

Detection and Quantitation of Cannabinoids in Biological Specimens

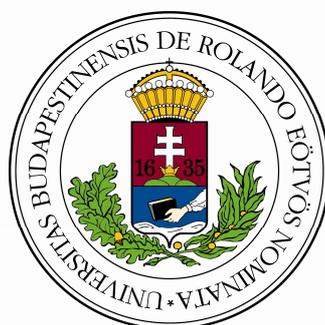
PhD Thesis

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Budapest
2013

1. Introduction

The amount of confiscated drugs of *Cannabis* origin (marijuana and hashish) and the number of positive blood and urine test results because of Cannabis consumption has been permanently high during the last 20 years in Hungary. According to the Penal Code, the use of the illicit *Cannabis* is a crime. One of the main functions of the **National Institute of Forensic Toxicology** (NIFT), where this dissertation was written, is to give expert opinion on criminal cases of human intoxication. Besides, most of the **blood and urine specimens** collected by the police are analyzed at the NIFT in order to **prove drug consumption** if misuse is suspected. Driving under the influence (DUI) of a drug that is detrimental to driving ability is also against the Penal Code. In these cases the **determination of impairment** is the task of an expert in forensic medicine who uses the results of the tests of the blood taken from the suspects, usually performed at NIFT. Because of the work of the forensic toxicologist experts at the NIFT involves serious legal consequences, the tests need to be as rapid, sensitive, extensive, and have as high a level of confidence as possible. Therefore the experts at NIFT also carry out research and development work. The study that is presented below also became necessary in the field of analysis of cannabinoids, the main pharmacologically active compounds in *Cannabis*.

Hemp is an annual, wind-fertilized, dioecious, short-day plant. Two species are distinguished within the genus: the wild grown *Cannabis (C.) ruderalis* (Janisch.) and the cultivated *C. sativa* L. However, *C. indica* is considered more and more as a distinct species as well. Both species contain terpenophenol type **cannabinoids**, however in different amounts and ratios. In particular, the glandular hairs on the surface of bract leaves covering the female flowers produce a resin, which contains mostly terpenes as well as cannabinoids. The resin contains the **psychoactive compound** **(-)-trans-(6aR,10aR)- Δ^9 -tetrahydrocannabinol (Δ^9 -THC)**. During the biosynthesis of cannabinoids, cannabinoid acids (e.g. Δ^9 -tetrahydrocannabinolic acid A, Δ^9 -THCA-A) are formed from cannabigerolic acid which is produced as a central compound. Homologues contribute to the high number of cannabinoids (>80 are known). For example, homologues may possess a butyl moiety at the C-3 position instead of a pentyl side chain. The cannabinoid content is determined mainly by genetic factors, but it depends on gene expression and the activity of biosynthetic enzymes as well. Only *C. sativa* containing high amounts of Δ^9 -THC is regarded as an illicit plant.

Marijuana is the most frequent form of the drug, prepared by drying flowering shoot tops of female *Cannabis* plants. In Hungary, 90 % of marijuana samples contain 0.02–12 % of

free Δ^9 -THC (2011). There are several types of *C. sativa* and drugs extracted from them for recreational use; most of them belonging to “sativa” and “indica” type. In addition, there are many hybrid types (*sativa* \times *indica*).

When abused as an illicit drug, mostly the smoke of the combusted drug is inhaled while cannabinoid acids undergo **thermal decarboxylation**. During this process Δ^9 -THC is formed from Δ^9 -THCA. The decarboxylated form of other cannabinoids and their metabolites may also be detected in the body fluids of *Cannabis* consumers. During metabolism the Δ^9 -THC is hydroxylated at C-11 and the hydroxyl group undergoes further oxidation to produce **(-)-11-nor-9-carboxy- Δ^9 -THC (Δ^9 -THC-COOH) as main metabolite**. The acid is then glucuronated, which helps excretion. The peak Δ^9 -THC concentration in blood, which may reach 200 ng/ml, occurs at about 9 minutes after smoking marijuana, and the concentration decreases quickly afterwards. However, the Δ^9 -THC-COOH concentration in blood reaches its maximal value only at about 30 minutes after smoking. The window of detection of Δ^9 -THC in blood plasma ranges from 3–27 hours if the limit of detection is 0.5 ng/ml. After it has reached its peak value in urine, the concentration of Δ^9 -THC-COOH decreases quite rapidly to 20–50 ng/ml, but then the rate of decrease becomes slower. Concentrations of Δ^9 -THC-COOH above 15 ng/ml may be detected in the urine of regular users for up to 2 weeks after the last administration of cannabis.

