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A method for CP 47, 497 a synthetic non-traditional cannabinoid in human urine using liquid chromatography tandem mass spectrometry

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ABSTRACT

A rapid method has been developed to analyse CP 47, 497 in human urine. Urine samples were diluted with water:acetonitrile (90:10, v/v) and sample aliquots were analysed by triple quadrupole tandem mass spectrometry with a runtime of 5 min. Multiple reaction monitoring (MRM) as survey scan was performed. The method was validated in urine, according to an in-house validation protocol based on the criteria defined in Commission Decision 2002/657/EC. Three MRM transitions were monitored. The decision limit (CCα) was 0.01 μg mL^{-1} and for the detection capability a (CCβ) value of 0.02 μg mL^{-1} was obtained. The measurement uncertainty of the method was 21%. Fortifying human urine samples (n = 18) in three separate assays, show the accuracy of the method to be between 95 and 96%. The precision of the method, expressed as RSD values for the within-lab reproducibility at the three levels of fortification (0.1, 0.15 and 0.2 μg mL^{-1}) was less than 10% respectively. The method proved to be simple, robust and time efficient. To the best of our knowledge there are no LC–MS methods for the determination of CP 47, 497 with validation data in urine.

1. Introduction

Synthetic cannabinoids are functionally similar to the main active ingredient in cannabis. These substances are more aptly called cannabinoid receptor agonists and were developed over the last 40 years as therapeutic agents for pain relief. However it was very difficult to isolate the desired therapeutic properties from the psychoactive effects of these drugs. These drugs were found to be considerably more potent than cannabis. Although the term synthetic cannabinoids is utilised, many of the substances are not structurally related to the classical cannabinoids. The synthetic cannabinoids consist of seven major structural groups including (a) naphthoylindoles (JWH-018, 073, 398), (b) naphthylmethylindoles, (c) naphthoylpyrroles, (d) naphthylmethylindenes, (e) phenylacetylindoles (JWH-250), (f) cyclohexylphenols (CP47, 497) and (g) classical cannabinoids (HU-210).

The cannabinoid receptor agonists mimic the effects of the cannabis main active ingredient by interacting with the CB1 receptor in the brain. These synthetic cannabinoids can get individuals very intoxicated and were never intended for human use. However certain manufacturers are marketing these substances for human consumption under the identity of "incense" while satisfying the authorities by writing in very small print "not for human consumption" on the packaging. Original testing of these products showed that no cannabis was present in these herbal products so authorities did not ban them. These products reputation quickly grew as legal alternatives to cannabis. A headshop is a retail outlet which specialises in drug paraphernalia for consumption of cannabis, other recreational drugs and new age herbs. A number of these herbal products are available for sale in headshops and Internet websites in Ireland and across the world. These herbal products are sold under trade names such as "Spice Gold", "Spice Diamond", "Spice Silver", "Spice Artic Synergy" to name but a few. Even though manufacturer's officially warn against human ingestion of spice it is usually smoked. In late 2008 the identification of synthetic cannabinoid compounds in these herbal products were recognised as causing cannabis like effects by German and Austrian authorities. In December 2008 the German company THC Pharma reported the presence of the synthetic cannabinoid JWH-018 as an active ingredient in a herbal product called "Spice" [1]. On the 20 January 2009 the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) reported that a team of German forensic experts from the University of Freiburg and the German Federal Criminal Police Office (BKA) identified a C-8 homologue of the synthetic cannabinoid CP, 47-497 (2-[(IR, 3S)-3-hydroxycyclohexyl]-5-(2-methyoctan-2-yD phenol) a synthetic cannabinoid receptor agonist in spice [2]. A team of researchers at the National Institute of Health Sciences Japan [3] also identified the synthetic cannabinoids CP47, 497 and JWH-018 in herbal products marketed as incense. Zimmerman et al. published a study in which a patient was admitted into a German hospital after daily consumption of Spice Gold' for 8 months [4]. The patient had increased the use of the product up

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to 3 g daily as a tolerance had been developed. Constant craving for the product was reported when the patient could no longer obtain the herbal product. The patient showed symptoms such as inner unrest, drug craving, headache, nocturnal nightmares, nausea, profuse sweating, elevated blood pressure and tremors. However when the patient smoked the herbal product again these symptoms vanished. The authors interpreted the symptoms observed as typical of physical dependence and marijuana addiction. The doctors postulated that an admixture of compounds such as CP47, 497 and JWH-018 which have been found in 'Spice Gold" in combination with the patient's daily consumption of very large amounts was responsible for the dependence. In 2010, Muller et al. presented a case of a 25 year old man whom had smoked "Spice" on 3 different occasions (3 g each). Immediately after Spice usage the patient experienced "imperative voices" and "recurrent paranoid hallucinations". Urine samples analysed were negative. The patient also had a history of psychotic episodes at an early age and a family history of psychosis however the symptoms were worse than previously experienced after the Spice consumption [5]. According to the EMCDDA there is very little known about the toxicology of these substances as few formal studies have been published [2]. In addition there is a risk of severe psychiatric problems because the type and amount of these synthetic drugs may vary considerably. There could be a higher potential for overdose from these substances compared with cannabis due to batch-to-batch variation even within the same product [1] and also these products contain a number of herbs on which the synthetic drugs are sprayed and information on the health effects of these herbs is limited. Indeed some herbs may themselves have psychoactive properties. In 2010 a review was published by Vardakou et al. entitled "Spice drugs as a new trend" [6]. The review detailed the difficulties encountered in indentification of these compounds due to the lack of availability of reference materials in order to aid toxicological analysis.

