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Determination of plasma and urine levels of Δ^9-tetrahydrocannabinol and its main metabolite by liquid chromatography after solid-phase extraction

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Abstract

Δ^9-Tetrahydrocannabinol is the most widespread drug of abuse in the world and it is also currently available as the active principle of formulations for the treatment of chronic pain. Its main metabolite, 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid, is the most important marker of Δ^9-tetrahydrocannabinol consumption. An original liquid chromatographic method has been developed for the determination of these two analytes in human plasma and urine. Separation was obtained on a C8 column using a mobile phase with 35% phosphate buffer at pH 2.7 and 65% acetonitrile. The UV detector was set at 220 nm and indomethacin was used as the internal standard. Sample pre-treatment was carried out by solid-phase extraction with C8 cartridges; urine samples were subjected to basic hydrolysis before extraction. Both extraction yields (>91%) and precision values were highly satisfactory. The method was successfully applied to biological samples collected from *Cannabis* users. Accuracy and selectivity results were satisfactory. This is the first HPLC-UV method developed for the simultaneous quantification of Δ^9-tetrahydrocannabinol and 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid in both plasma and urine for the monitoring of either therapeutic or recreational use.

Keywords: Δ^9-Tetrahydrocannabinol; 11-Nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid; Therapeutic drug monitoring; High-performance liquid chromatography; Solid-phase extraction

1. Introduction

Cannabinoids are tricyclic terpenoid derivatives bearing a benzopyran moiety [1] and the most important member of the class is Δ^9-tetrahydrocannabinol (THC; Fig. 1), a compound to which most of the pleasant effects of *Cannabis* are usually attributed [2]. Recently, cannabinoids have been proposed as a therapeutic option in the treatment of chronic pain [3] and the European Union currently funds research organisations to develop standardised extracts of *Cannabis* for the treatment of rheumatoid arthritis and migraine [4]. A sublingual spray derived from an extract of cannabis has been approved in Canada as the prescription drug Sativex® for the treatment of neuropathic pain in multiple sclerosis [5]; this drug may be legally imported into other countries as well, on prescription. Moreover, synthetic THC has been shown to possess anti-emetic properties useful in cancer therapy [6]. THC is rapidly absorbed by inhalation and by ingestion. It is very lipophilic and is largely bound to plasma proteins [7]. It is mainly metabolised in the liver by the cytochrome P450 system [8] and the most important metabolite is 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH; Fig. 1) [9]. The terminal half-life of THC and THC-COOH can be longer than 48 hours: this explains why they can be found in plasma and urine even days after the actual consumption [10,11]. However, only a small percentage of THC can be found as such in biological fluids, thus the main marker of cannabinoid use is THC-COOH [12]. Plasma levels of THC can vary from a few ng mL^{-1} for intrapulmonary-administered THC formulations [13] to 50–150 ng mL^{-1} for a normal mari-

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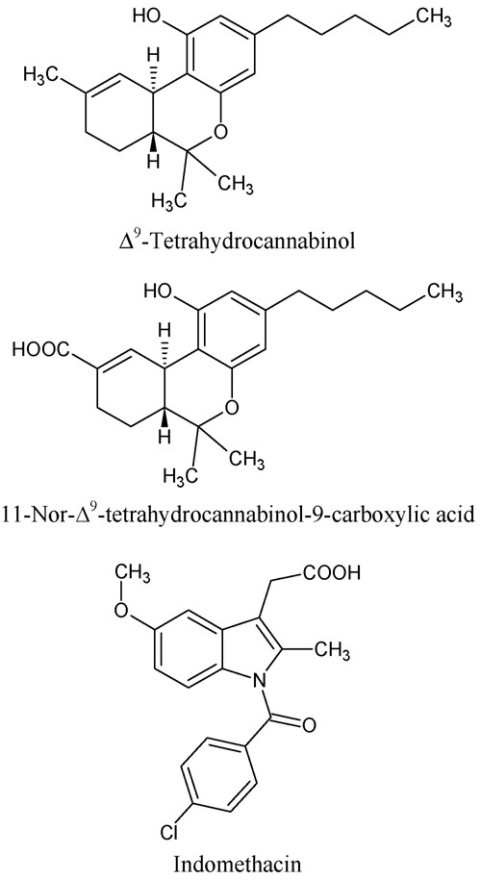

Fig. 1. Chemical structures of the analytes and the IS (indomethacin).

