

Time-Resolved Fluoroimmunoassay for Δ^9 -Tetrahydrocannabinol As Applied to Early Discrimination of *Cannabis sativa* Plants

Maria A. Bacigalupo,* Adriano Ius, Giacomo Meroni, Gianpaolo Grassi,[†] and Anna Moschella[†]

Istituto di Biocatalisi e Riconoscimento Molecolare, CNR, Via Mario Bianco 9, Milano 20131, Italy

In the cultivations of *Cannabis*, it is important to be able to distinguish fiber-type plants from drug-type plants by an easy observation of their phenotype. This study required the screening of many samples for their cannabinoid content. A simple and highly sensitive time-resolved fluoroimmuno-logical method was developed for the determination of Δ^9 -tetrahydrocannabinol in the leaf extracts. The useful range of the calibration curve was between 10 pg and 25 ng of standard. Matrix effects were minimized by a high dilution of samples.

Keywords: Δ^9 -Tetrahydrocannabinol; cannabinoids; *Cannabis*; TR fluoroimmunoassay; monoclonal antibody

INTRODUCTION

The cannabinoid concentrations of *Cannabis sativa* vary widely depending not only on the genotype but also on the vegetative phase and cultivation environment (Grassi et al., 1996; De Meijer et al., 1992). On the basis of their cannabinoid content, *Cannabis* plants are classified as either fiber-type or drug-type plants. According to Fetterman et al. (1971) a phenotype with a ratio of (% Δ^9 -THC + % cannabinol)/% cannabidiol that is >1 is classified as a drug-type plant (Δ^9 -THC is Δ^9 -tetrahydrocannabinol).

However, *Cannabis* is generally defined as a fiber-type plant (Avico et al., 1985) if the Δ^9 -THC (the major psychoactive component) content is <0.3%, and such a maximum limit is observed in the European Community (G.U. 121/4, Regulation CEE 1164/89 of 28-04-89) to qualify for subsidy.

In the context of a project for developing the cultivation of *Cannabis* fiber plants, high priority was given to introducing characteristic morphological features of a phenotypic mutant, enabling facile visual identification of the low cannabinoid content variety (Murari et al., 1983). A low Δ^9 -THC content fiber-hemp cultivar needed to be selected from among a large number of young sample plants (Barni-Comparini et al., 1984; Avico et al., 1985) using a mass screening method that had to be sensitive, simple, applicable on large numbers of samples, and, if possible, inexpensive. An immunological assay responds well to these requirements. It has been widely employed to measure cannabinoids in clinical and forensic settings (Owens et al., 1981; Colbert et al., 1987; Altunkaya et al., 1991; Goodal and Basteyns, 1995; Pelissier et al., 1997) and is easily transferred for extracts of *Cannabis* leaves (Tanaka et al., 1996; Grassi et al., 1997).

We developed a time-resolved fluoroimmunological assay (TR-FIA) for detecting Δ^9 -THC in *Cannabis* that offers the advantages of a broad range of linearity for

the calibration curve and a high sensitivity. It can also be applied to other plant extracts (Bacigalupo et al., 1998), for which the effect of the matrix can be reduced by using high rates of dilution.

EXPERIMENTAL PROCEDURES

Apparatus. A model 1232 Delfia fluorometer with time resolution (Wallac) was used for fluorescence measurements; a Bruker 300 AC instrument operating at 300 MHz was used for NMR measurements; and a GC 8000 TOP gas chromatograph with FID 80 flame ionization detector (CE-Instrument) was used for assay comparisons.

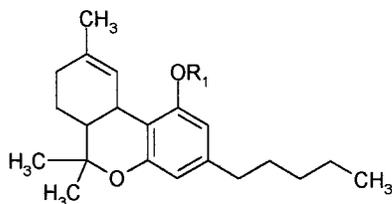
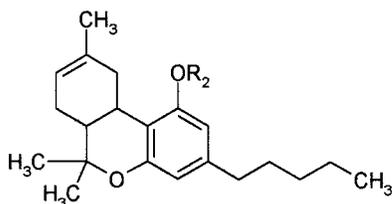
Materials. Polystyrene microtiter plates were obtained from Nunc (Roskilde, Denmark); Sephadex G-50 was purchased from Pharmacia (Uppsala, Sweden); mouse monoclonal antibody isotyping kit was purchased from Boehringer (Milan, Italy); bovine serum albumin (BSA), ovalbumin (OVA), porcine thyroglobulin (PT), Δ^9 -THC, Δ^8 -tetrahydrocannabinol (Δ^8 -THC), cannabinol (CBN), cannabidiol (CBD), 11-nor- Δ^8 -tetrahydrocannabinolic acid, 11-nor- Δ^9 -tetrahydrocannabinolic acid, and sheep anti-mouse IgG polyclonal antibody were from Sigma (Dorset, U.K.); silica gel and TLC plates were from Merck (Darmstadt, Germany). All other chemicals were from Aldrich (Milan, Italy), and 4,7-bis(chlorosulfonyl)-1,10-phenanthroline-2,9 dicarboxylic acid (BCPDA) was synthesized (Evangelista et al., 1988). Sheep anti-mouse IgG-BCPDA conjugate was obtained as described (Ius et al., 1990).

