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Detection of cannabigerol and its presumptive metabolite in human urine after *Cannabis* consumption

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Received December 06, 2009, accepted February 02, 2010

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Pharmazie 65: 408–411 (2010) *doi: 10.1691/ph.2010.0035R*

Kemp et al. (1995) could detect Δ^9-tetrahydrocannabinol (Δ^9-THC), cannabinol and cannabidiol, three neutral cannabinoids, and the metabolites of Δ^9-THC in urine samples of *Cannabis* consumers. In this study we aimed to identify cannabigerol (CBG), which in its acid form is one of the main intermediate compounds of the biosynthesis of cannabinoids in hemp, in authority urine samples of proved *Cannabis* consumers. For this reason we applied the modified method of Kemp et al. to test for CBG, since enzymatic hydrolysis seems to be necessary for the formation of free neutral cannabinoids from conjugates. After extraction, derivatisation with N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) and GC/MS analysis, peaks of characteristic fragment ions (m/z 337, 391, 377 and 460) of bis-trimethylsilyl derivative of CBG appeared at 12.48 minutes in both real sample and the urine spiked with CBG. It shows that CBG enters the body during *Cannabis* smoking and is excreted with urine in a conjugated form, like other neutral cannabinoids. Analysing the chromatograms of hydrolysed and trimethylsilylated extracts we checked for the presence of CBG-metabolites based on the study of Harvey and Brown (1990). We detected a compound in the *Cannabis* consumers' urine extracts, having fragment ions at m/z 425, 465 and 479 at the retention time of 14.19 min which is presumed to be the 4″-hydroxy-CBG or 5″-hydroxy-CBG. However, it could not be identified completely by GC/MS. This peak was absent in non-hydrolysed urine samples, indicating that it is also excreted in glucuronated form.

1. Introduction

Urine analysis for cannabinoids is a widely accepted detection method for recent abuse of hashish and marijuana. The target analyte of these analytical methods is usually 11-nor-9-carboxy-Δ^9-tetrahidrocannabinol (Δ^9-THC-COOH), because Δ^9-THC-COOH—as the main metabolite of Δ^9-tetrahydrocannabinol (Δ^9-THC), also known as the main psychoactive compound of *Cannabis*— can be liberated by simple alkaline hydrolysis from glucuronyl conjugates and can be found at the highest concentration in urine among other cannabinoids.

Although Δ^9-THC-COOH has the major importance, reports of identifications of other cannabinoids by techniques using on-line mass spectrometry are known as well. An early study demonstrated the presence of cannabinol (CBN) and 11-hydroxy-Δ^9-tetrahydrocannabinol (11-OH-THC) in urine of *Cannabis* smokers after enzymatic hydrolysis (Kelly and Arnold 1976). The glucuronide of Δ^9-THC-COOH was first identified by GC/MS after derivatisation (Williams and Moffat 1980). Cannabidiol (CBD) was first detected by GC/MS in 1983 (Hattori 1983) and later about 50 metabolites of CBD were identified (Harvey and Mechoulam 1990; Harvey et al. 1991a,b) in urine. It was also demonstrated that not only both precursors of Δ^9-THC-COOH during the oxidation process (that is Δ^9-THC, 11-OH-THC) but 4 more hydroxylated metabolites of Δ^9-THC may be also present in urine mainly in the form of glucuronyl conjugates (Kemp et al. 1995). The identification of a carboxylic acid metabolite of Δ^9-tetrahydrocannabivarin, the propyl homologue of Δ^9-THC, was reported by ElSohly et al. (1999). Finally it was also demonstrated that Δ^9-tetrahydrocannabinolic acid A (Δ^9-THCA-A), which is the precursor of Δ^9-THC in hemp, can be found in urine by LC/MS/MS, indicating that the decarboxylation process of Δ^9-THCA-A is not complete during smoking *Cannabis* (Jung et al. 2007).

