation (RSD), where the sample standard deviation (SD) was calculated for five replicates at each of the above concentrations for the intra-day precision and over 5 days for the inter-day precision.

Extraction efficiency was determined at the above three concentrations (0.2, 2.0 and 4.0 ng/mg) for each compound. Nine blank samples were fortified with the appropriate amounts of mixed standard solution and extracted as described. The extraction efficiency was calculated by comparing the peak areas obtained from the samples fortified before extraction, with the peak areas obtained from the sample fortified after extraction.

Stability under sample preparation conditions (1% HCl methanolic solution, 45°C) was monitored from 2 to 24 h. The stability was calculated by comparison against the initial analyte concentrations.

For analysis of human hair, the matrix effect and extraction efficiency were investigated at the same concentrations.

In vivo application

The developed method was applied to an *in vivo* study to determine hair accumulation of the target analytes in 7-week-old male, Lister Hooded outbred rats obtained from Harlan Laboratories (Indianapolis, IN, USA). Animals were allowed at least a 1-week acclimatization period in the laboratory animal facility and were kept on a standard rat chow and permitted tap water *ad libitum*. Rats were housed at constant room temperature (24 ± 1°C) and humidity (60 ± 5%) with a 12-h light/dark cycle, in separate, metabolic cages, to prevent urine and saliva contamination of hair. Before drug administration, dark gray and white hair were obtained from the nape and the dorsal region, respectively, using an electric shaver. Five rats were used in each experimental group. After 3 days, each group received an oral dose of a single phenethylamine (2.5 mg/kg) once a day for 5 consecutive days. Four weeks after the final treatment, newly grown hair was collected from the areas that had been shaved before compound administration, sampling separately dark gray and white hair. All samples were washed and extracted as described in the 'Sample preparation' section and analyzed in triplicate. All procedures were approved by the Local Animal Care and Use Committee.

Results and discussion

Sample preparation

Prior to extraction, hair samples were submitted to an initial decontamination by washing first with acetonitrile and then with Milli-Q water. Acetonitrile was chosen as the washing solvent because non-protic solvents do not swell the hair and thus do not extract substances from the matrix (29). Washing of hair samples has two main purposes: regarding human hair it removes hair care products, sweat, sebum or surface material, which could interfere with the analysis and regarding both human and rat hair, it is important to remove external contaminants from the environment.

For extraction, methanolic HCl has been used, taking in account the chemical properties of the target analytes: amphetamines are basic drugs and due to protonation, they are well extracted in acid media.

Method validation

Preliminary experiments were carried out by analyzing blank rat hair to evaluate the interference of the matrix. Selectivity was satisfactory, because no interfering, endogenous substances at the retention times of the test compounds or the IS were observed when blank samples were analyzed. A chromatogram of an extracted hair sample fortified with target analytes (1 ng/mg) and IS is shown in Figure 1. Qualitative analysis of the analytes was performed, according to their retention times and their two MRM transitions. As already explained, transitions with higher signal intensity were selected for quantitation of the target analytes. For additional confirmation of identity, another transition was chosen. For positive identification of each analyte, the ratio of the qualifier transition to the quantifier transition was required to be within ± 30% of the average, measured ratio from calibration curve standards (23). Retention times for all analytes are given in Table II. The retention time of the IS was 2.6 min.

With regard to validation data, regression equations were linear, over the tested concentration range (LOQ—5.0 ng/mg), with excellent correlation coefficients that exceeded 0.996 for all compounds (Table II). The LODs and LOQs ranged from 0.03 to 0.07 ng/mg and from 0.09 to 0.20 ng/mg, respectively (Table II). Considering that the hair cutoff value established for similar amphetamines is 0.2 ng/mg (11), the assay sensitivity reported here could be suitable for confirmation of the presence of