juana smoke [9,14]. Plasma levels of THC-COOH in *Cannabis* users can vary from 10 to 200 ng mL^{-1}, with mean values of about 50 ng mL^{-1} [9,15]. The consumption of 10–50 mg of cannabinoids (which roughly represent an "average dose") generate urine levels of THC between 2 and 20 ng mL^{-1} and of THC-COOH between 20 and 200 ng mL^{-1}. Higher doses give rise to urine levels of up to 50 ng mL^{-1} of THC and of up to 3 μg mL^{-1} of THC-COOH [16]. Both THC and THC-COOH are almost exclusively found in urine as their glucuronides. In clinical practice, four main immunochemical assays are used to carry out the screening of cannabinoids in biological fluids, usually in urine: enzyme multiplied immunoassay technique (EMIT); radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) and most frequently fluorescence polarisation immunoassay (FPIA). These kits most likely contain different antibodies, but often information concerning the preparation of the antibodies or their possible cross-reactivity is not available. Thus, while having high sensitivity (limit of detection in the nanograms per millilitre range), the FPIAs are not sufficiently selective and can only be used to eliminate negative samples. Positivity to the initial screening (i.e., measured concentration above a cut-off level usually fixed at 10 ng mL^{-1}) should always be confirmed by a separative assay. These confirmation assays are usually chromatographic and should have sufficient sensitivity and selectivity to confidently identify the analyte down to the cut-off level [17]. Furthermore, analytical methods for the determination of THC and its metabolite in biological samples can be useful for the study of chemical–clinical correlations of THC used for pain relief.

Some separative methods can be found in the literature for the determination of cannabinoids in biological fluids [18], mainly in whole blood, plasma and urine, but also in other matrices such as oral fluids [19–21], hair [22] and sweat [23]. Most analytical methods for the determination of THC and THC-COOH in plasma and/or urine are based on liquid chromatography–mass spectrometry [24–28] or gas chromatography–mass spectrometry [29,30]; some liquid chromatography methods use electrochemical [31,32] or spectrophotometric [33–37] detection. Most of these techniques [24–32], however, require expensive instrumentation, which is not always available in normal analysis laboratories. Sometimes, the sample pre-treatment is particularly complicated or unusual, such as an immunoaffinity extraction procedure [30]; sometimes, the method only analyses THC [35,36] or THC-COOH [28,33,34,37], or is only applied to urine [28,33,34,37] or to plasma [36]. Thus, the aim of this study was the development of a feasible and reliable analytical method for the determination of THC and THC-COOH in human plasma and urine, both for toxicological and clinical monitoring purposes. In fact, the quantitative determination of both compounds gives a more complete pharmacokinetic and toxicokinetic outline of the subjects; the analysis of both matrices (plasma and urine) also gives more complete information to the clinician and to the pharmacologist. Furthermore, the use of liquid chromatography with UV detection makes the method less expensive, simpler and more widely applicable in clinical laboratories.

2. Experimental

2.1. Chemicals and solutions

Methanolic stock solutions of Δ^9-tetrahydrocannabinol (1000 μg mL^{-1}) and 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (101.1 μg mL^{-1}) were purchased from Alltech Italy (Bologna, Italy). Methanol and acetonitrile (HPLC grade), 85% (w/w) phosphoric acid, potassium dihydrogen phosphate, 10 M potassium hydroxide, 99% (w/w) acetic acid, all pure for analysis, were produced by Carlo Erba (Milan, Italy). Indomethacin, used as the Internal Standard (IS; Fig. 1), was purchased from Sigma–Aldrich (St. Louis, MO, USA).

Ultrapure water (18.2 MΩ cm) was obtained by means of a MilliQ apparatus by Millipore (Milford, MA, USA).

The stock solutions of THC and THC-COOH were stored in stoppered low-actinic glass vials at −20 °C. The stock solutions of the IS (1 mg mL^{-1}) were prepared by dissolving 20 mg of the pure substance in 20 mL of methanol and were stored at −20 °C. Stock solutions were stable for at least 1 month. Standard solutions were prepared daily by diluting stock solutions with methanol in low-actinic glass vials, avoiding direct light.

2.2. Apparatus and chromatographic conditions

The chromatographic system was composed of a Jasco (Tokyo, Japan) PU-980 chromatographic pump and a Jasco UV-975 spectrophotometric detector.

Separations were obtained on a Varian Zorbax C8 reversed-phase column (150 mm × 4.6 mm i.d., 5 μm) coupled to a Phenomenex (Torrance, CA, USA) SecurityGuard C8 guard cartridge (4 mm × 3.0 mm i.d., 5 μm). The mobile phase was composed of a mixture of acetonitrile (65%, v/v) and a pH 2.7, 50 mM phosphate buffer (35%, v/v). The mobile phase was filtered through a Phenomenex membrane filter (47 mm membrane, 0.2 μm, NY) and degassed by an ultrasonic apparatus. The flow rate gradient was programmed as follows: from 0 to 8 min, linear gradient 0.3–2.5 mL min^{-1}; from 8 to 12 min, constant flow rate at 2.5 mL min^{-1}; from 12 to 14 min, linear gradient 2.5–0.3 mL min^{-1}. The injections were carried out through a 20 μL loop and absorbance signals were monitored at 220 nm (range: 0.0005 mAU, response: standard). If the preliminary FPIA assay detected medium-low cannabinoid levels, a 50 μL loop was used instead of a 20 μL loop. Data processing was handled by means of a Varian (Walnut Creek, USA) Star Chromatography 4.0 software.