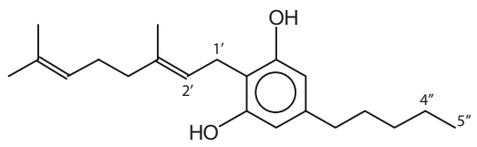

Fig. 1: Chemical structure of CBG

It is obvious that the previously mentioned reports cover the identification of several already known natural cannabinoids and also the identification of lots of their metabolites in human urine. However, the presence of cannabigerol (CBG, Fig. 1), which in its carboxylated form (cannabigerolic acid) is the first cannabinoid formed during the biosynthetic pathways of the natural cannabinoids is hemp (*Cannabis sativa* L., Shoyama et al. 1975), has not yet been studied. In this study we aimed to identify CBG in urine in the authority samples of proved *Cannabis* consumers.

Kemp and co-workers could detect CBN and CBD, two neutral cannabinoids having no psychotrophic effect after enzymatic

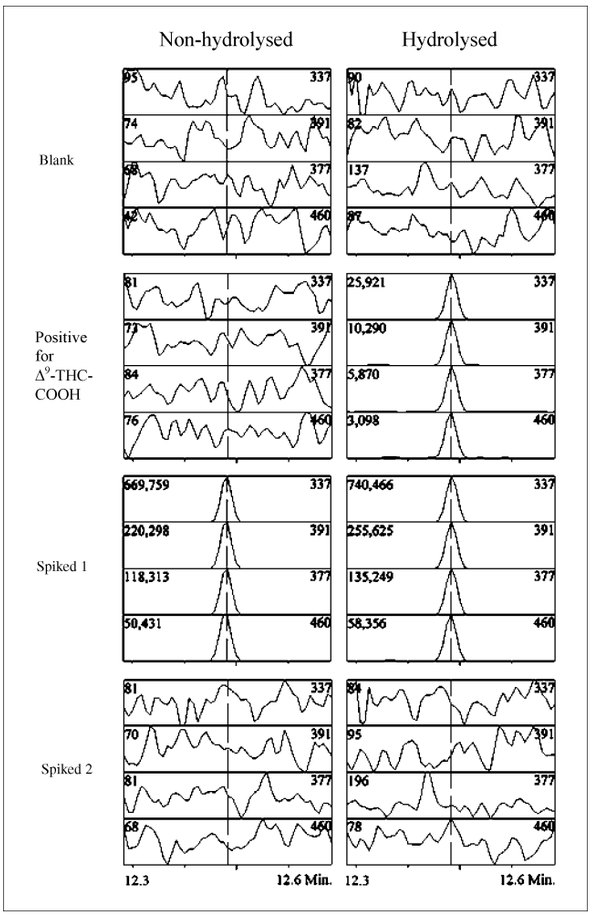

Fig. 2: Demonstration of the specific appearance of CBG in urine of a *Cannabis* consumer. Authentic sample contains CBG in glucuronated form therefore it could not be detected in not hydrolysed urine. Blank: blank urine sample, Spiked 1: urine sample spiked with CBG, Spiked 2: urine sample spiked with other cannabinoids (see text). Hydrolysis was performed by Type IX-A glucuronidase of *E. coli*. For m/z values of characteristic fragment ions: see right corner of mass chromatograms. Left corner: absolute intensities registered

