Journal of Chromatography B, 879 (2011) 2519–2526

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Gas and liquid chromatography–mass spectrometry studies on the metabolism of the synthetic phenylacetylindole cannabimimetic JWH-250, the psychoactive component of smoking mixtures

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ARTICLE INFO

Article history:
Received 27 February 2011
Accepted 7 July 2011
Available online 18 July 2011

Keywords:
JWH-250
Herbal mixture
Metabolites
GC–MS
LC–MS/MS

ABSTRACT

Prohibition of some synthetic cannabimimetics (e.g., JWH-018, JWH-073 and CP 47497) in a number of countries has led to a rise in new compounds in herbal mixtures that create marijuana-like psychotropic effects when smoked. The cannabimimetic JWH-250 (1-pentyl-3-(2-methoxyphenylacetyl)indole) was identified in May 2009 by the German Federal Criminal Police as an new ingredient in herbal smoking mixtures. The absence or low presence of the native compound in urine samples collected from persons who had consumed JWH-250 necessitates a detailed identification of their metabolites, which are excreted with urine and present in blood. Using gas and liquid chromatography–mass spectrometry (GC–MS and LC–MS/MS), we identified a series of metabolites in urine samples and serum sample from humans and urine samples from rats that were products of the following reactions: (a) mono- and dihydroxylation of aromatic and aliphatic residues of the parent compound, (b) trihydroxylation and dehydration of the N-alkyl chain, (c) N-dealkylation and (d) N-dealkylation and monohydroxylation. The prevailing urinary metabolites in humans were the monohydroxylated forms, while N-dealkylated and N-dealkyl monohydroxylated forms were found in rats. The detection of the mono- and dihydroxylated metabolites of JWH-250 in urine and serum samples by GC–MS and LC–MS/MS proved to be effective in determining consumption of this drug.

1. Introduction

According to tradition, herbal mixtures that create marijuana-like psychotropic effects when smoked are called "spices". Despite the fact that Internet sales of these mixtures have been tracked since 2004 [1], their composition has not attracted attention. However, the increased sales of such plant mixtures in 2008 led to the detection of the synthetic ingredient JWH-018, the agonist of the mammalian cannabinoid receptors CB_1 and CB_2 [2]. JWH-018 and many other compounds possessing similar psychoactive properties were synthesised during studies on selective CB_1- and CB_2-receptor agonists [3,4]. Since 2009, many European countries took legal action to control JWH-018 and some of its analogues [2].

Owing to the significant number of prepared and characterised cannabimimetics and the simplicity of their syntheses, their prohibition led to a shift in manufacturing of the smoking mixtures to new synthetic cannabimimetics [5]. Unlike the market for commonly abused drugs, the cannabimimetics market has been characterised by considerable variability.

JWH-250 (1-pentyl-3-(2-methoxyphenylacetyl)indole) was synthesised by Huffman et al. [6] and first identified in May 2009 by the German Federal Criminal Police [2,7] as an ingredient of herbal smoking mixtures. This match was confirmed in a series of publications [8–10]. JWH-250 has been identified and quantified by gas and liquid chromatography–mass spectrometry (GC–MS and LC–MS/MS). GC separation has been achieved on non-polar (DB-1 [7]) and low-polarity (BPX5 [5] or HP-5ms [8,9]) capillary columns. Reversed-phase columns operating in gradient mode with acid eluents have been used for LC separation. Since the columns were coupled to mass spectrometers, eluent pH was adjusted with acetic acid [5] or ammonium formate buffer [9]. Recently, a LC–MS/MS method of quantitation for JWH-250 and a number of other cannabimimetics in serum using a phenyl-hexyl column and gradient elution was reported [11].

The known methods of detection and identification of alkylindole cannabimimetic metabolites are limited to the investigation of naphthoylindole metabolites. *In vitro* metabolism of JWH-015 [12] and JWH-018 [13] has been explored by LC–MS. Identified analytes

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doi:10.1016/j.jchromb.2011.07.004

were separated on reversed phase columns with gradient elution. The wide range of metabolites detected included products of mono- and polyhydroxylation, carboxylation, N-dealkylation, dehydration of the N-alkyl chain and arene oxidation of the naphthalene moiety of the parent compounds. Nevertheless, the information obtained was too general, and the set of urinary or serum metabolites was not specified.

Urinary metabolites by GC–MS and LC–MS/MS in persons consuming JWH-018 have been identified in several works [14–18]. Generally, the gas and liquid separation conditions were similar to those described above. The acetylation [14,16,17] or trimethylsilylation [15–17] of the samples was required for GC separation. Recently, the identification of JWH-073 urinary metabolite and JWH-018 metabolites in serum was described [16,17]. The authors noted that considerable proportions of the metabolites consisted of mono- and polyhydroxylated compounds. Additionally, carboxylated metabolites and products of arene oxidation of the naphthalene moiety were also detected. The presence of N-dealkylated and hydroxylated N-dealkylated metabolites was also reported, although it was noted that these compounds were insignificant in human urine [16] and serum [17]. Additionally, N-dealkylation was more characteristic for rats. A major finding of these studies [14–18] was the absence or trace amounts of the native compounds contained in the body fluids of consumers of aminoalkylindoles, which is evidence of the nearly complete metabolism of these compounds and demands focus on their metabolic products.