Solid-phase extraction (SPE) was carried out on IST (Hengoed, UK) Isolute C8 cartridges (100 mg, 1 mL) by means of a Vac Elut (Varian) apparatus.

A Crison (Barcelona, Spain) Basic 20 pHmeter and a Hettich (Tuttlingen, Germany) Universal 32 R centrifuge were used.

2.3. Sample collection and pre-treatment

Plasma and urine samples were collected at the Laboratory of Clinical Pharmacology and Toxicology of the "S. Maria delle Croci" Hospital, Ravenna (Italy); they were samples which resulted positive to the fluorescence polarisation immunoassay (FPIA) for cannabinoids. For the FPIA, calibrator, control and patient urine samples were submitted to the Abbott Diagnostics (Abbott Park, IL, USA) Axsym® instrument for automated analysis. Calibrator and control materials were provided by the manufacturer. Each lot of calibrator sets is value-assigned on the Axsym® using reference material with values determined by the manufacturers' reference laboratory, using an independently validated HPLC-MS method. Each lot of control sets is value-assigned by FPIA analysis based on these calibrators.

"Blank" plasma and urine samples were obtained from healthy volunteers not subjected to any pharmacological treatment; all urine samples had physiological creatinine values (0.7–1.2 mg dL^{-1}). Both plasma and urine samples were stored at −80 °C until the analysis.

Before the SPE pre-treatment, urine samples were subjected to basic hydrolysis [38,39]. The urine sample was thawed and centrifuged at 2000 × *g*. Then, 50 μL of 10 M KOH and 50 μL of IS standard solution (final IS concentration injected into HPLC: 1 μg mL^{-1}) were added to 500 μL of urine. The mixture was vortexed for 1 min and left to rest for 45 min at 70 °C in a stoppered glass vial. The samples were cooled down to room temperature and acidified with 60 μL of 99% (w/w) acetic acid and 500 μL of 50 mM phosphoric acid.

The SPE procedure was carried out on Isolute C8 cartridges (100 mg, 1 mL). Cartridges were activated by passing 1 mL of methanol through the cartridge five times, and then conditioned by passing 1 mL of ultrapure water five times. To 500 μL of plasma, 1000 μL of ultrapure water and 50 μL of IS solution (final concentration injected into HPLC: 1 μg mL^{-1}) were added. This mixture, or the final solution obtained from urine hydrolysis, was loaded onto a previously conditioned cartridge. The cartridge was then washed twice with 1 mL of pH 2.7, 50 mM phosphate buffer, once with 1 mL of a pH 2.7, 50 mM phosphate buffer/methanol mixture (80/20, v/v) and once with 100 μL of methanol. The cartridge was then dried by vacuum (1 min at −30 mmHg) and the analytes were eluted with 1 mL of methanol; the cartridge was dried again at the end of the elution. The eluate was dried under vacuum (rotary evaporator), redissolved with 125 μL of methanol, then injected into the HPLC system. If the preliminary FPIA assay detected medium-low cannabinoid levels, 1000 μL of plasma or urine were used instead of 500 μL.

2.4. Method validation

Method validation was carried out according to "Crystal City" [40], United States Pharmacopeia (USP) [41] and International Conference on Harmonization (ICH) [42] guidelines.

Stability: To verify analyte stability, 50 μL of a standard solution containing THC, THC-COOH and the IS were added to 2.5 mL of blank plasma and to 2.5 mL of blank urine. One 500 μL sample aliquot was immediately subjected to the sample pre-treatment procedure. The remaining volume was divided into four 500 μL aliquots; two of them were stored in polypropylene vials, the other two in low-actinic glass vials. One of the former samples and one of the latter samples were analysed in 2 consecutive days.

Calibration curves: Aliquots of 50 μL of analyte standard solutions at seven different concentrations containing the IS at a constant concentration were added to 500 or 1000 μL of blank plasma or urine. Two linearity ranges were considered for each analyte and precisely: 2–50 ng mL^{-1} for THC and 0.8–20 ng mL^{-1} for THC-COOH (1000 μL of plasma or urine; 50 μL loop); 50–650 ng mL^{-1} for THC and 20–2500 ng mL^{-1} for THC-COOH (500 μL of plasma or urine; 20 μL loop). The IS concentration was maintained constant at 1 μg mL^{-1} (final concentration in the injected solution). The resulting mixtures were subjected to the previously described sample pre-treatment procedure and injected into the HPLC. The procedure was carried out in triplicate for each concentration. The analyte/IS peak area ratios (pure numbers) obtained were plotted against the corresponding concentrations of the analytes (expressed as ng mL^{-1}) and the calibration curves set up by means of the least-square method. The values of limit of quantitation (LOQ) and limit of detection (LOD) were calculated as the analyte concentrations which give rise to peaks whose heights are 10 and 3 times the standard deviation of the baseline noise, respectively.