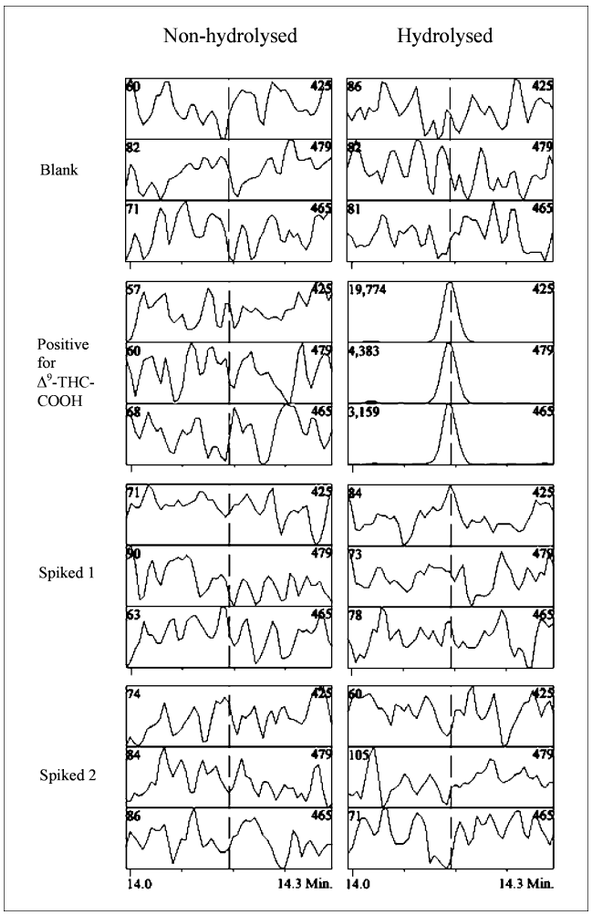

Fig. 3: Demonstration of the specific appearance of the presumptive CBG metabolite (OH-CBG) in urine of a *Cannabis* consumer. Authentic sample contains OH-CBG in glucuronated form therefore it could not be detected in not hydrolysed urine. Blank: blank urine sample, Spiked 1: urine sample spiked with CBG, Spiked 2: urine sample spiked with other cannabinoids (see text). Hydrolysis was performed by Type IX-A glucuronidase of *E. coli*. For m/z values of characteristic fragment ions: see right corner of mass chromatograms. Left corner: absolute intensities registered

hydrolysis besides the cannabinoids mentioned above (Kemp et al. 1995). For this reason we applied the modified method of their laboratory to test for CBG, since enzymatic hydrolysis seems to be necessary for the formation of free neutral cannabinoids from conjugates. The presence of CBG in urine may have diagnostic value in forensic cases and can help us to make the GC/MS analysis of an extensive urinary cannabinoid profile and the indirect investigation of the consumed plant source material. CBG and the related cannabinoids may have therapeutic potential for the treatment of glaucoma because of the activity in reducing intra-ocular pressure (Colasanti 1990). Thus, a further benefit of our study could be to find out whether CBG could be a parent compound to create a more active "soft" drug variety for the treatment of glaucoma (Buchwald et al. 2000, 2002), based on the "soft" drug approach of Nicholas Bodor (Bodor and Buchwald 2004).

2. Investigations, results and discussion

Human urine samples having a Δ^9-THC-COOH concentration of 1000 ng/ml or more were selected for the study, then hydrolysed with type IX-A glucuronidase of *E. coli*, and extracted by liquid-liquid extraction at alkaline pH prior to GC-MS analysis, underivatised and derivatised by several reagents (e.g. trimethylsilylation and methylation), CBG was identified in some of the analysed samples. The specific appearance of CBG is presented in Fig. 2. CBG was not detected in blank urine. It was also absent in urine spiked with Δ^9-THC, Δ^9-THC-COOH, cannabichromene, CBN and CBD at 0.8 μg/ml for each, indicating that it is not artificially formed from these cannabinoids during the extraction or derivatisation process. It was also absent if the samples of *Cannabis* consumers were not hydrolysed enzymatically. The identification of CBG in the enzymatically hydrolysed urine samples was based on the mass spectrum and retention time. The peaks of characteristic fragment ions (m/z 337, 391, 377 and 460 [$M^{\bullet+}$]) of bis-trimethylsilyl derivative of CBG appeared at 12.48 min in both the real sample and the urine spiked with CBG (0.8 μg/ml). A congruent result was obtained analysing underivatised sample extracts (characteristic fragment ions of CBG: m/z 193, 231, 219, 316 [$M^{\bullet+}$]) and samples methylated with MethElute® reagent (characteristic fragment ions of bismethyl-CBG: m/z 221, 275, 261, 344 [$M^{\bullet+}$]). Our results show that CBG enters the body during *Cannabis* consumption and is excreted with urine in a conjugated form.

