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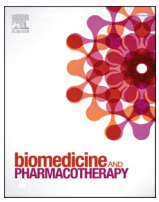

A new UHPLC-MS/MS method for cannabinoids determination in human plasma: A clinical tool for therapeutic drug monitoring

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ABSTRACT

Cannabinoid derivates have been largely used for different medical purpose. In the literature, several methods capable of separating THC and its principles metabolites are described, although Δ8- and Δ9-THC separation has not been completely achieved. THC metabolism has not been fully understood and metabolites plasma distribution in healthy and pathological patients remains to further deepen. The aim of this study was the validation of UHPLC-MS/MS method for the quantification of 10 cannabinoids in human plasma, as important tool for improving clinical efficacy of cannabis administration. Obtained results were in accordance with recommendations of ICH Harmonised Guideline for bioanalytical method validation, showing a good linearity, optimal accuracy as well as satisfactory results in terms of intra-day and inter-day precision and matrix effect. Furthermore, blood sampling study was performed to investigate the better collection method. Optimal separation of Δ-9-tetrahydrocannabinol (Δ9-THC), Δ8-tetrahydrocannabinol (Δ8-THC) was obtained. The present method showed optimal linearity and satisfactory results in terms of specificity and selectivity. Recovery was between 92.0% and 96.5% for all analytes. The matrix-effect showed good performance; no carry over was observed. Cannabinoid metabolites present in higher plasma concentrations were: 11–Hydroxy–Δ9–tetrahydrocannabinol, 11–Nor–9carboxy–Δ9–tetrahydrocannabinol and THC-COOH-glucuronide. Method performance makes it suitable for routine purposes and a potential tool for therapeutic ranges definition. The present work will be used to test several samples in a long-term clinical study, paving the way for further future works.

Abbreviations: Δ9-THC, Δ9-tetrahydrocannabinol; CBD, cannabidiol; PD, pharmacodynamic; GPCRs, G-protein-coupled receptors; CNS, Central Nervous System; PNS, peripheral nervous system; CBRs, cannabinoid receptors; UHPLC-MS/MS, ultra-high performance liquid chromatography coupled with tandem mass spectrometry; PK, pharmacokinetics; Δ8-THC, Δ8–tetrahydrocannabinol; TDM, therapeutic drug monitoring; CBN, Cannabinol; THCA, Tetrahydrocannabinolic acid; CBDA, Cannabidiolic acid; 11-OH-THC, 11–Hydroxy–Δ9–tetrahydrocannabinol; 11-COOH-THC, 11–Nor–9carboxy–Δ9–tetrahydrocannabinol; CBD-D3, Cannabidiol-D3; THC-D3, tetrahydrocannabinol–D3; 11-OH-THC-D3, 11–Hydroxy–Δ9–tetrahydrocannabinol–D3; 11-COOH-THC-D3, 11–Nor–9–carboxy–Δ9–tetrahydrocannabinol; QCs, quality control samples; ULOQ, upper level of quantification; LLOQ, lower level of quantification; IS, internal standard; ESI, electrospray ionization interface; LOD, limit of detection; LOQ, limit of quantitation; EQA, external quality assessment; EDTA-3K, ethylenediaminetetraacetic potassium salt 1:3; RF, response factor.

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1. Introduction

Cannabis plant has been used worldwide for medical scope for thousands of years [1–4].

The cannabis phytocomplex is made up of several substances (nearly 500), including steroids, flavonoids, mono- and sesquiterpenes, sugars, hydrocabons, nitrogenous compounds and amino acids [5].

The principal metabolites are Δ9-tetrahydrocannabinol (Δ9-THC) and cannabidiol (CBD) [6].

Focusing on metabolic pathway, THC is promptly converted by cytochrome P450 in 11–Hydroxy–Δ9–tetrahydrocannabinol (11-OH-THC), which is also a psychoactive substance [7] and reaches peak concentration within 15 min from consumption. 11-OH-THC is then metabolized in 11–Nor–9carboxy–Δ^9–tetrahydrocannabinol (11-COOH -THC) following oxidation; this oxidized product is then glucuronated to THC–COOH-glucuronide (THC-COOH-gluc) [8].

The Δ9-THC shows psychoactive action according to its percentage (8–14% w/w) in different pharmaceutical preparations. The Δ9-THC pharmacodynamic (PD) mechanism is based on the interaction with the endocannabinoid system receptors: two types (CB1 and CB2) of G-protein-coupled receptors (GPCRs) are part of this complex [9].

The CB1 is the most abundant GPCR in the central nervous system (CNS) and is expressed in presinaptic neurons in the neocortex, cerebellum and limbic system. It is present also in the peripheral nervous system (PNS), where it activates K^+ channels and causes inhibition of neurotransmitter release. CB2 has been identified in the immune system, such as in lymphocytes, mast cells and macrophages, and in the CNS on microglia cells and astrocytes [10].