CP47, 497 is a synthetic cannabinoid in which there is limited information available due to the lack of analytical methodologies and the structure is shown in Fig. 1. It cannot be ruled out however that it could be widely used as a legal alternative to cannabis. CP47, 497 and its homologue CP47, 497-C8 are potent CB1 and CB2 agonists with CP47, 497-C8 being the most potent. In a survey of member states carried out by EMCDDA the synthetic cannabinoids CP47, 497 and HU-210 were presumed to be more important than JWH-018 [2]. Studies have shown in a number of mouse models that analgesic properties of CP47, 497 were 5 to 10 fold higher compared with THC [7]. Research studies have also identified that CP 47, 497 has similar effects to THC at considerably lower doses [8]. Other studies have recognised that CP 47, 497 has similar pharmacology to THC but shows 3–28 times greater potency depending on the model used [9]. In this study work was undertaken to develop a quantitative confirmatory analytical strategy for the determination of CP, 47, 497 in human urine. Unfortunately at the time of carrying out this work at our laboratory no CP47, 497-C8 was available for inclusion in this study. There is limited information and analytical strategies available to regulatory laboratories for CP47, 497 or researchers in other fields. The need for such methods arises due to the higher potential of overdose from CP 47, 497 and other synthetic cannabinoid substances compared with cannabis due to the batch-to-batch variation. Furthermore CP, 47 497 has already been banned in a number of EU countries. A study was carried out by Auwarter et al. in various herbal products identified CP47, 497 in the herbal product "Sence" using GC–MS and LC–MS [1]. However the study has very limited instrument method parameters given, the method was qualitative and no information was given on the presence of this substance in urine although it was analysed. A study was carried out by Kraemer et al. using GC–MS and LC–MS [10]. The study showed that the parent compound CP, 47, 497 could be identified in urine and in addition several hydroxylated metabolites could also be identified [10]. This study describes for the first time a quantitative confirmatory method for the determination of CP, 47 497 in human urine based on spiking studies using dilute and shoot sample preparation and analysis by LC–MS/MS with a chromatographic run-time of 5 min and validation according to an in-house protocol [11].

Table 1
LC gradient profile for the determination of CP47, 497.

Time (min)	Component A (%)	Component B (%)
0.0	90	10
0.40	90	10
0.75	85	15
2.40	10	90
3.50	10	90
4.00	90	10
5.00	90	10

Component A: water:acetonitrile (90:10, v/v + acetic acid). Component B: acetonitrile.

2. Experimental

2.1. Materials and reagents

LC–MS grade water and acetonitrile (HPLC) were obtained from Reagecon and acetic acid was obtained from BDH (Merck, UK). CP 47, 497 and internal standard were purchased from LGC Standards (LGC, UK). An intermediate standard solution (stable for 6 months) of CP 47, 497 was prepared in methanol at a concentration of 10 $\mu g\,mL^{-1}$ (stable for 3 months) and a separate internal standard solution was prepared at this concentration also. Standard fortification solution (stable for 3 months) was prepared in methanol at a concentration of 2.5 $\mu g\,mL^{-1}$ from the 10 $\mu g\,mL^{-1}$ intermediate stock solution and at a concentration of 0.25 $\mu g\,mL^{-1}$ from the 2.5 $\mu g\,mL^{-1}$ stock. A working standard internal standard fortification solution was prepared at 2 $\mu g\,mL^{-1}$. A fortification solution is a solution used to spike the unknown samples or matrix matched calibration standard sample at different concentration levels with CP 47, 497 or with internal standard. All standards solutions were stored at 4 °C in the dark. Injection solvent was water:acetonitrile (90:10, v/v).

2.2. LC–MS/MS conditions

The LC consisted of an Agilent 1200 Rapid Resolution LC equipped with a G1312B Binary pump, G1316B-HiPALS SL autosampler and a G1316B-TCCSL column oven (Agilent Ireland). The drugs were chromatographed on a 1.8 μm Agilent Eclipse Plus C_{18} column (2.1 mm × 50 mm) (Agilent, Ireland) and the column temperature was maintained at 55 °C. A gradient was applied with water and acetonitrile (90:10, v/v +0.0001 M acetic acid) (A) and acetonitrile (B) (Table 1). The total run time was 5 minutes with a flow rate of 0.7 mL min^{-1}. The injection volume was 20 μL. The mass spectrometer used was a QTRAP 4000 with a TurbolonSpray source from Applied Biosystems (Applied Biosystems/MDS-Sciex, Canada). The MS was controlled by version 1.5 of Analyst software. The described LC–MS/MS system was shown to be suitable for the analysis of CP 47, 497 in this study (Figs. 2 and 3).