Analysing the chromatograms of hydrolysed and then trimethylsilylated extracts we checked for the presence of

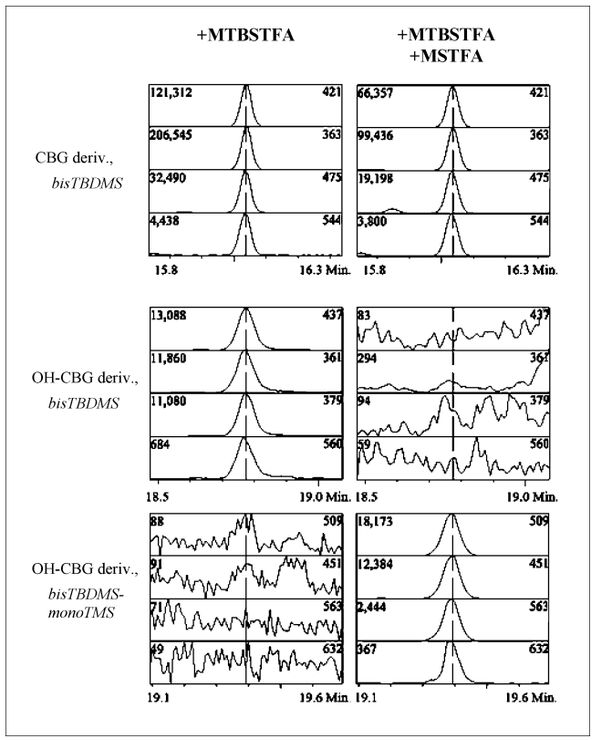

Fig. 4: Detection of the bisTBDMS derivative of CBG (1st row), the presumptive bisTBDMS derivative of OH-CBG (2nd row) and the presumptive bisTBDMS-monoTMS derivative of OH-CBG (3rd row) in human urine of a proved *Cannabis* consumer after enzymatic hydrolysis. For the sequential derivatisation 50 µl of MTBSTFA were added, then the derivatisation process was run, and after cooling down 50 µl MSTFA were added to run the second derivatisation (2nd column)

putative CBG-metabolites based on the study of Harvey and Brown (1990). We detected a compound in the trimethylsilylated (TMS) urine extract having fragment ions of m/z 425, 465 and 479 at the retention time of 14.19 minutes. This peak was only present in *Cannabis* consumers' urine samples positive for CBG (Fig. 3). It was absent in blank urine, and also in urine spiked with CBG or spiked with other cannabinoids mentioned above, showing that it occurs specifically in urine of *Cannabis* consumers. It was also not detected in any not hydrolysed urine samples, indicating that it is excreted as a glucuronyl conjugate, too. Since there is no reference substance available, only a presumptive identity of the component could be given. Based on ion fragments and the ion fragment ratios first reported by Harvey and Brown, the candidate structures are thought to be the 4″-hydroxy-CBG or the 5"-hydroxy-CBG (for numbering see the original paper). The complete identification of this metabolite needs more specific techniques.