Considering this data, medical cannabis presents several pharmacological potential applications.

In Italy, Ministerial Decree 9/11/2015 regulates medical cannabis use. It is administered in different cases: for chronic and neuropathic pain; as an appetite stimulant in cachexia caused by chemotherapy, radiotherapy, antiretroviral therapies, anorexia; in glaucoma and in the Gilles de la Tourette syndrome [11,12]. To date, few data are available in literature concerning cannabis pharmacokinetics (PK) [13], although a large number of methods for cannabinoids detection have been published over the last few years [3,14–18]. Particularly, no method performs an optimal chromatographic separation between Δ9-tetrahy drocannabinol (Δ9-THC), Δ8-tetrahydrocannabinol (Δ8-THC) and in a few minutes.

For this reason, aim of this study was to develop and validate an ultra-high performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS) method for the quantification of principal cannabinoids and their metabolites in human plasma, as a useful tool for PK studies. Furthermore, this study could be helpful in evaluating the efficacy and safety of medical cannabis in treated patients, laying the foundations for the therapeutic range definition.

2. Material and methods

2.1. Chemicals

Cannabinol (CBN), Tetrahydrocannabinolic acid (THCA), Cannabidiolic acid (CBDA), Δ9-THC, Δ8-THC, CBD, 11-OH-THC, 11-COOH-THC, THC-COOH-gluc, cannabidiol–D_3 (CBD-D_3), tetrahydrocanna binol–D_3, (THC-D_3), 11–Hydroxy–Δ9–tetrahydrocannabinol–D_3 (11-OH-THC- D_3), 11–Nor–9–carboxy–Δ9–tetrahydrocannabinol (11-COOH-THC-D_3), THC–glucuronide-D_3 solutions in methanol (≥99% purity) were purchased from Sigma-Aldrich (Milan, Italy).

HPLC grade acetonitrile was obtained from VWR International (Radnor, PA, USA).

HPLC grade water was produced with Milli-DI system coupled with a Synergy 185 system by Millipore (Milan, Italy).

Formic acid was purchased from Sigma-Aldrich (Milan, Italy).

Blank plasma from healthy donors were kindly supplied by the Blood Bank of the "Città della Salute e della Scienza" of Turin.

2.2. Standard and quality control

Stock solutions were used to independently spike blank plasma to obtain 6 levels of calibration standard and 2 different quality control samples (QCs): high and low (QC H and L, respectively). Calibration and QCs concentrations are: 250 ng/ml (ULOQ – upper level of quantification), 100 ng/ml, 50 ng/ml, 20 ng/ml, 10 ng/ml, 5 ng/ml (LLOQ – lower level of quantification) for standards and 150 ng/ml (QC H) and 7 ng/ml (QC L) for QCs, respectively.

2.3. Standards, QCs and patients' samples extraction

After thawing at room temperature, each sample was treated as follows: 10 μl of internal standard (IS) working solution were added to a volume of 100 μl of samples, standards and QCs and then, analytes extraction was obtained by addition of 300 μl of frozen-acetonitrile (−20 °C). Following, samples were stored at − 20 °C for 10 min to improve protein precipitation.

The samples were vortex-mixing for at least 10 s. Subsequently, all samples were centrifuged at 4000 rpm for 10 min at 4 °C.

Then, 180 μl of supernatant were diluted with 120 μl of water:acetonitirle 70:30 v/v, transferred in total recovery vials and 10 μl were injected in the chromatographic system.

2.4. Chromatographic conditions

The chromatographic system was an Acquity H-Class PLUS ® (Waters), composed of a Sample Manager FTN-H® autosampler and a column manager Acquity UPLC® column oven. The chromatographic separation was performed on a KINETEX® 2.6 μm Polar C18 100 Å LC column 100 × 2.1 mm (Phenomenex, Italy) at 40 °C.

The flow rate was settled at 0.5 ml/min; the used mobile phases were 0.1% v/v formic acid in water (Phase A) and 0.1% v/v formic acid in acetonitrile (Phase B), according to the gradient shown in Table 1.

The total run time was 10 min. The temperature of the sample manager was set at 15 °C. Water:acetonitrile 30:70 v/v was adopted as washing solution.

The separation efficiency was evaluated by Van Deemter model through N (number of theorical plates) and HETP (height equivalent to a theoretical plate) calculus, as follow: $N = 16 \bullet \left(\frac{t_R}{W_b}\right)^2$ and $H = \frac{L}{N}$ where t_R was retention time expressed in minutes, W_b was the width calculated at the base of peek and L was the length of column in millimeters.

These parameters were monitored for control analytical process and derives was prevented.

