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Article

A Study on Photostability of Amphetamines and Ketamine in Hair Irradiated under Artificial Sunlight

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Abstract: Background: Drug incorporated in hair are exposed to the environment and to cosmetic and chemical treatments, with possible decrease of their content. Knowledge concerning the effect of sun light on drug content in hair can be helpful to the forensic toxicologist, in particular when investigating on drug concentrations above or below pre-determined cut-offs. Materials and Methods: Twenty authentic positive hair samples were selected that had previously tested positive for amphetamines and/or ketamine. Washed hairs were divided into two identical strands: the former was exposed at 765 W/m² (310–800 nm spectrum of irradiance) for 48 hours in a solar simulator, the latter was kept in the dark. Hair samples were extracted and analyzed by LC-HRMS detection. The percent photodegradation was calculated for each analyte (amphetamine, methamphetamine, methylendioxyamphetamine methylendioxymethamphetamine, ketamine, norketamine). In parallel, photodegradation processes of standard molecules dissolved in aqueous and organic solutions were studied. Results: In 20 hair samples positive for the targeted analytes, exposure to artificial sun light induced an appreciable decrease of drug concentrations. The concentration ranges in the non-irradiated hair samples were 0.01–24 ng/mg; 65% of samples exhibited a decrease in post-irradiation samples, with reduction from 3% to 100%. When more drugs were present in the same hair sample (e.g, MDMA and ketamine) the degradation yields were compound dependent. A degradation product induced by irradiation of ketamine in aqueous and methanol solutions was identified; it was also found to be present in a true positive hair sample after irradiation. Conclusions: Ketamine, amphetamines and their metabolites incorporated in hair of drug users undergo degradation when irradiated by artificial sunlight. Only for ketamine a photoproduct was identified in irradiated standard solutions and in true positive irradiated hair. When decisional cut-offs are applied to hair analysis, photodegradation must be taken into account since sunlight may produce false negative results. Moreover, new markers could be investigated as evidence of illicit drug use.

Keywords: Hair; solar light; photodegradation; amphetamines; MDMA; ketamine

1. Introduction

The main advantage of hair as a testing matrix is the ability to provide information relating to historical drug exposure; hair analysis has many applications within forensic (drug-related deaths, drug-facilitated crimes (DFCs), child protection) and clinical toxicology (drug rehabilitation programs, workplace drug testing) [1-4]

The stability of drugs in hair, however, is affected by exposure to sunlight and weathering, cosmetic chemical treatments (oxidative dyeing, bleaching, or permanent wave) and also physical damage [5-7]. In particular, the exposure to sunlight and/or artificial light, for many hours per day,

can induce photodegradation reactions (photolysis) of drugs/illicit drugs through the formation of free radicals (produced by the drug itself or formed by eumelanin, pheomelanin and their oxidative products, oxyeumelanin, and oxypheomelanin) or photosensitization reactions by intermolecular energy transfer. [8]

Previous studies on this matter have been published by Skopp et al [9] in which cannabinoids detected in hair and affected by solar radiation showed to reduce their concentrations; more recently Favretto et al. [10] evaluated the effect of light exposure on methadone, cocaine and heroin metabolites in hair.

In order to better understand the role and the underlying mechanisms of solar light exposure in decreasing hair concentrations of drugs and following our previous [10] photodegradation studies on UVA and UVB induced changes, the aim of the present work was to evaluate photodegradation of some common stimulant drugs (amphetamines and ketamine, KET) in true positive hair samples exposed to the whole spectrum of sunlight in a solar simulator. The use of a solar simulator, including visible light (400 - 800 nm) and part of UV radiation from 400 nm to 310 nm (UVA = 400 - 315 nm and some wavelengths in the UVB = 315 - 280 nm) mimics the exposure to the environmental sunlight.

Amphetamine-based drugs are synthetic stimulant and illegal drugs that share a common structural backbone; the four amphetamines considered in the present work are the most used in Europe: amphetamine (AMF), methamphetamine (MA), 3,4-methylenedioxymethamphetamine (MDMA) and 3,4-methylenedioxyamphetamine (MDA).

AMF could be metabolized along two pathways, either by hydroxylation of the aromatic ring to 4-hydroxyamphetamine or by deamination of the side chain to benzyl methyl ketone, which could then be degraded to benzoic acid. MA is metabolized by cytochrome P450 (CYP), mainly by the CYP2D and CYP3A subfamilies, leading to the production of 4-hydroxyamphetamine and AMF. The half-life of MA is about 10 hours and 35%–45% of a dose is excreted unchanged in the urine over a period of several days; other metabolites, such as 4-hydroxymethamphetamine, norephedrine, and 4-hydroxynorephedrine, also are found in urine in substantial quantities [11,12, 13].

MDMA (3,4-methylenedioxymethamphetamine, ecstasy) is a widely abused psychostimulant drug that acts as a powerful releaser and/or reuptake inhibitor of serotonin (5-HT), dopamine (DA), and norepinephrine (NE). MDMA metabolism depends on the following main metabolic pathways: (1) O-demethylenation followed by catechol-O-methyltransferase (COMT) methylation and/or glucuronide/sulfate conjugation; and (2) N-dealkylation, deamination, and oxidation. MDMA N-demethylation gives rise to 3,4-methylenedioxyamphetamine (MDA). The elimination half-life of MDMA is about 8–9 hours, lower than those reported for MA (10–12 hours) or AMF (12–15 hours) [14, 15].

