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Sensitive and rapid quantification of the cannabinoid receptor agonist naphthalen-1-yl-(1-pentylindol-3-yl)methanone (JWH-018) in human serum by liquid chromatography–tandem mass spectrometry☆

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ABSTRACT

The current paper describes a validated method for the detection and quantification of naphthalen-1-yl-(1-pentylindol-3-yl)methanone (JWH-018), an ingredient of a herbal mixture called "Spice", by means of HPLC–ESI–MS–MS in serum. Lower limit of detection and lower limit of quantification were 0.07 and 0.21 ng/ml, respectively. In 2 subjects who consumed ca. 50 μg/kg of JWH-018 by smoking, the active ingredient was detected by means of the described method. Thereby, the serum concentrations reached values of approx. 10 ng/ml and dropped within 3 h very fast (<10% of the measured maximum concentrations).

1. Introduction

Since a few years herbal blends have been sold as incense under the brand names "Spice", "Smoke", "Sence" and others. To our knowledge, these appeared in different parts of the world, such as Europe, America and Japan. The mixtures became very popular in the middle of 2008 in Germany and were available at head shops and on the Internet. There were claimed to be different varieties of a mixture of herbs (*Canavalia maritima*, *Nymphaea caerulea*, *Scutellaria nana*, *Pedicularis densiflora*, *Leonotis leonurus*, *Zornia latifolia*, *Nelumbo nucifera* and *Leonurus sibiricus*) which should produce cannabis like effects. Since December 2008, however, laboratory tests identified ingredients which were able to explain the ambiguous symptoms as an effect of synthetic cannabinoids. Therefore, "Spice" was proposed as a new product of designer cannabinoids. The analysis found several potent ingredients: naphthalen-1-yl-(1-pentylindol-3-yl)methanone (JWH-018), naphthalen-1-yl-(1-butylindol-3-yl)methanone (JWH-073) and 2-[(1R,3S)-3-hydroxycyclohexyl]-5-(2-methyloctan-2-yl)phenol (CP 47,497) along with its dimethyloctyl homologues and further chemical additives (tocopherol, oleamide) [1–4]. In consequence of the investigations in January, 2009, JWH-018 and CP 47,497 were added to the German controlled drug schedules along with homologues [5].

Because of the growing popularity and the possible hazard potential, the analysis of "spice" ingredients becomes relevant in the field of forensic toxicology. However, reference substances for quantitative and qualitative investigations are rare, in Germany at the time of the current experiment only JWH-018 was commercially available.

Reported in the 1990s, JWH-018 belongs to a group of cannabimimetic indoles which acts as an agonist at cannabinoid receptors and shows the profile of cannabinoid effects in mice [6–8]. JWH-018 even acts as an agonist at cannabinoid receptors with a preference to the CB2 receptor. Compared to THC, a significant increase of binding affinity at cannabinoid receptors is described [9]. JWH-018 occurred in many of the "Spice" mixtures investigated and seems to be the only synthetic cannabinoid in "Smoke" [1].

The present study introduces a validated procedure for the detection of JWH-018 based on the tandem mass spectrometry. The method was used to examine the blood samples obtained after a self-experiment during which 2 test persons had smoked