2.5. Mass spectrometry conditions

Tandem mass spectrometry detection was carried out by means of tandem mass spectrometry XEVO TQ-S micro, Waters® (Milan, Italy) with an electrospray ionization (ESI) interface. The ESI source was set in

Table 1
Gradient phases concentration (%v/v) in chromatographic elution.

Time (min)	Flow (ml/min)	Phase A (%)	Phase B (%)
0.0	0.500	82.0	18.0
0.60	0.500	82.0	18.0
5.50	0.500	28.0	72.0
6.50	0.500	28.0	72.0
7.30	0.500	21.0	79.0
7.80	0.500	21.0	79.0
8.80	0.500	5.0	95.0
9.20	0.500	5.0	95.0
10.0	0.500	18.0	82.0

positive ionization mode (ESI+) for most of the analytes and in negative ionization (ESI-) for THC-COOH-glucuronide, CBN, THCA and CBDA (see Table 2) Optimization of the MS conditions was obtained by infusion of reference standards of each drug (100 ng/ml in acetonitrile and HPLC grade water 60:40) at 5.0 μl/min into the mass spectrometer, combined with the flow from the chromatographic system at medium concentrations phases (Phase A and Phase B 50% v/v) as reported in Table 2.

Nitrogen (>99.9%) produced with a Nitrogen LCMS 40–1 nitrogen generator (Claind, Italy) was used as nebulizing and heating gas, while argon was used as collision gas.

The general conditions for positive ionization are electrospray voltage at 4.0 kV; source temperature at 600 °C; nebulizing gas flow at 1000 L/h.

2.6. Validation

2.6.1. Analytical selectivity and specificity

The selectivity was evaluated analyzing blank sample (plasma sample without addition of analyte or IS) obtained from ten different lots of plasma. The detected response was evaluated as the percent deviation from LLOQ concentration level: these results were accepted with gap $\leq$ 20% (absolute value) for analytes responses and $\leq$ of 5% (absolute value) for IS responses.

2.6.2. Calibration curve and range

The calibration curve was performed with six concentration levels (measure range 5 – 250 ng/ml), in addition to blank sample, in order to represent the relationship between analyte concentration and peak area normalized for its IS. The interpolation of Area/IS Area and concentration was calculated by least square method. Correlation factor (R^2) > 0.995 was considered as acceptable criteria for linearity. The Δ% for each concentration level was accepted with value including to ± 20% for LLOQ and ± 15% for other concentration levels [19].

The limit of detection (LOD) was estimated by Hubaux-Vos algorithm [20].

The limit of quantification (LOQ) was defined theoretically as three times of the LOD. The LOQ corresponded to LLOQ for each analyte in calibration curve [21].

2.6.3. Repeatability and reproducibility

The repeatability and reproducibility were evaluated through ten repetitions at four levels of concentrations in different sessions, as reported in results paragraph. The statistical analysis of this data set was executed at 97.5% level of confidence t-student distribution. In this context, the intra-laboratory precision with limit repeatability was evaluated as follow $= \sqrt{2} \times t \times s_r$, where t represented the t-student at $(1-\alpha) = 0.975$ with $\nu = 9$ (degrees of freedom for ten repetition of experiment intra-day), then s_r was the standard deviation in repeatability conditions. The assessment of precision in repeatability and reproducibility conditions were evaluated by relative standard deviation $RSD\% = \frac{s}{\bar{x}} \times 100$, where s represented standard deviation in repeatability or reproducibility conditions, $\bar{x}$ is the mean value of ten measures executed by single operator on the same sample. The repeatability measures were conducted on the same day, while the reproducibility measure on three different days.

2.6.4. Accuracy, precision, recovery and uncertainty

The method extraction efficacy, identification and quantification of analytes was demonstrated by spiking plasma samples with standard solution at four levels of concentrations: LLOQ, 20% ULOQ, 40% ULOQ, ULOQ. These samples were processed as ten replicates on three different days in order to evaluate precision. The condition of acceptability for coefficient of variation (CV)% was: ± 15% for each level, except for the LLOQ ± 20%.

In these experiments the relative recovery was evaluated and described as $R(\%) = \frac{C_f - C_s}{C_a} \times 100$, where C_f was the mean concentration of spiked sample, C_s was the mean concentration of not spiked sample and C_a was the concentration of spiked sample. The calculation of relative recovery was performed through ten experiments executed by a single operator. The acceptability conditions have been the same reported for precision test.

The evaluation of accuracy in accordance with the ICH guidelines [22] was performed through Bias % calculus as $b(\%) = \frac{\bar{x} - x_{ref}}{x_{ref}} \times 100$, where $\bar{x}$ was the mean of the results and x_{ref} was the reference value obtained by proficiency test specimens (EQA, external quality assessment). The evaluation of measurement uncertainty was performed by Horwitz heuristic model. The conditions of applicability of Horwitz equation were verified on data distribution as follow: ratio between s_r and sR was to be comprised between 0.50 and 0.67. The sR (standard deviation in reproducibility conditions calculated by Horwitz equation) was calculated by $sR = \frac{C}{100} \bullet 2^{(1-0.5\mathrm{Log}C)}$ where C was the concentration level of single analytes reported as mass ratio.