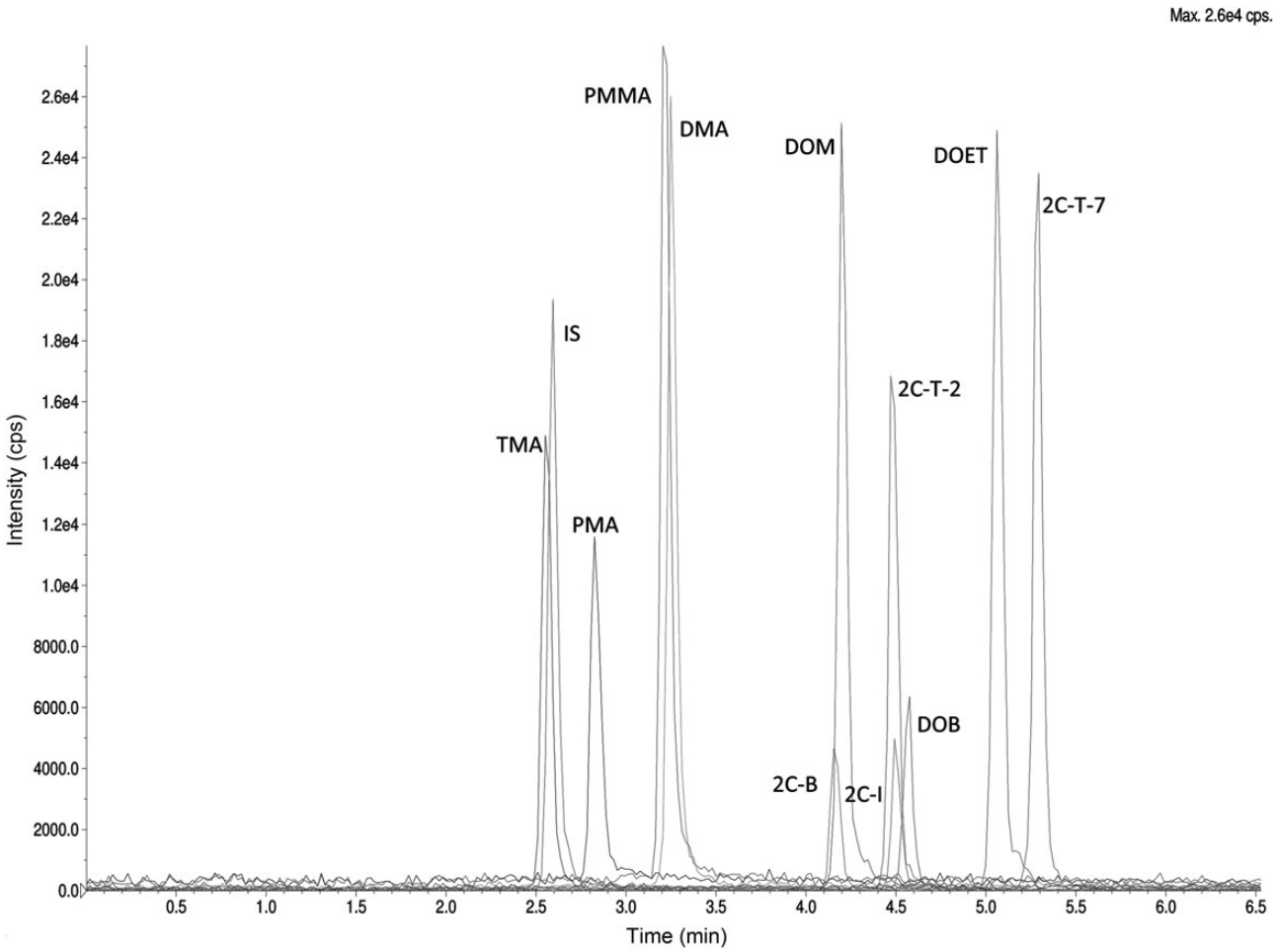

Figure 1. Chromatogram of an extracted hair sample fortified with target analytes (1 ng/mg).

Table II
Validation Parameters

Compound	t_R (min)	$r \pm$ SD ($n = 5$)	$y = ax + b$ ($n = 5$)	LOQ (ng/mg)	LOD (ng/mg)
TMA	2.55	0.997 ± 0.024	$y = 0.601x + 0.095$	0.13	0.04
PMA	2.81	0.998 ± 0.028	$y = 0.860x + 0.090$	0.19	0.06
PMMA	3.21	0.999 ± 0.023	$y = 2.04x + 0.144$	0.10	0.03
DMA	3.25	0.999 ± 0.015	$y = 1.83x + 0.122$	0.18	0.06
2C-B	4.16	0.996 ± 0.016	$y = 0.320x + 0.021$	0.20	0.07
DOM	4.20	0.998 ± 0.057	$y = 1.56x + 0.154$	0.09	0.03
2C-T-2	4.47	0.998 ± 0.031	$y = 1.14x + 0.092$	0.19	0.06
2C-I	4.49	0.997 ± 0.015	$y = 0.382x + 0.057$	0.19	0.06
DOB	4.56	0.999 ± 0.003	$y = 0.430x + 0.027$	0.13	0.04
DOET	5.06	0.999 ± 0.041	$y = 1.83x + 0.174$	0.12	0.04
2C-T-7	5.27	0.999 ± 0.008	$y = 1.33x + 0.133$	0.19	0.06

t_R, retention time; SD, standard deviation; LOQ, limit of quantitation; LOD, limit of detection.

the target analytes in toxicological and forensic samples. The matrix effect was always lower than 15% and recoveries ranged from 77 to 103% (Table III). The intra- and inter-day precision showed RSD values from 3.2 to 13.0% (Table III). No marked loss or deterioration was observed in the stability study.

Selectivity, matrix effect and recovery data for human and rat hair do not show significant differences (Table III). Also, LODs and LOQs for both matrices were similar.