The most suitable method for the **extraction of cannabinoids from plant material** is solid-liquid extraction using an organic solvent. For separation of cannabinoids, there are thin layer chromatography (TLC) and many other more modern chromatographic techniques available. Using gas chromatography, thermal decarboxylation takes place in the inlet and the combined amounts of the neutral cannabinoids and their corresponding acidic forms are measured, unless derivatization is applied. Nowadays, gas chromatography is the preferred approach in forensic chemistry. TLC and high pressure liquid chromatography (HPLC) are suitable for the determination of “free” Δ^9 -THC-content, hence the quantitative result will be lower than in the case of gas chromatography.

The tetrahydrocannabinoid content of **blood** samples are usually determined by gas chromatography-mass spectrometry (GC/MS) methods in the selected ion monitoring (SIM) mode. The extraction is often achieved by liquid-liquid extraction (LLE) or solid phase extraction (SPE) using C18-modified cartridges. In the latter case elution is carried out using organic solvents. Sample preparation can also be performed in two steps: first, by a simultaneous liquid-liquid extraction and protein precipitation, followed by a solid phase

extraction. After extraction and evaporation, the final derivatization step usually consists of methylation, silylation or acylation.

The most convenient method to prove hashish or marijuana usage within a few days of consumption is the detection of **cannabinoids in urine**. The target analyte is mostly Δ^9 -THC-COOH. However, identification of other cannabinoids¹ in urine has also been reported. The most widely used extraction methods are LLE and SPE. For instrumental analysis, HPLC/UV, HPLC/DAD, GC/MS and HPLC/MS are most commonly used. In the case of gas chromatographic assay, formation of acyl, alkyl or silyl derivatives of Δ^9 -THC-COOH is necessary. For HPLC analysis of cannabinoids, reversed phase systems using C8 or C18 stationary phases are common.

2. Aims of Research

In order to raise the standard and improve its reliability of the forensic experts' work, the aims and objectives of the research described in this thesis were the following:

1. To develop overpressured layer chromatographic (OPLC) methods with high sample capacity that provide qualitative and accurate quantitative analysis of Δ^9 -THC, CBN, CBD, CBC and CBG in drugs of *Cannabis* origin, since the presently known OPLC methods with adequate sample capacity don't provide satisfactory baseline separation for all of the critical pairs of analytes.

2. To develop a sensitive, accurate and precise GC/MS method following SPE extraction for the determination of Δ^9 -THC, 11-OH- Δ^9 -THC and Δ^9 -THC-COOH in human blood. The new method should help in the interpretation of police cases related to driving under the influence of drugs (DUID), because the methods reported in the literature are either lengthy or their extraction recovery is low.

3–5. Besides Δ^9 -THC and its metabolites, to study the presence of acidic and neutral cannabinoids and their metabolites in the urine of *Cannabis* users, which may have a diagnostic role in analytical forensic toxicology. More precisely, to compare the Δ^9 -THC-COOH/ Δ^9 -THCV-COOH ratio in urine samples from populations in two different geographical regions and to identify CBG and its metabolites in the urine of *Cannabis* users.

¹ e.g. 11-nor- Δ^9 -tetrahydrocannabivarin-9-carboxylic acid (Δ^9 -THCV-COOH)

3. Experimental Methods

Plant materials were extracted using a mixture of methanol and chloroform. Dry, evaporated extracts were redissolved in the extracting solvent.