Extraction yield (absolute recovery): The procedure was the same as that described under "calibration curve", above, except the points were at three different concentrations, corresponding to the lower limit, middle point and upper limit of the total linearity curves. The analyte peak areas were compared to those obtained injecting standard solutions at the same theoretical concentrations and the extraction yield values were calculated.

Precision: The assays described under "extraction yield" were repeated six times within the same day to obtain repeatability (intraday precision) and six times over different days to obtain intermediate precision (interday precision), both expressed as R.S.D.% values.

Selectivity: Blank plasma and urine samples from six different volunteers were subjected to the pre-treatment procedure and injected into the HPLC; the resulting chromatograms were checked for possible interference from endogenous compounds. The acceptance criterion was: no interfering peak higher than an analyte peak corresponding to its LOD. Furthermore, standard solutions of several different drugs active on the central nervous system were injected at concentrations higher than the respective therapeutic or usual levels; if the resulting chromatograms contained any interfering peak, the potentially interfering compounds were subjected to the SPE procedure and injected to ascertain whether they are extracted. During interference studies, run time was extended to 30 min in order to ascertain whether any possible interference would be carried over to subsequent analytical runs.

Accuracy: Accuracy was evaluated by means of recovery assays. The assays described under "extraction yield" were carried out adding standard solutions of the analytes and the IS to real plasma or urine samples, which had resulted positive to the cannabinoid FPIA assay. These assays were repeated three times and the mean recovery and S.D. of the results calculated.

3. Results and discussion

3.1. Choice of the chromatographic conditions

The analytes have similar UV spectra, with the main relative absorbance maxima near to 210 nm. However, preliminary chromatographic assays showed that at 210 nm heavy baseline noise is recorded, and that 220 nm gave the best signal/noise ratio, thus this wavelength was chosen for all subsequent assays.

Cannabinoids are quite lipophilic compounds. For their separation, a C18 column was initially tried as the stationary phase, however it retained the analytes too much, in particular the retention time of THC was too long. For this reason, a C8 column, which is less lipophilic, was tried: it gave a more adequate retention of the analytes and thus it has been chosen for the chromatographic analysis.

The mobile phase was initially composed of an acidic (pH 2.7) phosphate buffer and acetonitrile (50/50, v/v) at a flow rate of 1 mL min^{-1}. Under these conditions, the analytes were separated, however run times were still too long (30 min). A shorter column (150 mm) and an higher percentage of acetonitrile (50–70%) were thus tried. With 65% acetonitrile the retention of THC-COOH was adequate, however that of THC was still too high (retention time >15 min). In order to eliminate this inconvenience while still maintaining the retention of THC-COOH, a flow rate gradient was introduced. In particular, the flow rate increases linearly (from 0.3 to 2.5 mL min^{-1}) during the first part of the run and is kept constant at 2.5 mL min^{-1} during the second part (after 8 min). The retention time of THC-COOH is almost unaffected by the gradient, however that of THC decreases by about 25%, to 10.7 min. This flow rate gradient has no visible effects on baseline appearance nor on peak shapes; furthermore, it does not require any equilibration time after the flow rate has returned to the starting value (0.3 mL min^{-1}).

Several compounds were tested as possible ISs; among them, indomethacin, amiloride, chlorpromazine, loxapine and mianserin. Of these compounds, only indomethacin is sufficiently retained by the system and, like THC-COOH, has a carboxylic group; for these reasons, it was chosen as the IS. Besides this, the IS does not increase run times, since its retention time is shorter still than that of THC-COOH and a chromatographic run lasts 11 min.

3.2. Analysis of standard solutions

Good linearity ($r^2 > 0.9994$) was obtained over the following concentration ranges: 16–400 ng mL^{-1} for THC and 6.4–160.0 ng mL^{-1} for THC-COOH (50 μL loop); 400–2600 ng mL^{-1} for THC and 160–10000 ng mL^{-1} for THC-COOH (20 μL loop). Precision assays were carried out at three different levels (16, 1300 and 2600 ng mL^{-1} for THC; 6.4, 5000.0 and 10000.0 ng mL^{-1} for THC-COOH) and gave good results: the relative standard deviation (R.S.D.) was always lower than 5.8% for all analytes. The limits of quantification (LOQs) were 16 ng mL^{-1} for THC and 6.4 ng mL^{-1} for THC-COOH; the limits of detection (LODs) were 6 ng mL^{-1} for THC and 2.5 ng mL^{-1} for THC-COOH.