However, some features of the presumed hydroxyl metabolite were certified with the following experiments. Sample extract of enzymatically hydrolysed urine samples of a *Cannabis* consumer was derivatised using MTBSTFA resulting the bisTBDMS derivative of CBG (fragment ions at m/z 421, 363, 475, 544 [$M^{\bullet+}$]). However, the fragments of the hypothetic trisTBDMS-OH-CBG could not be detected, calculated based on those of the presumed trisTMS-OH-CBG. Instead, we detected peak possessing abundant fragment ions at m/z 437, 361, 379, 560 [$M^{\bullet+}$], suggesting that it could be the bisTBDMS derivative of OH-CBG (Fig. 4, 1st column). After a second derivatisation with MSTFA, the peak of bisTBDMS-OH-CBG disappeared and a new peak with fragment ions of m/z 509, 451, 563 and 632 [$M^{\bullet+}$] emerged, indicating that there still was an active hydrogen in the molecule (Fig. 4, 2nd column). This compound is supposed to be bisTBDMS-monoTMS-OH-CBG. The mass differences of molecular ions and the base peaks m/z 509 and 437 of bisTBDMS-monoTMS-OH-CBG and bisTBDMS-OH-CBG, respectively, gave the same result (72 Da), showing the substitutional reaction of one TMS group. These results also show, that the OH group is not attached to the C-2′–C-9′ carbons since the base peaks (m/z 437 and 509) are due to the cleavage between C-1′ and C-2′. It is known that MTBSTFA reacts mainly with phenolic hydroxyl groups (Quintana et al. 2004). Hence the hydroxylation of CBG probably does neither occur on the aromatic ring since then MTBSTFA would derivatise all three hydroxyl groups. For the mass spectra of several silyl derivatives of the presumptive CBG metabolite, see Fig. 5.

3. Experimental

3.1. Chemicals

The reference materials of the analytes cannabigerol, cannabinol, cannabidiol and cannabichromene were provided by the United Nations Drug Control Program. Δ^9-THC-COOH and Δ^9-THC were purchased from Cerilliant® (Round Rock, TX).

β-glucuronidase (Type IX-A from *Escherichia coli*) and N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) was purchased from Sigma-Aldrich (Saint Louis, MO).

MethElute™ Reagent was purchased from Pierce (Rockford, IL).

N-(*tert*-butyl-dimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA), containing 1 % of tert-butyldimethylchlorosilane was provided by Alltech (Deerfield, IL). Other chemicals were of analytical grade.

3.2. Selection of urine samples

All the samples investigated were previously screened with the Abbott AxSYM immunochemical analysing system. Positive results were confirmed by an HPLC/DAD method (not given here in detail) following alkaline hydrolysis and solid phase extraction according to Application Note PH-005 of J.T.Baker® (U.S. Patent No 4,650,784). For further analysis we selected urine samples containing high (1 mg/ml<) Δ^9-THC-COOH concentration.

3.3. Enzymatic hydrolysis

For enzymatic hydrolysis we applied the modified method of Kemp et al. (1995). Before application, 25,000 units of β-glucuronidase were reconstituted in 1 ml of purified water. Then 2.5 ml urine sample was diluted by 2.5 ml of 0.1 M potassium phosphate buffer (pH 6.8). After mixing the sample 200 µl of β-glucuronidase (5000 units) were added and mixed thoroughly. Hydrolysis was performed at 37 °C overnight. The treatment of samples not enzymatically hydrolysed was the same in terms of adding buffer and overnight incubation, only enzyme preparation was omitted.

3.4. Extraction

After hydrolysis the samples were cooling down and alkalized by 250 µl of 5 M KOH and then extracted by 7 ml of *n*-hexane–ethyl acetate (7:1, *v/v*) for 10 min. Organic solvent was evaporated under a mild stream of N_2 at room temperature.

3.5. Derivatisation

For trimethylsilylation of the residues 100 μl of MSTFA were added and incubated at 80 °C for 30 min. For the analysis of underivatised compounds, residues were dissolved in 100 μl of methanol. For methylation the residues were dissolved in 100 μl of MethElute™ reagent (0.2 M trimethylanilinium hydroxide, methanolic soln., used for on-line flash methylation), and injected to GC injector immediately. For producing *tert*-butyldimethylsilyl (TBDMS) derivatives, MTBSTFA was used in the same manner as MSTFA.