According to recent investigations [9–11] and information from Internet drug forums, JWH-018 and other cannabimimetics were substituted for JWH-250 after their prohibition. The variability in the market of synthetic cannabimimetics demanded a fast evolution of the methods for drug-intoxication diagnostics and, consequently, the identification of the metabolites of new cannabimimetics. The aim of this study was to identify JWH-250 metabolites as markers of the consumption of this drug using GC–MS and LC–MS/MS. One serum and eleven urine samples from humans, as well as four rat urine samples, from subjects who consumed JWH-250 were analysed.

2. Materials and methods

2.1. Reagents

HPLC-grade acetonitrile and methanol were purchased from Panreac Quimica S.A. N,O-Bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (BSTFA + 1% TMCS) was purchased from Acros Organics (Geel, Belgium), and pyridine (analytical grade) was supplied by Scharlau Chemie S.A. (Barcelona, Spain). The other solvents and chemicals for analysis were obtained from Ecos-1 (Moscow, Russia).

Three samples of substances containing JWH-250 (more that 90% of the target component) were purchased from anonymous distributors via the Internet. The authenticity and content of the JWH-250 components were estimated by thin layer chromatography, high performance liquid chromatography with diode-matrix detection and GC–MS [7,9]. AccuBond ODS-C18 (3 mL, 200 mg) solid-phase extraction (SPE) cartridges were supplied by Agilent Technologies (Santa Clara, CA, USA).

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

2.2. Urine and blood samples

Eleven urine samples were collected from persons with symptoms of drug intoxication who were brought to the hospital by police or ambulance orderlies. One blood sample was also collected from a member of this group. These persons confessed to recently having smoked herbal mixtures. No common drugs were detected by immunochromatographic assays, including cannabinoids, benzodiazepines, morphine, cocaine, amphetamines or their metabolites.

2.3. Drug administration (rats)

One of the purchased JWH-250 samples was used to obtain positive urine samples from rats. After a day of fasting, the JWH-250 suspension in a 1% starch solution was introduced into four Wistar rats by gastric intubation at a rate of 100 mg of JWH-250 per kg of body mass. Three hours after suspension introduction, the animals were placed in metabolic pans for 20 h with free access to water, and their urine was collected. An additional rat was given a placebo (1% starch) to obtain a blank urine sample.

2.4. Preparation of urine samples and serum sample

Liquid–liquid extraction (LLE) and SPE were used to prepare the urine samples. For LLE, hydrochloric acid (0.3 mL, 30%) was added to urine (2.5 mL) and heated at 90–95 °C for 60 min. After cooling, aqueous ammonia (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 under a stream of nitrogen at 45 °C or lower. The dry residue was dissolved either in 50 μL of ethanol (for GC) or in the same volume of acetonitrile in water (10% v/v, for LC) or derivatised.

For SPE, the sample (3 mL of hydrolysed urine at pH 8–9 plus 3 mL of water and 0.6 mL of acetonitrile) was loaded onto a cartridge. The sorbent was rinsed with acetonitrile in water (20% v/v, 3 mL) and dried under a stream of air for 30 s. The analytes were eluted with 3 mL of acetone.

Blood was centrifuged for 5 min at 3000 rpm. Next, 3 mL of water and 0.4 mL of hydrochloric acid were added to 1 mL of serum and heated at 90–95 °C for 60 min. The remaining steps were similar to those for the urine samples. Finally, the dry residue was trimethylsilylated for GC analysis.

Urine samples were derivatised for GC by either 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 30 min. For acetylation, the dry residue was dissolved in a mixture of acetic anhydride and pyridine (50 μL each) and heated at 70 °C for 30 min. The mixture was then evaporated in a vacuum concentrator (Concentrator 5301, Eppendorf, AG, Hamburg, Germany) and dissolved in 50 μL of ethanol. TFA derivatives were prepared in a mixture of trifluoroacetic anhydride and ethyl acetate (50 μL each) at 50 °C for 30 min. After evaporation, the dry residue was dissolved in 50 μL of ethyl acetate.

2.5. GC–MS

Two model 6890 GCs connected to model 5973 single quadrupole mass spectrometers from Agilent Technologies was used with the following columns: a HP-5ms (30 m × 0.25 mm × 0.25 μm) for separation of underivatised, TFA and AC samples; and a DB-17ms (15 m × 0.25 mm × 0.25 μm) for the same samples with considerable matrix influences. Additionally, another 6890 GC/5975VL MS (Agilent Technologies) was used with an EVDX-5ms column (25 m × 0.20 mm × 0.33 μm) for trimethylsilylated samples.