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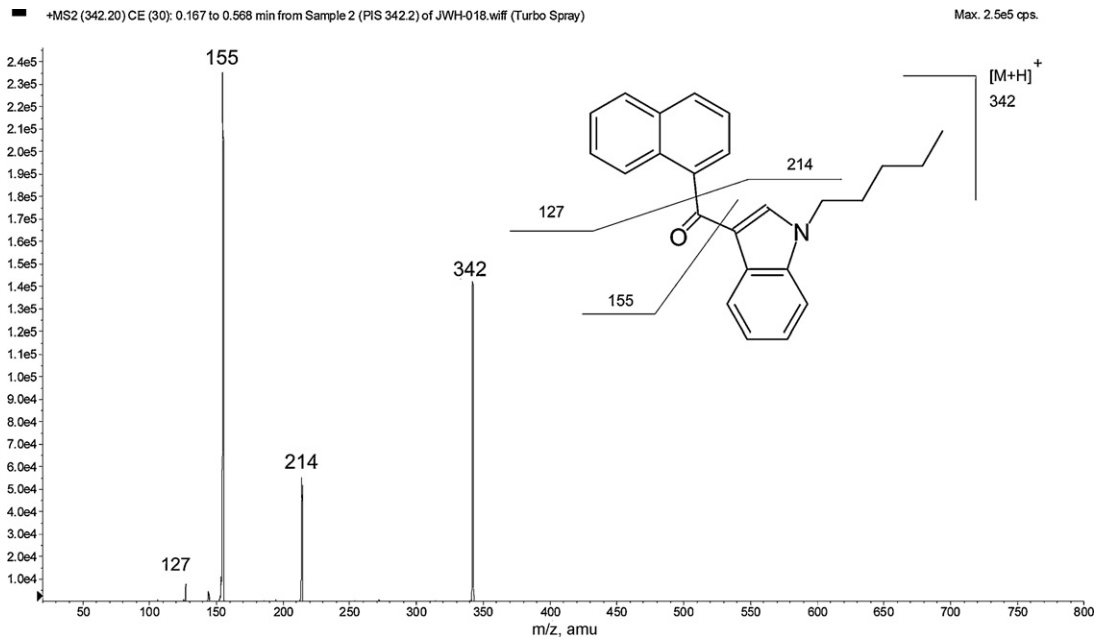

Fig. 1. ESI-product ion spectrum of JWH-018, precursor: *m*/*z*: 342, collision energy 30 V.

an incense of the specialty "Smoke". To our knowledge, there currently exist no comparable published data on a validated procedure for the detection of JWH-018.

2. Experimental

2.1. Analytical standards and reagents

HPLC-grade methanol was supplied by J.T. Baker (Deventer, The Netherlands). Ethyl acetate, hexane and sodium hydrogencarbonate were supplied by Merck (Darmstadt, Germany), ammonium acetate by Sigma-Aldrich (München, Germany), acetic acid by Riedel-de Haen, (Hannover, Seelze, Germany). JWH-018 and diazepam-d5 were obtained from LGC Promochem (Wesel, Germany).

2.2. Apparatus and conditions

An Applied Biosystems (Darmstadt, Germany) API 2000 tandem mass spectrometer equipped with ESI (Turboionspray®), a Shimadzu high pressure gradient system with 2 solvent delivery units (LC-10Advp), system controller (SCL-10Avp), auto injector (SIL-10ADvp), column oven (CTO-10Asvp) and degasser (DGU-14A) were used for LC/MS/MS analyses. The data were processed using Analyst 1.4 software.

Chromatographic separation was performed on a Phenomenex (Aschaffenburg, Germany) Luna 5 μm C18 (2) 100 A (150 mm × 2 mm) column. Both eluents contained 10mM ammonium acetate and 0.1% acetic acid (pH 3.2) and were composed of water/methanol 95 + 5 (v/v, eluent A) or water/methanol 5 + 95 (v/v, eluent B), respectively. The mobile phase was pumped at a flow rate of 0.3 ml/min and was programmed as follows: 70% B initially, ramping to 100% B from 0 to 2.5 min, hold for 1.5 min, ramping to 70% B from 4.5 to 5.5 min, hold for 2 min for column re-equilibration.

Under electrospray conditions JWH-018 forms positive charged $[M+H]^+$ pseudo molecular ions (Fig. 1). The fragmentation patterns were taken from [1]. ESI–MS/MS was performed in multiple reaction monitoring (MRM) mode with the following settings: source temperature 400 °C, ion spray needle voltage + 4000 V, curtain gas 25 psi (nitrogen), nebulizer gas 50 psi (nitrogen), auxiliary gas 50 psi (nitrogen), collision gas set on 4 (nitrogen), dwell time 200 ms and pause between mass ranges 5 ms. Further analyte-dependent MS/MS parameters (Table 1) were optimised by continuous infusion of standards dissolved in eluent B at a flow rate of 10 μl/min. The MRM-transitions *m*/*z* 342 → 155 and *m*/*z* 342 → 127 were chosen as quantifier and qualifier ions, respectively.

2.3. Preparation of standard solutions, quality control samples and calibration samples

Main stock solutions of JWH-018 and diazepam-d5 (1 ng/μl) were prepared by dissolving standard solutions of the drugs separately in methanol. The stock solution of JWH-018 was further diluted to give a working solution with a concentration of 0.1 ng/ml.