2.6.5. Blood sampling and matrix-effect

The following types of vacutainer tubes for plasma and serum collection were tested: lithium heparin, ethylenediaminetetraacetic potassium salt 1:3 (EDTA-3 K) and without additive. The evaluation of interactions between sampling tubes contents and the instrumental response was conducted by response factor (RF) calculated as follow: $RF_i = \frac{A_i}{C_i}$, where A_i was the single analyte area and C_i was the related concentration. The deviations by mean RF for each collection type and analyte were evaluated with percent difference of RF ($\Delta RF\%$) as follow: $\Delta RF\% = \frac{RF_i - \overline{RF}}{\overline{RF}} \times 100$, where the mean RF was calculated by relation

Table 2
MRM transitions. The Analytes detected in negative ionization presents negative collision energy and capillary voltage value.

Analyte	PARENT MRM [m/z]	Ion QUANTIFIER MRM [m/z]	Ion QUALIFIER MRM [m/z]	Cone Voltage [V]	Collision Energy [V]
CBN	311	293	223	-25	-18
CBD	315	193	259	25	20
Δ9-THC	315	193	259	25	20
Δ8-THC	315	193	259	20	20
11-OH- THC	331	313	175	30	12
THC-COOH	345	299	327	25	15
THCA	357	313	245	-30	-30
CBDA	357	313	245	-36	-12
THC-COOH-glucuronide	519	343	113	-25	-22
7-OH-CBD	331	175	201	25	12
THC-D_3	318	196	262	36	20
CBD-D_3	318	196	262	64	22
11-OH- THC-D_3	334	316	178	35	25
THC- COOH-D_3	348	302	330	25	30
THC-COOH-glucuronide-D_3	522	346	116	-25	-12

$\overline{RF} = \frac{\sum_{i=1}^{n} RF_i}{n}$. In the last equation, the n = 6 was the number of concentration levels in calibration curve of each analyte. Therefore, the $\Delta RF\%$ was computed for single concentration level related to single analyte respectively in three different collection conditions as reported before.

The study of the matrix was carried out by comparing the solubility chemical equilibrium and analytical distribution of single specimens in solvent and in plasma matrix. The quantitative deviations of matrix effect were evaluated by slope comparing between calibration curve executed in solvents and in plasma matrix. Furthermore, in accordance with ICH guideline the matrix effect was evaluated by three replicates of QC L and QC H in ten different plasma lots. The analytes average response was compared to theorical concentration with percent deviation in acceptability range of ± 15% for each concentration level upper LLOQ and ± 20% at LLOQ. The same acceptability values were considered for CV% in precision evaluation of response data in these experimental sessions.

2.6.6. Stability and incurred samples reanalysis

Stability study was conducted on bank plasma spiked at three different concentrations (5, 50 and 250 ng/ml), in order to evaluate the feasibility of samples and standards collection. Two different batches of blank plasma have been used also to assess matrix-effect.

Samples were stored at −20 °C and −80 °C and the selected timings for the stability study included 10, 30 and 90 days.

Stability was calculated as the percent difference between analytes concentrations found in samples freshly extracted and samples collected at −20 °C and −80 °C.

We also performed incurred samples reanalysis on authentic patient samples in three independent analytical sessions. The condition of acceptability for coefficient of variation (CV)% was ± 20%.

2.6.7. Clinical applications

Four samples obtained from patients using Marijuana and treated at the Amedeo di Savoia Hospital (Turin, Italy) for other pathologies were analyzed.

Four samples obtained from individuals using Marijuana, but without pathologies, were considered as negative controls; they all were marijuana users (3 times a week).

3. Results

The analytical method was fully validated in accordance with the recommendations of ICH Harmonised Guideline for bioanalytical method validation [22]. The following parameters were investigated: specificity and selectivity, linearity range, limit of detection (LOD), limit of quantitation (LOQ), accuracy, intra-day and inter-day precision, robustness, matrix-effect and sampling mode.

3.1. Chromatographic separation

The whole chromatographic run was completed in 10.0 min.

Retention times of the selected analytes were reported in Table 3.

Fig. 1 showed a chromatogram recorded from the middle point of the calibration curve (100 ng/ml) and the chromatographic parameters are reported in Table 3.

3.2. Analytical selectivity, specificity

The response detected in the processed blank samples did not report significative percent deviation (<2.5% as absolute value) compared to LLOQ default amount. While simultaneously, the chromatograms and mass spectra in SCAN mode of blank sample did not report interfering signal in analytes and IS acquisition window.