KET is a dissociative anesthetic drug that functions as an antagonist of the N-methyl-D-aspartate receptor and enhances the antinociceptive effects of conventional opioid analgesia, binding to μ opioid and σ receptors. KET is increasingly misused as a recreational and "club drug", because of its hallucinogenic and stimulant effects, and also as a "date-rape" drug (to facilitate sexual assault). KET is metabolized in the liver by the P450 system and CYP3A4 is the main enzyme responsible for N-demethylation into norketamine (NKET), 4-hydroxy-ketamine and 6-hydroxy-ketamine. The elimination half-life is about 2 h; the predominant metabolite of urinary excretion over a 72 h period is dehydronorketamine (DHNK) (16%), along with conjugates of hydroxylated ketamine metabolites [16, 17].

The consumption of alcohol and/or drugs is associated with an increased risk of being the victim of a sexual assault; a retrospective case series in London on 1014 cases of claimed drug-facilitated sexual assault (DFSA) showed that in 34% of samples (blood and/or urine) an illicit drug (with or without alcohol) was found, of which 10.8% contained cocaine, 4.6% “ecstasy” (MDMA), and 2.3% AMF [18, 19]. Because in DFC cases, the alleged victims are often unconscious about the events leading up to the assault because of the amnesiac effects of the drug(s) administered, a considerable amount of time may be spent before the victim reports the incident; consequently, hair analysis is of primary importance compared to blood and urine analysis, but the exposure to environment may affect the stability of drugs in keratin matrix.

For these reasons, reliable interpretation of the analytical results is fundamental for a correct interpretation of a positive or negative result and general knowledge on the photostability of drug analytes in the biological matrix must be considered. However, no data have been till now published on the effect of light on KET, amphetamines and their respective metabolites in the keratin matrix, while Morphine and 6-MAM photodegradation has been studied by UVA and UVB irradiation only [20].

In the present paper, levels of AMF, MA, MDMA, MDA, KET, and NKET, were determined by means of liquid chromatography-high resolution mass spectrometry (HPLC-HRMS) [21, 22] in authentic hair samples from drugs users, before and after irradiation under the whole spectrum of sunlight in a solar simulator.

Although the photodegradation of a molecule not only depends on the wavelength used, but also on its physical state (solid, liquid) and the environment in which it is irradiated (solvent, polarity, pH, presence of salts, oxygen and other compounds in the sample), we tested the same analytes in pure methanol and water solution irradiated under the whole spectrum of sunlight to gather general information on their behavior and to study their kinetics of photodegradation.

2. Experiments

2.1. Chemicals

AMF, MA, MDMA, MDA, KET, NKET, were purchased from LGC Promochem Cerilliant (Teddington, Middlesex, UK) as pure solutions in methanol at 1.0 mg/mL. The internal standards (IS) MA-D5, AMF-D5, MDMA-D5, MDA-D5, KET-D3, were methanol solutions also from LGC Promochem at 1.0 mg/mL.

Methanol, CH_2Cl_2, acetone and acetonitrile (Merck, Darmstadt, Germany) were high performance liquid chromatography (HPLC) grade. Ammonium acetate, of analytical grade, was from Merck also. Trifluoroacetic acid (TFA) was from Sigma (Sigma-Aldrich, Milan, Italy). HPLC water was prepared using a Milli-Q Plus (Millipore, Molsheim, France) system. All other reagents were from Sigma-Aldrich (St Louis, MO, USA).

2.2. Hair samples

Authentic positive hairs were selected from samples at the laboratory that had previously tested positive for amphetamines and/or KET. Hair samples were collected with scissors from the posterior vertex, and cut as close to the scalp as possible, wrapped in aluminum foil, and kept at room temperature until analysis.

2.3. Irradiation procedure

Irradiation was performed in a Suntest CPS+ (Atlas, Linsengericht, Germany) equipped with a 1.8 kW xenon lamp and a glass filter (cut-off 310 nm) according to Option 1 of ICH Guideline Q1B (European Medicines Agency. ICH Topic Q1B – Photostability testing of new active substances and medical products, 1998). The dark samples were maintained at solar box temperature during irradiation.

2.4. Photolysis experiments in solution (in vitro study)

Solutions of the compounds at concentrations ranging from 10^{-5} to 10^{-4} M in methanol and water were irradiated in the Suntest CPS+ with increasing time from 1 up to 3 hours.

Photolysis was evaluated by UV spectrophotometry (UV-Vis Varian Cary 50 Spectrophotometer) analyzing the change in the original spectrum upon irradiation, as already described,[20] and by high resolution mass spectrometry (HRMS). At selected UV doses, the solutions were diluted to 10^{-6} M in methanol and water and analyzed by direct injection HRMS, to measure the photodegradation of the analytes by recording the decrease of the ion signal of protonated molecules

obtained by electrospray ionization (ESI) of solutions kept in the dark. The results are the mean of at least three experiments.

2.5. Photolysis experiments in hair samples (in vivo study)

Hairs, 5–7 cm long, were divided into two approximately identical strands: the former was put between two 5 x 5 cm optical glasses and exposed at 765 W/m^2 (spectrum of irradiance: 310-800 nm) for 48 hours in the solar simulator, to an endpoint corresponding to two months exposure under the sunlight, and the latter was kept as a dark control in the same chamber of irradiation covered with an aluminum foil.