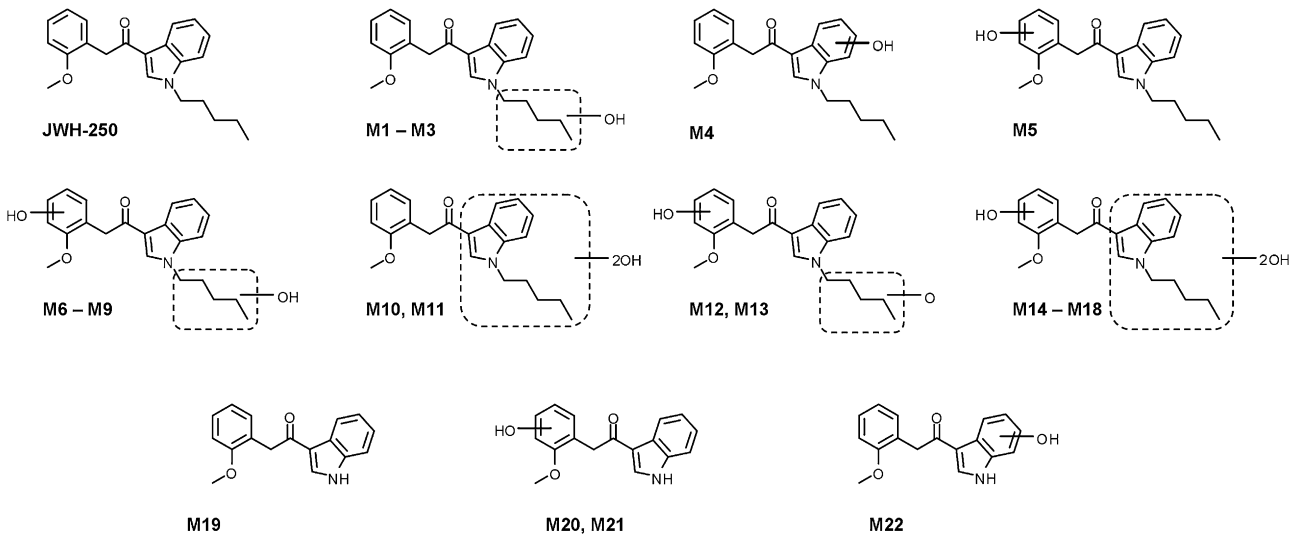

Fig. 1. Proposed structures of the identified JWH-250 urinary metabolites.

Separation was performed under the following temperature program on the low-polarity columns (HP-5ms and EVDX-5ms) with helium as a carrier gas at 1 and 0.8 mL/min, respectively: 50 °C held for 0.5 min, increased at 99°/min to 100 °C, and then held for 1 min; next, the temperature was increased at 35°/min to 300 °C and held for 15 min. The same temperature program was used on the mid-polarity column (DB-17ms) at a helium flow of 1 mL/min, except for the last gradient step, which was 20°/min.

The samples described in Section 2.4 (1 μL) were injected in splitless mode at 270 °C. The temperatures of the transfer line, ion source (electron ionisation mode, EI, 70 eV) and mass filter were 290, 230 and 150 °C, respectively. The mass spectra of identified compounds were isolated from the background by the AMDIS (Automatic Mass Spectral Deconvolution and Identification System) program (NIST, USA) in manual and auto modes. Variations in the intensity of the most critical *m*/*z* values were verified in selective ion monitoring (SIM) mode.

2.6. LC–MS/MS (QqQ)

This method was used in the product ion scan and multiple reaction monitoring (MRM) modes. The liquid microcolumn chromatograph (Milichrom A-02, Econova, Russia) with a ProntoSIL-120-5-C8 AQ column (2.1 mm × 75 mm, Bischoff Chromatography, Leonberg, Germany) was connected to a SCIEX API 365 triple-quadrupole mass spectrometer (AB Sciex, Foster City, CA, USA) in positive electrospray ionisation mode (ESI). AB Sciex Analyst workstation software was used with this system, and the injected sample volume was 10 μL. The following linear gradient was used with mobile phases A (0.1% v/v formic acid in water) and B (methanol) delivered at 0.1 mL/min: from 2% to 100% of B over 30 min, with a 2-min hold time at the end.

3. Results and discussion

3.1. GC–MS of rat and human urine samples

JWH-250 metabolites were primarily analysed and identified by GC–MS. Twenty-two metabolites were identified after examining all of the samples described in the experimental section. Their proposed structures are given in Fig. 1. All detected compounds could be structurally divided into six groups. There were products of monohydroxylation (M1–M5), dihydroxylation (M6–M11), trihydroxylation and dehydration of the N-alkyl chain (M12, M13), trihydroxylation (M14–M18), N-dealkylation (M19) and N-dealkylation with monohydroxylation (M20–M22). The linear retention indices (*n*-alkanoic scale) and occurrence of the compounds in human and rat urine are given in Table 1.

The analysis and identification of metabolites was based on general assumptions concerning the probable metabolic pathway of similar compounds and conclusions made in other investigations on the metabolism of naphthoylindolic compounds (JWH-015, JWH-073 and JWH-018) [12–18]. The metabolic transformations of the phenylacetylindole JWH-250 structure were similar to the metabolism of the specified naphthoylindoles. The consecutive oxidation of parent compound (metabolites M1–M18) without destruction of the aromatic skeleton or formation of low molecular weight products was the primary pathway of detoxification. Metabolic elimination of the parent compound consisted of only N-dealkylation (metabolites M19–M22) and was accompanied by hydroxylation. None of the urine or serum samples contained O-demethylated metabolites.

The general metabolic pathways for JWH-250 and JWH-018 differed in rats and humans. If oxidation products, especially monohydroxylation products, were more prevalent in humans than in rats, dealkylation with subsequent hydroxylation was more characteristic in rats. However, small amounts of dihydroxylated metabolites were also detected. None of the urine samples contained the native JWH-250 structure, which was consistent with similar studies on JWH-018 and JWH-073 [15–17].