Table 3
Chromatographic parameters according to van Deemter model. RT= retention time; Wb= width calculated at the base of peek; N = number of theorical plates; H= height equivalent to a theoretical plate.

Analyte	RT	Wb	N	H
THC-COOH-glucuronide	4,40	0,55	1024	0,0977
11-OH- THC	5,24	0,48	1951	0,0513
7-OH-CBD	5,25	0,52	1668	0,0599
THC-COOH	5,40	0,34	4036	0,0248
CBDA	5,80	0,42	3147	0,0318
CBD	5,30	0,36	4489	0,0223
Δ8-THC	6,10	0,31	6692	0,0149
CBN	6,48	0,3	7327	0,0136
Δ9-THC	6,75	0,32	6972	0,0143
THCA	7,06	0,34	6899	0,0145

3.3. Calibration curve and range

The calibration curve was analyzed through samples processed in three replicates on three different analytical sessions. The linearity results were consistent with acceptability criteria as reported in Table 4. The LOD and LOQ were calculated and reported in Table 4.

3.4. Repeatability and reproducibility

The results showed the stability of the analytical process over time and good compliance of precision and accuracy. The overall measure report was reported in Table 4.

3.5. Accuracy, precision, recovery and uncertainty

The precision results were reported in Table 4. The percent deviation compared to the default amount and the CV% were in accordance with acceptability criteria.

The recovery for each compound was calculated on three levels of concentrations (5, 50, 250 ng/ml) and was included between 92.0% and 96.5% (absolute value), while the Bias% was comprised between 8.00% and 3.5% (absolute value). The uncertainty of measure was evaluated with Horwitz equation and its values were comprised between 35.7% and 19.7% calculated with coverage factor as k = 1.0 (Table 4).

3.6. Blood sampling and matrix-effect

The sampling method for optimal results was chosen with lower ΔRF % for each analyte. The ΔRF% of sample collected in vacutainer tubes added with EDTA-3 K was < 5.0% (see Fig. 2).

The matrix effect was quantified in percent deviation of linear slope in matrix calibration compared to solvent calibration: the analytical protocol was efficient and suppressed matrix effect down to 5.0%. The CV% of QC H and QC L in evaluation of precision in matrix effect study was compliant with acceptability criteria (see Table 4).

3.7. Stability and incurred sample reanalysis

Analysis showed samples were stable for 10 days when kept at − 20 °C and up to 3 months when stored at − 80 °C.

As reported in EMA and FDA guidelines, samples have been re-analyzed to evaluate incurred samples reanalysis.

They showed acceptable bias: 14% for Δ9-THC, 17% for THC-COOH and 4% for THC-gluc.

3.8. Clinical applications

The presented method has been applied on 8 samples obtained from individuals using cannabis.

All samples were successfully quantified for each drug.

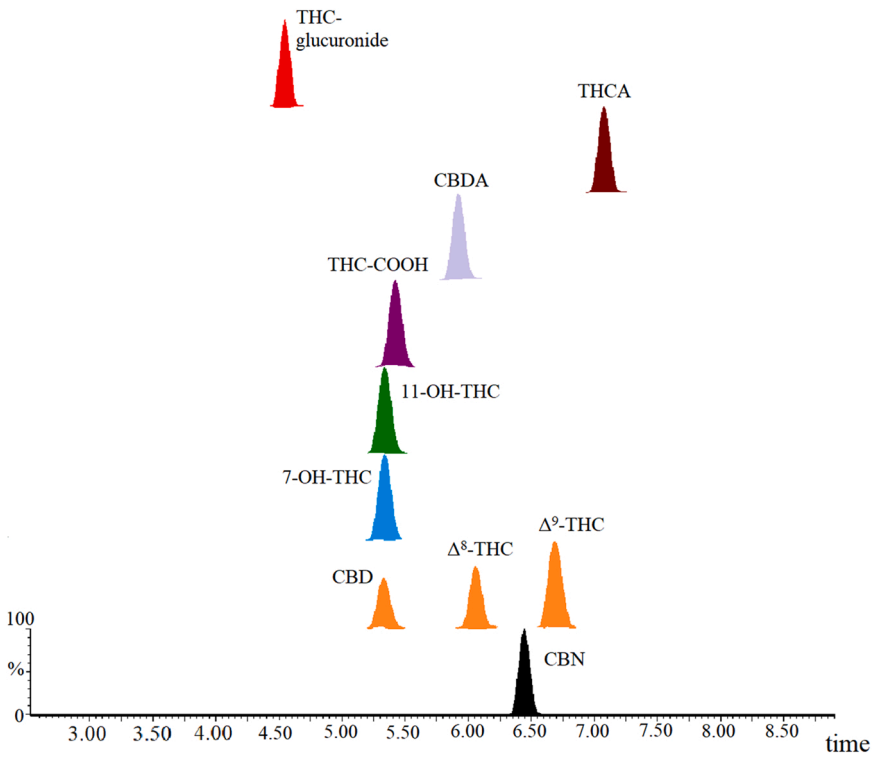

Fig. 1. Chromatogram recorded from the middle point of the calibration curve (100 ng/ml) reporting the considered analytes.

