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Chromatography–mass spectrometry studies on the metabolism of synthetic cannabinoids JWH-018 and JWH-073, psychoactive components of smoking mixtures

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ABSTRACT

The Russian Federation prohibited the distribution of herbal mixtures with synthetic aminoalkylindoles JWH-018 and JWH-073, agonist cannabinoid receptors, on January 22, 2010. The lack or low content of their native compounds in urine requires detailed identification of their metabolites, which are excreted with urine and are present in blood. Using gas and liquid chromatography–mass spectrometry, we identified a series of metabolites in urine samples from humans and rats that were products of the following reactions: (a) mono- and dihydroxylation of the parent compounds with hydroxyl groups located at aromatic and aliphatic residues, (b) carboxylation, (c) N-dealkylation and (d) N-dealkylation and hydroxylation. The prevailing urinary metabolites in humans are monohydroxylated forms, while N-dealkylated and N-dealkyl monohydroxylated forms are found in rats. Twenty-six samples of herbal smoking mixtures with JWH-018, purchased in Russia, were analysed.

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1. Introduction

The search for compounds possessing high affinity to cannabinoid receptors CB_1 and CB_2 led to the synthesis of a series of aminoalkylindoles and studies of their pharmacological activity [1]. High-affinity compounds from naphthalen-1-yl-(1-pentyl-1H-indol-3-yl)methanone (JWH-018) and its butyl homologue JWH-073 were reported in 1998 [2]. The affinity of these compounds to CB_1 receptors (located mainly in the central nervous system and associated with the generation of a psychoactive response) is rather high (affinity constant $K_i = 9$ nM and 8.9 nM for JWH-018 and JWH-073, respectively). These compounds showed about four times more potent affinity than did Δ^9-tetrahydrocannabinol ($K_i = 41$ nM), a psychoactive ingredient in marijuana [3,18].

The herbal mixtures made from exotic plant components (according to the labels on the packages) and called "spices" entered the market in 2004 [4]. These mixtures create marijuana-like effects when smoking them, although they contain neither Δ^9-tetrahydrocannabinol nor any other known psychoactive substances. The increased popularity of these mixtures in Western Europe has attracted much public attention and has consequently resulted in the study of their chemical composition. In December of 2008, THC Pharma (Germany) and AGES PharmMed (Austria) independently reported the detection of the synthetic component JWH-018 sprayed onto an herbal foundation in a number of these mixtures [5]. This result was confirmed by additional studies in Western Europe [6,7] and Japan [8,9]. The direct linkage between JWH-018 and the psychotropic effects caused by smoking the JWH-018-containing "spices" was established in [10].

The analysis of smoking mixtures, conducted by chromatography–mass spectrometry, revealed additional components, which were plant-based or added to the mixtures to modify their properties. Except for phytosterols, several aromatic additives (ethylvanilin, eugenol and eucalyptol), α-tocopherol and oleamide [6,9] were detected. The quantitative analysis of fifteen smoking mixtures showed that the average concentration of JWH-018 is ca. 15 mg/g of a mixture [9]. Products with high JWH-073 content are extremely rare. This compound occurs in mixtures as an impurity (or additive) [7,9]. According to several messages in specialised

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Table 1
List of components found in smoke mixtures with active JWH-018.

Product	JWH-018	JWH-073	CP 47, 497 C8	Tocopherol	Other substances
Afghan incense	+++	+	–	+	–
Chernobyl	+++	+	+	+	JWH-018 (–Cl)[a]
Genie blend	++	–	–	+++	–
Master Kush	+++	–	–	+	–
Mojo	++	–	–	+++	–
Smoke	++	–	+	+	Eugenol, oleamide (main component)
Spice arctic synergy	++	–	++	+++	Menthol, JWH-018 (–Cl)[a]
Spice diamond	+ (trace)	–	++	+++	Ethylvanillin
Spice tropical synergy	++	+	++	+++	Menthol, caffeine
Yucatan fire	++		++	+++	–
Unnamed Mixture 1	+++	–	–	+++	–
Unnamed Mixture 2	+	–	+++	+	–
Unnamed Mixture 3	+++	+	–	–	Menthol
Unnamed Mixture 4	+++	++	–	+	–
Unnamed Mixture 5	+++	–	–	–	Nicotine, 1-pentyl-1*H*-indole, pentyl 1-naphthoate
Unnamed Mixture 6	+++	+	–	–	1-pentyl-1*H*-indole
Unnamed Mixture 7	+++	–	–	–	Nicotine
Unnamed Mixture 8	++	–	–	+++	–
Unnamed Mixture 9	++	–	+	+++	–
Unnamed Mixture 10	+++	–	–	–	Oleamide
Unnamed Mixture 11	+++	+	–	–	Vanilin 1-pentyl-1*H*-indole
Unnamed Mixture 12	+++	–	–	–	–
Unnamed Mixture 13	++	–	+	+++	–
Unnamed Mixture 14	+++	+	–	++	–
Unnamed Mixture 15	+++	–	–	–	–
Unnamed Mixture 16	+++	–	–	–	Menthol, nicotine, indole, 1-pentyl-1*H*-indole, pentyl 1-naphthoate, 1-naphthamide

[a] Chlorinated JWH-018 components.

internet forums (and instructions on the use of smoking mixtures), 80–150 mg of these mixtures, corresponding to approximately 1–2.5 mg of JWH-018, must be smoked to induce psychogenic effects. The actual quantity absorbed by the lungs is significantly lower.

JWH-018 intake can be established by detecting the native compound in blood [11–13]. For this purpose, liquid chromatography–mass spectroscopy (LC–MS) and high-sensitivity MS methods were used to detect JWH-018 at concentrations of ng/mL and lower. The compound concentration in a smoker's serum decreases rapidly (90% of the maximal concentration has dissipated within three hours after smoking) [11].

JWH-018 and JWH-073 are poorly soluble in water and produce no hydrophilic conjugates. The first study of the metabolism of similar compounds were performed *in vitro* and showed that the main metabolic pathways of these compounds might be hydroxylation and N-dealkylation of the parent compound [14]. The authors in [15] reported the results of urine analyses of rats that had JWH-018 introduced by intragastric intubation. Although the native compound was found in the urine, the authors noted the prevailing content of the hydroxylated N-dealkylated forms. In a study on the identification of JWH-018 metabolites in human urine, hydroxylation of the initial structure was proposed as the main metabolic pathway; however, N-dealkylated metabolites were also found [16]. No native JWH-018 was observed in three human urine samples, which is a significant result of the study.

The aim of this study is to identify JWH-018 and JWH-073 metabolites as markers of JWH-018 and JWH-073 use by gas and liquid chromatography–mass spectrometry. Eleven urine samples from humans who took synthetic cannabinoids and four rat urine samples were analysed. In addition, a review of smoking mixtures with JWH-018 (and JWH-073) purchased in Russia is provided.

2. Materials and methods

2.1. Reagents

N,O-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (BSTFA + 1% TMCS) was purchased from Acros Organics (Geel, Belgium), pyridine (analytical grade) was

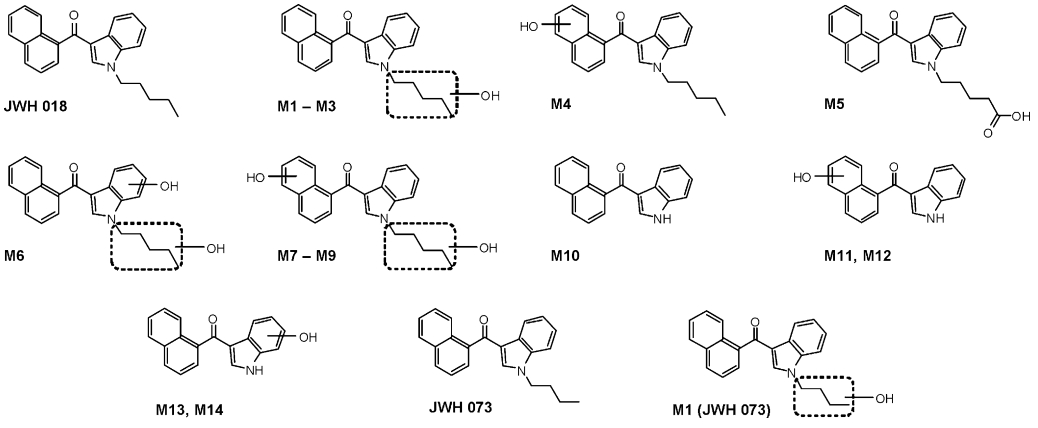

Fig. 1. The proposed structures of the identified JWH-018 metabolites.