In vivo application

The proposed LC–MS-MS method was successfully applied to an *in vivo* study in Lister Hooded outbred rats. Both dark gray and white hair were analyzed to investigate the effect of pigmentation on the distribution of the target analytes in pigmented and non-pigmented hair. With respect to the analysis of dark gray hair, all phenethylamines, except PMMA, were found at quantifiable concentrations ranging from 0.25 to 1.30 ng/mg (Table IV). PMMA was detected at concentration below the LOQ. Although, interestingly the dark gray hair from rats treated with PMMA, revealed the presence of PMA. This is most probably due to *in vivo* metabolism that causes N-demethylation of PMMA to PMA (30). In the case of the white hair analyzed, not one of the phenethylamine was detected. This result is consistent with that reported in the literature, since it is well known that pigmented hair incorporates basic xenobiotics more easily than non-pigmented hair. Indeed, incorporation of weak basic substances is dependent on hair pigmentation (acidic environment). Melanin, the component that determines the pigmentation of hair, is a polymer characterized by a high content of anionic sites (carboxyl groups). The influence of the ionic environment on the ability of an organic molecule to bind melanin is not negligible. Drugs with cationic properties (such as amphetamines) appear to bind melanin

Table III
Extraction Efficiency and Matrix Effect

Compound	Concentration (ng/mg)	Matrix effect rat hair (%)	Matrix effect human hair (%)	Extraction efficiency rat hair (%)	Extraction efficiency human hair (%)	Intra-day precision (RSD %)	Inter-day precision (RSD %)
PMA	0.2	−7	−11	77	89	5.3	6.6
	2.0	−5	−8	85	78	7.6	9.2
	4.0	−10	−8	95	93	9.1	12.5
PMMA	0.2	−8	−5	86	83	6.3	11.0
	2.0	−6	−9	92	88	6.0	7.2
	4.0	−11	−14	88	92	9.6	4.8
TMA	0.2	−9	−6	83	85	11.9	8.0
	2.0	−7	−4	88	96	4.2	8.6
	4.0	−8	−12	92	89	7.8	11.1
DMA	0.2	−3	−7	89	100	9.0	4.9
	2.0	−8	−13	85	95	4.5	10.6
	4.0	−6	−12	94	97	3.2	9.3
DOM	0.2	−3	−3	99	92	8.0	5.5
	2.0	−3	−4	96	101	3.6	9.3
	4.0	−9	−7	92	96	7.7	7.0
DOET	0.2	−7	−9	89	97	9.6	4.7
	2.0	−8	−5	91	99	8.0	9.1
	4.0	−5	−11	97	84	12.1	6.2
DOB	0.2	−11	−6	77	84	9.9	11.9
	2.0	−8	−14	79	84	5.8	5.2
	4.0	−9	−8	82	87	6.6	7.8
2C-B	0.2	−6	−5	96	92	7.4	6.2
	2.0	−4	−7	81	95	7.7	6.9
	4.0	−9	−3	98	101	13.0	7.0
2C-I	0.2	−3	−9	96	91	6.4	6.7
	2.0	−7	−10	97	103	11.5	9.9
	4.0	−6	−11	100	97	9.6	8.0
2C-T-2	0.2	−14	−11	86	81	8.2	5.2
	2.0	−12	−10	96	88	9.4	11.3
	4.0	−13	−7	97	100	3.5	12.0
2C-T-7	0.2	−15	−7	86	94	8.7	9.3
	2.0	−13	−9	81	89	11.9	8.9
	4.0	−10	−3	90	101	5.5	6.2

Table IV
Rat Hair Concentrations of 11 Phenethylamines After Oral Administration ($n = 5$)

Compound	Dark gray hair (ng/mg)	White hair (ng/mg)
PMA	0.87	–
PMMA	<LOQ[a]	–
TMA	0.72	–
DMA	1.30	–
DOM	0.70	–
DOET	0.90	–
DOB	0.85	–
2C-B	0.37	–
2C-I	0.45	–
2C-T-2	0.25	–
2C-T-7	0.30	–

[a]0.25 ng/mg of PMA.

through electrostatic forces resulting from the negative charges of the matrix and the positive charges on the molecule (31). Another characteristic that increases drug accumulation in hair is the presence of aromatic rings, which produce non-electrostatic interaction with the indolic nuclei of melanin. Amphetamines are organic amines having all the above-mentioned structural characteristics which explain why we found the phenethylamines investigated in this study only in pigmented hair.

Conclusion

To the best of our knowledge, this is the first report to identify and quantify simultaneously PMA, PMMA, DMA, TMA, DOM, DOET, DOB, 2C-B, 2C-I, 2C-T-2 and 2C-T-7 in hair. Compounds were detected by LC–MS-MS operating in positive ionization mode, developing an MRM method. The absence of matrix interferences, together with the excellent reproducibility of both retention times and relative abundances of the diagnostic transitions, allowed the correct identification of all target analytes. The method was linear over the tested concentration range (from LOQ to 5.0 ng/mg), with excellent correlation coefficients. With respect to the quantitative analysis, the developed method allows quantitation of very low amounts of the studied phenethylamines, considering the excellent LODs and LOQs values obtained. *In vivo* application of the method highlighted differences between the accumulation of target analytes in pigmented and non-pigmented rat hair. Concentrations above the cutoff have been detected only in pigmented hair. Not one of the phenethylamines analyzed has been found in non-pigmented hair. The developed method could potentially be used for forensic and toxicological purposes in the detection and quantitation of these illicit amphetamines in human hair in workplace drug testing; drug-facilitated crime investigation; driver re-licensing; determining drug abuse history and postmortem toxicology.

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