Concentrations were reported in Table 5, expressed as ng/ml.

4. Discussion

In this work, an analytical method performing the identification and quantification of cannabinoids and its metabolites was described.

The method was fully validated in accordance with the ICH Harmonised Guideline for bioanalytical method validation recommendations [22,23]. Following parameters were investigated: specificity and selectivity, linearity range, LOD, LOQ, accuracy, intra-day and inter-day precision, robustness, matrix-effect and sampling mode.

The present method novelty occurs in its simple method of extraction, shorter run-time and low plasma requirement than previous published LC–MS/MS assays.

Particularly, for the first time, an optimal chromatographic separation between Δ9-THC and Δ8-THC was obtained from what previously reported in the literature. For example Crippa et al. [24] described a partial co-elution between Δ8-THC and Δ9-THC (RT 1.90 and 1.97, respectively).

Δ8-THC is not well studied in patients, but it is marketed in cannabis preparations, making its chromatographic separation from its Δ9 analog and inclusion in panel analyses useful.

As reported by Tagen and Klumpers [25] the Δ8-THC oral absorption in humans is virtually unstudied: no pharmacokinetic studies have been performed with oral dosing.

For this reason, the capability of this novel method in Δ9-THC and Δ8-THC separation could be useful for clinical purpose.

Regarding the calibration curve linearity range, since one of the aims of the present study was the evaluation of plasma cannabinoids concentrations in patients treated with medical cannabis, we expected higher concentrations than those observed in toxicological analysis. Consequently, we decided to consider 5 mg/ml as LLOQ, a higher value compared to what reported in literature.

Since the present method was developed as a tool for Therapeutic Drug Monitoring (TDM) analysis, a blood collection study was mandatory to understand the potential variations in analytes measures. In particular, the vacutainer sampling types contain different anticoagulant substances probably interfering for mass spectrometer detection, confirming what reported in the previous published study of Jamwal et al. [26].

Our study highlights how lithium heparin tubes used for blood collection are involved in instrumental signal suppression.

The application of the validated method on clinical sample analysis was examined by quantification of cannabinoids in human plasma.

Moreover, the method was tested on eight biological samples: four obtained from patients using Marijuana and treated at the Amedeo di Savoia Hospital (Turin, Italy) and four samples obtained from individuals using Marijuana, but without pathologies.

Blood sampling in patients and volunteers was executed about 14 h after taking the drug (cigarette of cannabis); this study deepens the comprehension of cannabinoids metabolites in plasma after drug assumption.

11-OH-THC, THC-COOH and THC-COOH-gluc were the principal observed metabolites.

The CBD, Δ9-THC and Δ8-THC also featured at low to undetectable (n.d) concentrations in blood collected samples; a missing detection of these analytes, however, does not exclude recent intake[27].

As reported in the literature, plasma samples with THC-COOH (higher than 75 ng/ml) and THC (lower 5 ng/ml) probably indicate a chronic user sample, confirming our results [28].

The higher concentrations levels of THC-COOH-gluc were evaluated in patient groups and 11-OH-THC, THC-COOH were higher in controls.

It is important to consider that several factors influencing cannabinoid plasma concentrations are present: the drug use form, interindividual differences and pharmacogenetics might have a role in this field. The conversion of the acid precursors to the corresponding cannabinoids, depending on the reaction temperature, could have an impact on plasma concentration variability [28].

A limitation of this study is no information on exact times, forms or frequency of cannabis consumption was available for the tested plasma samples.

Despite this lack of information, the results obtained are in line with

Table 4
(A-B) – Evaluated parameters for analytical validation.