3.3. Development of the sample pre-treatment procedure

The analysis of highly complex biological matrices, such as human plasma and urine, by means of HPLC-UV requires the implementation of reproducible and reliable sample pre-treatment procedures in order to eliminate endogenous interference and, if necessary, to suitably concentrate the analytes. Since the analytes are excreted into the urine mainly as glucuronic acid conjugates, a preliminary hydrolysis step is clearly necessary when dealing with this matrix to obtain the free compounds. Basic hydrolysis was chosen for this purpose, since the analytes are not pH-sensitive and basic hydrolysis is certainly less complicated and less expensive than enzymatic hydrolysis.

Preliminary assays regarding hydrolysis time (15, 30, 45, 90 and 120 min) and temperature (50, 70, 90 and 100 °C) ascertained that hydrolysis reached a yield plateau at 70 °C for 45 min. These conditions were thus used for all subsequent assays.

The pre-treatment of plasma samples and of urine samples after hydrolysis was carried out by SPE, an established, feasible and highly reproducible technique, which also allows the

concentration of the analytes. The first tried sorbent was BondElut Certify, which is a mixed ionic exchange/lipophilic resin developed for abuse drug extraction and already successfully used for the analysis of cannabinoids by HPLC-mass [19,20]. However, heavy interference was detected with this kind of cartridges, probably it is because not sufficiently selective to be suitable for UV detection. The Oasis Hydrophilic–Lipophilic Balance (HLB) sorbent can retain compounds having different chemical–physical characteristics; however, it gave poor yields of the analytes. Another possibility was the use of C8 or C18 cartridges, due to the high lipophilicity of the analytes. The best results were obtained when using C8 cartridges, which possess the right degree of lipophilicity to retain the analytes while allowing them to be eluted with an average strength solvent, such as methanol. Using a C8 sorbent, the most immediate choice would be to load the samples in acidic environment, to keep THC-COOH undissociated. However, it was found from preliminary assays that neutral (pH 7) loading did not lead to any analyte loss; on the other hand, it led to strong interference when loading urine samples. Thus, urine was loaded in acidic environment ($pH \leq 5$) and plasma in neutral environment. The washing step was initially carried out with 2×1 mL of water and 1 mL of a water/methanol (80/20) mixture, however this procedure did not sufficiently purify the sample from endogenous interference. For this reason, water was substituted with an acidic phosphate buffer, which gives better elimination of basic compounds while keeping THC-COOH undissociated. Finally, it was found that a small volume (100 μL) of methanol could be passed through the cartridge prior to the elution to eliminate other strongly retained interference without eluting the analytes. This step was thus added as the last washing step. The methanolic eluate is dried and redissolved in 125 μL of the same solvent, in order to concentrate the analytes eight times when expected plasma or urine levels are medium-low or four times when expected plasma or urine levels are high.

The chromatograms of blank plasma and urine samples after SPE (eight-fold concentration, 50 μL loop) are shown in Figs. 2a and 3a, respectively; the chromatograms of blank plasma and urine samples after hydrolysis and SPE (four-fold concentration, 20 μL loop) are shown in Figs. 4a and 5a, respectively. As can be seen, the baseline is remarkably flat and no interference is apparent at retention times corresponding to those of the analytes and the IS. The chromatograms of blank plasma and urine samples spiked with the analytes and the IS (eight-fold concentration, 50 μL loop) are shown in Fig. 2b (5 ng mL^{-1} of THC, 5 ng mL^{-1} of THC-COOH) and Fig. 3b (10 ng mL^{-1} of THC, 20 ng mL^{-1} of THC-COOH), respectively. Again, no interference is apparent and peaks are symmetrical and well resolved.

3.4. Method validation

Some authors have reported that cannabinoids are only stable if they are kept shielded from direct light and in glass containers [43]. Since analyte stability is a critical issue in analytical method validation, this observation needed to be confirmed. Thus, the stability assays reported in the Experimental section have been carried out in order to evaluate the stability of cannabi-

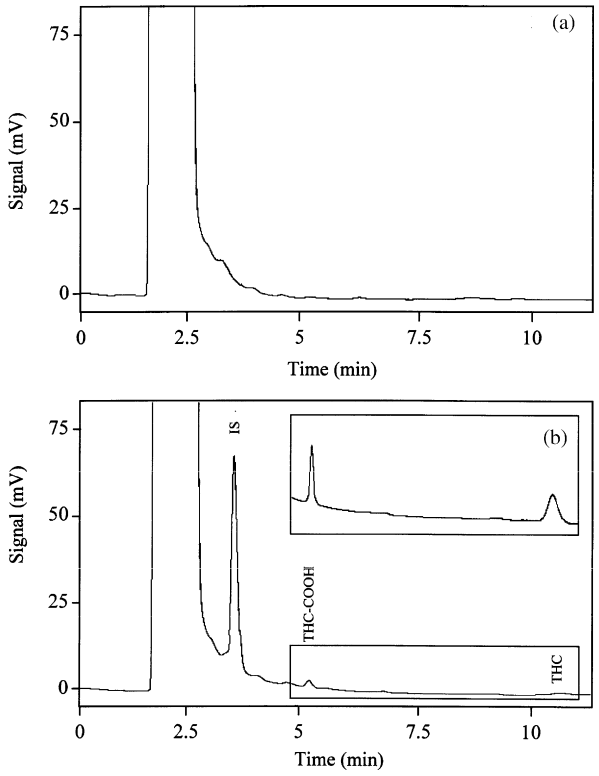

Fig. 2. Chromatograms of (a) a blank plasma sample from a healthy volunteer and (b) the same blank plasma sample spiked with 5 ng mL^{-1} of THC, 5 ng mL^{-1} of THC-COOH and the IS (1 μg mL^{-1}, concentration in the injected solution). Eight-fold concentration, 50 μL loop.