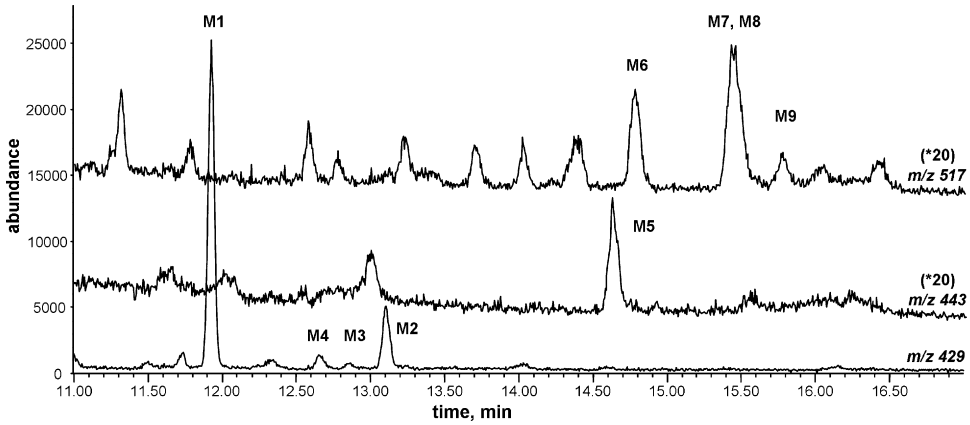

Fig. 2. Fragments of ion chromatograms of urinary JWH-018 metabolites (TMS). SCAN mode.

purchased from Scharlau Chemie S.A. (Barselona, Spain), and acetonitrile and methanol were supplied by Panreac Quimica S.A. (HPLC-gradient grade). The other solvents and chemicals (for analysis) were obtained from Ecos-1 (Moscow, Russia). Solid-phase extraction cartridges, AccuBond SPE ODS-C18 (3 mL × 200 mg), were supplied by Agilent Technologies (Santa Clara, CA, USA).

The immunochromatographic assays were applied to test strips (ICA-4-MULTI-FACTOR, ICA-MARIJUANA-FACTOR and ICA-TAD-FACTOR) supplied by Factor-Med (Moscow, Russia).

2.2. Herbal smoking mixtures and their processing

Twenty-six smoking mixtures (Table 1) with different labels (or without labels) were purchased via the internet from July 2009 to January 2010. The weight of these herbal products varied from 0.5 to 5 g.

The smoking mixtures (10–30 mg) were crushed into powder and extracted with 3 mL of ethanol under ultrasonication for 10 min at room temperature to extract the sprayed compounds. The extract was then centrifuged and filtered through 0.45 μm filters (Millipore, Bedford, MA). The extracts were analysed by GC–MS.

Unnamed Mixture 4, containing (apart from JWH-018) a considerable amount of JWH-073, was used to study JWH-018 metabolism in rats. Five grams of this mixture was extracted twice with ethanol (2 × 100 mL) under ultrasonication. After filtration, the solution was evaporated in a vacuum rotary evaporator. The resulting tarry residue (TR, about 110 mg) was applied to study the metabolism of the active components without any further purification.

2.3. Urine samples (of drug-intoxicated persons detained by police)

The urine samples were collected from eleven individuals with symptoms of drug intoxication who were brought to the drug service by police and confessed to having smoked herbal mixtures. No common drugs were found by immunochromatographic assay (including detection of cannabinoids, benzodiazepines, morphine, cocaine and amphetamines).

2.4. Experimental procedures with rats

The TR suspension in 2% Tween-80 was intraperitoneally introduced into four Wistar rats after a day without feeding at a rate of 15 and 30 mg of TR/kg of body mass (that corresponds to approximately 0.7–1.4 g of the original smoking mixture). Symptoms of suspension influence are typical for cannabinoids cataleptic activity [17]. Three hours after suspension introduction, the animals were placed in metabolic pans for 20 h with free access to water to collect their urine. An additional rat was given a placebo (2% Tween-80 in water) to obtain a blank urine sample.

2.5. Preparation of urine samples

Liquid-liquid extraction (LLE) and solid-phase extraction (SPE) were used for the preparation of the urine samples.

For LLE, 2.5 mL of urine was added to 0.3 mL of hydrochloric acid (ca. 30%) and heated at 90–95 °C for 60 min. After cooling, aqueous ammonia (ca. 25%) was added to reach a pH of 8–9. The samples were extracted with 3 mL of chloroform and centrifuged. The organic phase was evaporated by nitrogen stream at 45 °C or below. The dry residue was dissolved either in 50 μL of ethanol (for GC) or in the same volume of 10% v/v acetonitrile in water (for LC).

For SPE, the sample (3 mL of hydrolysed urine, pH 8–9 plus 3 mL of water and 0.6 mL of acetonitrile) was loaded onto a cartridge. The sorbent was rinsed by 10 and 40% v/v acetonitrile in water (3 mL) and dried by air stream for 30 s. The analytes eluted 3 mL of acetone. The recovery was determined by adding an extract with high concentrations of the components to the blank hydrolysed urine. For three measurements, the average recovery was 102% (RSD 10%).

Derivatisation of the samples for GC was performed by trimethylsilylation (TMS), acetylation (AC) or trifluoroacetylation (TFA). TMS was carried out in a mixture of BSTFA and ethyl acetate, 25 μL each, at 60 °C for 60 min. For acetylation, the dry residual was dissolved in the mixture of acetic anhydride and pyridine, 50 μL each, and heated at 70 °C for 30 min. Then the mixture was evaporated in the vacuum concentrator (Concentrator 5301, Eppendorf AG, Hamburg, Germany) and dissolved in 50 μL of ethanol. The TFA derivatives were prepared in a mix of trifluoroacetic anhydride and ethyl acetate, 50 μL each (50 °C, 30 min). After evaporation, the dry residual was dissolved in 50 μL of ethyl acetate.

2.6. GC–MS

Gas chromatographs 6890 connected to single-quadrupole mass spectrometers 5973 and 5975VL were equipped with HP-5ms (30 m × 0.25 mm × 0.25 μm) and EVDX-5ms (25 m × 0.20 mm × 0.33 μm) columns (Agilent Technologies). The separation was performed by applying the following temperature program: 50 °C (0.5 min), 99°/min (100 °C, 1 min), and 60°/min (320 °C, 15 min). Helium was the carrier gas (1 and 0.8 mL/min for both columns, respectively). The column temperature was reduced for the acquisition of spectra of poorly separated compounds (35 °C/min and 300 °C at the last steps). The sample (1 μL) was injected in splitless mode (270 °C). The temperatures of the transfer line, ion source (electron ionisation mode, 70 eV) and mass filter were 290, 230 and 150 °C, respectively. The mass spectra