3.6. Gas chromatographic–mass spectrometric (GC/MS) analysis

Analyses were performed by Shimadzu GC-2010 Plus gas chromatograph mass spectrometer equipped with GCMSsolution software. The gas chromatographic parameters were as follow: capillary column: HP-1MS of 25 m length, 0.2 mm inner diameter and 0.33 µm film thickness. Injector: 270 °C, oven initial temperature: 90 °C, hold time: 1 min, rate: 20 °/min, final temperature: 290 °C. Carrier gas: He (6.0), carrier gas pressure: 120 kPa, splitless time: 0.7 min, total flow: 23.4 ml/min, split ratio: 25, interface temperature:

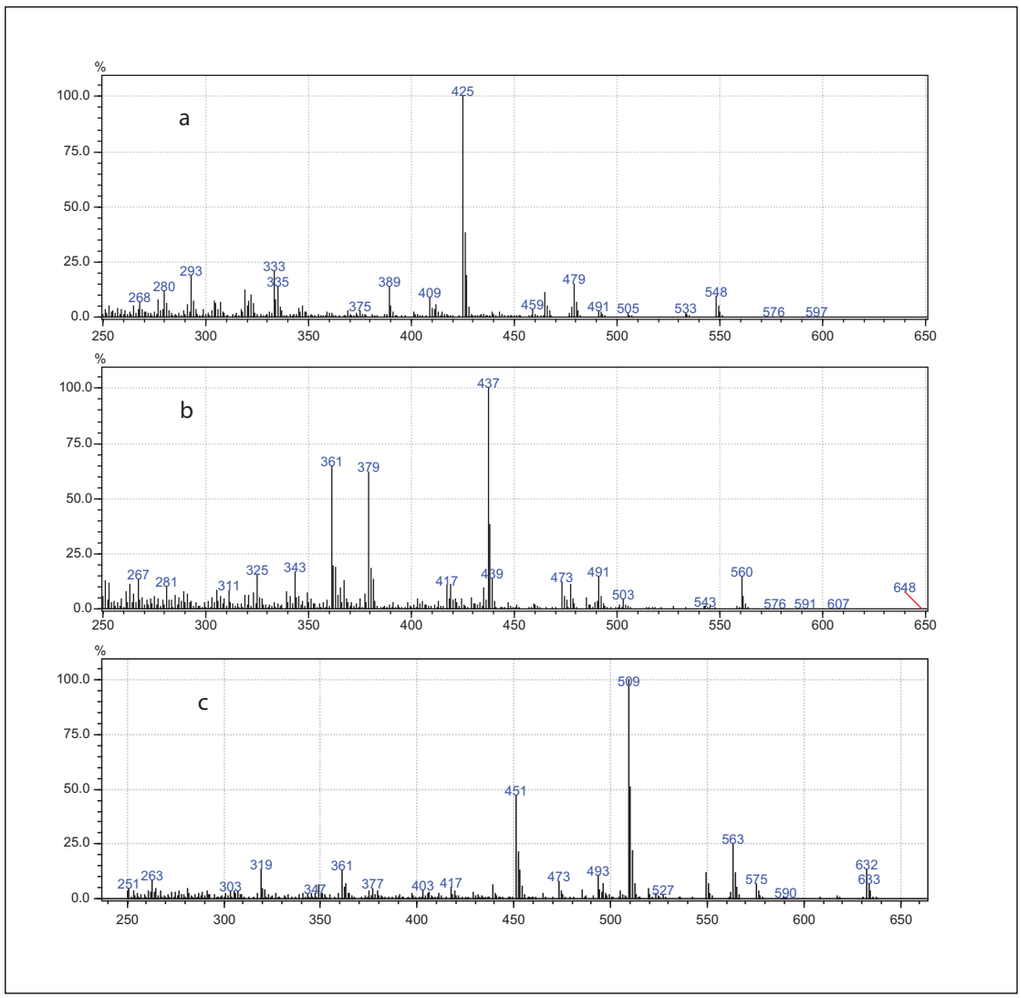

Fig. 5: EI mass spectra of three different silyl derivatives of the presumptive 4″-OH or 5″-OH metabolite of CBG. a) trisTMS, b) bisTBDMS, c) bisTBDMS-monoTMS. Mass range: m/z 250–650

270 °C, ion source temperature: 270 °C, ionization: EI at 70 eV. Compounds were mainly analysed in SCAN mode in the range of m/z range 50–500, unless indicated. 1 µl of samples were injected for all analyses. For the analysis of TBDMS derivatives a SIM method was developed monitoring the following fragment ions: m/z 361, 363, 379, 421, 437, 451, 475, 509, 544, 560, 563 and 632. During these analyses the following parameters were changed: splitless time: 0.5 min, split ratio: 30.

