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POST-COLUMN DERIVATIZATION PROCEDURE FOR THE COLORIMETRIC ANALYSIS OF TISSUE CANNABINOIDS SEPARATED BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A new method for quantitating cannabidiol (CBD) and Δ^9-tetrahydrocannabinol (THC) in mouse plasma and brain involves (1) the separation of CBD and THC from their major metabolites by the use of isocratic, reversed-phase high-performance liquid chromatography (HPLC), and (2) the on-line reaction of the cannabinoids with Fast Blue Salt B (FBB) as the former elute from the column; the colored cannabinoid–FBB derivatives are then detected at 490 nm in a spectrophotometer with a sensitivity of less than 50 ng. In addition to this HPLC–FBB analytical procedure, a method for extracting CBD and THC from brain and plasma is described, and selected examples illustrate the procedure's application to the analysis of CBD and THC in mouse plasma and brain samples taken from animals injected with these two cannabinoids.

INTRODUCTION

The psychoactivity of Δ^9-tetrahydrocannabinol (THC) has stimulated considerable medical and legal interest in the pharmacodynamic and pharmacokinetic properties of this cannabinoid. As a consequence, several sensitive analytical methods have been developed and directed toward the problems of detection and quantitation of very low concentrations of THC in plasma. The major analytical procedures which have been devised can be categorized into five different groups: gas chromatography–mass spectroscopy (GC–MS)[1–3], radioimmunoassay[4–6], radioimmunoassay combined with high-performance liquid chromatography (HPLC)[7], HPLC of fluorescent dansyl derivatives of the cannabinoids[8] and HPLC combined with the detection of the cannabinoids at 220 nm[9,10].

Although cannabidiol (CBD) is not psychoactive, it, too, can produce functional changes in the central nervous system, as exemplified by its anticonvulsant properties and motor toxicity[11,12]. Unfortunately, the development of specific analytical procedures for measuring tissue concentrations of this cannabinoid has not received much attention, compared with that of THC; and an evaluation of the THC methods described above revealed that all of them suffer from inadequacies which

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precluded their application to pharmacological and pharmacokinetic investigations of CBD in our laboratory. For example, although the development of a specific GC–MS method for CBD is feasible, the necessary equipment was not available to us. Aside from the problem of access to GC–MS equipment, this approach requires some expertise in the synthesis and purification of a suitable deuterated internal standard in order to achieve reliable quantitation; in addition, particular attention must be paid to the removal of lipids and other endogenous substances in tissue which could potentially interfere with this very sensitive analytical tool. Radioimmunoassay is another sensitive procedure, but none exists for CBD; the radioimmunoassays currently available are oriented toward THC. Even if a radioimmunoassay for CBD were developed, there would undoubtedly be a cross-reactivity problem similar to that encountered in the THC assay. The cross-reactivity problem might be circumvented by initially isolating CBD with HPLC before assaying tissue extracts[7], but this procedure introduces an additional step and an additional time element into the analysis. The HPLC approach based upon the chromatography and detection of a fluorescent dansyl derivative of CBD is flawed because at least two different derivatives of CBD are formed resulting in two distinct peaks on the chromatogram. Finally, of the two HPLC methods that monitor the column eluate at 220 nm, one has been applied to the quantitation of cannabinoids in urine[10]; however, this particular procedure requires an initial separation step using thin-layer chromatography (TLC). Whether this approach could be successfully applied to plasma and tissue extracts is unclear, but there are grounds for pessimism because of the ubiquitous presence of UV-absorbing substances in such extracts.