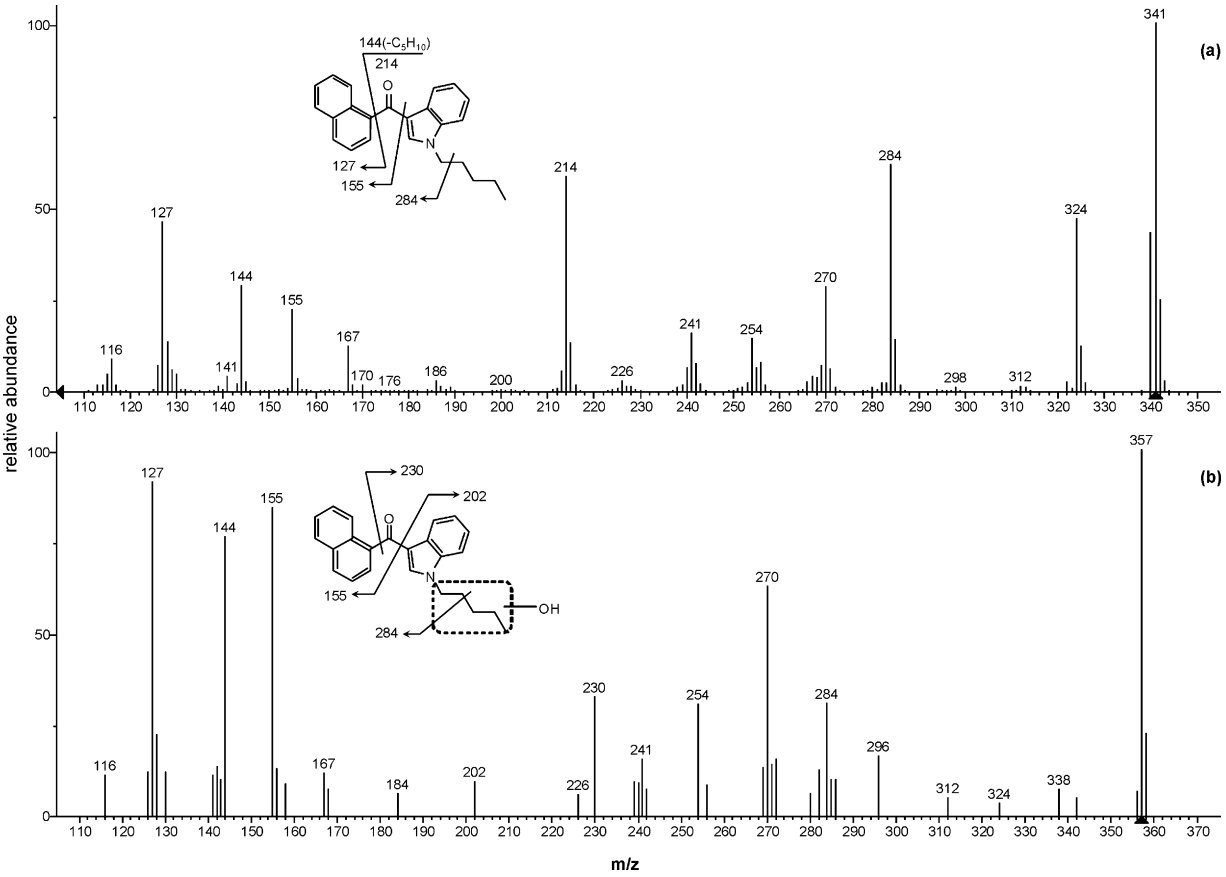

Fig. 3. Mass spectra of JWH-018 (a) and its main metabolite M1 (b).

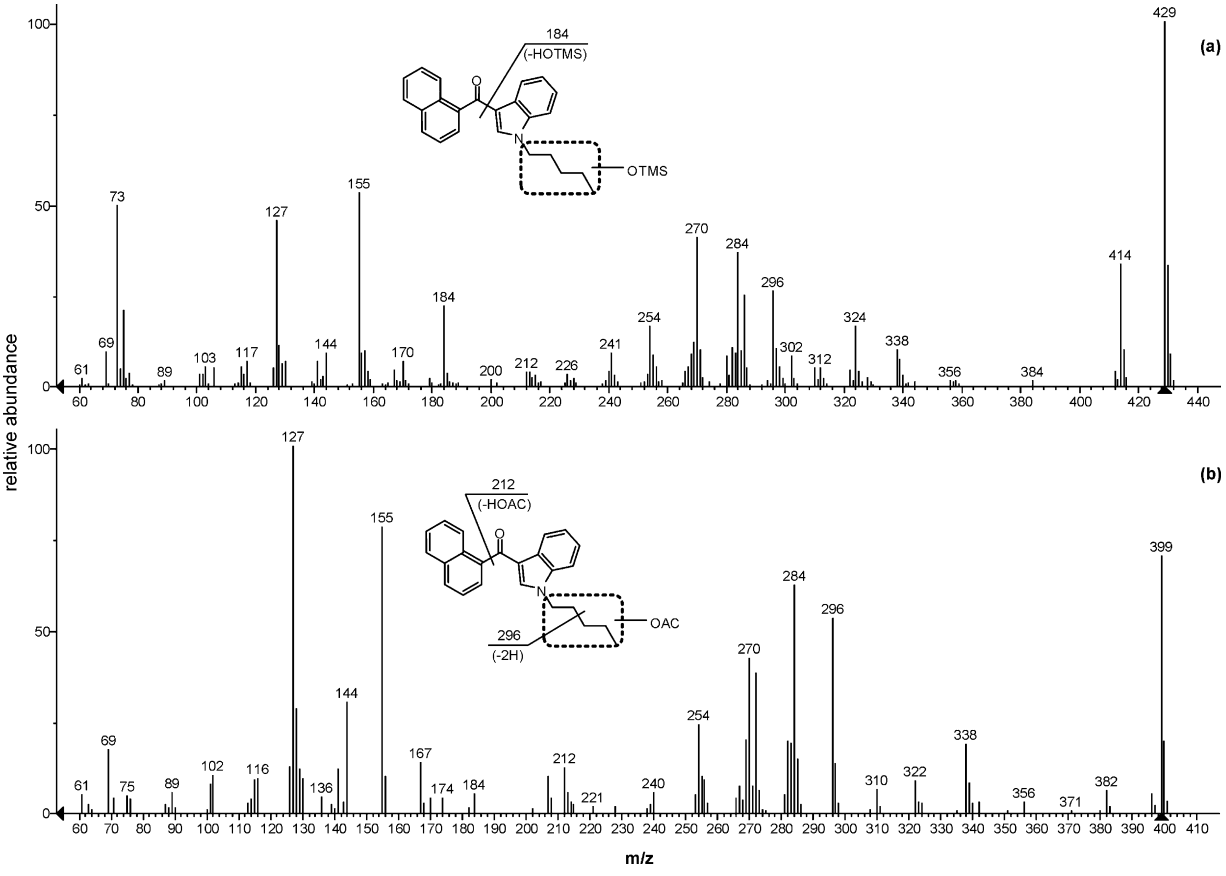

Fig. 4. Mass spectra of M1 after derivatisation by TMS (a) and AC (b).

Table 2
Linear retention indices (*n*-alkanoic scale, EVDX-5ms column) and occurrence of detected metabolites.

No.	Compound	Net formula	$[M]^{+\bullet}$	Index	Detection	
					Human	Rat
1	JWH-018	$C_{24}H_{23}NO$	341	3316	–	–
2	M1	$C_{24}H_{23}NO_2$	357	3543	+	–
3	M1 TMS	$C_{27}H_{31}NO_2Si$	429	3498		
4	M1 AC	$C_{26}H_{25}NO_3$	399	3580		
5	M1 TFA	$C_{26}H_{22}F_3NO_3$	453	3328		
6	M1 TFA/artefact	$C_{24}H_{21}NO$	339	~3313		
7	M2 TMS	$C_{27}H_{31}NO_2Si$	429	3591	+	–
8	M2 AC	$C_{26}H_{25}NO_3$	399	3702		
9	M2 TFA	$C_{26}H_{22}F_3NO_3$	453	3454		
10	M3 TMS	$C_{27}H_{31}NO_2Si$	429	3571	+	–
11	M4 TMS	$C_{27}H_{31}NO_2Si$	429	3557	+	–
12	M5 TMS	$C_{27}H_{29}NO_3Si$	443	3689	+	–
13	M5 Me	$C_{25}H_{23}NO_3$	385	3630		
14	M6 2TMS	$C_{30}H_{39}NO_3Si_2$	517	3699	+	–
15	M7 2TMS	$C_{30}H_{39}NO_3Si_2$	517	3735	+	–
16	M8 2TMS	$C_{30}H_{39}NO_3Si_2$	517	3735	+	–
17	M9 2TMS	$C_{30}H_{39}NO_3Si_2$	517	3752	+	–
18	M10 AC	$C_{21}H_{15}NO_2$	313	3129	–	+
19	M11 2AC	$C_{23}H_{17}NO_4$	371	3451	+	+
20	M12 2AC	$C_{23}H_{17}NO_4$	371	3493	+	+
21	M13 2AC	$C_{23}H_{17}NO_4$	371	3499	+	+
22	M14 2AC	$C_{23}H_{17}NO_4$	371	3479	+	–
23	JWH-073	$C_{23}H_{21}NO$	327	3234	–	
24	M1 (JWH-073) TMS	$C_{26}H_{29}NO_2Si$	415	3397	+	
25	M1 (JWH-073) AC	$C_{25}H_{23}NO_3$	385	3478		

of the identified components were background, corrected by the AMDIS (automatic mass spectral deconvolution and identification system) program (NIST, USA) in manual and auto modes. Variations in intensity of the most critical *m*/*z* values were checked in selected ion monitoring (SIM) mode.