2.3. MS/MS parameters

The analysis was performed using negative ion electrospray MS/MS in multiple reaction monitoring (MRM) mode. Three transitions were used and the collision energy was optimised as shown (Table 2). The transitions monitored were 317.3 > 299, 317.3 > 245 and 317.3 > 159.1. The MRM MS/MS detector conditions were as fol-

Table 2
MS/MS parameters for determination of CP 47, 497 in negative mode.

Compound	Transition	Declustering potential (V)	Collision energy (eV)	Collision Cell exit potential (V)
CP 47, 497	317.3 > 299.0 (1)	140	35	6
	317.3 > 245.0 (2)	140	45	4
	317.3 > 159.9 (3)	140	71	10
D9-THC–COOH	352.0 > 308	100	30	10

Note: Ion 2 was used for quantitiation as more selective.

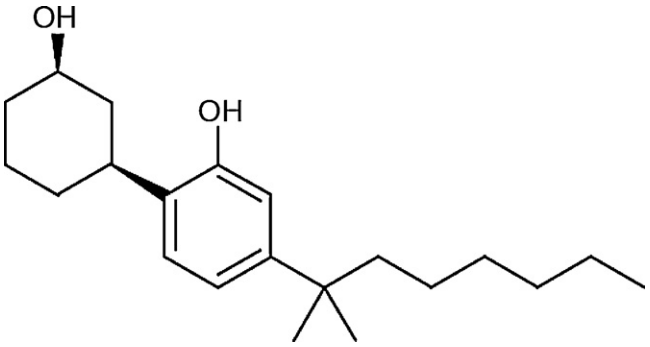

Fig. 1. Structure of CP47, 497.

lows: ion mode electrospray negative; curtain gas 45 psi; ion spray voltage 4500 V; temperature 650 °C; ion source gas 1 70 psi; ion source gas 2 70 psi; interface heater on; entrance potential 10 V; resolution Q1 unit; resolution Q2 unit; collision-activated dissociation CAD gas = medium.

2.4. Urine samples

Urine obtained for use as negative controls were separated into 50 mL aliquots and stored at −20 °C. The urine was analysed in previous batches and urine found to contain no detectable residues of CP 47, 497 was used as negative controls. A pool of urine was used in the validation from 10 different donors. 5 donors were male and 5 were female.

2.5. Sample preparation

Urine samples (100 μl) were aliquoted into 15 ml polypropylene tubes. Samples were fortified with internal standard at levels corresponding to 0.1 μg mL^{-1} by adding a 100 μL portion of a 2 μg mL^{-1} solution of d_9-THC–COOH. Samples were fortified at levels corresponding to 0.10, 0.15 and 0.20 μg mL^{-1} by adding 40, 60 and 80 μL portions of a 0.25 μg mL^{-1} CP47, 497 fortification solution. After fortification, samples were held for 15 min prior to the next analytical step. Water:acetonitrile (90:10, v/v) (1800 μL) was added to the urine and samples were vortexed (30 s), centrifuged (3568 g, 5 min, 4 °C) and the supernatant was transferred to an autosampler vial. An aliquot (20 μL) was injected on the LC column.

2.6. Matrix-matched calibration

Matrix matched calibration curves were prepared and used for quantification. Control urine previously tested and shown to contain no residues was prepared as above (2.4). Control urine sample (2 mL) was diluted with water: acetonitrile (90:10, v/v) to 40 mL. A single urine sample was used for each calibration standard level. Urine samples (mL) were aliquoted into 50 mL polypropylene tubes. Samples were fortified with internal standard at levels corresponding to 0.1 μg mL^{-1} by adding a 100 μL portion of a 2 μg mL^{-1}