Synthesis. Δ^9 -Tetrahydrocannabinol porcine thyroglobulin (Δ^9 -THC-PT) and Δ^8 -tetrahydrocannabinol bovine serum albumin (Δ^8 -THC-BSA) conjugates were synthesized as shown in Chart 1.

Δ^9 -THC-1-O-Ethoxycarbonyl Propyl Ether (**2**). Tetraethylammonium fluoride (75 mg) was added to a solution of Δ^9 -THC (50 mg) in *N,N*-dimethylformamide (1 mL). The solution was warmed at 80 °C under vacuum with a water pump for 10 min. Ethyl 4-bromobutyrate (22 μ L) was added, and the solution was stirred at room temperature in the dark. After 24 h, 10 mL of aqueous NaHCO₃ (1%) was added, and the reaction mixture was extracted with ethyl acetate and dried (MgSO₄). Evaporation of the solvent followed by purification by column chromatography (2 × 30 cm) on silica gel (230–400 mesh) eluted with 20% acetone in hexane produced 26 mg of **2**. This compound was unequivocal on TLC plates (silica gel F₂₅₄) with an *R*_f = 0.73 (acetone/*n*-hexane = 1:4): ¹H NMR (300 MHz, CDCl₃) δ 6.30 (1H, s), 6.27 (1H, s), 6.21 (1H, s), 4.01 (2H, t), 2.56 (2H, t), 2.46 (2H, t), 1.67 (3H, s), 1.40 (3H, s), 1.07 (3H, s), 0.87 (3H, t).

* Author to whom correspondence should be addressed (e-mail bacigalupo@ico.mi.cnr.it; fax ++39 02 28500036).

[†] Present address: Istituto Sperimentale per le Colture Industriali, Via di Corticella 133, Bologna 40129, Italy.

Chart 1. Structure of THC Isomer Analogs1 - R₁ = H2 - R₁ = (CH₂)₃-COOCH₂CH₃3 - R₁ = (CH₂)₃-COOH4 - R₁ = (CH₂)₃-CONH-PT5 - R₂ = H6 - R₂ = (CH₂)₃-COOCH₂CH₃7 - R₂ = (CH₂)₃-COOH8 - R₂ = (CH₂)₃-CONH-BSA

Δ^9 -THC-1-O-Carboxypropyl Ether (3). The ethyl ester (2) (24 mg) was dissolved with 50 mg of NaOH in 2 mL of methanol and 0.5 mL of H₂O; the solution was heated at reflux for 1 h. The alkali was neutralized by addition of diluted sulfuric acid. The methanol was evaporated, water added, and the product extracted with ethyl acetate. After drying with MgSO₄, the solvent was removed to yield the crude product (chromatographically unequivocal) in an essentially quantitative yield.

Δ^9 -THC-PT (4). Ten milligrams of 3 was dissolved in 1 mL of freshly distilled dimethylformamide, cooled to 4 °C, and 8 μ L of tri-*n*-butylamine was added. After 10 min, 5 μ L of isobutyl chloroformate was added, and the solution was left for 30 min at 4 °C. This mixture was added to 20 mg of porcine thyroglobulin dissolved in 2 mL of 40% dimethylformamide in water, and then 40 μ L of 1 N NaOH was added. The pH was kept at 8.5–9.5 and the reaction mixture left overnight at 4 °C. Subsequently, the reaction mixture was dialyzed for 48 h against 0.05 M NH₄CO₃ and lyophilized.

Δ^8 -THC-1-O-Carboxycarbonyl Propyl Ether (6) and Δ^8 -THC-1-O-Carboxypropyl Ether (7). The reaction procedure and molar quantities used were the same as for 2 and 3. The starting material was Δ^8 -THC (5).

Δ^8 -THC-BSA (8). The reaction procedure and molar quantities used were the same as for 4. The starting material was compound 7.

Antibody Production. BALB/c mice were injected with 100 μ g of Δ^9 -THC-PT conjugated (Thorpe, 1994; Knott et al., 1997) in physiological solution and 1:2 v/v Freund's complete adjuvant. After 2 weeks, the mice were treated with a booster injection of 200 μ g of the immunogen emulsified with Freund's incomplete adjuvant. Another 200 μ g of conjugate was injected 2 days before cell fusion and subsequent hybrid cell selection (Fazekas de Saint Groth and Sheiddegger, 1980) and assayed by competitive ELISA for antibody characterization. The selected anti-THC monoclonal antibody was characterized for the isotype using isotyping kit according to the instructions

Table 1. Specificity of Anti- Δ^9 -THC Monoclonal Antibody (F10 Clone)

compound	cross-reaction, %
Δ^9 -THC	100
Δ^8 -THC	96
11-nor Δ^9 -tetrahydrocannabinolic acid	10
11-nor Δ^8 -tetrahydrocannabinolic acid	1.8
cannabinol (CBN)	0.15
cannabidiol (CBD)	<0.01

of the manufacturer. The F10 clone (IgG1 isotype) was selected for use in the immunoassay.