Calibration standards of JWH-018 (0, 0.5, 1.0, 2.5, 5.0, 7.5, 10 and 20 ng/ml) were prepared by adding appropriate amounts of the working solution and 100 ng/ml diazepam d5 in drug free serum samples. Quality control samples were prepared at concentrations

Table 1
Optimised MS–MS parameters for the detection of JWH-018.

	M1 (*m*/*z*)	M2 (*m*/*z*)	DP (V)	FP (V)	EP (V)	CEP (V)	CE (V)	CXP (V)	t_R (min)
JWH-018	342	155	71	310	12	14	37	6	4.9
	342	127	71	310	12	14	67	4	4.9
Diazepam-d5	290	198	36	360	10	16	47	4	3.4

M1 = precursor ion, M2 = product ion, DP = declustering potential, FP = focusing potential, EP = entrance potential, CEP = collision cell entrance potential, CE = collision energy, CXP = collision cell exit potential.

Table 2
Accuracy and precision.

Concentration added (ng/ml)	Concentration found (ng/ml)	%RSD	% Bias
Intra-day assay precision and accuracy			
0.5	0.52	3.9	4.0
5.0	5.50	2.4	10.0
10.0	10.5	4.8	5.0
Inter-day assay precision and accuracy			
0.5	0.45	13.5	−9.1
5.0	4.86	13.7	−2.8
10.0	9.43	14.8	−5.7

of 0.5, 5 and 10 ng/ml JWH-018 and 100 ng/ml diazepam-d5 using drug free serum.

2.4. Sample preparation

An aliquot of 0.2 ml human serum was transferred to a 2 ml polypropylene vial prepared with 100 μl water, 20 μl of the internal standards (1 ng/μl diazepam-d5) and 10 mg $NaHCO_3$. Liquid–liquid extraction (LLE) was performed twice with 1 ml hexane/ethyl acetate 99 + 1 (v/v) by gentle shaking for 15 min. After centrifugation and evaporation of the organic layers, the residue was reconstituted in 150 μl HPLC eluent and 25 μl was injected.

2.5. Method validation

The calibration and control samples were obtained by adding 200 μl of drug free human serum, an aliquot of fresh diluted stock solution of JWH-018 and 20 μl of fresh diluted stock solution of diazepam-d5 (internal standard). Calibration curves were generated using linear regression and duplicate measurements of each level. The selectivity was checked by extracting six different serum samples and screening for interfering peaks. In addition, three of the serum samples were spiked with the internal standard. Accuracy and precision experiments were performed to evaluate bias and repeatability at three concentration levels (0.5, 5 and 10 ng/ml JWH-018) with eight replicates per level. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated from the signal to noise values of spiked serum samples containing 0.5 ng/ml JWH-018. Those samples were extracted and analyzed five times.

Studies of ion suppression/enhancement and matrix effects were performed according the procedure published by Matuszewski et al. [10]. For that purpose, three sample sets were used: standard solutions diluted in HPLC eluent (batch A), 5 different lots of human serum spiked with JWH-018 after extraction (batch B), and human serum spiked before extraction (batch C). These tests were performed at two concentration levels of 1 and 15 ng/ml JWH-018, respectively.

2.6. Design of the self-experiment

The smoking experiment was performed by two healthy subjects, a female (volunteer 1: 33 years old, 178 cm, 72 kg, regular smoker) and a male (volunteer 2: 47 years old, 182 cm, 86 kg, occasional smoker). Each subject smoked a cigarette containing 100 (volunteer 1) or 150 mg (volunteer 2) of the incense "Smoke". They were instructed to choose inhalation frequency and inhalation depth in a typical manner. Blood samples were obtained 5, 15 and 60 min as well as 3, 12, 24 and 48 h post-experiment and were examined for the detection of JWH-018. The collection of a pre-experiment blood sample had been omitted because a former contact of the subjects with JWH-018 could be plausibly excluded.

Before the experiment, the potency of the incense was determined. For that purpose, 50 mg of "Smoke" were extracted as a double set using a 20 min ultrasonic bath with 50 ml methanol. Afterwards, a 1:2000 dilution of the extract was examined by means of LC/MS/MS. The JWH-018 potency value was 2.9%.