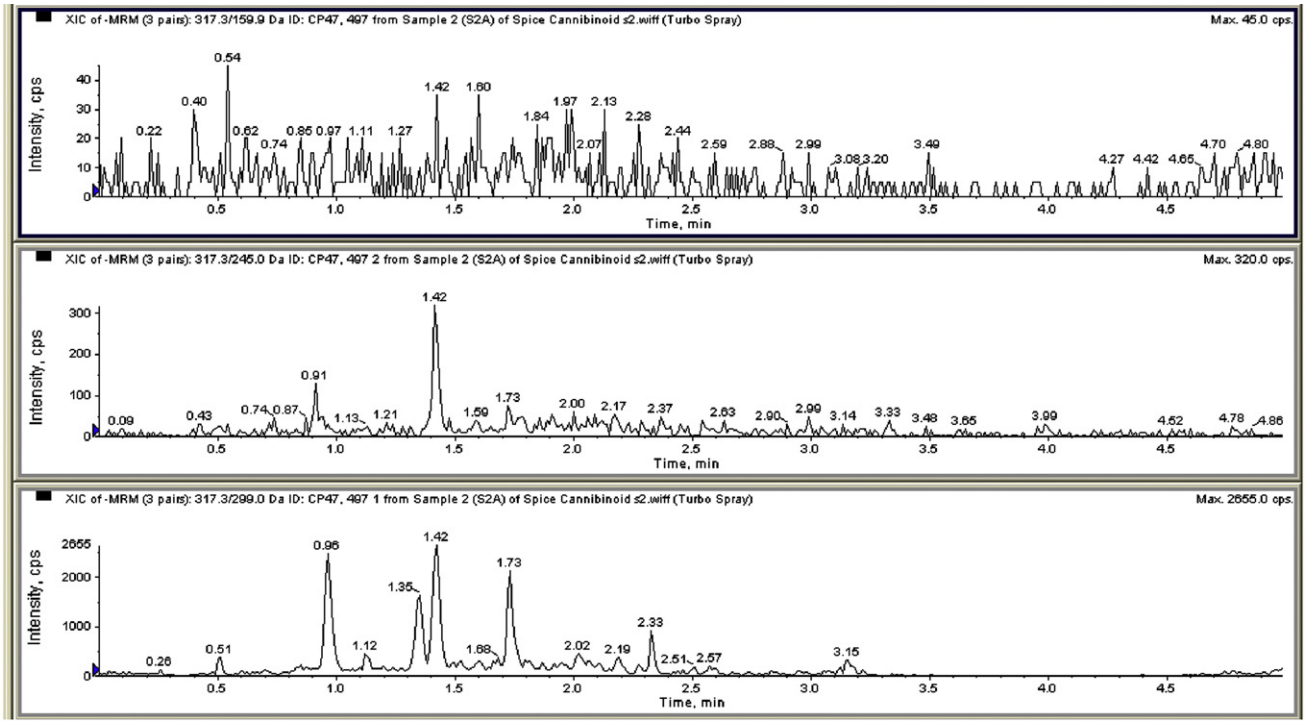

Fig. 2. Chromatogram of negative control urine.