2.7. LC–MS/MS (Q-TOF)

The liquid chromatograph 1200 in tandem with a 6510 quadrupole – time-of-flight (Q-TOF) mass spectrometer and Chip Cube ionisation source (Agilent Technologies) was used in nanoflow mode. The separation was performed on a HPLC-Chip (Agilent) containing enriching (Zorbax 80SB-C18, 40 nL, 5 μm) and analytical (Zorbax 80SB-C18, 75 μm × 43 mm, 5 μm) columns. The enriching column was loaded and washed with 0.1% formic acid in water (3 μL/min). The sample components were eluted by a linear gradient with a mobile phase A (0.1% formic acid in water) and B (20% v/v phase A in acetonitrile) from 2 to 100% of phase B for 48 min and held with the final solution over 2 min. The mobile phase flow rate was 0.4 μL/min.

MS/MS-analysis was made by electrospray ionisation (ESI) and positive ion detection. The temperature of the drying gas (nitrogen, flow rate 5 L/min) was 325 °C at a capillary voltage of 2000 V. The scanning range was from 100 to 1000 Da, and auto MS/MS components search mode (registration of precursor and product ions spectra) with Agilent MassHunter workstation software was used.

2.8. LC–MS/MS (QqQ)

This method was used in the product ion scan and selected reaction monitoring (SRM) modes. The liquid micro-column chromatograph Milichrom A-02 (Econova, Russia) with 2.1 mm × 75 mm column (ProntoSIL-120-5-C8 AQ; Bischoff Chromatography, Leonberg, Germany) was connected to SCIEX API 365 triple-quadrupole mass spectrometer (AB Sciex, Foster City, CA, USA) in positive ESI mode. AB Sciex Analyst workstation software was used. The following gradient system was used with mobile phase A (0.1% v/v formic acid in water) and B (methanol) delivered at 0.1 mL/min: from 2% to 100% of solvent B within 30 min and then solvent B for 2 min.

3. Results and discussion

3.1. Smoking mixtures composition

The Russian market for smoking mixtures is distinguished by the diversity of available products. Originally, smoking mixtures were imported to Russia under known trademarks, and their composition corresponded to the published data [6–9]. The relative simplicity of the mixture-making procedure as well as the quality and variety of the ingredients resulted in a large number of home-made products. These products were then sold under Russian trademarks (or not marked) via the internet or anonymous distribution networks. The publication of synthetic formulas in scientific journals facilitated the synthesis of JWH-018 at home or in a small laboratory. The prohibition of JWH-018 in Russia (effective as of January 22, 2010) has led to sales of sets of legal components, allowing home-made JWH-018 synthesis. Therefore, the composition of the smoking mixture eventually became dependent on a particular maker's (seller's) capacities, personal features and mixture history (Table 1).

The components extracted from smoking mixtures can be divided into five groups.

(1) Psychoactive components added to a mixture according to the adopted technology or while making or using the product (homologous aminoalkylindoles JWH-018, JWH-073 and cyclohexylphenol CP-47, 497 C8 (2-[(1R,3S)-3-hydroxycyclohexyl]-5-(-2-methyloctan-2-yl)phenol, $C_{22}H_{36}O_2$) [6]).
(2) Ingredients improving or modifying the quality of a product. Tocopherol is present in nearly all of the considered mixtures and is probably added as an antioxidant. Tocopherol is the main ingredient observed in "Genie Blend", "Mojo", "Spice Arctic Synergy", "Spice Diamond", "Spice Tropical Synergy", "Yucatan Fire" and Unnamed Mixtures 8, 9 and 13 (based on the GC method). Oleamide, which apparently modifies the psychoactivity of aminoalkylindoles, is rare. "Smoke" (except oleamide with a prevailing concentration) also contains considerable amounts of saturated and unsaturated amides C_{14}–C_{20}.

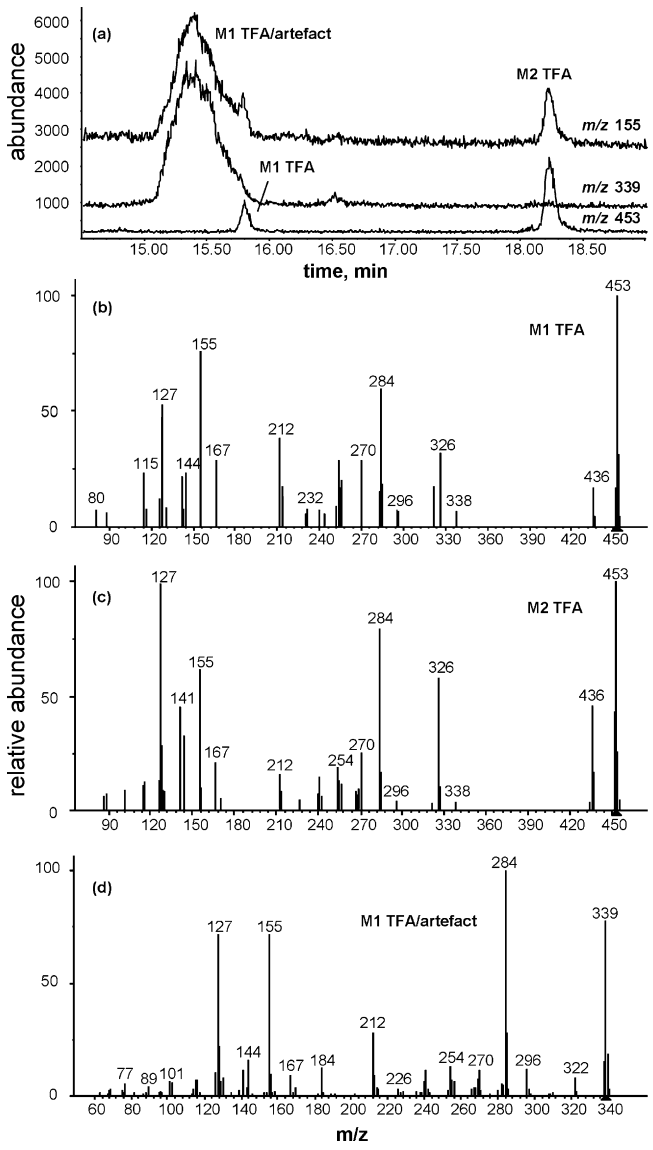

Fig. 5. Pyrolysis of M1 TFA in chromatography column.

(3) Aromatic additives (eugenol, menthol and vanillin derivatives).
(4) Numerous substances typical of an herbal basis for a mixture, including nicotine.
(5) Possible synthesis of half- or by-products of active components and impurities (indole, 1-pentyl-1*H*-indole, pentyl 1-naphthoate and 1-naphthamide). Atypical impurities were found in "Chernobyl" and "Spice Arctic Synergy" and identified as substances containing chlorine atoms in the JWH-018 naphthalene residue. In total, two similar components are detected, and it is impossible to state whether one of them is identical to the known chlorinated aminoalkylindole of JWH-398 ((4-chloronaphthalen-1-yl)(1-pentyl-1*H*-indol-3-yl)methanone, $C_{24}H_{22}ClNO$) [3].

The original mixture composition can be distorted by the chemical degradation of its components. Our observations show that for an allegedly home-made mixture (Unnamed Mixture 3, without tocopherol) that had been stored about one month at room temperature, JWH-018 almost completely disappeared. Nevertheless, monitoring of Unnamed Mixture 4 (an alcoholic extract) that was stored in a room for stability assessment did not result in any decrease in concentration of aminoalkylindoles within at least a month.

