DEVELOPMENT OF THE UHPLC-DAD METHOD FOR SIMULTANEOUS DETERMINATION OF FOUR ACTIVE PHARMACOLOGICAL INGREDIENTS IN CANNABINOID PLANT MATERIAL

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Abstract: A stability-indicating ultra-high-performance liquid chromatography (UHPLC) with DAD detector was developed and validated for the simultaneous determination of Δ^9 -tetrahydrocannabinol (THC), cannabidiol (CBD), Δ^9 -tetrahydrocannabinolic acid (THC-COOH) and cannabidiolic acid (CBD-COOH) in extracts of *Cannabis sativa* L. The separation was achieved on a Kinetex, C18, 100 x 2.1 mm, 2.6 μ m. The gradient separation was started with a mixture of methanol and 12 mM ammonium acetate in ratio 75 : 25 (%/%). The methanol was increased to 100% from 0.0 min to 10.0 min, then maintained 100% for the next 1.0 min, then decreased to 75% from 11.0 min to 15.0 min and held at 75% from 15.0 min to 20.0 min. The injection volume was 5.0 μ L and the wavelength of detection was controlled at 228 nm.

Keywords: cannabinoid extract, UHPLC-DAD, validation

Extract of *Cannabis sativa* L. can contain many groups of C₂₁ terpenophenolic compounds (1). Actually, one can observe growing interest in the medical use of cannabinoids, particularly predominant natural cannabinoids. From the point of view of pharmacological activity, the most important are: Δ^9 -tetrahydrocannabinol (THC) and cannabidiol (CBD). Due to the psychoactive properties of THC, its use in drugs is limited. In contrast to THC, CBD is not a psychoactive compound and have promising properties for the therapeutical application such as anti-seizure, antioxidant, neuroprotective, anti-inflammatory, analgesic, anti-tumor, anti-psychotic, and anti-anxiety (2). In the plant tissues, those cannabinoids are biosynthesized from acidic forms: Δ^9 -tetrahydrocannabinolic acid (THC-COOH) and cannabidiolic acid (CBD-COOH) because carboxylic forms are easily susceptible to degradation under the influence of temperature, light and oxidation factor (Fig. 1) (3). Moreover, THC under the

influence of heat and light can also be easily transformed by oxidation into cannabinol (CBN). Aforementioned degradations can take place during heating, drying of harvested plant material during its storage as well as during preformulation and formulation studies with their participation (4). Therefore the development of analytical methods for simultaneous determination of THC, THC-COOH, CBD, CBD-COOH in plant materials being the source for development of pharmaceutical dosage forms is so important.

In previous studies, chromatographic methods: GC, TLC, and HPLC have been applied for determining of selected cannabinoids in plant materials (5). The application of GC methods for the simultaneous analysis of THC/THC-COOH and CBD/CBD-COOH is limited due to significant instability of carboxylic forms and their conversion even in condition of analysis. The usage of TLC method in combination with fast blue B spray

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Figure 1. Chromatograms of a non-degraded mixture of THC, THC-COOH, CBD, CBD-COOH (a), chromatograms of cannabinoid extracts (b-c)

reagent for distinguishing and detecting 10 cannabinoids was reported but determination of concentration changes of them in plant materials has not been presented yet (6). In the literature, a few papers have presented a HPLC method for determination of selected cannabinoid compounds (7, 8). However all of these methods utilized classical stationary phase known from reversed-phase liquid chromatography and mass spectrometers as detectors. Disadvantages of these solutions are long time of analysis, high-cost resulted from the usage of mass spectrometer detector and finally organic solvents as ingredients of the mobile phase. It is worth noting that most of them were not validated according to recent ICH guidelines, especially in regards to selectivity of the method in the presence of degradation products formed during acidic-basic hydrolysis, oxidation, and thermolysis. So far only one report has presented an UHPLC procedure with tandem mass spectrometry for cannabinoid determination in blood (9). Therefore the aim of the presented study was to develop and validate fast UPLC-DAD method for the quantification of THC, THC-COOH, CBD and CBD-COOH in extracts of *Cannabis sativa* L. allowing to separate them successfully.