2.6. Hair sample preparation and extraction

Hair samples were decontaminated with 3 mL of CH_3OH, 3 mL H_2O and 3 mL CH_3OH. Pulverization was applied by an automatic homogenizer (Precellys® Evolution, Bertin Technologies) at speed 6000 RPM, cycle 9 x 30s, pause 30 s. Solid phase extraction (SPE) was performed by Oasis MCX (Waters) cartridges. Sample preparation consisted in the addiction of appropriate amounts of IS and 3 mL of a methanol/TFA (90:10, v/v) solution to 25 mg powdered hair samples, ultrasonication for 1 h and incubation overnight at 45 °C. After centrifugation, the methanolic solutions were dried under stream of nitrogen at 40 °C. The residues were reconstituted in 3 mL of 0.1 M phosphate buffer pH 6 and subjected to SPE. After cartridges conditioning with 3 mL methanol and 3 mL 0.1 M phosphate buffer pH 6, sample was loaded. Cleanup was accomplished by sequential washes with 3 mL water, 3 mL 0.1 N HCl and 3 mL methanol. Cartridges were dried for 10 min under vacuum before elution with 2 mL dichloromethane:2-propanol (80:20, v:v) 2% NH_4OH. Eluates were evaporated to dryness with nitrogen at 40 °C, reconstituted in 200 µL of water (0.1% formic acid)/acetonitrile 9:1 mixture, and 25 µL were injected into the LC–HRMS system.

2.7. HRMS

All measurements were performed on an LTQ-Orbitrap (Thermo Fisher Scientific, Bremen, Germany) high accuracy, high resolution mass spectrometer operating in positive ESI mode and equipped with a Surveyor MS Pump.

2.8. ESI-HRMS

For the analysis of pure standard solutions, direct injection analysis was performed with a syringe pump delivering solutions at 10 µL/min directly into the ESI source; the positive ion ESI parameters were as follows: capillary voltage 10 V, sheath gas flow rate 20 (arbitrary units, a.u.), auxiliary gas (N_2) flow rate 5 (a.u.), sweep gas flow rate 5 (a.u.), and capillary temperature 275 °C; profile full scan mass spectra were acquired in the Orbitrap in the *m*/*z* range 120–700 with a target mass resolution of 100.000 (FWHM as defined at *m*/*z* 400) and a scan time of 0.65 s.

2.9. HPLC-HRMS

For the determination of drug concentrations in hair and in standard solutions, 10 µL of solutions or extracts were injected into an Atlantis T3 (150 × 1.0 mm, 3 µm) column (Waters Corporation, Milford MA, USA). HPLC separation was achieved by gradient elution at a constant flow rate of 300 µL/min. HPLC conditions: A (water, 0.1% formic acid HCOOH) and B (methanol, 0.1% HCOOH); initial conditions 10% B, linear gradient to 25% B in 4 min, 25% B hold from 4 to 7 min, then ramped to 40% B in 5 min, to 60% B in 4 min and to 90% B in 2 min; 90% B hold from 18 to 26 min. The column temperature was 40 °C. MS conditions were: positive ion ESI; capillary voltage 10 V, sheath gas flow rate 50 (arbitrary units, a.u.), auxiliary gas (N_2) flow rate 5 (a.u.), sweep gas flow rate 5 (a.u.), and capillary temperature 275 °C; profile full scan mass spectra were acquired in the Orbitrap in the *m*/*z* range 120–700 with a target mass resolution of 60.000 (FWHM as defined at *m*/*z* 400) and a scan time of 0.45 s. Detection of the analytes and the IS was based on retention time, accurate mass measurements of MH^+ ions, and correspondence of the observed isotopic pattern to

the calculated one. Drug concentrations were determined from peak area ratios of analyte to its IS compared to calibrator curves of peak area ratios to concentrations. The method was fully validated exhibiting a linear range from 0.1 to 50 ng/mg (determined from regression with $1/x^2$ weighting utilizing six calibration points), lower limits of quantification of 0.01 ng/mg and limits of detection of 0.005 ng/mg for all the target analytes, intra-day imprecision and inaccuracy always lower than 19% and 20% and inter-day imprecision and inaccuracy always lower than 21% and 22%, respectively.

3. Results

3.1. Photodegradation in methanol and water solutions exposed to Suntest CPS+

Preliminary, stock methanol and water solutions for all the analytes diluted to 10^{-4} M were irradiated inside the photostability test chamber from 1 to 3 h (each hour corresponding to about 70 J/cm^2), and their absorption spectra before and after irradiation were recorded (Supplementary Figures S1A1–S1F2).

AMF dissolved in methanol is characterized by the only band at around 205-210 nm, mainly corresponding to the solvent, thus remaining unmodified in all the irradiated solutions (see Supplementary Figure S1A1); however, a shift to 205 nm and a small increase in spectral intensity were detected by increasing irradiation; when irradiated in water, AMF is characterized by two bands (at around 220 nm and 240 nm) decreasing as irradiation dose increases (see Figure S1A2).

MA both in methanol and water (Figure S1B1 and S1B2 respectively) presents curves similar to AMF, the unique difference being the absorption at 205 nm in water which increases under irradiation.

MDA in methanol (Figure S1C1) is characterized by three bands at 208 nm, 235 nm and 285 nm; the irradiated solutions present similar curves with increasing absorption and a small blue shift at 230 nm and 280 nm. Similar spectra are obtained for MDA in water (Figure S1C2): two bands at 230 nm and 285 nm increasing in relation to irradiation time.

MDMA in methanol shows two bands centered at 235 nm and at 285 nm showing a small absorption increase and a small blue shift (Figure S1D1); a new band of absorption (300-340 nm) appears that increases upon irradiation. In water, MDMA presents the same absorption band with a higher increase at 235 nm than at 285 nm and the appearance of a band at 320 nm (Figure S1D2).

KET in methanol is characterized by a single absorption band (205-230 nm); when irradiated, this band slightly increases and a shoulder (230-270 nm) appears, as evidenced in supplementary Figure S1E; on the contrary, in water KET doesn't change its absorption spectrum upon irradiation (Figure S1E2). Absorption spectrum of NKET in methanol increases upon irradiation at 210-220 nm and in the range 240-250 nm (Figure S1F1); in water, NKET shows a similar behavior upon irradiation for the band 240-250 nm, with slight changes (Figure S1F2).