Both JWH-250 and its metabolic oxidation products (as derivatives) were characterised by lower retention indices compared to similar products for JWH-018 and JWH-073. These lower retention times could be attributed to the fact that JWH-250 metabolites are more easily detected by GC–MS. Many urine samples collected from hospitalised persons possessed high enough concentrations of the JWH-250 metabolites for reliable detection, even without derivatisation. Nevertheless, the applied analytical procedure included derivatisation to increase sensitivity. TMS yielded the highest sensitivity and lowest retention out of the three applied derivatisation techniques (i.e., TMS, AC, TFA). Acetylation was also applicable to routine analyses, though the sensitivity was slightly reduced. Trifluoroacetylation is not recommended because some TFA derivatives of JWH-250 metabolites had low thermal stability. According to our estimates, over 90% of the hydroxylated metabolites were present in urine as conjugated forms, and enzymatic or acidic decompo-

Table 1
Linear retention indices (*n*-alkanoic scale) and occurrence of detected JWH-250 metabolites and their derivatives.

Compound	$[M]^{+\bullet}$	Derivatisation (column)				Detection	
		No (HP-5ms)	AC (HP-5ms)	AC (DB-17ms)	TMS (EVDX-5ms)	Human	Rat
JWH-250	335	3044	–	–	–	–	–
M1	351	3256	3323	4036	3225	+	–
M2	351	3352	3432	4195	3312	+	–
M3	351	3230	3288	3968	3203	+	–
M4	351	3329	3357	4056	3249	+	–
M5	351	3320	3345	4058	3179	+	–
M6	367	–	3505	4221	3333	–	+
M7	367	–	3580	–	3350	+	–
M8	367	3545	3534	4285	3357	+	+
M9	367	3400	3608	4398	3372	+	+
M10	367	–	–	–	3417	+	–
M11	367	–	–	–	3438	+	–
M12	365	–	3444	–	3339	+	–
M13	365	3497	3516	–	3354	+	–
M14	383	–	–	–	3455	+	+
M15	383	–	–	–	3483	+	+
M16	383	–	–	–	3499	+	+
M17	383	–	–	–	3471	+	–
M18	383	–	–	–	3536	+	+
M19	265	–	2893	3539	–	–	+
M20	281	–	3095	3793	–	–	+
M21	281	–	3178	3919	–	+	+
M22	281	–	3233	3965	–	+	+

sition was required. Sample preparation by SPE and LLE isolated nearly identical amounts of the matrix compounds in the extracts. Therefore, a reduction-of-measurements cost was applied to sample preparation by LLE.

The EI mass spectra of all the studied compounds possessed a small number of features. The primary breaking of the $CO–CH_2$ bond with charge retention at the pentylindole fragment was the general molecular ion fragmentation pathway. This led to the appearance of an ion corresponding to the pentylindole residue and its fragments.

For JWH-250 (Fig. 2a), such breakages led to formation of an ion with at *m*/*z* 214, which is a marker of an unaltered pentylindole moiety together with products of the consecutive elimination of the N-alkyl chain and carbonyl group (*m*/*z* 144 and 116, respectively). The ion at *m*/*z* 121, presumably corresponding to a methoxybenzyl moiety, had a low intensity. The molecular ions and ions corresponding to their methoxybenzyl moieties were also of low intensity for all of the other compounds considered and could only be detected at high concentrations. These spectral features resulted in difficult metabolite identification.

The identification features of each group's detected metabolites will be considered in the following subsections. Extracted ion chromatograms (EICs) of urine samples from humans who smoked JWH-250 are shown in Fig. 3a. The choice of *m*/*z* values corresponded to the most intense ions in the spectra of these compounds (TMS derivatives).

3.1.1. Monohydroxylation (M1–M5), $[M^+]_{TMS} = 423$

The following compounds were found in human urine only. Three of the five monohydroxylated metabolites (M1–M3) had a hydroxyl group in the N-alkyl chain. M1 was the main component detected under our conditions. Because all of the spectra were similar, we will only consider the features of M1 fragmentation (Fig. A.1). For underivatised M1–M3, the hydroxyl location was clearly identified from *m*/*z* 230, corresponding to the increase of 16 from the unchanged pentylindole moiety, and *m*/*z* 144 and 116, representing the unaltered indole bicycle. This line of evidence was also applicable to the TMS and AC derivatives (Figs. 2b and A.2, respectively) taking into account addition of the trimethylsilyl and acetyl group masses.

For all of these spectra, the fragmented ion with *m*/*z* 212 was formed by either dehydration of the hydroxylated alkyl side chain or trimethylsilanol and acetic acid elimination for derivatives. A similar reaction for TFA derivatives occurred in the GC at increased temperatures and resulted in the formation of dehydrogenised JWH-250. This process is typical for one of the monohydroxylated metabolites of JWH-018 (TFA derivative) [16]. Metabolite M1 (as a derivative) was the most convenient compound for establishing consumption of JWH-250 by GC–MS, though its relative concentration decreased during the time between consumption and urine collection.