A	THCA			THC-COOH			CBDA			Δ9-THC			Δ8-THC		
	LEV 1	*LEV 2*	*LEV 3*	*LEV 1*	*LEV 2*	*LEV 3*	*LEV 1*	*LEV 2*	*LEV 3*	*LEV 1*	*LEV 2*	*LEV 3*	*LEV 1*	*LEV 2*	*LEV 3*
mean value of calculated concentration (Xm)	5,45	49,26	239,94	5,20	47,70	245,86	4,81	51,45	243,95	4,80	50,01	244,04	5,08	48,12	243,54
standard deviation on repeatability (Sr)	0,70	4,60	22,70	0,55	4,80	9,34	0,75	7,50	33,00	0,72	6,95	26,30	0,82	5,72	38,25
confidence interval (CI)	0,50	3,29	16,24	0,39	3,43	6,68	0,54	5,36	23,61	0,51	4,97	18,81	0,59	4,09	27,36
repeatability coefficient (r)	2,24	14,72	72,62	1,76	15,35	29,88	2,40	23,99	105,57	2,30	22,23	84,13	2,62	18,30	122,37
relative standard deviation on repeatability (RSDr)	12,85	9,34	9,46	10,58	10,06	3,80	15,60	14,58	13,53	14,98	13,90	10,78	16,14	11,89	15,71
relative standard deviation on reproducibility (RSDR)	13,20	11,23	11,74	11,70	9,10	7,11	10,50	5,50	12,00	13,01	17,80	16,42	15,02	12,62	13,30
correlation factor (R^2)	0998			0997			0998			0997			0997		
angular coefficient of solvent calibration curve (m)	0,12739			0,04391			0,22623			0,05658			0,04722		
angular coefficient of matrix calibration curve (m')	0,13359			0,04192			0,21685			0,05402			0,04913		
matrix deviation in percentage; (Δm%)	4,87			-4,53			-4,15			-4,52			4,04		
limit of detection (LOD)	0,45			0,45			0,22			0,87			0,86		
limit of quantification (LOQ)	5,00			5,00			5,00			5,00			5,00		
recovery (R%)	92,0			95,0			93,2			94,6			94,3		
uncertainty of measure (U(x))	35,1	25,2	19,8	35,3	25,3	19,8	35,7	25,0	19,8	35,7	25,1	19,8	35,4	25,3	19,8
B	CBD			CBN			11-OH-THC			THC-COOH-gluc			7-OH-CBD		
	LEV 1	*LEV 2*	*LEV 3*	*LEV 1*	*LEV 2*	*LEV 3*	*LEV 1*	*LEV 2*	*LEV 3*	*LEV 1*	*LEV 2*	*LEV 3*	*LEV 1*	*LEV 2*	*LEV 3*
mean value of calculated concentration (Xm)	4,86	54,69	250,15	5,45	48,53	245,37	4,99	53,23	220,72	4,87	53,85	239,79	5,56	47,72	245,39
standard deviation on repeatability (Sr)	0,74	8,81	26,95	0,84	6,93	30,65	0,61	4,50	23,83	0,72	6,94	22,84	0,76	5,55	24,25
confidence interval (CI)	0,53	6,30	19,28	0,60	4,95	21,93	0,44	3,22	17,04	0,52	4,96	16,34	0,55	3,97	17,34
repeatability coefficient (r)	2,38	28,19	86,20	2,70	22,15	98,06	1,95	14,40	76,22	2,30	22,19	73,08	2,44	17,74	77,56
relative standard deviation on repeatability (RSDr)	15,26	16,11	10,77	15,47	14,27	12,49	12,23	8,45	10,79	14,79	12,88	9,53	13,70	11,62	9,88
relative standard deviation on reproducibility (RSDR)	15,91	14,90	15,00	8,20	14,00	15,11	9,60	8,90	7,03	9,74	13,40	15,07	12,00	12,89	13,50
correlation factor (R^2)	0997			0997			0998			0999			0997		
angular coefficient of solvent calibration curve (m)	0,07658			0,06335			0,09658			0,05568			0,26273		
angular coefficient of matrix calibration curve (m')	0,07292			0,06642			0,09198			0,05842			0,25221		
matrix deviation in percentage; (Δm%)	-4,78			4,85			-4,76			4,92			-4,00		
limit of detection (LOD)	0,86			0,15			0,55			0,24			0,17		
limit of quantification (LOQ)	5,00			5,00			5,00			5,00			5,00		
recovery (R%)	95,1			95,0			96,2			96,5			92,4		
uncertainty of measure (U(x))	35,7	24,8	19,7	35,1	25,2	19,8	35,5	24,9	20,1	35,7	24,8	19,8	35,0	25,3	19,8