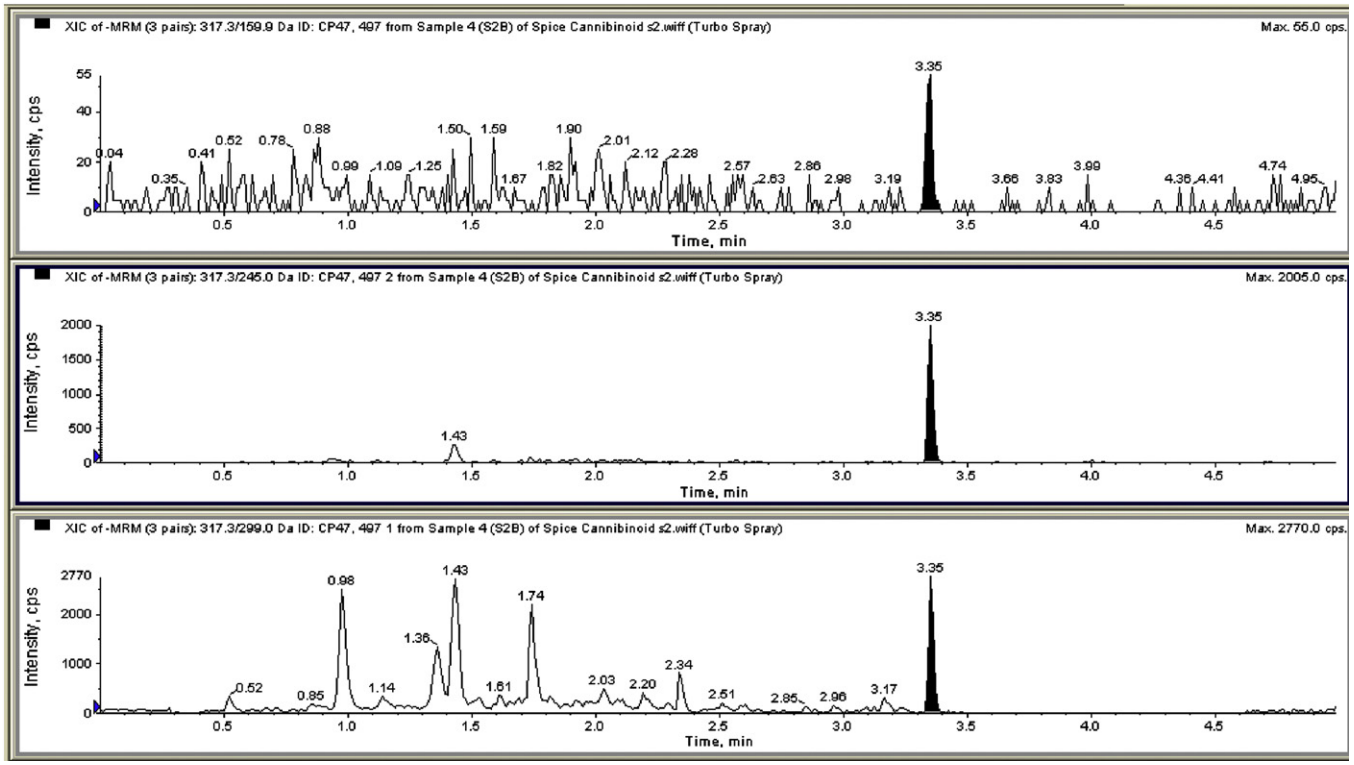

Fig. 3. Chromatogram of negative control urine fortified at 0.05 μg mL^{-1} with CP 47, 497 (3 transitions monitored).

solution of d_9-THC–COOH. Calibration standard levels were fortified at levels corresponding to 0, 0.05, 0.1, 0.25, 0.5, 1.0 and 2.0 μg mL^{-1} by adding 0, 20, 40, 100 μL portions of a 0.25 μg mL^{-1} fortification solution and 20, 40 and 80 μL portions of a 2.5 μg mL^{-1} standard solution of CP 47, 497. After fortification, samples were held for 15 min prior to procedure as described above (2.5). The concentration of the drug (μg mL^{-1}) was determined from the matrix matched calibration curves. The calibration curves were calculated by linear regression, plotting the response factor (peak area analyte/internal standard peak area of the strong transition as a function of analyte concentration.

2.7. Method validation

The EU Council Directive 96/23/EC describe measures to monitor certain substances and residues thereof in live animals and animal products in the veterinary drug residue field. This directive defines whether a substance is Group A (banned) or Group B (authorised with a maximum residue limit (MRL)). The setting up of analytical methods and validation criteria required to implement EC Council Directive 96/23/EC are laid out in Commission Decision 2002/657/EC. These guidelines for validation were implemented in this study for a Group A substance, as CP47, 497 has been recently banned in Ireland and other EU countries. Many similarities exist whether monitoring for drugs in animal tissue or in human tissues so the validation of the veterinary drug field was adopted as an in-house validation protocol for CP47, 497 at our laboratory. The EU drug legislation states if no incurred material is available then matrix fortified with the analyte should be used. This approach was utilised in this study as an alternative. The decision states criteria on the agreement of retention times, base peak and diagnostic ions, relative abundances between the standards and the analytes is essential. The decisions are based on the calculation of identification points (IPs) which depend on the analytical technique and methodology employed. In the case of banned substances the minimum number of IPs for a methodology to be considered reliable has to be equal to 4 or higher. To achieve this number, it is necessary to monitor a minimum of 4 selected ions per compound when working with single MS and a minimum of two selected reaction monitoring (SRM) transitions per compound in tandem MS therefore running two MRM transitions gives 4 IPs.

According to Commission Decision 2002/657/EC, 18 aliquots of blank material are selected and six aliquots are fortified at 1, 1.5 and 2 times the minimum required performance limit. The minimum required performance limit is for substances in which no permitted limit has been established and is the minimum content of an analyte in a sample which at least can be detected and confirmed. There is no level set legislatively and depends on the performance of the analytical method used. The estimation of accuracy, precision including repeatability and within-laboratory reproducibility, decision limit and detection capability were calculated from analysis of the six test portions at each of the three fortification levels at 0.1, 0.15 and 0.20 μg mL^{-1} corresponding to 1, 1.5 and 2 times the minimum required performance limit.

Analysis of the 18 test portions was carried out on three separate occasions. The terms decision limit and detection capability are terms utilised in Commission Decision 2002/657/EC and replace limit of detection and limit of quantitation respectively. The decision limit (CCα), is defined as the limit at and above which it can be concluded with an error probability of α (usually 5%) that a sample is non-compliant, and is a crucial parameter for confirmatory methods. The detection capability, CCβ is defined as the smallest content of the substance that can be detected and/or quantified, with an error probability of β in a sample. β is 1% for group A or banned substances and 5% for group B or authorised substances. The decision limit (CCα) of the method according to the guidelines of Commission Decision 2002/657/EC was calculated according to ISO 11843 calibration curve procedure using the intercept (value of the signal, y, where the concentration, x is equal to zero) and 2.33 times the standard error of the intercept for a set of data with 6 replicates

at 3 levels. The detection capability (CCβ) was calculated by adding 1.64 times the standard error of the CCα. ISO 11843 calibration curve procedure [12] is an official document from the International Organisation for Standardisation (ISO) body referring to detection limits from calibration curves in a particular matrix. Matrix effects were investigated by infusion of CP 47, 497 (2.5 μg mL^{-1}) by an external syringe pump to a tee-connector at 10 μl mL^{-1} between the electrospray probe and the outlet of the analytical column with simultaneous injection of water:acetonitrile (90:10, v/v) only and subsequently with blank matrix diluted in water:acetonitrile (90:10, v/v) onto the analytical column. The specific ion transitions of the analyte were recorded and any signal decreasing or increasing at the retention time of CP 47, 497 was compared with the water: acetonitrile (90:10, v/v) injection.