3.2. Identification of new photoproducts

All the irradiated solutions in the solar simulator, including the control solutions kept in the dark, were diluted to 10^{-5} M either in methanol or water, and analyzed by direct infusion HRMS, with the aim of identifying the compounds eventually formed upon irradiation. Interestingly, for KET and NKET only some new species could be observed in irradiated solutions. In HRMS spectrum of KET irradiated in water solution, its $[M+H]^+$ ion at *m/z* 238.0993 ($C_{13}H_{17}ClNO$) is accompanied by a new ionic species at *m/z* 220.0888 ($C_{13}H_{15}ClN$), corresponding to the loss of H_2O. In methanol two species are observed, at *m/z* 220.0888 and at *m/z* 252.0786 ($C_{13}H_{15}ClNO_2$), this last corresponding to the loss of H_2 and to the photoaddition of one oxygen atom. The proposed structures of KET photoproducts are shown in Figure 1 and Figure 2. It must be highlighted that the ionic species identified are new protonated molecules present in solution and they are not fragment ions produced by collisional experiments on precursor ions.

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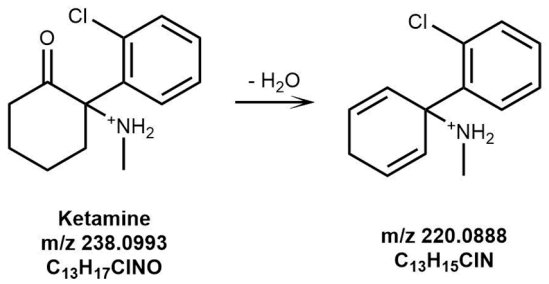

Figure 1 Proposed structures of KET photoproduct formed in water solution upon irradiation for 3h.

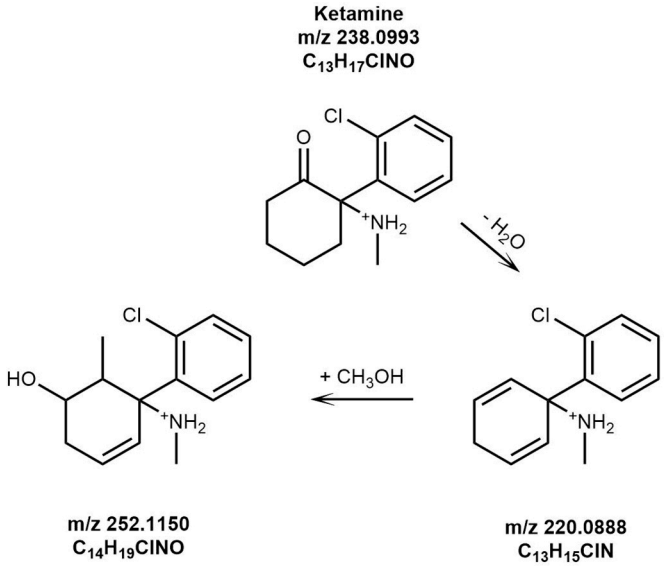

Figure 2 Proposed structures of KET photoproducts formed in methanol solution upon irradiation for 3h.

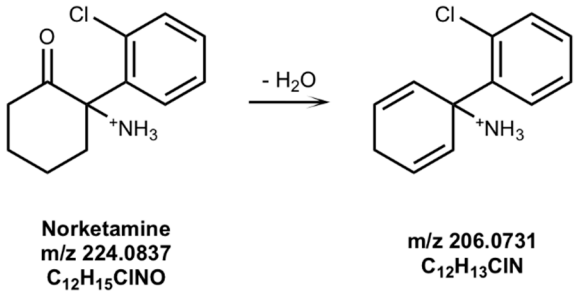

Figure 3 Proposed structures of NKET photoproduct formed in a methanol solution upon irradiation for 3h.

In HRMS spectrum of the methanol solution of NKET irradiated in the solar simulator, $[M+H]^+$ ions at *m/z* 224.0837 ($C_{12}H_{15}ClNO$) are accompanied by a new species at *m/z* 206.0731 ($C_{12}H_{13}ClN$), corresponding to the loss of H_2O. The proposed structure of NKET photoproduct formed in methanol is shown in Figure 3 analogously to KET. However, differently from KET, no photoproduct was detected in HRMS spectrum of NKET irradiated in water solution.

Vice versa, for all the other analytes (AMF, MA and MDMA), both in water and methanol solutions, no photoproduct could be evidenced by direct HRMS analysis. To avoid ionization suppression phenomena that could occur in a mixture and shield the presence of less abundant species, irradiated solutions were also analyzed by HPLC-HRMS. The ratios of peak areas observed

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for samples upon irradiation *vs* analogous kept in dark were calculated as 'percent degradation.' In Figure 4 the yields of photodegradation obtained for all the analytes after 1h, 2h and 3h of irradiation are reported.

As may be observed, AMF and MA in water show the highest photostability: the photodegradation is 1% and 4% respectively, while in methanol it increases up to 15%. MDMA and MDA in methanol and water both present similar photodegradation with a linear relationship with irradiation time: MDMA from 23% to 42%, MDA from 13% to 36%. For KET and NKET the photodegradation yield is significantly higher in methanol (KET 61%; NKET 36%) than in water (KET 16%; NKET 13%), after 3 h.