Table 3
Matrix effect, recovery and process efficiency according to Matuszewski [10].

Concentration [ng/ml]	1.0	15 .0
Matrix effect [%]	45.1	56.6
Recovery [%]	88.9	101.2
Process efficiency [%]	40.1	57.3

3. Results and discussion

3.1. Linearity and selectivity

Calibration curves were generated using linear regression for the quantifier MRM transition and found to be linear over the range investigated. The equations were $y = 0.0994x + 0.0517$ ($R^2 = 0.997$). Blank samples did not yield any interference at the elution time for detection of JWH-018 and diazepam-d5.

3.2. Accuracy and precision

Results of the precision (expressed as %RSD) and the accuracy (expressed as % bias) studies are summarised in Table 2. The RSD values were between 2.4–4.8% (intra-day) and 13.5–14.8% (inter-day). The accuracy values were found to be ≤10%. All values met the acceptance criteria of less the 15%.

3.3. Limits

The LOD is the lowest amount of JWH-018, for which identification criteria can still be fulfilled. LOD was calculated from three times the noise value of the qualifier transition (*m*/*z*: 342/127) and was estimated to be 0.07 ng/ml. The LOQ is the lowest concentration that can be quantitatively determined, and was calculated from ten times the noise value of the quantifier transition (*m*/*z*: 342/155) if identification criteria were fulfilled. The calculated LOQ value using this method is 0.21 ng/ml.

3.4. Matrix effects

By comparing the peak areas matrix effect (quotient of batch B/batch A × 100%), recovery of the extraction procedure (quotient of batch C/batch B × 100%) and process efficiency (quotient of batch C/batch A × 100%) were calculated. The results are summarised in Table 3.

The data obtained show significant ion suppressions for JWH-018. The matrix effects were between 45.1 and 56.6%, the recoveries were >88% and process efficiency was between 40.1 and 57.3%. However, the sensitivity is high enough for the determination of JWH-018 in serum samples.

Table 4
Serum concentrations of JWH-018; * exact time 1.33 h, ** half-quantitative value below LOQ, p. = present, qualitative verification in the area of LOD; n.p. = not present.

Post-smoking time	Volunteer 1 Concentration [ng/ml]	Volunteer 2 Concentration [ng/ml]
5 min	8.1	10.2
15 min	4.6	6.1
1 h	1.7*	1.8
3 h	0.41	0.25
6 h	0.16**	0.13**
24 h	p.	p.
48 h	p.	n.p.