3.2. GC–MS: urine samples from rats and humans

JWH-018 and JWH-073 metabolites were searched and identified mainly by GC–MS. For this purpose, all of the samples described in the experimental section were used. The spectra specified in this subsection were obtained by electron ionisation. No investigated urine sample showed native JWH-018 and JWH-073. These results are consistent with earlier observations [16] but contradict another study [15]. Such disagreement may have two reasons: (1) the low solubility of JWH-018 (according to our evaluation) in water and (2) the application of more sensitive analytical method (LC–MS/MS) by the authors [15]. Due to its structural features, JWH-018 cannot be conjugated by urea acids (as a native compound) and is therefore excreted as a water-soluble conjugate. Application of more sensitive methods of analysis could make it possible to detect trace concentrations of JWH-018 in human urine.

The search and identification of metabolites was based on general assumptions about the probable metabolic pathways of similar compounds, which suggested oxidation and N-dealkylation of the parent structure, and on conclusions from previous investigations [14–16]. Fourteen JWH-018 metabolites were found in human and rat urine samples. Their proposed structures are shown in Fig. 1. The linear retention indices and the occurrence of the studied compounds in the human and rat urine samples are given in Table 2.

The physiological activity of new compounds is generally first studied in laboratory animals. For this study, we can conclude that the general metabolic pathway of rats and humans differs. Only N-dealkylated (and N-dealkylated with monohydroxylation) metabolites were found in rat urine. The human urine samples showed a prevalence of primary and secondary hydroxylation products, and the content of N-dealkylated forms was insignificant upon routine urine analysis by GC–MS.

According to our estimates, over 90% of the hydroxylated metabolites are present in urine as conjugated forms, and enzymatic or acid decomposition is therefore required. Sample preparation by SPE and LLE isolated nearly identical amounts of the matrix compounds in extracts that were associated with rather low selectivity by reversed-phase SPE. Therefore, a reduction-of-measurements cost was applied to sample preparation by LLE.

The choice of a derivatisation method is a major issue in sample preparation. The best method of the three applied (TMS, AC, TFA), in terms of sensitivity and number of observable metabolites, was TMS. Acetylation is also applicable to routine analyses, though the sensitivity is slightly reduced. Trifluoroacetylation cannot be recommended because some derivatives have low thermal stability.

All of the detected metabolites may be structurally divided into four groups: monohydroxylation, dihydroxylation, carboxylation and N-dealkylation. The identification of each group's compounds will be described in the following subsection.

3.2.1. Monohydroxylation (M1–M4)

Ion chromatograms of the JWH-018 metabolites in human urine are shown in Fig. 2. The main JWH-018 (and JWH-073) metabolite observed under our conditions is the monohydroxylated form of M1, and the hydroxyl group is supposed to substitute for one of protons on the N-pentyl chain. This assumption corresponds to the observations suggesting that the prevailing oxidation occurs at the aliphatic part and not the aromatic part of the parent compounds. The mass spectrum of M1 is given in Fig. 3. The parent compound spectrum is provided for comparison. The spectra of both compounds possess typical ions. Both for the parent compound and for M1, the naphthalene residue can be identified by *m*/*z* 127 and

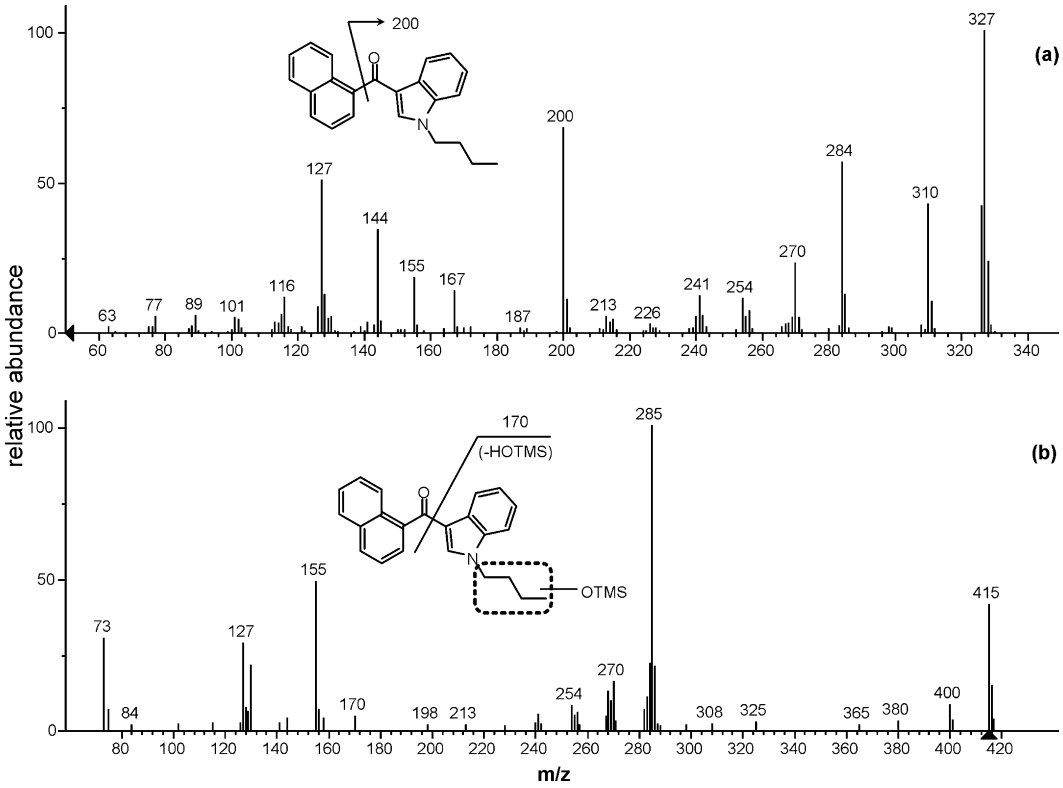

Fig. 6. Mass spectra of JWH-073 and its monohydroxylated metabolite (TMS).

155. The relevant ions for the indole residue of JWH-018 are 186 and 214, while similar ions with *m*/*z* 202 and 230 for M1 suggest the presence of hydroxyl groups. Ions with *m*/*z* 116 and 144 correspond to dealkylated indole fragments. The proposed location of a hydroxyl group on the N-pentyl chain (instead of on the indole bicycle) is based on the presence of ions with *m*/*z* 144, 116 and 284 ([$M1^{+\bullet}$ $-H_2O$, $-C_4H_7$]) and 296 ([$M1^{+\bullet}$ $-H_2O$, $-C_3H_7$]) in the M1 spectrum. Ions with *m*/*z* 212 indicate dehydration accompanied by double bond formation. Additional arguments in support of the hydroxyl location will be described below.

The mass spectra of the TMS and AC derivatives of M1 are given in Fig. 4. These spectra are similar to the underivatised M1 spectrum. For both derivatives, we note the presence of ions with *m*/*z* 184 and 212 formed through the elimination of trimethylsilanol (or acetic acid) with double bond formation in the N-pentyl chain. *m*/*z* 296 has the same parentage as underivatised M1.

The pyrolysis of the TFA derivative of M1 (and of some other metabolites) in a chromatographic column can serve as additional proof of the proposed hydroxyl localisation (Fig. 5). The formed chromatographic artefact corresponds to an N-pentenyl analogue of JWH-018.

The main metabolite of JWH-073 (observed under our conditions) is apparently a product of side chain monohydroxylation. Because JWH-073 is usually present in smoking mixtures in small concentrations (compared to JWH-018), detecting its minor metabolites is difficult. The parent compound and its metabolite spectra (as a TMS derivative) are given in Fig. 6.

Metabolite M2 (Fig. 7a) is the second mixture component in terms of the observable content. The mass spectra of its TMS and AC derivatives are very similar to the corresponding M1 spectra, and it may be identified as an isomer due to the location of a hydroxyl group in the N-pentyl chain.

The spectrum of the TMS derivative of the M3 metabolite (Fig. 7b) contains ions with *m*/*z* 127 and 155 (which suggests a lack of a hydroxyl group in the naphthalene residue), and it apparently does not contain *m*/*z* 116 and 144 (corresponding to a unchanged indole residue). Nevertheless, the intensive *m*/*z* 270 and 284 suggest that this compound is also an M1 isomer with regards to the location of a hydroxyl group in the N-pentyl chain.