Blood specimens were precipitated and extracted with acetonitrile. Solid phase extraction of supernatants was achieved using C18-modified silica cartridges after dilution with aqueous phosphoric acid. Cartridges were eluted with a mixture of *n*-hexane/ethyl acetate.

Basic hydrolysis preceded the extraction of **acidic cannabinoids from urine**. The acidic analytes were extracted with a mixture of *n*-hexane/ethyl acetate at an acidic pH. The conjugates of **neutral cannabinoids** were hydrolyzed using β -glucuronidase at pH 6.8. The extraction was then performed at basic pH using a mixture of *n*-hexane/ethyl acetate. A portion of the enzymatically hydrolysed specimens were also extracted at acidic pH.

Some of the **dried blood and urine extracts** were dissolved in **methanol**. In other cases, *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide, *N*-(*tert*-butyl-dimethylsilyl)-*N*-methyl-trifluoroacetamide, or *N,N,N*-trimethylanilinium hydroxide were used for **derivatization**.

OPLC analysis of **plant extracts** was performed using unmodified or amino modified HPTLC silica gel plates. In the first case, a Personal OPLC BS50 instrument was used in bidirectional mode, using dichloromethane as mobile phase. In the case of HPTLC silica gel plates, a Chrompres 25 instrument was used to optimize the separation by applying several solvents, solvent switching and overrunning mode. Densitograms were obtained using a Desaga CD 60 slit scanner at 200 nm wavelength.

A Shimadzu GCMS QP5000 instrument was used to perform the analysis of cannabinoids in **blood**. Carboxylated cannabinoids in **urine** were analysed using a Varian STAR 3400/Varian MS Saturn 2000 ion trap GC/MS instrument. Analysis of neutral cannabinoids in urine was performed with a Shimadzu GCMS-2010 Plus GC/MS instrument equipped with an HP-1MS capillary column and helium carrier gas. In each case, samples were introduced in splitless mode.

4. Results

1/a. An OPLC-separation method for neutral cannabinoids (Δ^9 -THC, CBD, CBN, CBG and CBC) was designed and validated, and was applied to the detection and quantitative determination of these cannabinoids in hemp samples using amino modified HPTLC layers and dichloromethane as mobile phase. The chromatographic resolution was at

least 0.8 for every critical analyte pair. The relative standard deviations of retention factors (R_F values) were less than 6.5 %. The regression factors (r^2) of the calibration lines were greater than 0.99. Signal-to-noise ratios of 3.0 were obtained for each analyte when 50 ng was applied to the plate (Limit of Detection). The precision within development was less than 4.3 % in each case. The reproducibility of the assay was less than 5.3%. The average recovery values ranged between 92–98 %. **Simultaneous analysis of 30 specimens was feasible within 4 minutes.** Quantitative analysis of the investigated plant specimens resulted in CBC content of 0.02–0.24 %; CBG: 0.01–0.08 %, CBN: 0.01–0.30 %, Δ^9 -THC: 0.02–3.54 % and CBD: 0.00–0.59 %.

1/b. An alternative OPLC separation method for neutral cannabinoids using unmodified silica gel layers has been optimized. The separation of the neutral cannabinoids was achieved by use of a stepwise gradient. Initially, 25 neat organic solvents and 25 mixtures of a solvent and *n*-hexane were tested in normal chamber developments. Based on their selectivity, two of these were chosen for further optimization: chloroform and a mixture of methyl ethyl ketone/*n*-hexane (1:9, *v/v*). For the latter, the *n*-hexane content was optimized during OPLC development. The final optimal separation was carried out by applying 0.6 ml of chloroform at first, then suddenly switching to a mixture of methyl ethyl ketone/*n*-hexane (3.5/96.5; *v/v*) (stepwise gradient) which was overrun on the plate. The switching point corresponded to a chloroform migration distance of 30 mm. Development time was 21 minutes. The method was compared to other OPLC separation techniques which do not use overrun developments. My method was more selective and showed higher resolution than those. **The resolution of each pair of cannabinoids was higher than 1.9** [$R_S=2.0$ for the critical pair (CBG–CBC)].