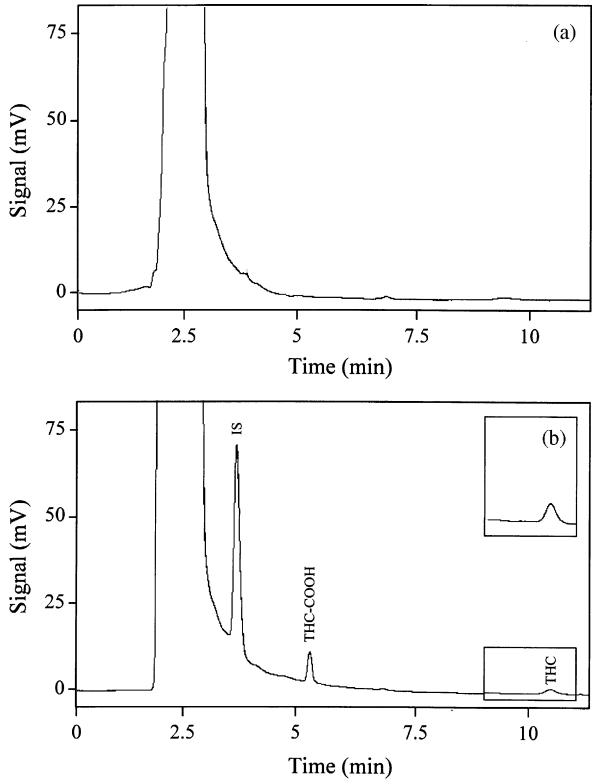

Fig. 3. Chromatograms of (a) a blank urine sample from a healthy volunteer and (b) the same blank urine sample spiked with 10 ng mL^{-1} of THC, 20 ng mL^{-1} of THC-COOH and the IS (1 μg mL^{-1}, concentration in the injected solution). Eight-fold concentration, 50 μL loop.

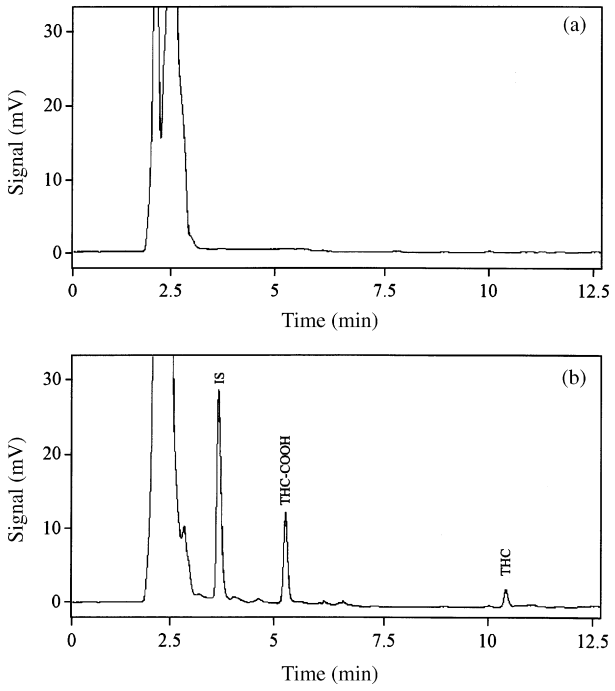

Fig. 4. Chromatograms of (a) a blank plasma sample from a healthy volunteer and (b) a plasma sample from a *Cannabis* user spiked with the IS (1 μg mL^{-1}, concentration in the injected solution). Four-fold concentration, 20 μL loop.