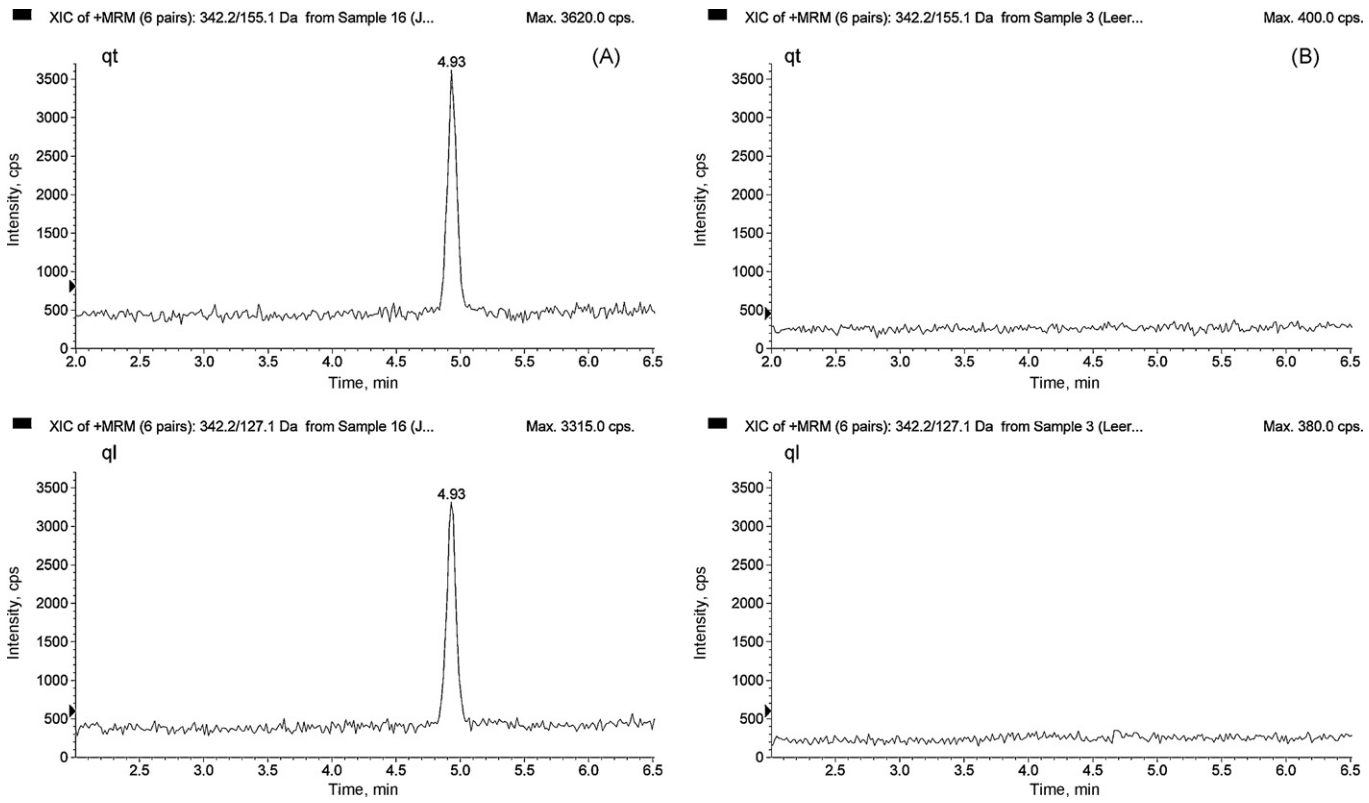

Fig. 2. Quantifier (qt) and qualifier (ql) MRM-transitions of JWH-018 in serum. (A) Volunteer 2, 3 h post-smoking (c = 0.25 ng/ml), (B) drug free serum.

3.5. Results of the self-experiment

The subjects reported sickness, sedation and xerostomia immediately after the smoking. Hot flushes, burning eyes and a subjectively felt thought disruption were also partially experienced. A rise of the pulse rate was observed, while the blood pressure was hardly raised. The pupil reaction was not noticeably affected, the pupil width was at most easily increased. Later, the symptoms went over in a state of light tiredness and exhaustion attenuating 6–12 h post-experiment. The serum samples of both subjects were examined by means of the described method. The detected concentrations of JWH-18 are summarised in Table 4.

Fig. 2 shows the chromatograms of the quantifier (qt) and qualifier (ql) MRM-transitions in a serum sample of Volunteer 2 as well as in a drug free serum sample.

The results present experiences of an exemplary investigation with 2 subjects. The influence of edge conditions such as pyrolysis attrition or inhalation depth could not be controlled. According to the obtained measurements, after the application via inhalation of approx. 50 μg/kg BM, the maximum concentrations were in the range of 10 ng/ml 5 min post-smoking. Within 3 h, the serum level rapidly dropped to values below 10% of the measured maximum concentration. After 24 or 48 h, merely trace findings were present in the LOQ area. In Volunteer 2, the active ingredient was not detectable after 48 h. It is currently unknown whether the consumption frequency affects the blood concentration. As with THC [11,12], the regular consumption will presumably cause an accumulation in the fatty tissue and a delayed elimination.

4. Conclusions

The introduced method can be applied to detect JWH-018 in serum samples by means of LC/MS/MS. Using 2 MRM-transitions and in due consideration of the retention data, a safe forensic identification and quantification is possible up to the pg/ml concentration area. Since the identified active ingredients and several homologues were recorded in the German controlled drug schedules in January, 2009, the importance of Spice-products would probably have decreased. Overall, there hardly exist any data on the prevalence, pharmacology or analytical methods for the assessment of real cases.

According to the data ascertained in the current paper, it can be assumed that in practice a consumption of Spice-products containing JWH-018 will only be provable by means of very sensitive procedures unless the consumption has taken place immediately before the blood withdrawal. There already exist first investigations and publications on the metabolism of cannabimimetic indoles [13]. However, it is still unclear to what extent the JWH-018 metabolites can be used in future for the assessment and evaluation of cases. Further investigation is required.

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