Acknowledgements: This research paper was presented during the 7th Conference on Retrometabolism Based Drug Design and Targeting, May 10–13, 2009, Orlando, Florida, USA.

References

Bodor N, Buchwald P (2004) Designing safer (soft) drugs by avoiding the formation of toxic and oxidative metabolites. Mol Biotechnol 26: 123–132.

Buchwald A, Browne CE, Wu WM, Ji F, Bodor N (2000) Soft cannabinoid analogues as potential anti-glaucoma agents. Pharmazie 55: 196–201.

Buchwald A, Derendorf H, Ji F, Nagaraja NY, Wu WM, Bodor N (2002) Soft cannabinoid analogues as potential anti-glaucoma agents. Pharmazie 57: 108–114.

Colasanti BK (1990) A comparison of the ocular and central effects of delta 9-tetrahydrocannabinol and cannabigerol. J Ocul Pharmacol 6: 259–269.

ElSohly MA, Feng S, Murphy TP, Ross SA, Nimrod A, Mehmedic Z, Fortner N (1999) Delta 9-tetrahydrocannabivarin (delta 9-THCV) as a marker for the ingestion of cannabis versus Marinol. J Anal Toxicol 23: 222–224.

Harvey DJ, Brown NK (1990) *In vitro* metabolism of cannabigerol in several mammalian species. Biomed Mass Spectrom 19: 545–553.

Harvey DJ, Mechoulam R (1990) Metabolites of cannabidiol identified in human urine. Xenobiotica 20: 303–320.

Harvey DJ, Samara E, Mechoulam R (1991) Urinary metabolites of cannabidiol in dog, rat and man and their identification by gas chromatography-mass spectrometry. J Chromatogr 562: 299–322.

Harvey DJ, Samara E, Mechoulam R (1991) Comparative metabolism of cannabidiol in dog, rat and man. Pharmacol Biochem Behav 40: 523–32.

Hattori H (1983) Detection of cannabinoids by gas chromatography/mass spectrometry (GC/MS). Part II. Quantitation of cannabidiol and cannabinol in human urine and blood plasma by GC/MS. Nihon Hoigaku Zasshi 37: 764–769.

Jung J, Kempf J, Mahler H, Weinmann W (2007) Detection of Delta9-tetrahydrocannabinolic acid A in human urine and blood serum by LC-MS/MS. J Mass Spectrom 42: 354–360.

Kelley JA, Arnold KP (1976) Detection of urinary *Cannabis* metabolites: a preliminary investigation. J Forensic Sci 21: 252–262.

Kemp PM, Abukhalaf IK, Manno JE, Manno BR, Alford DD, Abusada GA (1995) Cannabinoids in humans. I. Analysis of Δ^9-tetrahydrocannabinol and six metabolites in plasma and urine using GC-MS. J Anal Toxicol 19: 285–291.

Quintana JB, Carpinteiro J, Rodríguez I, Lorenzo RA, Carro AM, Cela R (2004) Determination of natural and synthetic estrogens in water by gas chromatography with mass spectrometric detection. J Chromatogr A 1024: 177–185.

Shoyama Y, Yagi M, Nishioka I (1975) Biosynthesis of cannabinoid acids. Phytochemistry 14: 2189–2192.

Williams PL, Moffat AC (1980) Identification in human urine of delta 9-tetrahydrocannabinol-11-oic acid glucuronide: a tetrahydrocannabinol metabolite. J Pharm Pharmacol 32: 445–448.