Although the prospects for acquiring pharmacokinetic data on CBD with the available THC methods initially appeared bleak, the reports on the reaction of cannabinoids with the azo dye Fast Blue Salt B (FBB) by Korte and Sieper[13] and Chia *et al.*[14] suggested that this reaction, which results in the formation of colored cannabinoid–dye compounds, might be integrated with HPLC techniques to quantitate CBD in tissue. Until now, the only analytical application of FBB in marihuana research has been as a spray reagent for detecting cannabinoids separated by TLC; the present report, however, describes a new application for this dye, in which a FBB solution is added continuously to the eluate from a reversed-phase microparticulate column; the cannabinoid–FBB compounds are formed within the tubing connecting the column to a spectrophotometer, and the colored derivatives are detected at a visible wavelength. The sensitivity of this HPLC–FBB method is not as great as that of either GC–MS or radioimmunoassay techniques; nevertheless, the sensitivity has been found to be more than adequate for obtaining pharmacokinetic data on CBD and THC in experimental animals. Furthermore, the HPLC–FBB analytical method reported here is capable of isolating and detecting the major metabolites of these two cannabinoids.

EXPERIMENTAL

Apparatus

For liquid chromatography the following equipment manufactured by Waters Assoc. (Milford, MA, U.S.A.) was used: two Model 6000A solvent delivery systems, a Model 660A solvent programmer, a Model U6K valve injector fitted with a 2-ml injection loop and a 30 cm × 3.9 mm I.D. stainless-steel column packed with either

μPorasil or μBondapak C_{18}. Changes in the absorbance of the column effluent were monitored with either a Model LC-55B spectrophotometer (Perkin-Elmer, Norwalk, CT, U.S.A.) or a Model 440 absorbance detector (Waters Assoc.). The output from the spectrophotometer or detector was recorded on an Omniscribe recorder (Houston Instruments, Austin, TX, U.S.A.); peak retention times, areas and heights were determined with a Minigrator (Spectra-Physics, Santa Clara, CA, U.S.A.) connected in series between the spectrophotometer (or detector) and the recorder. Hamilton 800-series syringes, modified for use with the U6K injector, were purchased from All-tech (Arlington Heights, IL, U.S.A.). Tissue homogenates were made in tissue grinders with conical ground-glass mortars and pestles (size 21 Duall; Kontes, Vineland, NJ, U.S.A.); and tissue extracts were prepared from the homogenates with the aid of IEC Models K and CRU-5000 centrifuges (Damon, Needham Heights, MA, U.S.A.). The Lang-Levy type micropipettes used in various steps of the extraction and assay procedure were purchased from H. E. Pedersen (Copenhagen, Denmark).

Chemicals

The grades of the solvents used for cannabinoid tissue extraction and for chromatography were: Chromar (Mallinckrodt, St. Louis, MO, U.S.A.), HPLC (J. T. Baker, Phillipsburg, NJ, U.S.A.) and double-distilled water. FBB (tetrazotized *o*-dianisidine chloride) was obtained from Matheson, Coleman & Bell (Norwood, OH, U.S.A.); analytical-reagent grade sodium nitrite was purchased from Mallinckrodt. The cannabinoids [THC, 11-hydroxy-Δ^9-tetrahydrocannabinol (11-OH-THC), CBD and cannabinol (CBN)] were supplied by the Biomedical Research Branch, Division of Research of the National Institute on Drug Abuse (Rockville, MD, U.S.A.). A set of standard solutions (5–100 μg/ml in 95% ethanol) was prepared for each cannabinoid and kept in tightly sealed, screw-cap tubes which were stored in a refrigerator at 0°C. There were no detectable changes in the composition or concentration of these standards over a 5-month period of use; care was taken to keep the standards on ice while removing aliquots, and to reseal and return them to the refrigerator immediately afterwards. All glassware, with the exception of the tissue grinders, was treated with a 10% solution in hexanes of Dri-Film SC-87 (Pierce, Rockford, IL, U.S.A.) followed by several rinses with acetone and double-distilled water and finally dried in a 100°C oven.