The hydroxyl group of M4 (Fig. 7c) is located in the naphthalene fragment because the spectrum of its TMS derivative contains ions with *m*/*z* 116, 144 and 214 (unchanged indole residue). The formation of *m*/*z* 358 and 372 is caused by the fragmentation or elimination of the N-pentyl chain.

3.2.2. Carboxylation (M5)

The TMS derivative of a carboxylated metabolite M5 can be identified by the *m*/*z* value for the molecular ion (443) and the presence of *m*/*z* 127, 144 and 155. A compound appearing in a mixture of metabolites after etherification with methanol in an acidic medium would support the given structure. The identified compound was a methylate of a carboxylated metabolite M5 (Fig. 8). No M5 was revealed in a mixture of TMS derivatives by additional derivatisation (TMS) after etherification.

3.2.3. Dihydroxylation (M6–M9)

Four strongly retained compounds (M6–M9) contain *m*/*z* 517 (proposed molecular ion) in their spectra and can be referred to as dihydroxylated metabolites of JWH-018 (Fig. 9). The interpretation of their structures is complicated due to low concentration and poor separation. The lack of ions with *m*/*z* 127 and 155 and the low intensity of *m*/*z* 243 indicate localisation of a hydroxyl group on a naphthalene residue that is valid for M7–M9. The ion with *m*/*z* 184 in the spectra of these compounds is formed by the elimination of trimethylsilanol with multiple bond formation that allows interpreting M7–M9 as the products of hydroxylation of the naphthalene residue and N-pentyl chain. Metabolites M7 and M8 can be partially separated if the column temperature gradient is reduced. The spectra of these compounds are generally similar. The intensive

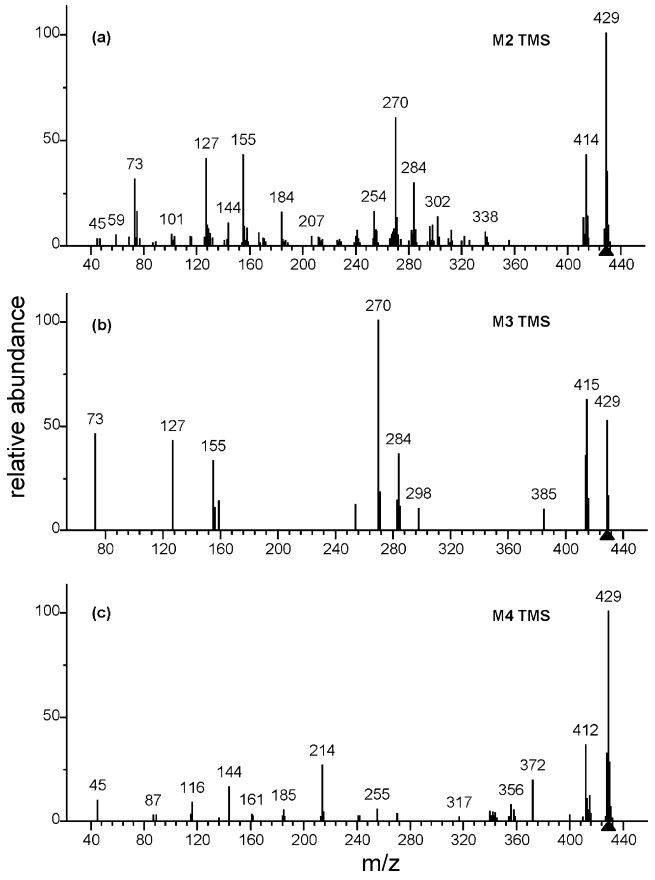

Fig. 7. Mass spectra of minor monohydroxylated JWH-018 metabolites (TMS).

m/*z* 127 and 155 is typical for M6. In this compound, the hydroxyl groups are likely to localise at the indole bicycle and N-pentyl chain. For all three compounds, an ion with *m*/*z* 358 results from the full elimination of a hydroxylated alkyl chain, and the group of ions with *m*/*z* 372–426 may be explained by multiple bond formation upon elimination of trimethylsilanol and the stepwise fragmentation of a chain.

3.2.4. Dealkylation (M10–M14)

The dealkylated metabolites of JWH-018 were originally identified in rat urine. These compounds were detectable due to the relative compositional simplicity of these samples and the smaller matrix influences. The entire group of dealkylated metabolites was identified only as AC derivatives because of difficulties with silylation, which is related to the specific behaviour of the indole nitrogen in these compounds. The simplest member of this group (M10) is present at a considerable concentration in rat urine, and it was not detected in human urine.

The monohydroxylated forms of depentyl JWH-018 are hardly separable under our chromatography conditions but can be satisfactory separated if the column temperature gradient is reduced. A comparison of the fragments from the ion chromatograms for the rat and human urine samples is shown in Fig. 10. Metabolites M11–M13 are present in both samples, while M14 is in human urine only. All of the dealkylated metabolites are present in human urine at low concentrations and cannot be detected in the majority of the samples.

Except for the location of the hydroxyl groups in the corresponding residue, decoding the structures of these metabolites is not problematic. According to the spectra in Fig. 11, M11 and M12 have hydroxyl groups located at the naphthalene residue. *m*/*z* 127 and

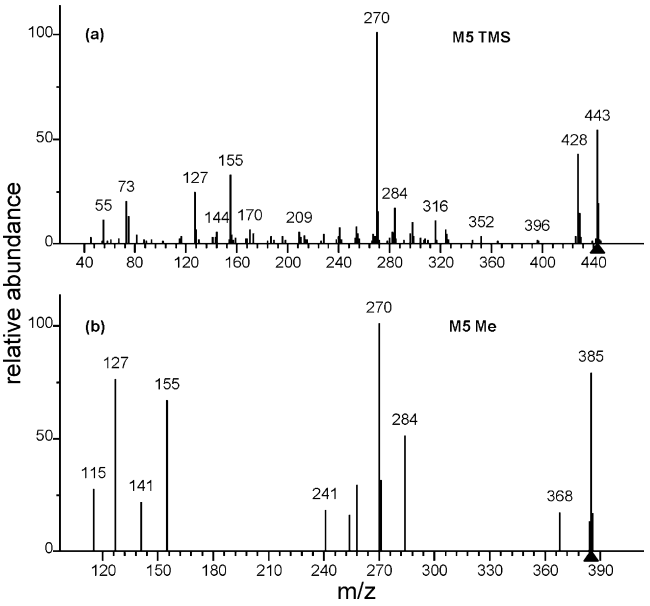

Fig. 8. Mass spectra of TMS and methyl derivatives of the M5 metabolite.

155 are not present, but intensive 270 and 286, which are formed by the elimination of ketene molecules, as well as *m*/*z* 144 occurs. For M13 and M14, an intensive ion with *m*/*z* 160 (naphthalene eliminating) indicates that the hydroxyl is localised at the indole residue.

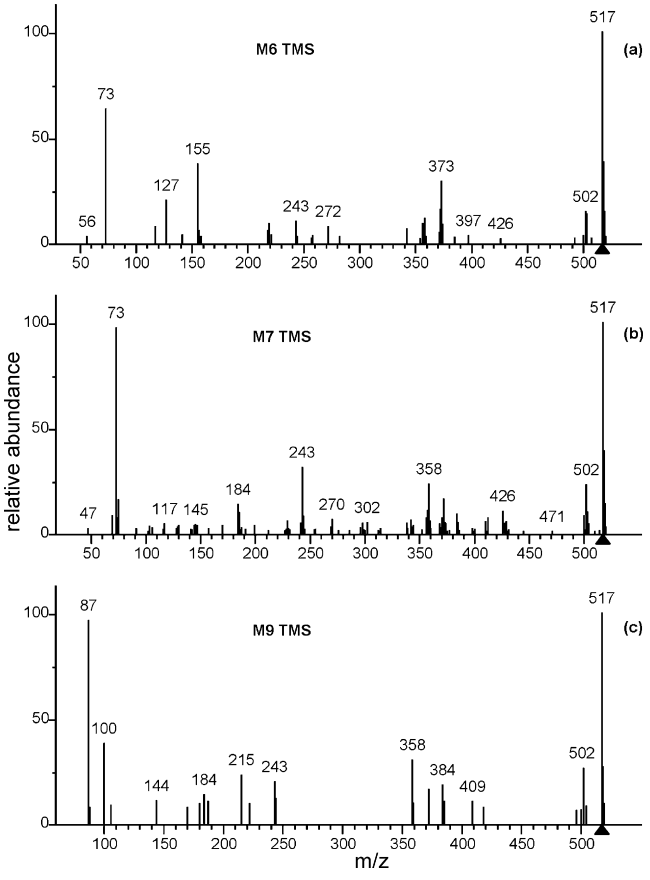

Fig. 9. Mass spectra of dihydroxylated JWH-018 metabolites (TMS), M6 (a), M7 (b) and M9 (c).