EXPERIMENTAL

Chemicals and reagents

Cannabinoid reference standards of THC and THC-COOH were purchased from THC Pharm GmbH (Frankfurt, Germany) with chromatographic purity = 99%. Cannabinoid reference standards of CBD and CBD-COOH with chromatographic purity = 99% were applied from LOGiCal (Middlesex, UK).

Hydrochloric acid, sodium hydroxide solution, hydrogen peroxide, ammonium acetate and all other chemicals were obtained from P.O.Ch. (Poland). Methanol of an HPLC grade was supplied by Merck KGaA (Germany) and formic acid (100%) by P.O.Ch. (Poland). High-quality pure water was prepared using an Exil SA 67120 Millipore purification system (France).

Sample preparation of cannabinoid extracts

During the first stage of the studies, the cannabinoid extracts were obtained in the result of maceration of *Cannabis sativa* L. materials with the use of different solvents (methanol, ethanol, water in selected ratios). The influence of extraction parameters such as temperature, shaking, ultrasounds, and darkness on the efficiency of applied method were tested. The content of THC/THC-COOH and

CBD/CBD-COOH in obtained extracts should be determined because they were genetically modified to achieve a lower level of THC and a higher level of CBD.

UHPLC equipment and chromatographic conditions

The Dionex UltiMate 3000 analytical system was used for determination and identification of THC, THC-COOH, CBD, and CBD-COOH. The chromatograph was composed of a Dionex UltiMate 3000 RS Pump (LPG-3400RS), Dionex UltiMate 3000 RS Autosampler (WPS-3000TRS), Dionex UltiMate 3000 RS Column Compartment (TCC-3000RS) and Dionex UltiMate 3000 Diode Array Detector (DAD-3000). As a stationary phase the Kinetex, C18, 100A, 100 × 2.1 mm column (Phenomenex, Torrance, USA) with 2.6 µm particle size was used. The mobile phase was composed of methanol and 12 mM ammonium acetate with gradient elution. The gradient separation was started with a mixture of methanol and 12 mM ammonium acetate in ratio 75 : 25 (%/%). The methanol was increased to 100% from 0.0 min to 10.0 min, then maintained 100% for the next 1.0 min, then decreased to 75% from 11.0 min to 15.0 min and held at 75% from 15.0 min to 20.0 min. The mobile phase flow rate was 0.5 mL min⁻¹. The wavelength of DAD detector was set at 228 nm and the column temperature was 30°C. Before the UHPLC-DAD analysis, each sample of cannabinoids extract and standard solution was filtered by FilterBio® Nylon Syringe Filters with 0.45 µm pore size and cooled to 5°C. During forced degradation studies, the cannabinoid reference standards of THC, THC-COOH, CBD and CBD-COOH and extracts of *Cannabis sativa* L. were tested separately and in a mixture.

Method validation

The method was validated according to the International Conference on Harmonization Guidelines (10). It comprised selectivity, linearity, precision, limits of detection (LOD) and quantitation (LOQ) in regards to THC, THC-COOH, CBD, and CBD-COOH determination. All measurements were performed triplicates. The selectivity of simultaneous determination of THC, THC-COOH, CBD, and CBD-COOH was examined for non-degraded and degraded samples. The selectivity of cannabinoid determination was examined after forced degradation studies in acidic conditions (0.1 M HCl), in a solution of sodium hydrochloride (0.1 M NaOH) and after oxidation (2% H₂O₂ at 353 K). The forced degradation studies of solvents of plant mate-