HPLC–FBB cannabinoid assay

The HPLC–FBB cannabinoid assay that evolved from the experiments described in the Results and discussion section consists of the following procedures and equipment. Aliquots of standards and of tissue extracts are injected onto a μBondapak C_{18} column which has a short guard column attached to its inlet. The guard column is constructed from a 5-cm length of 3.9 mm I.D. stainless-steel tubing fitted with a Swagelok (Crawford Fitting, Solon, OH, U.S.A.) zero dead volume reducing union (1/4 in. to 1/16 in. tubing) at each end. The union on the outlet end has a removable 2-μm stainless-steel frit with a Waters Assoc. gasket assembly (filter and PTFE washer) on top; this gasket assembly and frit combination serves as the support for the μBondapak C_{18}/Corasil (37–50 μm particle size; Waters Assoc.) packing material. When numerous brain extracts are to be analyzed, the addition of a guard column helps to prolong the life of the main column, because these extracts contain material that appears to bind irreversibly to the μBondapak C_{18} type

column packing; as a consequence, the normal operating pressure of the system (approx. 1000 p.s.i.) rises gradually as a function of the number of samples assayed. When the operating pressure exceeds 1500 p.s.i., the guard column is replaced; this criterion is arbitrary and the replacement is generally made after 100–150 samples have been analyzed.

The cannabinoids are eluted from the column with an 85% methanol solution at a flow-rate of 1.5 ml/min. Prior to each analysis the methanol and water are filtered through a Whatman GF/C glass filter (Reeve Angel, Clifton, NJ, U.S.A.) into 1-l Erlenmeyer flask reservoirs; the eluting solvent is either premixed and delivered with just one of the 6000A pumps or the mixing and solvent delivery are effected with both pumps and the 660A solvent programmer. The solvents are withdrawn from the reservoir(s) through a cylindrical, sintered, stainless-steel filter (20–30 μm pore size). The eluate from the μBondapak C_{18} column flows through a short length of PTFE tubing (0.3 mm I.D.) connected to a 3-way PTFE 1XP valve (Hamilton, Reno, NV, U.S.A.). A second 5-cm length of PTFE tubing, with dimensions identical to the first, couples the 3-way valve to an acrylic plastic "T" (Pierce). The FBB dye solution (either 0.1 or 0.25%) is introduced into the column eluate at the T at the rate of 0.5 ml/min and the combined solutions are carried from the T to the spectrophotometer through a 4-m coil of PTFE tubing (0.8 mm I.D.). The coil serves as a mixing device and provides the dwell time required for the FBB to react with the cannabinoids before they reach the flow cell of the spectrophotometer; the reaction products are detected at 490 nm.

Introduction of the dye solution into the column eluate is achieved by using a Milton-Roy Simplex single-piston, reciprocating pump Model 396-31 (Laboratory Data Control, Riviera Beach, FL, U.S.A.) connected to the plastic T with a length of PTFE tubing (1.5 mm I.D.). In addition to the pump, two 3-way valves, two 2-way valves and a pressure gauge are connected in series with the pump using fittings which allow operation at back pressures in excess of 500 p.s.i.; these valves and fittings are assembled in such a way as to permit the use of two solvent reservoirs. The ability to draw from two different solvent reservoirs is a convenience, especially during the shutdown procedure when the system has to be flushed with acetone, as will be discussed below. All of the HPLC experiments described in this report were carried out at room temperature (24–26°C).

Preparation of dye solution and shutdown procedure

A 0.1 or 0.25% FBB solution is prepared by dissolving the dye in 160 ml of water followed by the addition of 200 ml of methanol and 40 ml of a 10% aqueous solution of sodium nitrite. Although the dye forms a clear yellow solution in water, the addition of methanol and sodium nitrite results in a rapid darkening of the solution and the formation of a precipitate. The dye solution is stirred for approximately 30 min and filtered through a Whatman GF/C filter disc followed by filtration through a Metricel GA-6 membrane filter (0.45 μm; Gelman Instruments, Ann Arbor, MI, U.S.A.). The filtered dye solution is placed in a reservoir of amber-colored glass and withdrawn by the pump through a sintered, stainless-steel filter identical to the type used in the methanol and water reservoir(s). The dye solution continues to darken, but remains clear for more than 5 h. During the initial hour of an HPLC analysis, there is a baseline drift of 0.05 absorbance units/h which requires a readjustment of the spectro-