The two remaining metabolites were hydroxylated in the aromatic portions of the pentylindole (M4) and methoxybenzyl (M5) residues. Additional evidence for this hydroxylation location was found in the molecular ions, *m*/*z* 230 and 160, corresponding to an oxygen addition to *m*/*z* 144 and *m*/*z* 214 and 144 in the spectra of underivatised M4 and M5, respectively.

3.1.2. Dihydroxylation (M6–M11), $[M^+]_{TMS} = 511$

Dihydroxylation of JWH-250 was more typical in human metabolism, although small amounts of these compounds (M6, M8 and M9) were detected in rat urine.

Four metabolites (M6–M9), shown in Figs. 3a and 4a and b, possessed similar spectra. Since the spectra of these compounds were similar to the spectrum of M1 taking into account the *m*/*z* of the molecular ion, their hydroxyl groups should be localised in the N-pentyl chain and methoxybenzyl moiety (Fig. 2c). Metabolite M6 was only detected in rat urine, and M8 and M9 were the main dihydroxylated components in humans. M7 was only detected in human urine. This can be explained by the low concentration of this compound in rat urine, taking into account small concentrations of dihydroxylated metabolites.

Two metabolites (M10 and M11) were retained the most out of this group of compounds (Fig. 3a). Their spectra were characterised by intense ions at *m*/*z* 390 (Fig. A.3), which directly pointed to dihydroxylation of the pentylindole moiety. Further elucidation of the structures of these metabolites was complicated because of their low concentration. Nevertheless, the absence of *m*/*z* 144 and 116 in their spectra suggested localisation of at least one hydroxyl group in the indole bicycle.

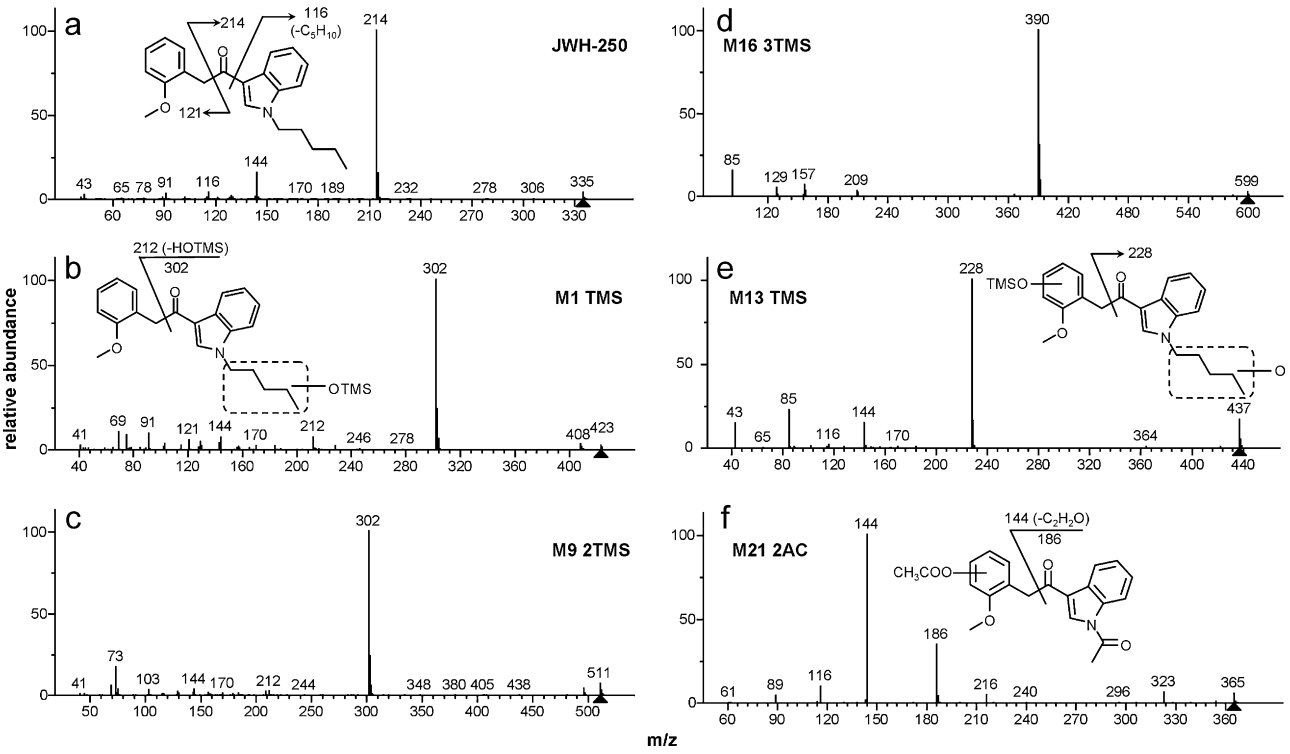

Fig. 2. Mass spectra of JWH-250 (a), silylated metabolites M1 (b), M9 (c), M16 (d), M13 (e) and acetylated M21 (f).