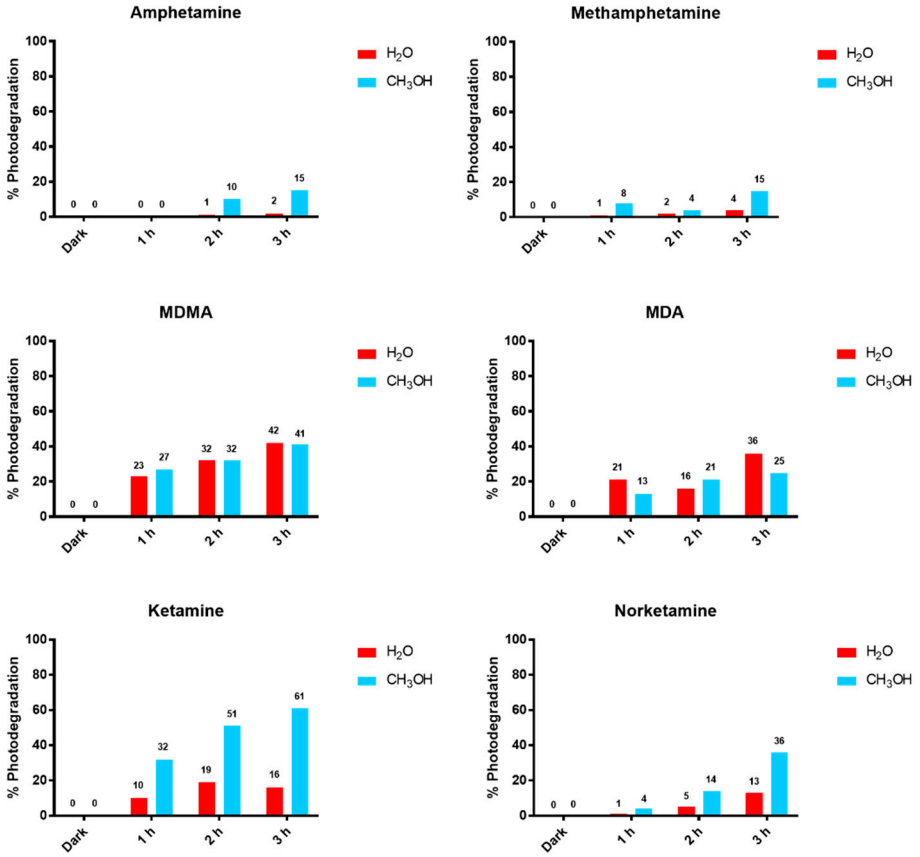

Figure 4: Relative degradation of target drugs in methanol and water solutions with increasing time of irradiation in the solar simulator (% photodegradation calculated using the peak area of protonated molecules in solutions kept in the dark as controls for 0% degradation).

3.3. Photodegradation in hair exposed to Solar box

The main goal of the study was to observe the photoinduced degradation of drugs in hair. In Table 1, the concentrations of each drug for all seventeen hair samples irradiated in the solar simulator or kept in the dark (control) are presented.

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Table 1. Drug concentrations in hair samples with their physical characteristics calculated by HPLC-HRMS in both aliquots kept in the dark or irradiated in the solar simulator. *-: absent at Limit of Detection (LOD, 0.005 ng/mg).

Physical characteristics				Dark						Irradiated					
Color	Thickness	Straight	Curl	MET (ng/mg)	AMP (ng/mg)	MDMA (ng/mg)	MDA (ng/mg)	KET (ng/mg)	NKET (ng/mg)	MET (ng/mg)	AMP (ng/mg)	MDMA (ng/mg)	MDA (ng/mg)	KET (ng/mg)	NKET (ng/mg)
Brown 1	Thin	●		-*	-	-	-	0.02	-	-*	-	-	-	0.02	-
Brown 2	Thin	●		-	-	-	-	0.04	0.02	-	-	-	-	0.26	0.01
Brown 3	Thin	●		-	-	2.34	0.34	23.8	3.17	-	-	0.34	0.0	4.16	0.85
Brown 4	Thick		●	-	-	-	-	0.01	-	-	-	-	-	0.05	-
Brown 5	Thin	●		-	0.06	-	-	0.10	0.03	-	0.04	-	-	0.07	0.01
Brown 6	Thick	●		-	-	0.20	-	-	-	-	-	0.12	-	-	-
Brown 7	Thin	●		-	-	0.69	0.05	-	-	-	-	0.76	0.04	-	-
Brown 8	Thick	●		-	-	0.09	-	-	-	-	-	0.07	-	-	-
Brown 9	Thick		●	-	-	-	-	0.06	0.06	-	-	-	-	0.04	0.04
Brown 10	Thin	●		-	-	-	-	0.15	0.02	-	-	-	-	0.08	0.01
Brown 11	Thick	●		-	-	-	-	0.28	0.02	-	-	-	-	0.20	0.03
Brown 12	Thick	●		-	-	-	-	0.05	0.01	-	-	-	-	0.02	0.01
Dark Brown 1	Thick		●	-	-	-	-	0.34	0.02	-	-	-	-	0.1	0.01
Black 1	Thin	●		-	0.99	1.40	0.07	14.3	2.43	-	0.6	1.63	0.06	8.85	2.40
Black 2	Thin	●		0.97	-	-	-	-	-	0.8	-	-	-	-	-
Black 3	Thin	●		24.11	0.93	-	-	-	-	12.68	0.7	-	-	-	-
Black 4	Thin	●		-	-	0.45	-	-	-	-	-	0.3	-	-	-

In Table 2, the percentage of photodegradation are reported. Calculations were made using the concentrations of sample kept in the dark as control:

% photodegradation = (drug conc.$_{dark}$ - drug conc.$_{irradiatied}$)/ drug conc.$_{dark}$ * 100.

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278 **Table 2.** Percentage of photodegradation, average, standard deviation and range of drugs in hair
279 samples irradiated at 765 W/m² in the solar simulator.