photometer between sample injections; this baseline drift cannot be attributed to the electronics of the detector or the recorder. After the first hour, the drift decreases to approximately 0.005 absorbance units/h. There is no obvious explanation for the gradual decrease in the drift; the dye darkens progressively, yet there are no marked differences in the peak height or area of standards analyzed during the first hour and then reexamined after the first hour. Moreover, if a dye solution which has been used for several hours is replaced with a freshly prepared solution, the initial large baseline drift does not appear.

At the conclusion of an experiment, the 3-way valve between the column and the T is switched to shunt the column eluate to a waste solvent reservoir. The dye pump and its attendant tubing and valves, the T, the 4-m mixing loop and the spectrophotometer flow cell are flushed with a 50% methanol solution followed by acetone and then with the 50% methanol solution a second time. The acetone wash is important in order to remove all traces of the remaining dye; if not removed, the residual dye eventually precipitates within the system and can block the lines during a subsequent analysis. Another precaution that must be exercised is to avoid the introduction of the FBB solution into a column eluate which is either 100% methanol or water; precipitation will result and a sudden block in the post-column part of the system will occur.

Extraction of cannabinoids from brain

The following procedure was developed to extract cannabinoids from the brains of adult mice. Fresh or frozen brain samples (approx. 0.5 g wet weight) were placed in tissue-grinder mortars containing 1 ml of ice-cold methanol and a 10–20-μl aliquot of a CBN standard solution (55 μg/ml); CBN was used as the internal standard in the HPLC–FBB cannabinoid assay. An additional 2-ml volume of methanol was added to each mortar and the brains homogenized while the mortars were kept in an ice–water bath during the actual homogenization. After the homogenization, the mortars were centrifuged at 1000 *g* for 5 min at 0°C; the supernatants were decanted into 12-ml, graduated, conical glass tubes. The pellets were resuspended in 3 ml of ice-cold methanol and centrifuged as before; the supernatants were combined with the first and the pellets discarded.

The combined supernantants were reduced in volume to approximately 1.5 ml; to increase the rate of solvent evaporation, the 12-ml tubes were placed in a water bath (65°C) and air was blown over the solvent surface through a manifold. After the volume was reduced, the tubes were placed in an ice–water bath for 10–20 min to promote precipitation; the samples were then centrifuged for 5 min at 1000 *g* at 0°C. The clear supernatants were transferred to 15-ml, stoppered, conical glass tubes and the pellets resuspended in 0.5 ml of ice-cold 80% methanol. The tubes were recentrifuged, the resulting supernatants added to the first and the combined supernantants extracted by the sequential addition of 2 ml of dichloromethane, 1 ml of water and 5 ml of hexanes; the tube contents were well mixed on a vortex-type mixer after each addition. The resulting two phases were separated by centrifuging for 5 min at 500 *g* at room temperature; the upper organic solvent phases were transferred with a Pasteur pipette to glass scintillation vials. The methanol–water phases were reextracted with 2 ml of dichloromethane and 5 ml of hexanes and the second organic phase combined with the first. The transfer of the organic phases was facilitated by chilling the tubes in ice,

which reduced the tendency for the organic solvent to leak from the pipettes during the transfer.