3.1.3. Trihydroxylation combined with dehydration of the N-alkyl chain (M12, M13), $[M^+]_{TMS} = 437$

Two metabolites with a hydroxyl group in the methoxybenzyl moiety and a carbonyl group in the N-alkyl chain were only detected in human urine. These compounds formed monoacetyl and monotrimethylsilyl derivatives and did not form methyl esters after esterification by methanol in acid medium. Overall, their spectra were quite similar. According to Figs. 2e and A.4, the pentylindole residue of this molecule was 14 Da higher (*m*/*z* 228) than for JWH-250. This evidence, together with the invariability of this residue after derivatisation and the presence of *m*/*z* 144, suggests the presence of a carbonyl group in the N-alkyl chain. A similar metabolic pathway was not reported by the authors of reference [13], who investigated the metabolism of JWH-018 *in vitro*, or in our previous works [16,17] examining JWH-018 metabolites in human serum and in human and rat urine. Despite the identification of similar compounds in the urinary metabolites of JWH-018, as reported by other investigators [15], we believed this metabolic pathway to be atypical because of the structural features of M12 and M13 and their high concentrations in the metabolite mixtures (Fig. 3a).

We did not detect carboxylated JWH-250, although carboxylated JWH-018 has been reported [15,16].

3.1.4. Trihydroxylation (M14–M18), $[M^+]_{TMS} = 599$

Five trihydroxylated metabolites were detected in human urine (Fig. 3a). The metabolite M17 was present in trace amounts in human urine but was not detected in rat urine (Fig. 4a and b).

Detailed structural information was difficult to obtain for this group because of its low concentration. All trihydroxylated metabolites possessed intense ions at *m*/*z* 390 in their spectra (Fig. 2d), but we could not identify *m*/*z* 144 and 116, corresponding to the unchanged indole bicycle. Based on these observations, we assumed that the three hydroxyl groups were localised in the methoxybenzyl moiety, the indole bicycle and the aromatic or aliphatic residue of the pentylindole moiety, respectively.

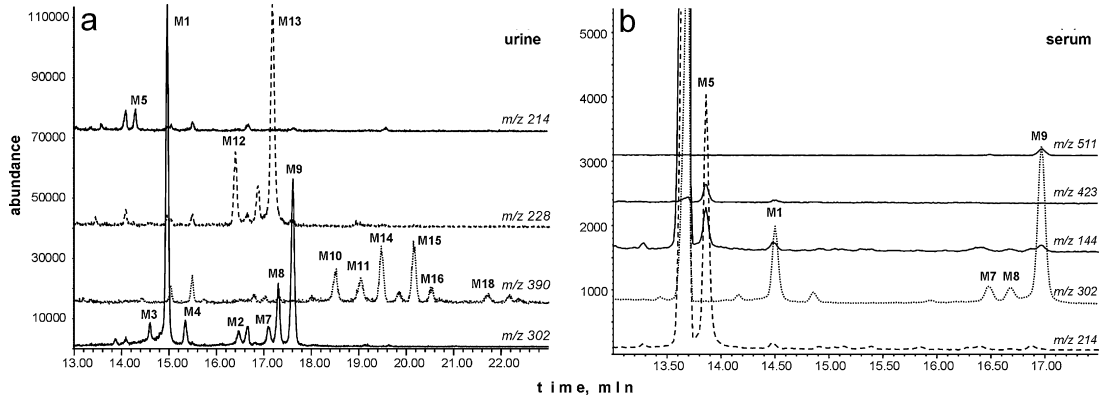

Fig. 3. The extracted ion chromatograms of JWH-250 metabolites in human urine (a) and serum (b) samples (TMS derivatisation, column = EVDX-5ms, SIM mode). The intense signal at the beginning of chromatogram (b) corresponded to cholesterol.

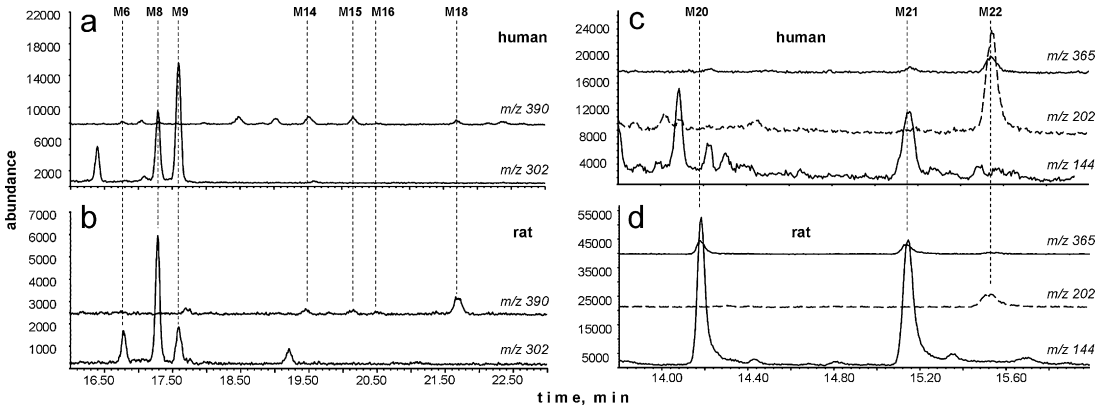

Fig. 4. Comparison of the allocation of the dihydroxylated metabolites in samples of human (a) and rat (b) urine (TMS derivatisation, column = EVDX-5ms), along with dealkylated metabolites in human (c) and rat (d) urine (AC derivatisation, column = DB-17ms).