	Photodegradation %											Average %	SD	RANGE %
	BROWN 5	BLACK 1	BLACK 2	BLACK 3										
MET	-	-	14	47								31	24	14-47
AMF	37	35	-	25								32	6	25-37
	BROWN 3	BROWN 6	BROWN 7	BROWN 8	BLACK 1	BLACK 4								
MDMA	85	41	- 11	20	- 16	30						25	37	(-)16-85
MDA	100		19		3							41	52	3-100
	BROWN 1	BROWN 2	BROWN 3	BROWN 4	BROWN 5	BROWN 9	BROWN 10	BROWN 11	BROWN 12	DARK BROWN1	BLACK 1			
KET	5	34	82	58	23	30	43	21	65	73	38	43	24	5-82
NKET		24	73		56	40	19	- 45	30	24	40	29	33	(-)45-56

280 Four of the seventeen samples were in sufficient amount to allow a second experiment, in order
281 to verify the role of the individual matrix on drug degradation or protection. Thus, they have been
282 pulverized; each pulverized hair was divided into two identical aliquots and put in Petri plates: one
283 was irradiated at 765 W/m² for 48 hours; the other one was covered by an aluminum foil (dark). The
284 drug concentrations are reported in table 3.
285

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Table 3. Physical characteristics and drug concentrations of pulverized hair samples analyzed by HPLC-HRMS.

	Dark		Irradiated	
Color	KET (ng/mg)	NKET (ng/mg)	KET (ng/mg)	NKET (ng/mg)
Brown 3 - Pulverized	0.04	0.007	0.2	0.008
Brown 10 - Pulverized	0.06	0.008	0.04	0.009
Brown 12 - Pulverized	0.41	0.017	0.31	0.023
Dark Brown 1 - Pulverized	31.8	7.78	30.8	4.44

In Figure 5 the percentages of photodegradation, calculated using the concentrations of the same sample kept in the dark as control, are ordered by increasing starting concentrations of KET.

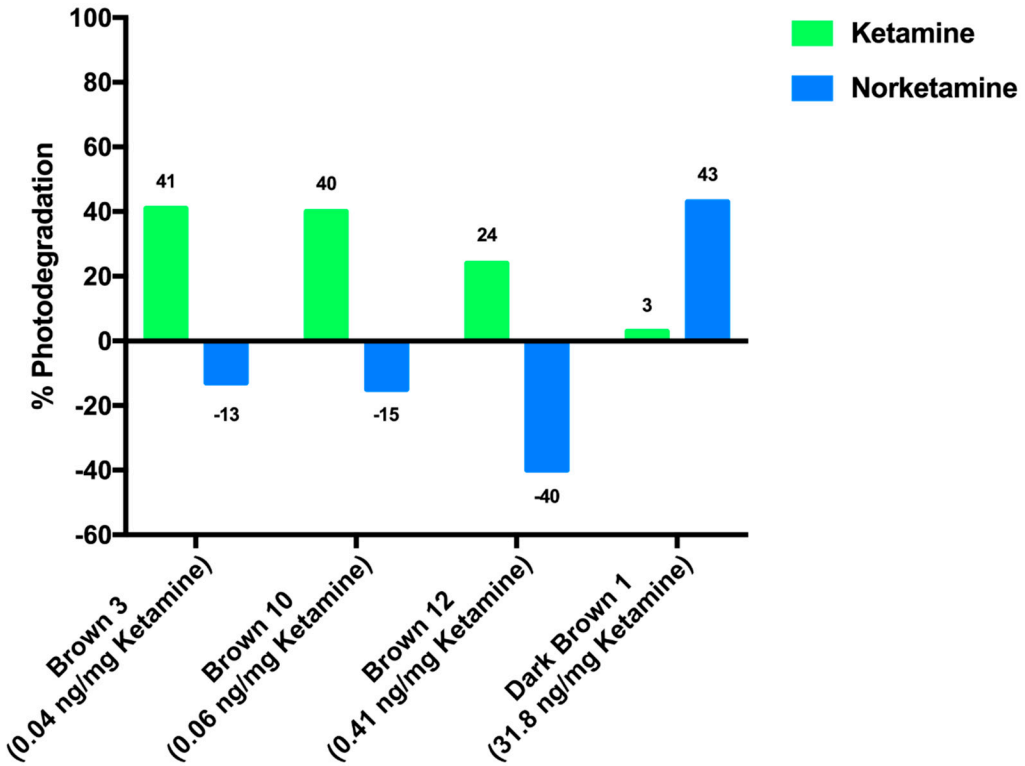

Figure 5. Percentage of photodegradation in four pulverized hair samples

For KET, a degradation of 41% was observed for an initial concentration of 0,04 ng/mg, while a degradation of only 3% was observed for a starting concentration of 31,8 ng/mg. *Vice versa*, for NKET, an apparent increase of concentration was observed, with the exception of the sample with the highest concentration.