The solutions in the scintillation vials were evaporated to dryness under a hood by putting the vials in a water bath (65°C) and by passing a small air stream into each vial through an air manifold. The use of air rather than nitrogen did not induce any detectable breakdown of the cannabinoids; however, such cannabinoid breakdown, especially of THC, does occur if drying takes place in non-siliconized vials, regardless of whether air or nitrogen is used. The dried extracts were dissolved in 1 ml of hexanes; the resulting solutions were then transferred to 10 × 75 mm glass culture tubes and evaporated to dryness (approx. 15 min) in a small vacuum centrifuge (Speed Vac, Savant Instruments, Hicksville, NY, U.S.A.)[15]. After redissolving the extracts in 200 μl of hexanes, the samples were dried a second time in order to concentrate the recovered material into a small pellet. Although some residue remained on the tube walls, most of the material appeared in the form of an amber-colored pellet. These pellets were redissolved in 20 μl of methanol which, if necessary, was slightly warmed; 100 μl of acetone was added, the solution mixed vigorously and the tubes placed in an ice–water bath for 10 min. The samples were centrifuged for 5 min at 1000 *g* at 0°C and the supernatants transferred to 6 × 50 mm glass culture tubes (Corning Glass Works, Corning, NY, U.S.A.) containing 75 μl of absolute ethanol; the resulting precipitate was discarded. Although a small gel-like pellet remained after drying the samples in the vacuum centrifuge, the material (probably phospholipid and cholesterol) in this pellet did not interfere with the assay for the cannabinoids. The pellet was redissolved in 100 μl of methanol and a 75-μl aliquot was chromatographed on the μBondapak C_{18} column.

Extraction of cannabinoids from plasma

Although hexanes are frequently used to extract cannabinoids from plasma, the recovery of CBD and THC after extracting 1 ml of plasma twice with 10 ml of this solvent was considered unsatisfactory (<25%). A more efficient, though more time-consuming, procedure similar to that described for the brain samples was adopted. In this method a 1- or 2-ml aliquot of plasma was pipetted into a 12-ml glass, screw-cap culture tube, followed by the addition of a 10–20-ml aliquot of CBN standard solution (55 μg/ml). The tubes were placed in an ice bath and 5 ml of methanol added; the samples were mixed vigorously and centrifuged for 5 min at 1000 *g* at 0°C. After transferring the supernatants to 12-ml conical glass tubes, the white, gel-like precipitate was resuspended in 3 ml of ice-cold methanol, the tubes centrifuged a second time and the supernatants combined with the first. These supernatants were then processed according to the procedure outlined for the brain samples, with these exceptions: the initial methanol extract was concentrated to 2 ml; there was no 80% methanol wash of the pellet resulting from chilling and centrifuging the initial methanol extract; and water was not added prior to the dichloromethane and hexanes extraction step.

For each group of brain or plasma sample analyzed, CBD or THC was quantitated by preparing a four- or five-point calibration curve using control brain or plasma samples from untreated mice. A fixed aliquot from CBD or THC standard solutions was added to each control sample and a constant amount of CBN was added to the control samples and unknowns; the controls and unknowns were processed

together. The quantity of cannabinoid used to establish the calibration curve ranged from 0.2 to 3.7 μg. Along with the set of standards prepared with tissue, an identical set of standards was prepared in 6 × 50 mm culture tubes containing 100 μl of 95% ethanol; these samples were stored at 0°C, dried with the unknowns and calibration-curve samples at the last step in the extraction procedure and redissolved in 100 μl of methanol prior to the HPLC analysis. The recovery of CBD and other cannabinoids was determined from the peak heights or areas measured for cannabinoid standards extracted from tissue and their corresponding non-extracted samples. Representative recovery values are presented in Table I.

TABLE I

RECOVERY OF CANNABINOID STANDARDS FROM MOUSE BRAIN AND PLASMA

Values are mean ±S.D.

Cannabinoid	*N*	*Brain*	*N*	*Plasma*
THC	10	72.3 ± 5.1	7	76.2 ± 9.9
11-OH-THC	11	79.9 ± 5.9	8	73.5 ± 6.1
CBD	12	83.7 ± 4.3	9	81.5 ± 8.3
CBN	18	74.2 ± 4.9	16	78.1 ± 8.1

RESULTS AND DISCUSSION

Spectral characteristics of the cannabinoid–FBB compounds

The absorption spectra in Fig. 1 were determined after reacting an aliquot of a standard solution of either CBD, THC or CBN in 4.5 ml of 90% methanol with a 1.5-ml aliquot of a 0.1% FBB solution containing sodium nitrite. The cannabinoids and dye were allowed to react for 10 min before the spectra were determined in a Cary

