Cannabinoid analysis in humans

Methodology used in evaluation of cannabinoids for publication in Journal of Medical Phyto Research (ISSN: 2577-6541) Vol. 2, Article 1, (1-13)

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Preparation of standard solutions

Stock solutions (1mg/mL in methanol) of the analytes (THC, CBD, 11-OH-THC) were diluted in methanol to obtain working solutions at ten different concentration levels in the range of 10–1000 pg/L for THC/11-OH-THC and 5–1000 pg/L for CBD. An IS was also prepared by diluting a stock solution (0.1 mg/mL in methanol) of THC-d3 , 11-OH-THC-d3 with methanol up to the concentration of 50 pg/L for THC-d3 and 11-OH-THC-d3. All solutions were stored at −20 C until use.

LC–MS/MS conditions

LC analyses were performed on an Agilent 1200 LC system consisting of a binary pump, a thermostated auto sampler, an on-line degasser and a thermostated column compartment (Agilent, Waldbronn, Germany). Samples were analyzed on a Kinetex EVO C18 column (100 × 2.1 mm; 5 m particle size) (Phenomenex, Bologna, Italy). The mobile phase was composed of (A) 2.0mM aqueous ammonium acetate and (B) acetonitrile using the following gradient program: 0.0–10.0 min, linear gradient from 30 to 90% (B); 10.0–15.0 min, isocratic at 90% (B), 15.0–18.0 min, linear gradient from 90 to 30% (B). A pre-equilibration period of 2.0 min was applied between each run. The flow-rate was 0.35 mL/min and the column temperature was 40 C. The injection volume was 25 L and the injector needle was washed with methanol/0.05%formic acid in water (1:9 v/v); the autosampler was maintained at room temperature. The chromatographic conditions were optimized by analyzing the standard solutions and extracts of whole blood spiked with the target analytes.

Tandem mass spectrometry was performed using a SCIEX API 4000 QTRAP mass analyzer equipped with a Turbo Ion Spray source (SCIEX, Toronto, Canada) operating in electrospray ionization (ESI) positive/negative mode. The Analyst

Software (version 1.5.2) was used for instrument control, data acquisition, qualitative and quantitative data analyses. Detection and quantitation of all analytes were accomplished using multiple reaction monitoring mode (MRM) due to the achieved high selectivity and sensitivity. Optimized instrument settings were as follows: ionization mode, positive and negative; curtain gas, 10 psi; CAD gas, 4 psi; nebulizer gas (GS1), 35 psi; heater gas (GS2), 45 psi; ion spray voltage, 4000 V; temperature, 450 ○ C. The nitrogen flow was produced by a gas gen- eration system (Nitrogen Generator model 75–72, Whatman Inc., MA, USA). A dwell-time of 100 msec was used for all transitions. MS/MS parameters were optimized by direct infusion of each indi- vidual analyte at 100 ng/mL in the initial LC mobile phase at a flow rate of 10 L/min. The validity of the chosen MRM transitions was also verified by LC–MS/MS analyses of blank whole blood samples spiked with the individual analytes at 100 ng/mL. The mass spectrometer was calibrated to <2.0 mDa mass error prior to each batch analysis.

Blood extraction and sample preparation

Aliquots of whole blood (200 L) were transferred into a 1.5 mL polypropylene centrifuge tube. Then 20 L of IS solution and 900 L of 0.1% (v/v) formic acid solution in acetronitrile:methanol 70:30 (v/v) was added to each sample. The samples were vigorously shaken for 1.3 min using an automatic shaker and centrifuged for 7 min at 6000 rpm. The supernatant was collected and loaded for the purification onto a PhreeTM phospholipid removal tube (1 mL). The extract obtained was evaporated to dryness under nitrogen stream at 55 ○ C. The residue was dissolved in 150 L of 2.0 mM aqueous ammonium acetate:acetonitrile (70:30, v/v). The samples were transferred to auto sampler vials for HPLC analysis. A 25 L aliquot of each sample was injected into the chromatographic system.

Validation

The present study was validated in compliance with Scientific Working Group of Forensic Toxicology (SWGTOX) standard practices for method validation in forensic toxicology [26].

The following parameters were evaluated: selectivity, calibration model, limit of detection (LOD), lower limit of quantitation (LLOQ), precision, accuracy, carryover, matrix effect, recovery and dilution integrity.

Calibration and quality control samples

To aliquots of blank whole blood (200L) were added 20L of the IS solution and an appropriate volume of working solution. The samples were subjected to the described sample processing in order to have 6 calibration levels in the range 1–100pg/L for THC, 11-OH-THC, and 0.5–100 pg/L for CBD. Quality control (QC) samples were prepared by spiking blank whole blood (200L) with 20 L of the IS solution and an appropriate volume of the working solution at two concentration levels. These QC samples were processed as described above; the final concentration in the samples was 2 and 50 ng/mL for CBD, THC, and 11-OH-THC whole blood for low (LQC), and high (HQC) levels, respectively (n = 5 for each level).

Selectivity

Aliquots (200 L) of blank whole blood specimens were added to 100L of methanol and processed as described above. These blank samples were individually assessed for the presence of any interference across the retention window of each analyte and the IS.