Extract Preparation. Samples were obtained from 200 mg of dried *Cannabis* material extracted for 20 min with 4 mL of methanol in sealed glass tubes. After filtration, the samples were stored at -20 °C and diluted in buffer (0.05 M Tris-HCl, pH 7.5, containing 0.9% NaCl, 0.25% gelatin, and 0.05% NaN₃) before use.

Coating of Microwells. The Δ^8 -THC-BSA conjugate was adsorbed on polystyrene microtiter wells; the wells were coated overnight at 27 °C with 250 μ L of 10 μ g/mL conjugated solution in 0.1 M sodium carbonate (pH 9). After washing, a second coat was laid with 300 μ L of 2% ovalbumin solution in carbonate buffer. After 4 h at 27 °C, the wells were washed with Tris-HCl assay buffer, dried, and stored at 4 °C until used.

TR-FIA. Δ^9 -THC standard dissolved in methanol was diluted in a pooled methanol extract with low cannabinoid content and Tris-HCl buffer (1:5000 v/v) in serial concentrations between 10 pg and 25 ng in 50 μ L. Standard or sample extracts (diluted 1:5000 with the buffer) were incubated in duplicate for 30 min at room temperature in the coated wells with 100 μ L of specific antibody. The wells were washed, and 150 μ L of a rabbit anti-mouse IgG labeled with BCPDA was added, followed by a further 30 min of incubation. After a 0.15 M NaCl wash, the bound BCPDA was determined by adding 170 μ L of dissociation solution (4 M urea, 1% sodium dodecyl sulfate, and 10⁻⁶ M Eu³⁺) to each well. Fluorescence was measured after 20 min, at an excitation wavelength of 345 nm. The delay time was 400 μ s after excitation, the emitted light being at 615 nm.

Gas Chromatography. To compare analytical data, all samples were analyzed by GC, and the gas chromatographic results were computed using Chrom-Card (version 1.21) software. A glass column (length = 1.2 m and i.d. = 3 mm) was packed with 2% DV-17 on 80–100 mesh Chromosorb WHP. The following conditions were employed: column temperature, 235 °C; injector temperature, 265 °C; detector temperature, 245 °C; gas flow (helium), 30 mL/min. Sensitivity was 1 ng/mL of standard.

RESULTS AND DISCUSSION

The F10 selected clone was tested for cross-reactions with the compounds reported in Table 1.

To avoid interference from the matrix, standard curves were carried out in a pooled extract, without detectable Δ^9 -THC and Δ^8 -THC contents. All samples were assayed in duplicate. Nonspecific bonds assayed in microwells with mouse nonimmune IgG were <3% of total fluorescence. The coefficients of variation for two samples with 25 pg and 6 ng of Δ^9 -THC were, respectively, 8.6 and 10.7% (means of six assays carried out in 2 weeks). The solid phase was prepared with the Δ^8 -THC derivative because its affinity for the antibody was a little lower than that of the Δ^9 -THC, which results in a greater sensitivity of the standard curve (Allen and Redshaw, 1978; Mitsuma et al., 1986). Under these conditions, the upper limit of quantitation was 25 ng of Δ^9 -THC/g of dried *Cannabis* material. Samples were assayed in duplicate, and the results were compared

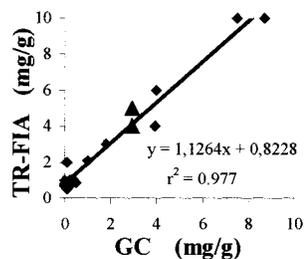


Figure 1. Correlation graph for Δ^9 -THC assays in leaf extracts: (▲) false positive points.

with GC findings. TR-FIA values plotted against GC values result in the linear regression graph given in Figure 1.

The graph (Figure 1) shows that our method gives an overestimate in comparison with GC, probably because of interfering compounds in the *Cannabis* extracts. However, all negative samples were correctly evaluated. Despite two false positive results, TR-FIA seems to be useful for a rapid screening of samples before GC confirmation. A breeding program for THC reduction in hemp cultivars needs to detect plants with a lower THC content as soon as possible at flowering. For our purposes, the fluoroimmunological method described is an improvement over traditional cannabinoid detection methods. It can also be applied to a wider program of plant analysis. The advantages of this method are its easy and rapid (100 extracts can be tested in 90 min) implementation and the number of GC analyses that can be reduced to only the few positive samples, with considerable savings of cost and time.

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