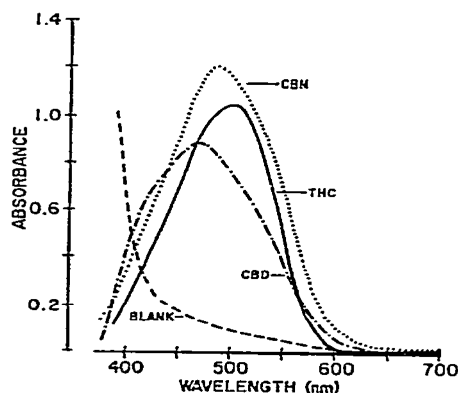


Fig. 1. Spectral scans of cannabinoid–FBB compounds in 80% methanol. Cannabinoid–FBB compounds were prepared by adding 1.5 ml of FBB reagent to a 4.5 ml solution of CBD, CBN or THC in 90% methanol; the mixture was allowed to stand for 10 min before an aliquot was transferred to a cuvette and scanned. The reference cuvette contained a blank solution prepared in a similar way, with the exception that no cannabinoid was present. This blank solution was also scanned using water in the reference cuvette. Cannabinoid concentrations (μg/ml): CBD = 10.2, CBN = 9.2, THC = 8.3. Scanning parameters: speed = 2 nm/sec; slit width = 0.05 mm; time constant = 1 sec. FBB reagent: 0.1% FBB and 1% sodium nitrite in 50% methanol.

118 recording spectrophotometer (Varian, Downey, CA, U.S.A.). The final methanol, dye and nitrite concentrations were 80, 0.025 and 0.25%, respectively; the final THC, CBD and CBN concentrations were 8.3, 10.2 and 9.2 μg/ml, respectively. Although not included in Fig. 1, the 11-OH-THC spectral scan is essentially identical to that of the THC scan. The spectra in Fig. 1 are very similar to those reported by Korte and Sieper[13], who formed the colored complex on paper chromatograms. Table II is a summary of the absorption maxima of CBD, THC, CBN and 11-OH-THC determined in several of the solutions used in the experiments described below. The methanol, nitrite and dye concentrations in the HPLC–FBB procedure are similar to the solvent conditions under which the maxima listed on the last line of Table II were measured.

TABLE II

ABSORBANCE MAXIMA (nm) OF VARIOUS CANNABINOIDS IN DIFFERENT ALCOHOL SOLUTIONS

Solvent	*Nitrite**	*THC*	*11-OH-THC*	*CBN*	*CBD*
Isopropanol	—	465	465	475	450
50% Methanol	—	505	505	490	480
50% Methanol	+	505	505	495	475
88% Methanol	—	505	505	495	485
80% Methanol	+	500	495	490	465

* —, Absent; +, present in 0.25% concentration.

Effect of sodium nitrite

The incorporation of sodium nitrite into the dye solution was found to produce a significant increase in the rate of the reaction of FBB with the cannabinoids (Fig. 2). Although the nitrite produced the same effect whether the cannabinoid was THC,

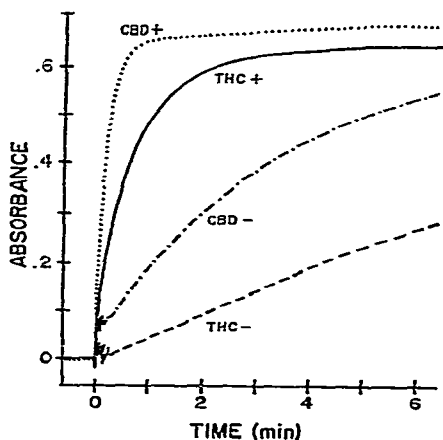


Fig. 2. Effects of nitrite on the reaction of FBB with CBD and THC. CBD (15 μg) or THC (13 μg) was added to a cuvette containing 2 ml of either 85% methanol (—) or a 0.4% solution of sodium nitrite in 85% methanol (+). The cuvette was placed in the spectrophotometer and the reaction was initiated by injecting 1 ml of a 0.1% solution of FBB in 50% methanol into the cuvette. Wavelength = 490 nm.