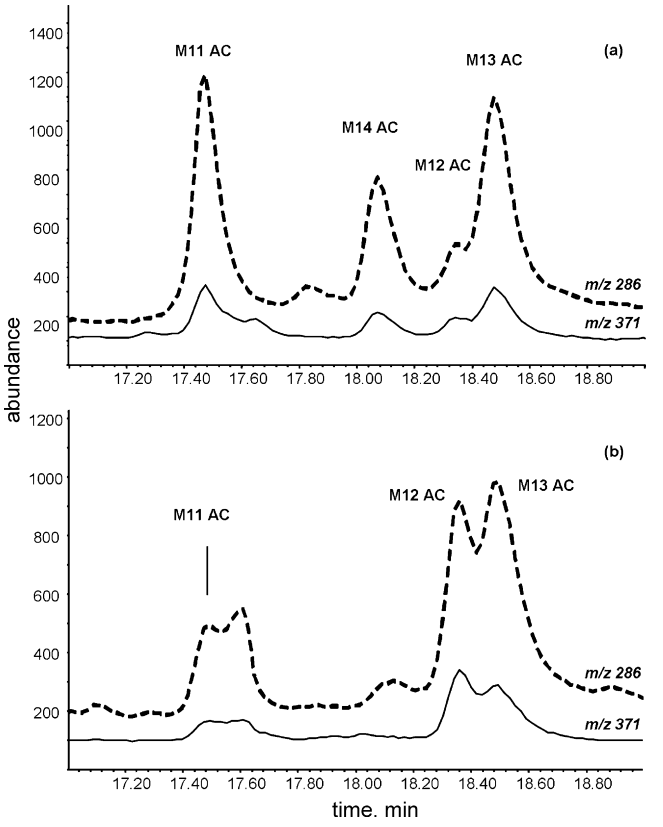

Fig. 10. Comparison of the allocation of metabolites M11–M14 in human (a) and rat (b) urine samples. The last steps of the temperature program: 35 °C/min and 300 °C, SIM mode.

3.3. LC-Q-TOF: rat and human urine

Liquid chromatography with a tandem Q-TOF mass spectrometer was used only to confirm the identification of some of the discovered metabolites and to clarify their structures (Fig. 12). The mass spectra of the JWH-018 product ions (t_R – 37.11 min, Fig. 12f) contains the same characteristic ions as the spectrum of electron ionisation.

The peak with t_R about 26.26 min (Fig. 12a and g) is the major peak on the chromatogram, registers within 358.1000–358.2000 and can be identified as metabolite M1 ($C_{24}H_{23}NO_2$). The difference between the calculated and measured m/z of the precursor ion is 1.60 ppm. The spectrum of the product ions indicates that the hydroxyl group is at the indole residue (230.1152, $C_{14}H_{16}NO_2$), and m/z 284.1061 ($C_{20}H_{14}NO$, the difference is 3.26 ppm) is a direct indication of hydroxylation of the N-alkyl chain. M1 is observed in human urine but is nearly absent in rat urine.

The carboxy metabolite M5 is detected in human urine only. The spectrum of its precursor ion corresponds to the molecular formula $C_{24}H_{21}NO_3$ (difference is −0.73 ppm).

The dihydroxylated metabolites were poorly separated in the applied chromatography conditions, and their detailed identification is therefore hindered. Nevertheless, the m/z of the single precursor ion from the chromatographic band corresponds to the gross formula $C_{24}H_{23}NO_3$ (difference is −4.60 ppm). The spectrum of product ions registered near the intensity maximum of the chromatographic band contain m/z 143.0514 ($C_{10}H_7O$) and 171.0459 ($C_{11}H_7O_2$), corresponding to the location of the hydroxyl group at the naphthalene residue (Fig. 12h). These metabolites are detected in human urine only.

The group of the hydroxylated N-dealkyl metabolites detected in rats and human urine (Fig. 12d, e and i) is also poorly separable in the provided conditions. The m/z of the precursor ion is consistent with the gross formula $C_{19}H_{13}NO_2$ (difference is −7.53 ppm).

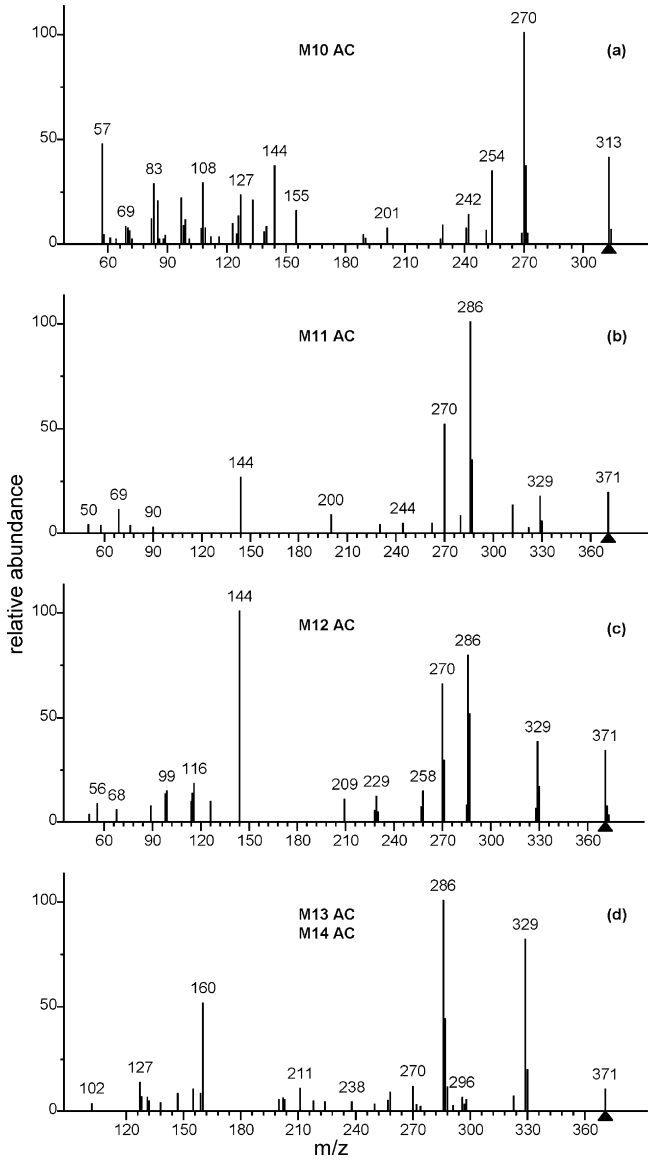

Fig. 11. Mass spectra of dealkylated metabolites (acetylation).

3.4. LC-QqQ: human urine

The high cost and rather low sensitivity of LC systems with tandem TOF mass spectrometers makes them impractical for routine analysis. Therefore, a triple-quadrupole mass spectrometer was mainly used for the LC analyses of the urine samples. The sensitivity of this method is sufficient to properly detect minor metabolites after a considerable time following JWH-018 administration. Thus, two targets of analysis may be acquired during one chromatographic run: establishment and confirmation of JWH-018 administration. The chromatograms of human urine samples are shown in Fig. 13a–c. Because metabolites with an ion at 155 in the spectrum prevail in the mixture and the intensity of this ion in the spectra of product ions is high (Fig. 13d–f), transitions [M+H] → 155 were used for SRM mode.