3. Results and discussion

3.1. Preliminary experiments

In this study an analytical strategy was developed to analyse urine samples. The LC–MS/MS method using MRM mode was developed to provide unequivocal confirmatory data for the analysis of CP 47, 497. The ionisation of CP 47, 497 was studied in positive mode and negative mode. Negative mode was the only mode suitable for analysis upon evaluation. The optimum conditions (declustering potential, collision energy, collision cell exit potential) for precursor and product ions was determined by direct infusion of the analyte solution (1 μg mL^{-1} in methanol) and the best diagnostic ions for MS/MS analysis can be seen in Table 2. For a method to be deemed confirmatory 4 identification points must be achieved. In MRM (multiple reaction monitoring) mode this is obtained by monitoring one precursor ion (parent mass) and two daughter ions (corresponding to strong and weak ion) which meet the commonly used guidelines for a confirmatory method [11]. In our method in order to improve specificity and confirmation, three daughter ions were monitored. Chromatographic tests were carried out using a 1.8 μm Agilent Eclipse Plus C_{18} column according to the method previously developed at the authors laboratory [13] however the method gradient was altered and a higher portion of component B was used in the gradient, the LC flow rate was reduced from 750 μL min^{-1} to 700 μL min^{-1}, the runtime of the LC method was reduced from 6.5 min to 5 min and the injection volume was increased from 15 μL to 20 μL. CP 47, 497 was eluted at a retention time of 3.35 min with a good peak shape when using a mobile phase of water and acetonitrile (90:10, v/v +0.0001 M acetic acid) (A) and acetonitrile (B). Dilute and shoot of urine was used during method development for the sample preparation procedure with a sample size of 100 μL based on the sensitivity achieved by the QTRAP. The urine samples were diluted 20 fold with water: acetonitrile (90:10, v/v). Carry over was investigated by injecting a blank solvent before and after each sample. No carry over was observed in the study. Matrix effects studies were carried out to evaluate the effect of the sample preparation procedure. The approach used in the study to overcome matrix effects was dilution of the samples 1/20. There is no concensus on the best way to evaluate matrix effects and post-column infusion method was evaluated. No matrix effects were observed as illustrated in Figs. 4 and 5. To further overcome matrix effects a second approach involving using an internal standard to compensate for alteration of the signal was applied. A number of different internal standards were evaluated in the study including d_3-meloxicam, d_3-ibuprofen, d_{10}-phenylbutazone, d_9-11-nor-9-Carboxy-THC (THC–COOH), d_9-tetrahydrocannabinol (THC), d_3-naproxen and d_3-ketoprofen. D_9-THC–COOH was used in this study for reliable quantitation as a result.

3.2. Validation study

Validation of the method was according to procedures described in Commission Decision 2002/657/EC [11] covering specificity, calibration curve linearity, accuracy, precision, decision limit (CCα) and detection capability (CCβ).

3.2.1. Specificity

The technique of liquid chromatography tandem mass spectrometry itself offers a very high degree of selectivity and specificity. To establish the selectivity/specificity of the method, urine samples were fortified with CP 47, 497 and also non-fortified samples were analysed. No interfering peaks were observed at the retention time of the analyte in the chromatograms of the non-fortified samples (Figs. 2 and 3).

3.2.2. Linearity of the response

The linearity of the chromatographic response was tested with matrix matched curves using 7 calibration points in the concentration range of 0–2.0 μg mL^{-1}. The regression coefficients (r^2) for all the calibration curves used in this study were ≥0.98.

3.2.3. Accuracy

The accuracy (n = 18) of the method was determined using human urine samples fortified at 0.10, 0.15 and 0.20 μg mL^{-1} in three separate assays was between 95 and 96%.

3.2.4. Precision

The precision of the method, expressed as RSD values for the within-lab reproducibility at the three levels of fortification (0.10, 0.15 and 0.20 μg mL^{-1}) was less than 10% (Table 3).

3.2.5. CCα and CCβ

The decision limit (CCα) is defined as the limit above which it can be concluded with an error probability of α, that a sample contains the analyte. In general, for non-MRL substances an α equal to 1% is applied. The detection capability (CCβ) is the smallest content of the substance that may be detected, identified and quantified in a sample, with a statistical certainty of $1 - \beta$, were β = 5% CCα and CCβ were calculated using the intercept (value of the signal, y, were the concentration, x is equal to zero) and the standard error of the intercept for a set of data with 6 replicates at 3 levels (0.10, 0.15 and 0.20 μg ml^{-1}). CCα is the concentration corresponding to the intercept + 2.33 times the standard error of the intercept. A CCα value of 0.01 μg mL^{-1} was obtained. CCβ is the concentration corresponding to the signal at CCα + 1.64 times the standard error of the intercept (i.e. the intercept + 3.97 times that standard error of the intercept). A CCβ value of 0.02 μg mL^{-1} was obtained for CP 47, 497.