Calibration Model

The processed calibration samples were analyzed in triplicate (injection volume: 25 L). Calibration curves were generated from the peak-area ratio of each analyte quantifier transition to the deuterated IS; the ratio was then plotted on the y-axis against the nominal analyte concentration to generate the standard curves by the method of least squares using a weighed (1/x) linear regression model.

LOD and LLOQ

Analyte identification criteria included a symmetric peak eluting within ±0.2min of average calibrator retention times with signal:noise of at least 3:1 ratio qualifier/quantifier MRM transition peak area ratio within ±20% average calibrator ratios. The sensitivity of the developed analytical procedure was evaluated by determining the LOD and the LLOQ. Fortified samples at decreased concentrations (n=3) were prepared and subjected to the described sample processing. LOD value for each analyte was estimated as the lowest concentration that achieved acceptable predefined detection and identification criteria. The LLOQ value was the lowest concentration fulfilling LOD criteria, while maintaining a bias of ±20% and CV < 20%.

Precision and accuracy

The precision and accuracy were evaluated at two levels, LQC (2ng/mL for THC, CBD, and 11-OH-THC) and HQC (50 ng/mL for THC, CBD, and 11-OH-THC).

Method precision and accuracy were determined by replicate analyses of the QC samples spiked at low and high levels (n = 5, each). Each sample was analyzed repeatedly three times within a single day to determine the intra-day precision, and

three times a day for five successive days (n = 15) to determine the inter-day precision. The precision was expressed as the coefficient of variance (%CV).

Accuracy of the method was evaluated by comparing the levels found in whole blood samples after clean-up procedure with the nominal analyte concentration; the obtained values are expressed as percent of the estimated concentration (bias).

Carryover

Carryover effect was evaluated by injecting extracts of blank whole blood samples after analyses of calibration samples spiked at the upper limit of quantitation. For acceptance, the peak areas of the blank sample should not exceed 10% of the peak areas obtained for the lowest calibrator.

Matrix effect and recovery

Matrix effect was evaluated for each analyte analyzing the QC samples spiked at low, medium and high concentrations. ME values were calculated by dividing the analyte peak area in QC samples spiked after the clean-up procedure (post-spiked samples) to the response for neat standards.

Recovery values were determined by comparing the levels found in QC samples spiked before the sample processing with the levels found in the post-spiked samples.

Dilution integrity

To validate the dilution integrity, blank whole blood samples (200 L) were spiked at 5 times the highest validation sample and mixed with additional blank blood to achieved a 5-fold (n = 6), 10- fold (n = 6), 15-fold (n = 6) dilution; internal

standard was added and samples were processed. The obtained samples were evaporated, reconstituted in the LC mobile phase and analyzed against the calculated calibration curves to assess if the performance criteria were still met.

Stability

The short-term stability was determined for LQC and HQC sam- ples in processed samples for 24 h at room temperature (n = 3). Stability of the drugs in processed samples was assessed after 24 h in the autosampler at 10 ○ C (n = 3). The drugs were considered sta- ble if the mean concentration was within ±15% of the nominal concentration.

Liquid Chromatography

One aim of this work was to develop a simple and rapid analytical method for the quantitative determination of CBD, THC, and 11-OH-THC in whole blood. To this end, the best performance for the determination of cannabinoids in biological matrices have been achieved employing C18 columns with gradient elution. In this study, the chromatographic performance of different columns was evaluated. The presence of ammonium acetate (2 mM) in the mobile phase provided a better reproducibility of retention times. Given the better chromatographic performance (in terms of both resolution and sensitivity), a shorter analysis time, Kinetex 5 u EVO (100 × 2.1 mm, 5 m, Phenomenex) column was finally selected for this study.

Using the gradient elution described in the experimental section the observed retention times were: 10.52 min for THC, 9.44 min for CBD and 7.68 min for 11-OH-THC. The relative standard deviation (RSD%) values less than 0.3% for all analytes and the IS indicated a good repeatability.

The retention time (RT) was the mean of at least 50 different individual analyses.

Mass Spectrometry

Multiple reaction monitoring (MRM) mode was used to carry out the quantitative analyses due to the achieved high selectivity and sensitivity. For each analyte, one precursor ion and one (or two) MRM transitions were set up, monitoring the more abundant product ion for quantitation (quantifier ion) and the less abundant product ions as qualifier ions for confirmation. The LC–MS/MS chromatograms of a blank whole blood sample spiked with IS in positive and negative ionization mode are in the reports attached.

Clean up procedure

The clean-up procedure was quick, efficient and reproducible as shown by the results of the method validation described in the following paragraph.

Partnerships

This report describes the development and full in-house validation of a high throughput method based on an optimized rapid PL removal procedure combined with a robust and highly sensitive LC–MS/MS detection technique, designed for the simultaneous determination and quantification of CBD, THC and 11-OH-THC in whole blood.

As far as we know, we are the first to use the rapid and highly selective technologies of solid-phase PL removal tubes. The efficiency of the proposed analytical procedure was confirmed and validated in compliance with SWGTOX standard practices for method validation in forensic toxicology. We have shared

these techniques with medical researchers at the FSSAI, CFTRI, Vellore Medical Hospital, Anresco Labs, the US DEA, University of Modeno (Italy), Singapore Health and Otsuka Medical and they have all reported results consistent with ours.