3.1.5. N-Dealkylation (M19), $[M^+]_{AC}$ = 307 and N-dealkylation combined with monohydroxylation (M20–M22), $[M^+]_{AC}$ = 365

We did not detect any O-demethylated metabolites. Nevertheless, N-dealkylated forms of JWH-250 were detected in both rat and human urine. Although the concentrations of these compounds in human urine were low, this metabolic pathway was prevalent in rats. Detection of the dealkylated metabolites was carried out after acetylation because the TMS derivatives of compounds with free indole nitrogens had low thermal stability (Fig. 4c and d).

The simplest member of this group (M19, spectrum given in Fig. A.5) was only detected in rat urine, although the concentration was very low compared to the N-dealkylated monohydroxylated forms (M20–M22). In M19's spectra, the ion at *m*/*z* 144 was formed by the elimination of the ketene molecule from the indole fragment (*m*/*z* 186).

Two of the three monohydroxylated forms (M20 and M21, Fig. 2f) contained a hydroxyl group in their methoxybenzyl moiety, and their spectra were similar to the spectrum of M19. Metabolite M20 was only detected in rat urine. The concentration of the third form (M22, the product of monohydroxylation of the indole bicycle) in samples of rat urine was less than that of the first two forms. The spectrum of this compound (Fig. A.6) was characterised by intense ions at *m*/*z* 202 and 160, which were formed by the consecutive elimination of two ketene molecules from the diacetylated M22.

3.2. GC–MS of human serum sample

Five metabolites of JWH-250 were detected in a smoker's serum, as shown in Fig. 3b. The monohydroxylated M1 and M5 and the dihydroxylated M9 were prevalent in this mixture. Two more metabolites (dihydroxylated M7 and M8) were present in small concentrations. Additionally, there were traces of metabolite M13 (not shown in Fig. 3b). Native JWH-250 was not detected. This seems contrary to a previous study [11] that used LC linked to tandem mass spectrometry to quantify the native JWH-250 in serum. Such disagreement can be explained by the difference in sensitivity of the applied analytical methods. This assumption, combined with findings [17,19] on the rapid metabolism of naphthoylindoles, has resulted in the conclusion that detection of metabolites is preferred over the native compounds themselves.

Dome distinction could be made in the relative quantities of metabolites detected in human urine and serum, especially with

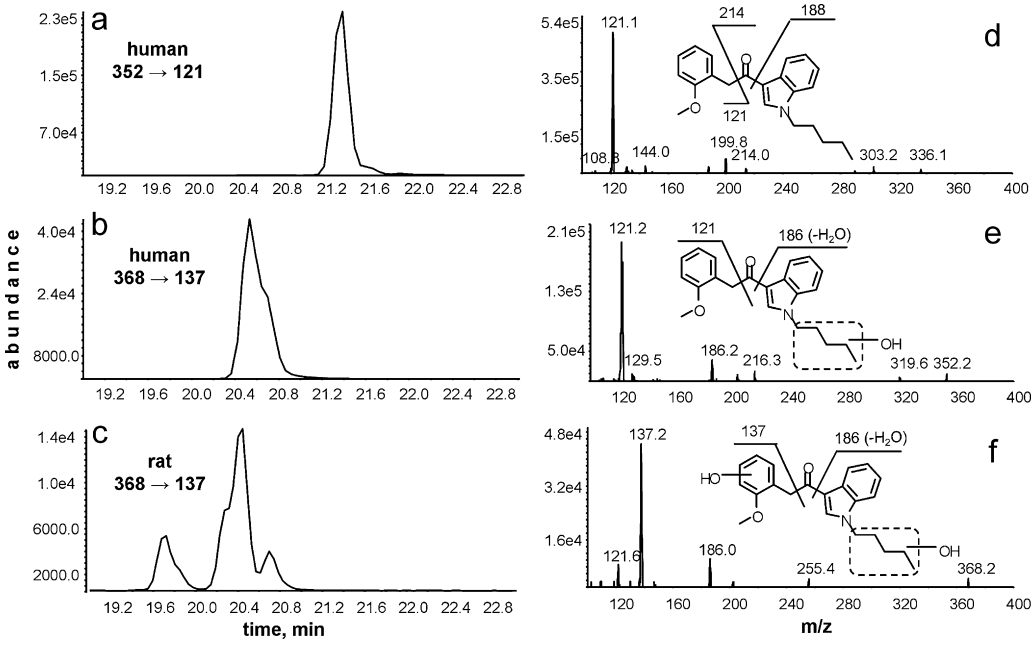

Fig. 5. MRM chromatograms of samples of human (a and b) and rat (c) urine; mass spectra of the product ions of JWH-250 (d) and metabolite M1 (e); spectrum of a mixture with poorly separated dihydroxylated metabolites (f) registered according to intensity maxima of the chromatographic band of the human urine sample chromatogram.