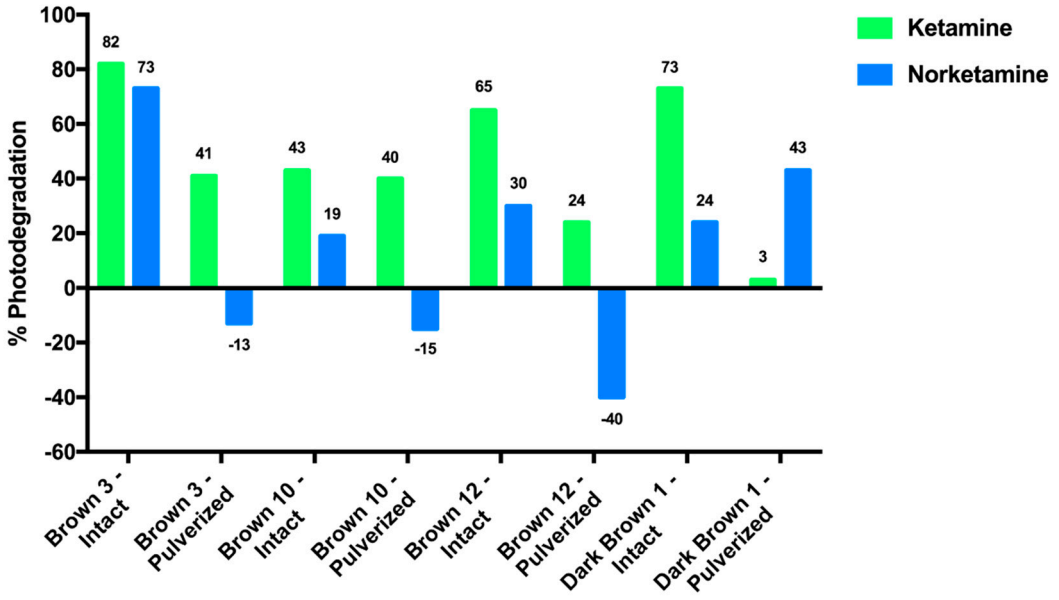

Figure 6. Percentage of photodegradation of KET and NKET in 4 whole *versus* pulverized hair samples

For the same four samples, the photodegradation yields observed in whole hair were compared to those obtained for the corresponding pulverized hair, as presented in Figure 6.

In the intact hair, KET and NKET show an average photodegradation of 66% and 37% respectively, while in pulverized hair the average photodegradation is 27% for KET and - 6% for NKET respectively.

In irradiated pulverized hair, two phenomena likely play together in opposite directions: a greater lability of the pulverized keratin would produce a larger release of analytes (as observed in previous studies [10]) whereas a physical weakening of the matrix and thus of its photoprotective effect allows a larger degradation of the drug molecules and possibly induce production of the metabolites. As a matter of fact, in three cases out of four, the metabolite NKET was found at a higher level in the pulverized hair than in the intact ones.

When comparing results from three polydrug abusers, photodegradation of all the analytes was generally obtained, with the highest, comparable photodegradation yields observed in sample # Brown 3 (see Figure 7).

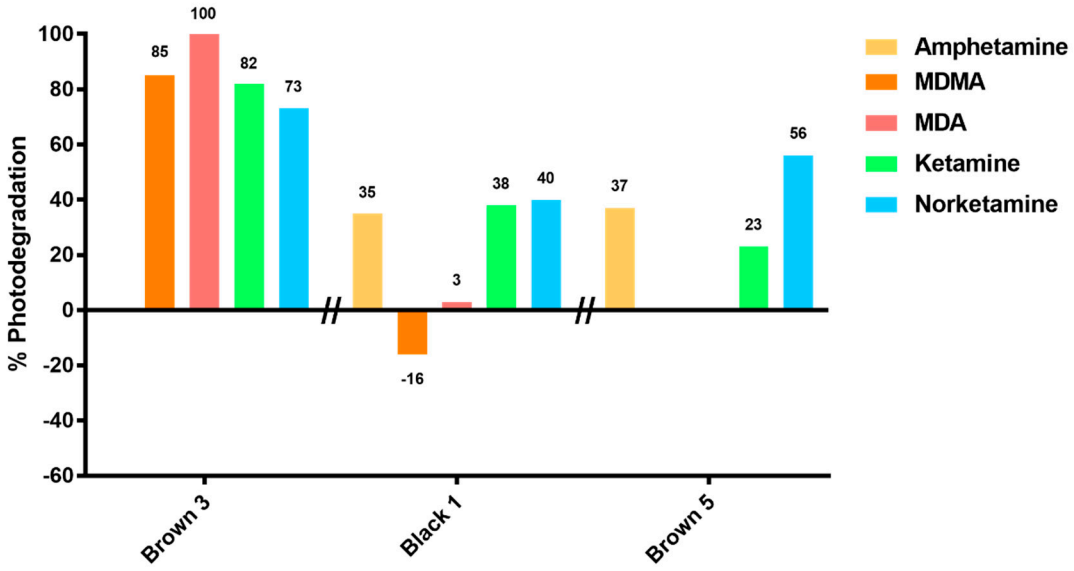

Figure 7. Percentage of photodegradation of AMF, KET and NKET in three different hair samples

In these three samples, the photodegradation seems to depend on the hair owner.

4. Discussion

From our results, a clearly different behavior of KET and NKET was evidenced when compared to all the other drugs and metabolites under study. Indeed, these drugs seem photo-unstable both in solution and in hair matrix. In particular, in experiments in solution, KET and NKET photoinduced products have been identified; in hair samples their degradation was higher (average 43 and 30 %) than all the other compounds (average 15 - 30 %).

Interestingly, no color effect was observed (although no fair hair samples were present in this study). It's well known that the color of hair depends on the relative amount of pheomelanin (red) and eumelanin (black), the first defense against UV in human hair and skin [24-26]. Generally, a part of the light is absorbed by the hair matrix itself without any photochemical effect. In dark hair, the eumelanin could protect the drugs/metabolites with a higher degree than in fair hair. However, melanin may also react with oxygen under irradiation, producing reactive species, such as superoxide anion, that can induce photolysis of melanin itself [27], thus weakening the photoprotective effect of the pigment. In this context, no clear-cut interpretation of the role of hair color can be made.

Regarding AMF and MA, on the basis of the *in vitro* experiments they were expected to photodegrade less readily than KET and NKET. Unexpectedly, the experiments in hair revealed an average degradation of 30% and 29% for MA and AMF respectively, with a range of 14-47% and 25-35%.

The presence of photoproducts was also investigated in irradiated hair samples. In one sample the species at *m/z* 220.08750, corresponding to the photoproduct of KET shown in Figure 1, could be identified by LC-HRMS as evidenced in Figure 8.