rials were carried in the same conditions. The linearity was tested at six concentration levels (THC and THC-COOH) and eleven concentration levels (CBD, CBD-COOH). For each concentration level, the appropriate amount of cannabinoid compounds were dissolved and introduced into the column. The linearity was studied in the concentration ranges from 0.064 – 0.12 µg (20-120% of nominal concentration) for THC and THC-COOH and 0.10–0.60 µg (20-120% of nominal concentration) for CBD and CBD-COOH. The precision of the assay of THC, THC-COOH, CBD, CBD-COOH was determined in relation to repeatability (intra-day). In order to evaluate the repeatability of the method, nine samples were determined during the same day for three concentrations of cannabinoid compounds. LOD and LOQ parameters were determined from the regression equations of THC, THC-COOH, CBD, and CBD-COOH. $LOD = 3.3 S_y/a$, $LOQ = 10 S_y/a$; where S_y is a standard error and a is the slope of the corresponding calibration curve. The impact of the composition of the mobile phase: percentage of methanol in the mobile phase and ammonium acetate, the flow rate of mobile phase (1.0 ± 0.1 mL/min), absorption wavelength (228 ± 5 nm) and column temperature ($25 \pm 1^\circ\text{C}$) on the peak area and shape of THC, THC-COOH, CBD, and CBD-COOH were determined.

RESULTS AND DISCUSSION

Method validation

The UHPLC procedure was developed in regards to stability-indicating assay of THC, THC-

COOH, CBD and CBD-COOH in plant materials of *Cannabis sativa* L. which were genetically modified to achieve lower level of THC and higher level of CBD. The chromatographic separation of THC, THC-COOH, CBD, CBD-COOH and their degradation products was achieved on a Kinetex, C18, 100A, 100×2.1 mm column with 2.6 µm particle size was used at 25°C . A mixture of 12 mM ammonium acetate and methanol with gradient change at flow rate of 0.5 mL/min was used as a mobile phase. The gradient separation was started with a mixture of methanol and 12 mM ammonium acetate in ratio 75 : 25 (%/%). The methanol was increased to 100% from 0.0 min to 10.0 min, then maintained 100% for the next 1.0 min, then decreased to 75% from 11.0 min to 15.0 min and held at 75% from 15.0 min to 20.0 min. The wavelength of detection was set at 228 nm. The chromatograms of non-degraded and degraded samples of THC, THC-COOH, CBD, CBD-COOH, and extracts of plant materials are shown in Figure 1. On chromatograms of non-degraded mixture of cannabinoid reference standards and extracts of plant materials, the following peaks originating from THC, THC-COOH, CBD, CBD-COOH were observed at retention times, $t = 4.58$ min, $t = 0.81$ min, $t = 2.55$ min, $t = 0.41$ min, respectively. The degradants of THC, THC-COOH, CBD, CBD-COOH were not interfering with the main peaks although on the chromatograms of cannabinoid reference standards after addition of oxidizing factor any peaks were not observed. In our the UHPLC-DAD method, it was possible to obtain shorter retention times for THC, THC-COOH, CBD, CBD-COOH ($t_{\text{analysis}} < 5$ min) in comparison to

Table 1. Validation parameters of determination of THC, THC-COOH, CBD, CBD-COOH.

	THC (4)	THC-COOH (2)	CBD (3)	CBD-COOH (1)
Retention time (min)	4.58	0.81	2.55	0.48
Range of linearity (µg)	0.064-0.12	0.064-0.12	0.10-0.60	0.10-0.60
Regression equation (y):				
Slope ($a \pm S_a$)	73.32 ± 0.28	33.45 ± 0.92	70.63 ± 1.49	7.41 ± 0.51
Correlation coefficient (r)	0.9999	0.9997	0.9984	0.9981
n	6	11	6	11
LOD (µg/mL)	0.0070	0.014	0.113	0.037
LOQ (µg/mL)	0.021	0.045	0.034	0.113
Intra-day precision (RDS, %)				
(80%)	1.03	0.30	2.20	2.05
(100%)	1.18	0.55	1.56	0.98
(120%)	0.79	0.043	2.27	0.83