3.3. Measurement uncertainty

According to SANCO/2004/2726 rev 1 [14], a document published in the EU for all regulatory laboratories gives guidelines for revised technical criteria in Commission Decision 2002/657/EC. The document states that the within laboratory reproducibility can be regarded as a good estimate of the combined measurement uncertainty of individual methods. The within laboratory reproducibility should be multiplied by a value of 2.33 and this should be used when determining the CCα, corresponding to a confidence level of 99%. The document states that for the calculation of the extended uncertainty a safety factor is required. At our laboratory according to our quality system requirements based on the IS0 17025 standard for accreditation in which we operate, a safety factor of 3.0 is used instead of 2.33 was agreed with auditors from this accreditation body. This value was chosen as during validation only the

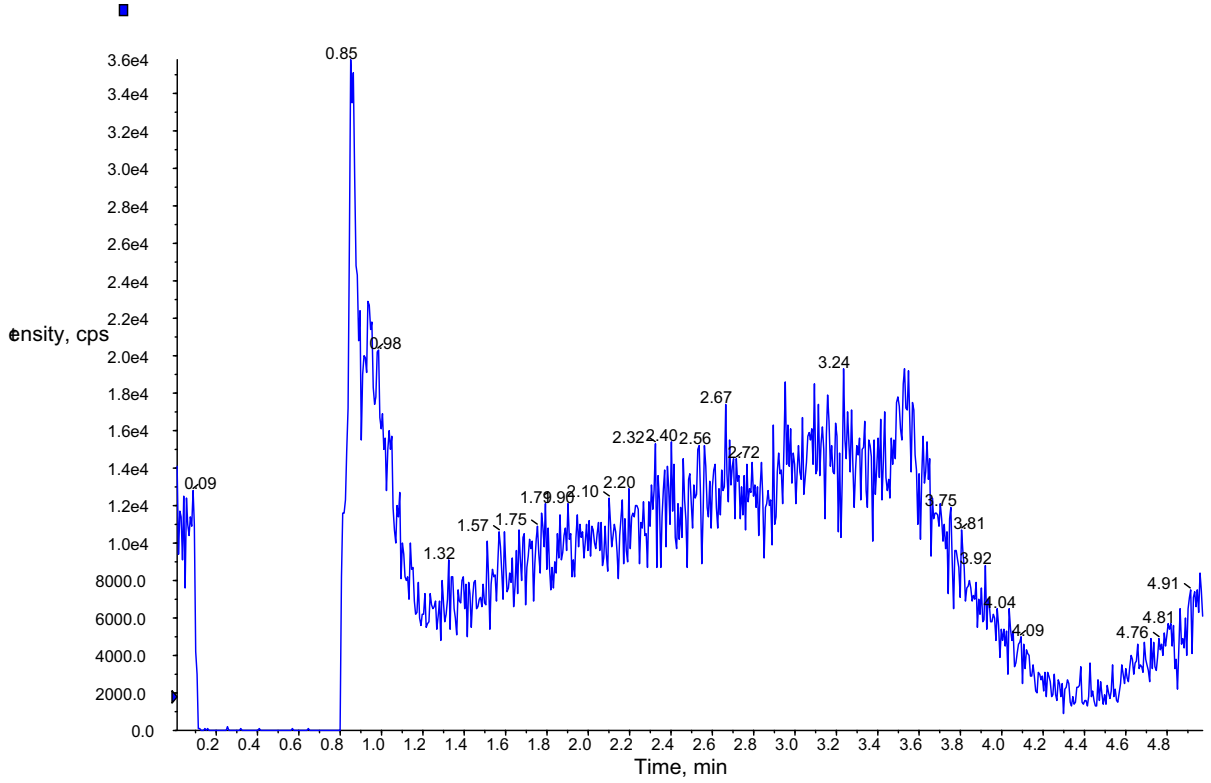

Fig. 4. Post-column infusion of CP 47, 497 (2.5 μg mL^{-1}) and injection of water:acetonitrile (90:10, v/v) solvent only.