2. Validation of a GC/MS method of determination of tetrahydrocannabinoids in human blood: the method has been proven selective and there were no interfering component in the blank sample. The detection limits were 0.6 ng/ml for Δ^9 -THC, 0.2 ng/ml for 11-OH- Δ^9 -THC, and 1.4 ng/ml for Δ^9 -THC-COOH, respectively. The correlation coefficient of the linear regression calibration line was >0.999 for each analyte. Relative precision and bias of quantitative determinations, calculated for concentrations of 10 and 50 ng/ml, ranged between 1.1–5.3 % and 101–104 %, respectively. **Extraction recovery was 85–99 %** for the three analytes at a concentration of 10 ng/ml.

3. The presence of Δ^9 -THCV-COOH in urine of cannabis users was proven indirectly. First, *bis*(*O*-TMS) derivatives of Δ^9 -THC-COOH and the presumed Δ^9 -THCV-COOH were synthesized, then the fragmentation patterns of these two homologues were compared. The

ratio of Δ^9 -THCV-COOH to Δ^9 -THC-COOH was studied. For this purpose, the peak area ratios of the corresponding components were calculated. Calculating relative observed standard deviation and using regression analysis, the eligibility of the Δ^9 -THC-COOH/ Δ^9 -THCV-COOH ratio for interpretation purposes was validated. The ratio proved stable in time and after sample dilution. **The Δ^9 -THC-COOH/ Δ^9 -THCV-COOH ratio was then determined for urine samples positive for Δ^9 -THC-COOH. 35 samples came from Hungary and 67 samples from Portugal.** The ratios showed lognormal distributions within both sample populations. This was proven by logarithmic transformation and using a Kolmogorov–Smirnov test. Next, it was proven using an F-test that the variance of the two normalized distributions was different at least at a 95 % of confidence level. Because of different variances, the mean values of two data sets were compared using a heteroscedastic T-test. This test resulted in rejecting hypothesis H_0 which assumes that the difference between mean values is 0 at a 99.999 % significance level.

4/a. For testing the presence of cannabigerol and its presumed metabolites in urine of *Cannabis* users, urine samples with high Δ^9 -THC-COOH concentrations were selected. **In the portions of the samples hydrolysed with glucuronidase, CBG was detected.** The identification of CBG was based on the EI mass spectrum and retention time in underivatized form and as *bis*-trimethylsilyl, *bis*(*O*-TBDMS)- and *bis*-methyl derivatives. CBG could not be detected in the following samples: blank urine, urine spiked with other cannabinoids, or unhydrolyzed urine samples from *Cannabis* users. The concentration of CBG was determined in 38 cases. The mean CBG concentration was 31 ng/ml.

4/b. During the analysis of the chromatograms of hydrolyzed and trimethylsilylated urine extracts, **one compound has been detected** that was present only in the urine of *Cannabis* users also positive for CBG, but was absent in blank urine, urine spiked with other cannabinoids and unhydrolyzed urine from *Cannabis* users. Based on fragment ions, their ratios and scientific references presenting the *in vitro* CBG metabolites it is inferred that **this compound was either 4''-hydroxy-CBG or 5''-hydroxy-CBG.** The unambiguous determination of the site of the hydroxy group could not be done due to the lack of appropriate reference materials. An attempt was made to solve this problem indirectly. Hence, the mass spectra of several derivatives of the detected metabolite were analyzed. During GC/MS measurements **two of the silyl derivatives of hydroxy-CBG (OH-CBG),** where the hydroxy group in question was trimethylsilylated, **showed an easily detectable methyl loss $[M-\bullet\text{CH}_3]^+$.** For further comparative investigations, two model compounds (4-phenyl-1-

butanol and 4-phenyl-2-butanol) were selected, which incorporate two potential forms of the required molecular fragment (hydroxy group either at chain-end or at position 2). The *O*-TMS derivative of 4-phenyl-1-butanol showed an intensive fragment ion at *m/z* 104, while for the *O*-TMS derivative of 4-phenyl-2-butanol, the relative intensity of the ion at *m/z* 117 was higher. For two of the silyl derivatives of OH-CBG² the *m/z* 117 fragment ion peak had higher relative intensity compared to *m/z* 104.