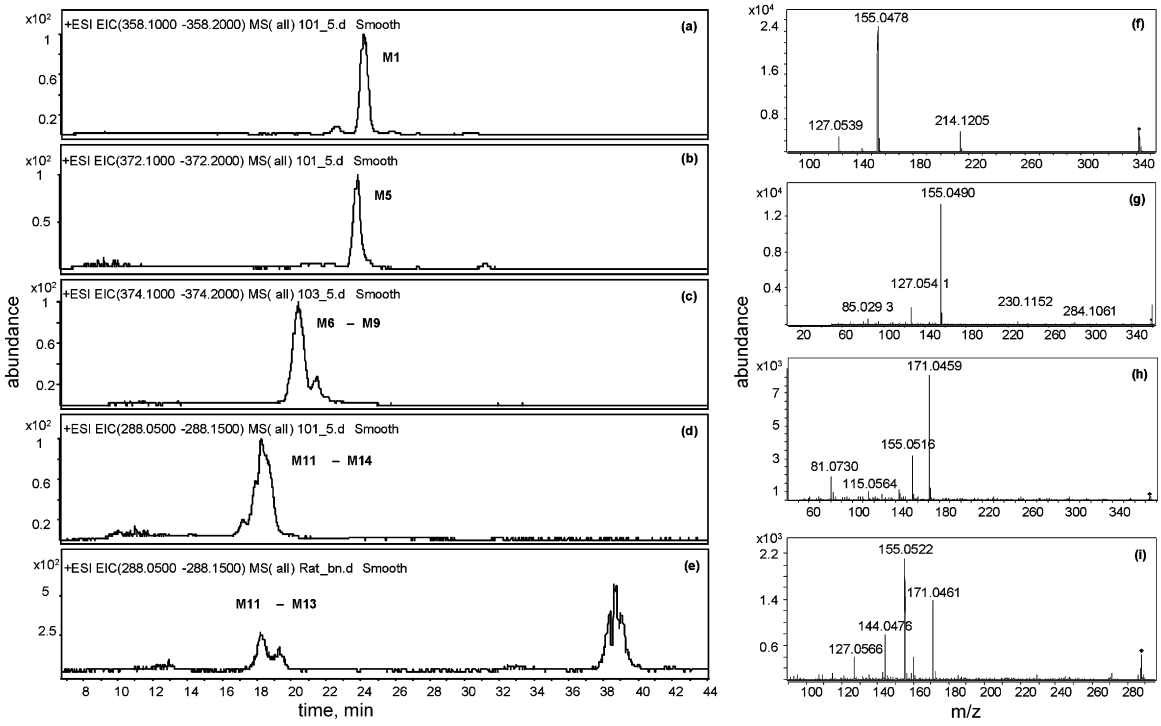

Fig. 12. Mass chromatograms of human (a–d) and rat (e) urine samples. Mass spectra of product ions of JWH-018 (f) and M1 (g). Spectra of M6–M9 (h) and M11–M14 (i) registered according to the intensity maxima of the chromatographic band of the chromatogram.

3.5. Comparison of concentrations of JWH-018 metabolites in human urine samples

The relative concentrations of metabolites in urine samples collected from individuals delivered to drug service due to inadequate behaviour may be of interest. The concentrations of three heterogeneous metabolites (M1, M5 and M6) in eleven urine samples, arranged in decreasing order of M1 concentration, are shown in Fig. 14. The analysis was carried out by GC–MS with a sample preparation, including liquid–liquid extraction and TMS. The molecular

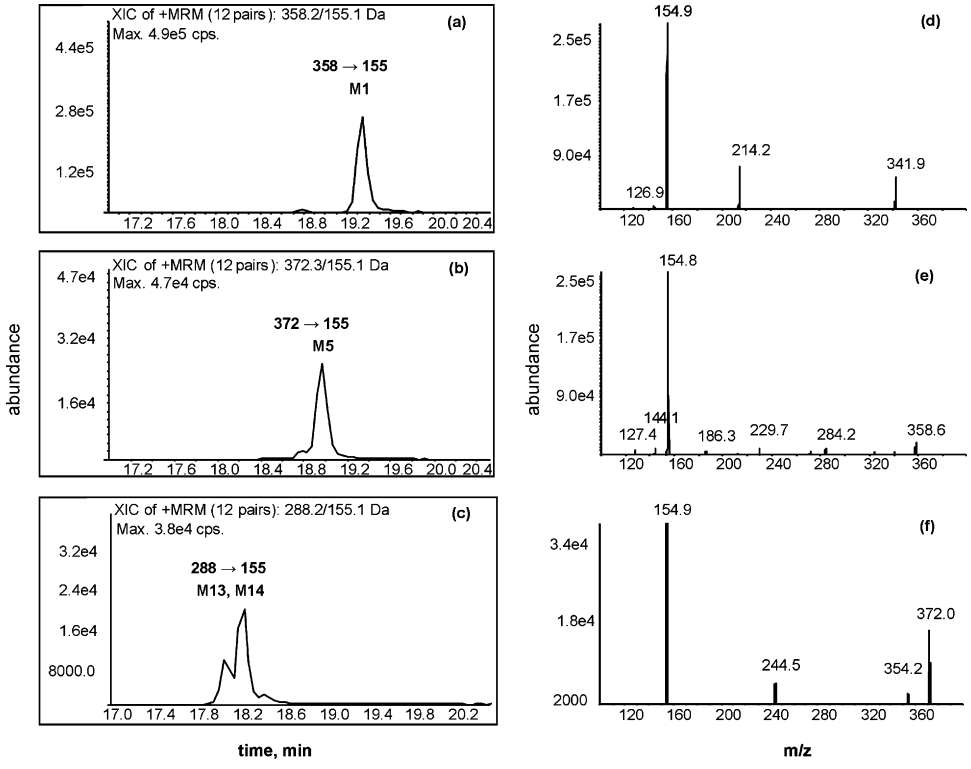

Fig. 13. SRM chromatograms of human urine (a–c). Product ion spectra of JWH-18 (d), M1 (e) and M5 (f).

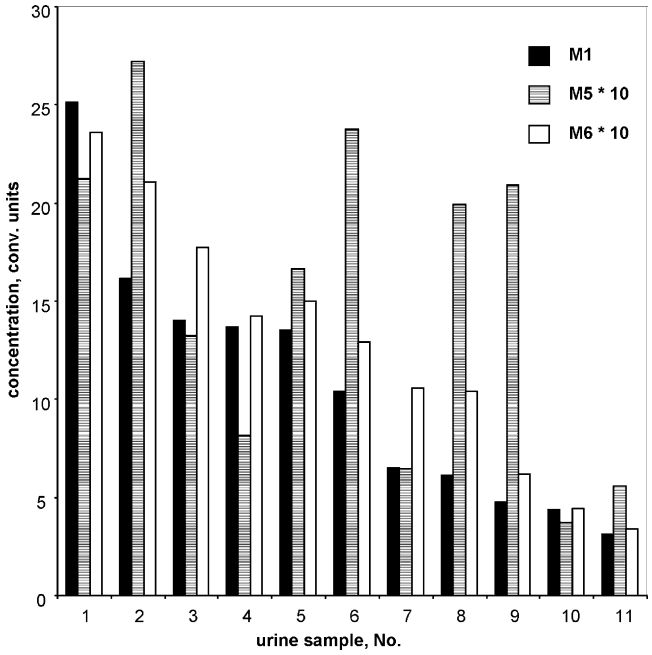

Fig. 14. Concentrations (conventional units) of three metabolites in urine samples collected from persons delivered to drug services. The M5 and M6 concentrations were multiplied by a factor of 10.

ions of TMS derivatives are intensive for all of the determined compounds, and their peak areas were used as analytical responses. The highest M1 concentration is about eight times higher than the lowest one, which may be due to different time periods between JWH-018 administration and urine collection.

4. Conclusion

Fourteen JWH-018 metabolites (hydroxylated, carboxylated and dealkylated products) were identified in human and rat urine samples. One monohydroxylated metabolite JWH-073 was detected in human urine. For JWH-018, the main observable components in human and rat urine are the monohydroxylated metabolites and the dealkylated with monohydroxylation metabolites, respectively.

Sample preparation by SPE and LLE resulted in nearly identical amounts of matrix compounds in the extracts. For the detection of minor (dealkylated and dihydroxylated) metabolites in human urine, LC–MS/MS was more convenient.

None of the investigated samples showed native JWH-018 or JWH-073. Therefore, the abusive consumption of synthetic cannabimimetics JWH-018 and JWH-073 can only be established by the detection of their metabolites in urine by GC–MS and LC–MS/MS.

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