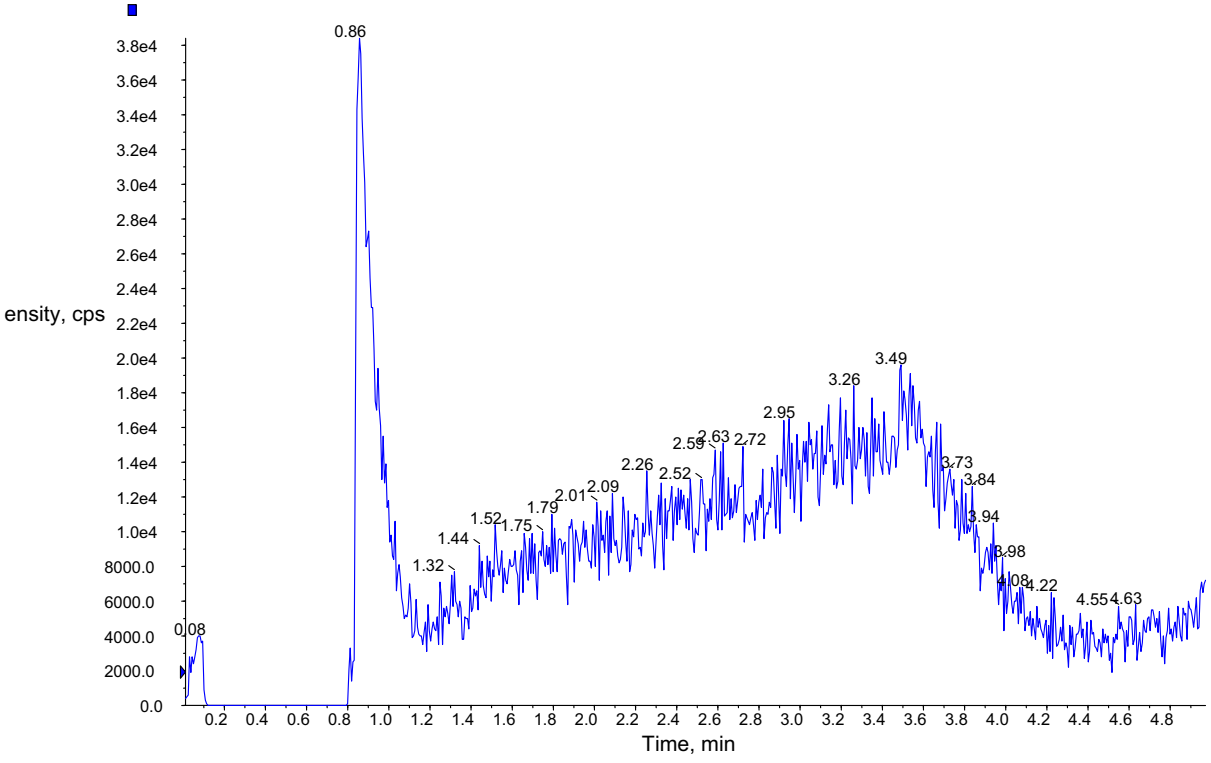

Fig. 5. Post-column infusion of CP 47, 497 (2.5 μg mL^{-1}) and injection of urine 20-fold diluted with water:acetonitrile (90:10, v/v).

Table 3
Intra- and inter-assay variation for accuracy of CP 47, 497.

Analyte	Fortification level (μg mL^{-1})	Accuracy (%)	Within run CV (%)	Between run CV (%)	Total CV (%)
CP 47, 497	0.10	96	5.646	6.957	8.960
	0.15	96	3.067	2.078	3.704
	0.20	95	4.636	5.839	7.455
Combined variance	0.10, 0.15, 0.20				7.06

different days and different urine sourced from different humans was utilised.

The measurement uncertainty of the method was estimated at 21% for CP 47, 497. This was determined by calculating the within laboratory reproducibility of the method, followed by multiplication of the within laboratory reproducibility by a safety factor of 3.0.

4. Conclusions

The advantages of the sample preparation method are that only a small volume of urine is required and the method is rapid. The advantages of the LC–MS method is the fast run-time of 5 min per injection and the monitoring of three LC–MS transitions which were previously not available. To date there have been limited reports of the application of LC–MS/MS as a detection technique in urine testing for CP 47, 497. The present investigation confirms that dilution of the urine samples 20-fold and direct injection into a triple quadrupole mass spectrometer can be used for the determination of CP 47, 497 in human urine from spiking studies. There are no LC–MS methods in the literature to the best of our knowledge for the determination of CP 47, 497 in urine with a run-time of 5 min and this study describes the first available methodology. Matrix effects studies were carried out and results have shown that no effects were identified. In conclusion the method shows that simple dilution of urine and analysis by LC–MS technology can present a rugged analytical strategy for this substance. Therefore the aim of developing a fast, simple and reliable sample preparation and detection method for CP 47, 497 has been achieved successfully. This is the first available quantitative confirmatory method for the determination of CP 47, 497 in human urine to the best of our knowledge. The analysis of headshop drugs is a very new area but difficulties in their analysis include the availability of incurred material, reference standards, appropriate isotopically labelled reference standards to aid quantification and very limited availability of analytical methodologies and knowledge of measurement ranges in human tissues for these substances. This manuscript describes an alternative validation protocol for new headshop products.

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