5. The method used for the analysis of CBG was applied to the analysis of other cannabinoids³ in 58 urine samples. No quantitative analyses were performed. However, the most characteristic fragment peaks of each analyte were integrated, the peak area values were converted to logarithms and correlated to each other, and then linear regression analysis was applied. It was found that the amount of CBG correlated with the amount of all other cannabinoids.⁴

5. Conclusions

1/a. High capacity, rapid and selective OPLC-separation of neutral cannabinoids⁵ is feasible on amino modified HPTLC layers using bidirectional development, as has been demonstrated by the system validation parameters. The method is suitable for determination of the free Δ^9 -THC content of confiscated hemp samples. By determining the other neutral cannabinoids as well, the hemp species (subspecies, type) can be ascertained. Specimens under investigation can be classified into two groups: marijuana samples with high Δ^9 -THC-content and hemp samples with high CBD-content. **The main advantage of the method is the special bidirectional development**, where the mobile phase application from two distal edges of the layer towards the midline does not result the overrunning of the solvent fronts since the overpressure sensor stops solvent delivery automatically. Hence the capacity of the assay increased as well: up to 30 samples can be analyzed in 190 seconds.

1/b. The studied neutral cannabinoids can be separated with high resolution using an OPLC method optimized for silica gel HPTLC layers which applies stepwise gradient and overrunning of the mobile phase. The method is suitable for analyzing extracts of

² *tris(O-TMS)* and *bis(O-TBDMS)-mono(O-TMS)-*

³ CBD, CBN, Δ^9 -THC, 11-OH- Δ^9 -THC, Δ^9 -THCV-COOH and Δ^9 -THC-COOH

⁴ The correlation with Δ^9 -THC was the highest ($r^2=0.71$), and with Δ^9 -THC-COOH the lowest ($r^2=0.21$).

⁵ Δ^9 -THC, CBD, CBN, CBG and CBC

confiscated marijuana samples. The optimized method comprises some innovations (new eluent composition, eluent switching and development by overrunning the mobile phase).

2. The GC/MS method validated in this work provides outstanding extraction recoveries, sensitive detection and accurate quantification of Δ^9 -THC, 11-OH- Δ^9 -THC and Δ^9 -THC-COOH in blood samples. The method fulfils the GTFCh guidelines.⁶ Besides, it achieves acceptable bias, precision and linearity and shows higher extraction recoveries than other methods. The measured concentrations of the analytes in forensic cases correlated well with clinical symptoms observed during the withdrawal of blood samples. This method is used by the NIFT as an accredited routine assay for the analysis of hundreds of specimens annually.

3. A comparison of two sets of urine samples from different geographical areas based on Δ^9 -THC-COOH/ Δ^9 -THCV-COOH ratios found that the **mean values of the distributions are significantly different** at the 95 % level of confidence. The reason for the difference is likely to be that the marijuana available in these two districts has different Δ^9 -THC/ Δ^9 -THCV ratios. Thus conclusions can be drawn indirectly on the consumed drugs based on the analysis of the cannabinoid profile of the urine samples.

4/a. Urine analyses show that **CBG enters the human body** during *Cannabis* use and it can be detected in urine. It is **excreted mainly in glucuronidated form**.