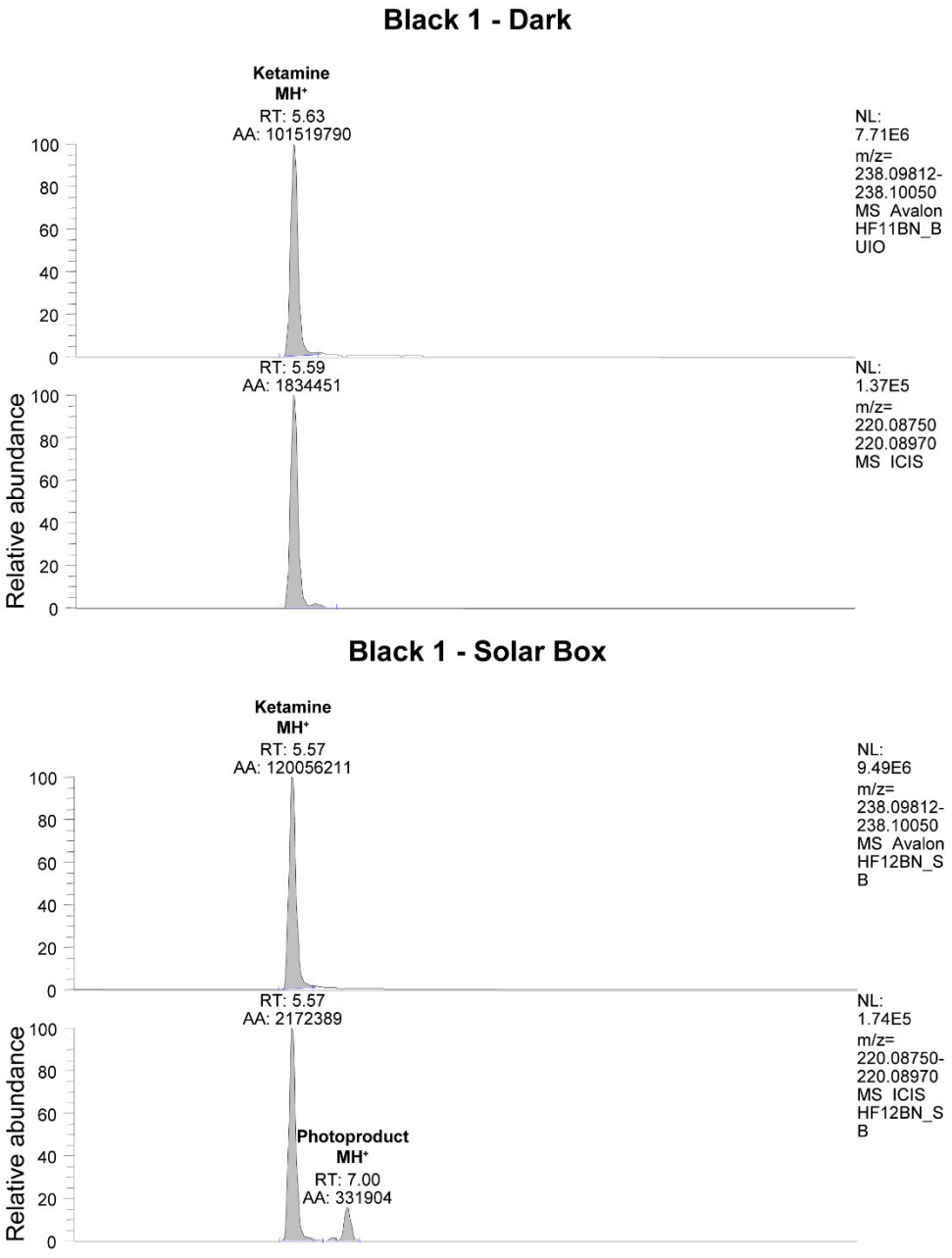

Figure 8. LC-HRMS analysis of Black 1 hair sample taken in the dark and after irradiation in the solar simulator. In the irradiated sample, the presence of a KET photoproduct with m/z 220.08750 is evident.

The importance of this finding must be highlighted, since no previous study identified stable photoproducts in hair for KET nor for any other substance previously investigated: cannabinoids, cocaine, opiates, methadone [9-10, 20-21].

As regard the experiment on pulverized *versus* intact hair samples, it's clear that KET photodegradation yield is not concentration-dependent but matrix-dependent and that the physical characteristics of the hair have influenced both the photostability and the extraction yield of the drug. The "false production" of NKET during irradiation was at first sight surprising, but could be reasonably related to the physical decomposition of the matrix, leaving KET more labile during the irradiation experiment and favoring its transformation to the metabolite. Furthermore, as already demonstrated in previous studies, light can lead to a greater lability of the keratin matrix with consequent greater yield during the extraction of the analytes in the liquid acid phase.

5. Conclusions

AMF, MA MDA, MDMA, KET and NKET incorporated in hair undergo degradation when irradiated by artificial sunlight suggesting that they can suffer photodegradation under natural sunlight. With this work, for the first time, the presence of a photoproduct of KET was evidenced in one true positive sample.

Since the detection of drugs in hair is often used as evidence of illegal acts, with consequences on the freedom of persons, the UV-Vis effects on the integrity of the drugs and their metabolites should be considered when single administration need to be evidenced. When decisional cut-offs are applied to hair analysis (e.g. for granting a driving license, a job, or a child custody), it must be taken into account that hair exposed to sun light may produce false negative results and lead to misjudgment. When possible, the detection of photoproducts of a drug under investigation could be a key factor in a case management.

Supplementary Materials: The Supplementary Materials available online.

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