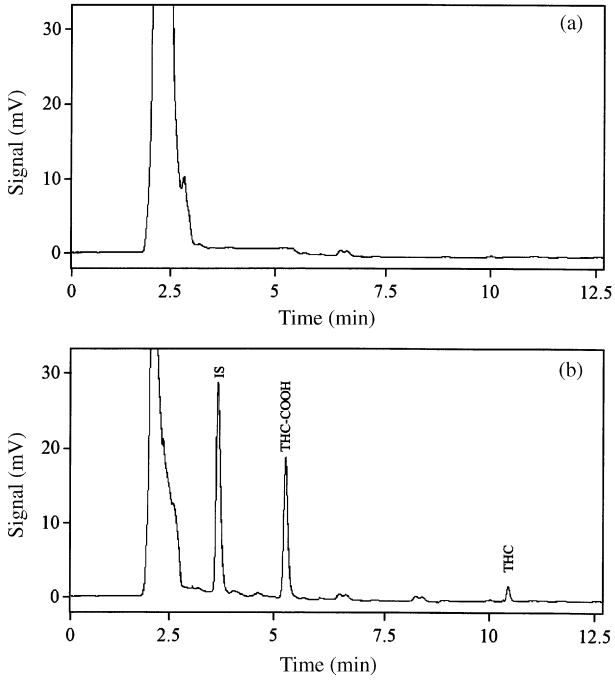

Fig. 5. Chromatograms of (a) a blank urine sample from a healthy volunteer and (b) a urine sample from a *Cannabis* user spiked with the IS (1 μg mL^{-1}, concentration in the injected solution). Four-fold concentration, 20 μL loop.

noids in biological samples stored in low-actinic glass containers as opposed to polypropylene containers.

It was found that plasma and urine samples stored in polypropylene vials gave extraction yields of THC and THC-COOH, which were about 60% the first day and 40% the second day, with respect to those obtained from samples analysed immediately. The samples stored in glass, on the contrary, gave extraction yield results, which were constantly higher than 90%. The extraction yields of the IS were constant in all kinds of containers. These results confirm that all samples should be kept in glass containers until analysis. On the other hand, it was confirmed that simply putting the samples in polypropylene tubes or vials for a few minutes during sample pre-treatment did not influence the results of the analysis.

Freeze–thaw stability was not checked because the samples were subdivided into small aliquots when collected; each aliquot was thawed once and analysed, without further freezing.

Calibration curves were set up on blank plasma and urine by adding standard solutions of the analytes to the samples at different concentrations and of the IS at constant concentration and subjecting the resulting mixture to SPE (plasma) or to hydrolysis and SPE (urine). Two linearity ranges were considered, depending on the expected plasma or urine levels and based on the results of the preliminary FPIA assays: one for medium-low expected levels and one for high expected levels. Good linearity was obtained over the following concentration ranges in both matrices: 2–50 ng mL^{-1} for THC and 0.8–20 ng mL^{-1} for THC-COOH (1000 μL of plasma or urine; 50 μL loop); 50–650 ng mL^{-1} for THC and 20–2500 ng mL^{-1} for THC-COOH (500 μL of plasma or urine; 20 μL loop). The LOQ was 2 ng mL^{-1} for THC and 0.8 ng mL^{-1} for THC-COOH, while the LOD was 0.8 ng mL^{-1} for THC and 0.3 ng mL^{-1} for THC-COOH. Both values were calculated according to the United States Pharmacopoeia [41]. Linearity parameters are reported in detail in Table 1.

Extraction yield (absolute recovery) and precision assays were carried out on blank plasma and urine spiked with analyte concentrations corresponding to the lower limit, middle point and upper limit of the calibration curves, namely: 2, 350 and 650 ng mL^{-1} for THC; 0.8, 1250 and 2500 ng mL^{-1} for THC-COOH. The results of these assays are reported in Table 2.

As one can note, mean extraction yields were very good, always being higher than 91% for both analytes (98% for the IS). Precision results were also satisfactory: R.S.D. values for repeatability were always lower than 6.9% for both analytes; for intermediate precision they were lower than 7.0%.

Selectivity was evaluated with respect to both exogenous and endogenous compounds.

To assess exogenous compound selectivity, standard solutions of several drugs commonly taken by *Cannabis* users, or which could interfere due to their physico-chemical properties, were injected into the HPLC: other abuse drugs (such as amphetamines and opiates), antidepressants and anxiolytics–hypnotics, which can be coadministered during THC therapy. The interference test runs lasted 30 min in order to detect possible late-eluting compounds, which could interfere in subsequent analytical runs. The complete list of the tested drugs is reported in Table 3. As can be seen, none of the compounds was detected: thus, none was subjected to SPE to ascertain whether they could be extracted. Anxiolytics–hypnotics, however, were subjected to hydrolysis and SPE, in order to check for possible

Table 1
Linearity parameters

Compound	Linearity range (ng mL^{-1})	Equation coefficients, $y = a + bx$[a]		r^2	LOQ (ng mL^{-1})	LOD (ng mL^{-1})
		a	b			
THC	2–50	0.0050	0.0307	0.9990	2	0.8
	50–650	0.0036	0.0151	0.9995		
THC-COOH	0.8–20	0.0061	0.0813	0.9991	0.8	0.3
	20–2500	0.0048	0.0423	0.9995		

[a] y = analyte/IS peak area ratio, pure number; x = analyte concentration, ng mL^{-1}; a = intercept, pure number; b = slope, mL ng^{-1}.