4/b. **One of the monohydroxylated metabolites of CBG can be detected in extracts of hydrolyzed urine from *Cannabis* users.** Based on published data and mass spectrometric analysis (loss of a methyl group in the mass spectra of two different *O*-TMS derivatives and comparison with the fragmentation of model compounds) it is **tentatively concluded that the detected metabolite is 4'-hydroxi-CBG**. However, no reference standards of any of the suspected forms of the metabolite are available. This metabolite is also excreted mainly in glucuronidated form.

5. Cannabinoids of acidic and basic character can be investigated and detected simultaneously in urine of *Cannabis* users. The relative amounts of cannabinoids (“cannabinoid spectrum”) differed widely in the 50 urine samples studied.

⁶ Namely that the assay has to be able to determine all three tetrahydrocannabinoids and that the LOD values are <1.5 ng/ml.

6. Papers having impact factor underlying dissertation

1. Szabady, B.; **Hidvégi, E.**; Nyiredy, Sz. (2002): Determination of neutral cannabinoids in hemp samples by overpressured-layer chromatography. *Chromatographia Suppl.* 56, S165-S168. **Impact Factor (IF)=1,230**
2. **Hidvégi, E.**; Somogyi, G. P. (2010): Detection of cannabigerol and its presumptive metabolite in human urine after *Cannabis* consumption. *Die Pharmazie* 65: 408-411. **IF=0,869**

7. Other papers underlying dissertation

1. **Hidvégi, E.**; Szabady, B. (2002): Some aspects of OPLC development using mobile phase switching. Proceedings of the 8th International Symposium on Separation Sciences, Toruń, Poland, 8-12 September.
2. **Hidvégi, E.** (2005): Fontosabb tetrahidrokannabinoidok meghatározása humán vérplazmában gázkromatográf-tömegspektrometriás módszerrel. Záródolgozat, Budapesti Műszaki és Gazdaságtudományi Egyetem, Vegyészmérnöki Kar.
3. **Hidvégi, E.**; Kerner, Á.; Mikó-Hideg, Zs.; Somogyi, G. (2005): Determination of main tetrahydrocannabinoids in human plasma by use of GC/MS. 6th Balaton Symposium on High Performance Separation Methods, Siófok, Hungary, 7-9 September.
4. **Hidvégi, E.**; Penczi, E.; Hideg, Zs.; de Boer, D.; Somogyi, G. (2006): Analysis of certain cannabinoid metabolites in urine samples from Hungary by GC/MS after alkaline and enzymatic hydrolysis. TIAFT 44th International Meeting, Ljubljana, Slovenia, 26 August-1 September.
5. **Hidvégi, E.**; Hideg, Zs.; Somogyi, G. P. (2007): Identification of cannabigerol in human urine after *Cannabis* consumption. 7th Balaton Symposium on High Performance Separation Methods, In Memoriam Szabolcs Nyiredy, Siófok, Hungary, 5-7 September.

8. Other publications in the field of dissertation

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2. de Boer, D.; Bosman, I. J.; Hidvégi, E.; Manzoni, C.; Benkő, A. A.; dos Reys, L. J. A. L.; Maes, R. A. A. (2001): Piperazine-like compounds: a new group of designer drugs-of-abuse on the European market. *Forensic Sci. Int.* 121: 47-56. **IF=1,520**
3. Hidvégi, E.; Fábrián, P.; Hideg, Zs.; Somogyi, G. (2006): GC-MS determination of amphetamines in serum using on-line trifluoroacetylation. *Forensic Sci. Int.* 161: 119-123. **IF=1,397**
4. Hidvégi, E.; Hideg, Zs.; Somogyi, G. P. (2008): Different reactivity of amphetamines with N-methyl-bis(trifluoroacetamide) in heated gas chromatographic injectors. *Die Pharmazie* 63: 233-234. **IF=0,858**
5. Dobos, A.; Hidvégi, E.; Somogyi, G. P. (2012): Comparison of five derivatising agents for the determination of amphetamine type stimulants in human urine by extractive acylation and gas chromatography-mass spectrometry. *J. Anal. Toxicol.* 36: 340-344. **IF=2,022**