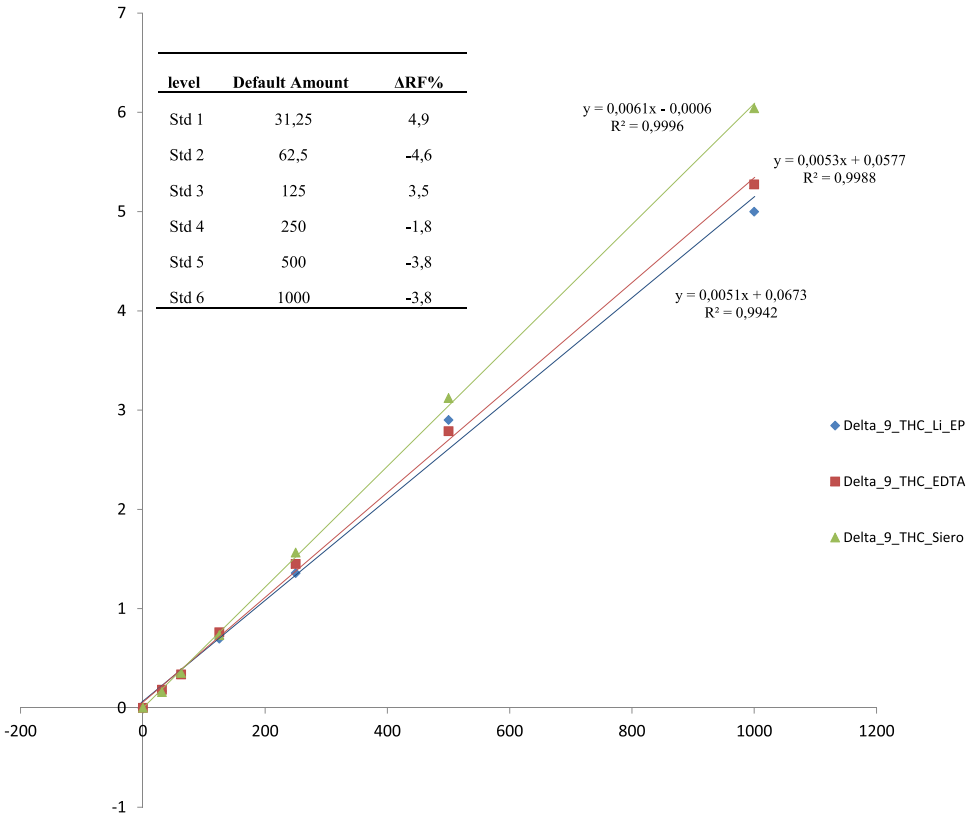

Fig. 2. The matrix effect study for Δ9-THC, calibration curves in lithium heparin, EDTA-3K and without additive.

Table 5
Summary of cannabinoids concentrations in human samples. P2 and N4 were slightly lower than LOQ (5 ng/ml) for Δ8-THC and Δ9-THC respectively.

Sample ID	Δ9-THC (ng/ml)	Δ8-THC (ng/ml)	11-OH-THC (ng/ml)	THC-COOH (ng/ml)	THC-gluc (ng/ml)	CBD (ng/ml)	CBDA (ng/ml)
Patients							
P1	n.d.	n.d.	**19.5**	**5.8**	**18.0**	n.d.	n.d.
P2	**10.0**	**4.2**	**27.2**	**60.1**	**191.7**	n.d.	n.d.
P3	n.d.	n.d.	**27.1**	**18.4**	**106.7**	n.d.	n.d.
P4	n.d.	n.d.	**24.2**	**27.7**	**218.9**	n.d.	n.d.
Controls							
N1	n.d.	n.d.	**832.4**	**702.1**	**371.6**	n.d.	**246.6**
N2	n.d.	n.d.	**931.6**	**710.2**	**403.1**	n.d.	**261.9**
N3	**5.2**	n.d.	**513.3**	**722.3**	**32.5**	n.d.	**557.9**
N4	**4.2**	n.d.	**181.9**	**654.1**	**316.1**	n.d.	**805.4**

the literature and suggest the suitability of the validated method for clinical intent.

5. Conclusions

In this work, we focused our attention on cannabis metabolites, aiming at developing a fast, specific, robust and accurate method to quantify them in plasma through of UHPLC-MS/MS.

The method was validated in accordance with the ICH Harmonised Guideline for bioanalytical method validation recommendations.

The obtained results suggest method could be suitable for routine purposes: it could be helpful in evaluating the efficacy and safety of medical cannabis, opening the way for therapeutic ranges definition.

The present work will be used to test several samples in a long-term clinical study with known cannabis use history and timings, also considering pharmacogenetics.

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Data availability

Data are available on request by the corresponding author.

CRediT authorship contribution statement

A.M. and F.C.: Conceptualization. **A.M. and F.C.**: Methodology. **J.M., A.P. and S.Z.**: Software. **A.M. and F.C.**: Validation. **A.M. and F.C.**: Formal analysis. **A.M. and F.C.**: Investigation. **A.D.A.**: Resources. **D.M., A.D.N. and A.M.**: Data curation. **A.M., F.C., J.M.**: Writing – original draft. **J.C., A.D.A., D.D.C., G.D.F.**: Writing – review & editing. **J.M. and S.Z.**: Visualization. **J.C., A.D.A., I.D.**: Supervision. **A.M., F.V., A.D.A.**: Project administration. **A.D.A.**: Funding acquisition. All data were generated in-house, and no paper mill was used. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy. All authors have read and agreed to the published version of the manuscript.

Conflict of interest statement

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Alessandra Manca reports a relationship with CoQua Lab srl that includes: equity or stocks. Antonio D'Avolio reports a relationship with CoQua Lab srl that includes: equity or stocks. No patents.

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