S_a standard deviation of slope; S_b standard deviation of intercept, t , calculated values of Student's t test, $t_{\alpha,f} = 2.1709$ critical values of Student's test for degrees of freedom $f = 11$ and significance level $\alpha = 0.05$

values received with HPLC ($t_{\text{analysis}} > 20$ min). The use of the UHPLC-DAD method ensuring a high selectivity of THC, THC-COOH, CBD, CBD-COOH determination in the presence of products without using buffers allowed an analysis of degradation products by means of an EIS-Q-MS mass spectrometer.

The method was also validated for linearity, intra-day, and precision, robustness, LOD, LOQ for the assay of THC, THC-COOH, CBD, and CBD-COOH in plant materials of *Cannabis sativa* L. The calibration plots were linear for cannabinoid reference standards in the ranges 0.064 – 0.12 µg (THC, THC-COOH) 0.10–0.60 µg (CBD, CBD-COOH). They were described by the equations $y = ax$. The b values, calculated from the equation $y = ax + b$, were insignificant because of being lower than the critical value $t_b = b/S_b$ (Table 1). Precision of the UHPLC-DAD method was characterized by relative standard deviation (RSD, %) which was calculated for six/eleven consecutive measurements at three concentration level 80%, 100% and 120% of standard solutions during the system suitability test. All values which characterize the intra-day precision of determination of THC, THC-COOH, CBD, and CBD-COOH were less than 2% and meet the criteria required for the precision of the method. A comparison of validation parameters obtained in the present study with those reported previously demonstrated that the newly developed method is satisfied sensitive, provides a wider range of linear relation between analyte concentration and a detector signal, and allows receiving limits of quantitation and detection suitable for THC, THC-COOH, CBD and CBD-COOH determinations in plant materials. With regard to % RSD reflecting the intra-day precision of the methods compared, HPLC procedures proved superior. The robustness of the UHPLC-DAD procedure was evaluated after changing the mobile phase composition methanol and ammonium acetate, their contents and changes in mobile phase, flow rate (0.5 ± 0.1 mL/min), absorption wavelength (224 ± 5 nm) and column temperature ($25 \pm 1^\circ\text{C}$). Significant differences were established for those parameters studied in the investigations, therefore, optimal parameters were chosen for our determinations.

Analysis

The present method was applied to the analysis of aqueous and alcoholic cannabinoid extracts. The preparation of these extracts was the same as described above. Under applied chromatographic conditions, it was possible to determine the THC, THC-COOH, CBD, and CBD-COOH levels in pres-

ent degradation products which were formed in the result of degradation of cannabinoid reference standards and other ingredients present in extracts. Cannabinoid concentrations of these samples of cannabinoid extracts were possible to determine after comparison with calibration, linear plots of cannabinoid reference standards. It was possible to notice that way of extraction (usage of selected solvents/temperature) significantly influences on the levels of THC and CBD while TH-COOH and CDB-COOH determinations did not change. Together with decrease of THC peak, a new peak appeared on the chromatogram at $t_r = 1.75$ min. Based on literature reports on the direction of THC degradation and elution order of analytes (CBD-COOH > THC-COOH > CBD > THC) in the applied chromatographic separation, it is possible to suggest that cannabinol is formed from THC under selected applied conditions of extraction (Backer et al., 2009).

CONCLUSIONS

A simple and cost-efficient the UPLC-DAD method for the simultaneous determination four important ingredients: THC, THC-COOH, CBD, and CBD-COOH of cannabinoid extracts have been successfully developed, validated and applied to determination of their determination in cannabinoid extracts of *Cannabis sativa* L.

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Conflicts of interest

Authors declare that they have no conflict of interest.

This article does not contain any studies with animals performed by any of the authors. This article does not contain any studies with human participants or animals performed by any of the authors.

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