Table 2
Extraction yield and precision results

Compound	Concentration (ng mL^{-1})	Plasma			Urine		
		Extraction yield, %[a]	Repeatability, R.S.D.%[a]	Intermediate precision, R.S.D.%[a]	Extraction yield, %[a]	Repeatability, R.S.D.%[a]	Intermediate precision, R.S.D.%[a]
THC	2	92	6.0	6.9	92	6.5	6.9
	350	95	4.6	4.8	94	5.3	5.4
	650	94	3.0	3.5	96	3.7	4.0
THC-COOH	0.8	92	6.8	6.4	94	6.0	6.3
	1250	94	3.9	4.6	96	4.6	4.7
	2500	96	2.3	2.4	97	2.8	3.1
IS	1000	98	2.1	2.5	98	2.2	2.5

[a] $n = 6$.

Table 3
Compounds tested as possible interference

Therapeutic class	Compound	t_R (min)
Analytes	Indomethacin (IS)	3.7
	THC-COOH	5.4
	THC	10.8
Antidepressants	Amitriptyline	n.d.
	Citalopram	n.d.
	Fluoxetine	n.d.
	Imipramine	n.d.
	Mirtazapine	n.d.
	Sertraline	n.d.
	Venlafaxine	n.d.
Anxiolytics–hypnotics	Brotizolam	n.d.
	Clobazam	n.d.
	Clonazepam	n.d.
	Diazepam	n.d.
	Flurazepam	n.d.
	Lorazepam	n.d.
Abuse drugs	Amphetamine	n.d.
	Buprenorphine	n.d.
	Codeine	n.d.
	MDMA (Ecstasy)	n.d.
	Methadone	n.d.
	Morphine	n.d.

n.d. = not detected within a 30 min chromatographic run.

interference from degradation products such as benzophenones. Again, no interfering peak was found.

To assess endogenous compound selectivity, the plasma and urine of six different non-consumer volunteers were analysed. None of the blank samples showed any peak, which could interfere with the analysis. Therefore, the method has demonstrated to be very selective.

3.5. Application to plasma and urine samples

Having thus validated the method, it was applied to the analysis of plasma and urine samples collected at the Toxicological Analysis Laboratory of the "S. Maria delle Croci" Hospital, Ravenna (Italy), from subjects who were suspected of consuming *Cannabis* and who resulted positive to the FPIA test for cannabinoids. As examples, the chromatograms of a plasma sample and of a urine sample from one of these subjects after SPE (four-fold concentration, 20 μL loop) are reported in Figs. 4b and 5b, respectively. Again, peak shapes and resolution are very satisfactory and no interference is present. The analyte concentrations found in these samples were: plasma sample, 68 ng mL^{-1} of THC and 180 ng mL^{-1} of THC-COOH; urine sample, 78 ng mL^{-1} of THC and 175 ng mL^{-1} of THC-COOH.

Plasma and urine samples from several other subjects were analysed with the method proposed and they always resulted free from interference.

3.6. Accuracy

Accuracy was evaluated by means of recovery assays. Standard solutions of the analytes at three different concentrations were added to plasma and urine samples of *Cannabis* users: the concentrations added were 2, 150 and 300 ng mL^{-1} for THC and 0.8, 500.0 and 1000.0 ng mL^{-1} for THC-COOH. Then,

mean analyte recovery and S.D. values were calculated. The results (mean recovery ± S.D.) were: (86 ± 4)% for THC and (89 ± 3)% for THC-COOH in plasma; (87 ± 2)% for THC and (88 ± 2)% for THC-COOH in urine. Thus, method accuracy is satisfactory.

4. Conclusion

The HPLC method with UV detection presented here for the simultaneous analysis of THC and THC-COOH in human plasma and urine is simple, sensitive and selective.

The SPE procedure implemented for the sample pre-treatment, based on C8 cartridges, allows obtaining very good extraction yields (>91% for both analytes) and purification from endogenous and exogenous interference. The method has been successfully applied to the analysis of real samples from *Cannabis* users, also giving satisfactory accuracy results.

When compared to the other methods found in the literature, the proposed method is certainly less expensive and more widely applicable in clinical laboratories than those which use HPLC-mass spectrometry [19,20,24–30] or HPLC-electrochemical detection [31,32]. With respect to other HPLC-UV methods [33–37], which use more complicated SPE procedures and have lower extraction yield values (75–85%), the presented method has the further important advantage of simultaneously analysing THC and THC-COOH. In fact, the quantitative determination of both compounds gives a more complete pharmacokinetic and toxicokinetic outline of abusers and patients. Moreover, this method can be applied to both plasma and urine and thus gives more complete information to the clinician and to the pharmacologist.

Thus, the developed method is suitable for the determination of THC and THC-COOH in plasma and urine of users. Furthermore, it seems to be also suitable for the therapeutic drug monitoring (TDM) of patients undergoing therapy with THC for the control of chronic pain. A research project related to the therapeutic application to oncologic patients is now in progress.

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