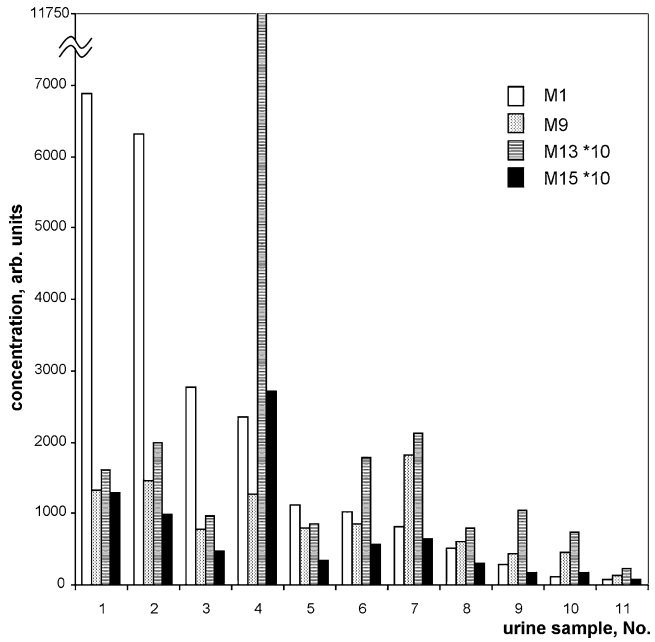

Fig. 6. Comparison of four metabolite (monohydroxylated M1, dihydroxylated M9, trihydroxylated with dehydration of the N-alkyl chain M13 and trihydroxylated M15) concentrations (arbitrary units) in urine samples collected from hospitalised individuals. The M13 and M15 concentrations were multiplied by a factor 10.

regard to metabolite M5, the product of monohydroxylation of the methoxybenzyl moiety. The relative concentration of M5 in serum was high, but it was a minor component of the urinary metabolites. It is possible that M5 was exposed to additional oxidation before urinary excretion.

3.3. LC–MS/MS of rat and human urine samples

LC with a triple-quadrupole mass spectrometer was used to confirm the structural elucidation of metabolites and evaluate the suitability of this method for routine urine screening.

The spectra of JWH-250 metabolite product ions registered in ESI mode considerably differed from those in EI mode, which is atypical for naphthoylindoles. In a JWH-250 sample (Fig. 5d), there was a good correlation between the most intense ion at m/z 121 and the presence of methoxybenzyl fragments, while the product ions corresponding to the pentylindole moiety (m/z 214 and 144) had low intensities. This difference was likely caused by enolisation of the phenylacetylindole molecules and conjugation between the aromatic moieties, which created a charge redistribution.

The formation of ion m/z 186 in the spectrum of the monohydroxylated metabolite M1 (Fig. 5e) could be related to the dehydration and formation of a double bond in the N-alkyl chain. The MRM chromatogram for the [M+H]→121 ion transition is depicted in Fig. 5a.

Because the dihydroxylated metabolites of JWH-250 were poorly separated under the applied chromatographic conditions (Fig. 5b and c), the spectrum derived from the protonated precursor ion at m/z 368 (Fig. 5f) did not correspond to the pure compound. Nevertheless, the ion at m/z 137 corresponded to hydroxylation of the methoxybenzyl moiety, and the considerable amount of dihydroxylated metabolites (M7–M9) in human urine could be used to confirm consumption of JWH-250 using the ion transition, [M+H]→137.

3.4. Comparison of JWH-250 metabolite concentrations in human urine samples

A comparison of the concentrations of metabolites in the urine samples collected from hospitalised individuals displaying suspicious behaviour was of particular interest. The concentrations (arbitrary units) of four heterogeneous metabolites in 11 urine samples, arranged in decreasing order of M1 concentration, are shown in Fig. 6. Analysis was carried out by GC–MS with a sample preparation that included LLE and TMS. To obtain an analytical response, peak areas from EICs were measured for the most intense ions in the spectra of TMS derivatives: 302, 302, 228 and 390 for M1, M9, M13 and M15, respectively. Metabolite M1 was highest in samples that contained the greatest general metabolite concentration (urine samples 1–4). However, the observable M1 concentration was less than (samples 7–11) or comparable to (samples 5–6) the M9 concentration for samples with a low content of metabolites. Considering the uncertainty in the amount of time between JWH-250 administration and urine collection, we assumed that [M1] ≤ [M9] for all older urine samples. Detection of these compounds was the most convenient method for establishing JWH-250 administration by GC–MS.

4. Conclusions

Twenty-two metabolites of the synthetic cannabimimetic JWH-250, products of oxidation and N-dealkylation of the parent compound, were identified in human urine and serum samples and in rat urine. The consumption of JWH-250 can be established by detection of these metabolites in urine collected within a day of consumption. The detection of the monohydroxylated metabolite was the most convenient for diagnosis of drug intoxication, although in older urine samples, its concentration approached that of the dihydroxylated metabolite.

At least nineteen JWH-250 metabolites (products of mono- and polyhydroxylation, trihydroxylation with dehydration of the N-alkyl chain and N-dealkylation with monohydroxylation) could be detected in human urine, and at least five mono- and dihydroxylated products could be detected in serum. The primary urinary metabolites detected in humans included the monohydroxylated components excreted as conjugates with urinary acids. At least eleven metabolites could be detected in rat urine: N-dealkylated, dihydroxylated and N-dealkylated combined with monohydroxylation components (high concentration).

Overall, both GC–MS (after derivatisation of samples by TMS or AC) and LC–MS/MS (MRM mode) can be used to establish JWH-250 consumption.

Acknowledgments

The authors wish to thank Anzhelika Barbatunova and Ekaterina Bozhko for their support.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.07.004.

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