11-OH-THC, CBD or CBN; the CBN rate of reaction in the absence or presence of nitrite was always slower than the other three (see Fig. 4).

Optimal FBB–cannabinoid ratios

Concentration values of the dye solutions given in the present paper are based upon the initial weight of FBB used. Unfortunately, the number and relative proportions of reactive compounds in the dye are not known; moreover, during the preparation of a FBB solution, the filtration step removes a significant amount of material; consequently, a critical examination of the stoichiometry of the cannabinoid–FBB reaction was not made. Nevertheless, an imprecise stoichiometric relationship can be demonstrated (Fig. 3). These data result from an experiment in which a progressively larger volume of a 0.4% FBB solution was added to test tubes containing a fixed amount of cannabinoid (0.7–0.8 μg) dissolved in 150 μl of methanol. After adding a sufficient volume of water–methanol–nitrite diluent to each tube to bring the final reaction volume to 200 μl, the cannabinoid and FBB were allowed to react for approximately 10 min at room temperature; the final methanol and nitrite concentrations were 88% and 0.025%, respectively. The resultant cannabinoid–FBB compounds were extracted by adding 200 μl of 0.1 *N* HCl and 200 μl of chloroform to the reaction medium followed by vigorous mixing and centrifugation of the samples to separate the phases. The upper phases were discarded and the lower chloroform phases containing the cannabinoid–FBB compound were washed with 200 μl of 0.1 *N* HCl, dried in the vacuum centrifuge, and the dark-red residue at the bottom of the tubes dissolved in 10 μl of acetone. The acetone solutions were diluted with 200 μl of isopropanol and were transferred to microcuvettes; the absorbance of each solu-

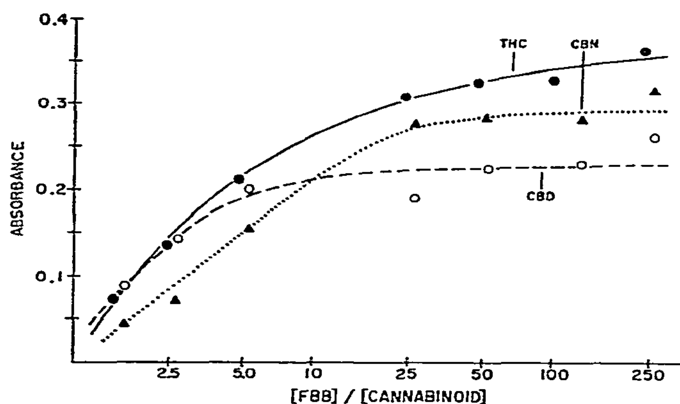


Fig. 3. Optimal FBB to cannabinoid ratio. Increasing volumes of a FBB reagent were added to a series of microtubes containing a fixed amount of cannabinoid dissolved in methanol; the final reaction volume was held constant by the addition of variable volumes of an appropriate diluent. After completion of the reaction, the cannabinoid–FBB compounds were extracted with chloroform, the chloroform extract dried, the residue redissolved in isopropanol and the optical density of the solution measured at the absorbance maximum wavelength for the particular cannabinoid. The amount of cannabinoid used: CBD and THC = 0.8 μg; CBN = 0.7 μg. The FBB reagent was a 0.4% FBB solution in 50% methanol containing 1% sodium nitrite.

tion was measured with a Gilford (Oberlin, OH, U.S.A.) Model 222 spectrophotometer at the isopropanol absorption maximum appropriate for the particular cannabinoid (see Table II). As illustrated in Fig. 3, the effect of varying the concentration of FBB, relative to a fixed concentration of CBD, THC or CBN, suggests that a 10- to 25-fold greater concentration of dye, relative to the concentration of the cannabinoids, is required to obtain maximum cannabinoid–FBB compound formation.

The method for synthesizing and isolating cannabinoid–FBB compounds outlined above has also been applied as a microassay for cannabinoids. In the microassay, a 50-μl aliquot of a 0.2% FBB solution in 50% methanol containing 1% sodium nitrite is added to unknowns and standards dissolved in 150 μl of methanol. Calibration curves for the cannabinoids were linear to 5 μg, and CBD, THC, CBN and 11-OH-THC could be measured in amounts smaller than 0.1 μg.

Stability of cannabinoid–FBB compounds and FBB solutions

After the cannabinoid–FBB compounds form, they are stable for more than 5 h when dissolved in isopropanol or in methanol–water solutions which have a 10–50% water content; a red precipitate gradually appears in methanol solutions with concentrations of water outside this range. The question of the stability of FBB solutions was approached in two ways. In the first approach, the slopes of standard curves developed for CBD, THC, 11-OH-THC and CBN were determined at different times after the preparation of a FBB solution. The curves were constructed by adding varying amounts of each cannabinoid to 2 ml of 85% methanol followed by the addition of a 1-ml aliquot from a 0.1% FBB solution prepared in 50% methanol containing 1% sodium nitrite; the samples were allowed to react approximately 10 min before measuring their absorbance at 490 nm. Five different cannabinoid concentrations ranging from 1 to 12 μg/ml were used for each curve. The curves were linear over the concentration range examined, and their slopes were calculated by linear regression analysis. The results of this experiment are presented in Table III; the absence of any significant change in the slopes suggests that the FBB reagent is stable for at least 5 h. The cannabinoid concentrations were converted to mM values prior to the regression analysis, and the slope values in Table III represent the virtual extinction coefficients for each cannabinoid. These are not true extinction coefficients because the concentration of the cannabinoid, rather than the actual concentration of the cannabinoid–FBB compound, was used in the calculations. Chia *et al.*[14] have reported that two

TABLE III

EFFECT OF TIME ON REACTIVITY OF A FBB SOLUTION*

Time (h)	*CBD*		*11-OH-THC*		*THC*		*CBN*	
	*C***	*%****	*C*	*%*	*C*	*%*	*C*	*%*
1	34.6	100	35.7	100	43.8	100	41.1	100
3	33.3	96	35.1	98	42.8	98	40.0	97
5	32.9	95	34.7	97	42.9	98	39.0	95

* FBB solution: 0.1% in 50% methanol solution containing 1% sodium nitrite.

** Virtual extinction coefficient determined as slope of standard curve for particular cannabinoid; expressed as mM^{-1} cm^{-1}. See text for details.

*** Slope as a percentage of 1-h value.

molecules of THC couple to each molecule of FBB; however, the reaction conditions used to form cannabinoid–FBB compounds in the present paper yield a more complex mixture of products, as revealed by TLC and HPLC analysis. When chloroform-extractable cannabinoid–FBB reaction products are separated by TLC, one predominant and several minor, but not insignificant, colored spots are seen. The results of the TLC experiments are corroborated by the appearance of multiple peaks when the reaction products are subjected to HPLC analysis, as will be described below. Although the complexity of the cannabinoid–FBB mixture precludes any simple correction to obtain a true extinction coefficient, the values in Table III do provide a relative estimation of the coefficients which may be of interest for comparison with other colorimetric assays.

The second approach for evaluating the stability of a FBB solution was to measure the rate of cannabinoid–FBB compound formation at different times after the preparation of a 0.05% and a 0.025% FBB reagent; the lower concentrations were chosen in order to amplify any decrease in the potency of the dye solution. The results of these rate experiments are presented in Fig. 4. The rate recordings illustrate that there are no marked changes in the cannabinoid–FBB reaction rate as a function of the age of the dye except at the lower (0.025%) concentration; in the HPLC–FBB analysis, the concentration of the dye solutions was 4–10 times greater than those in the experiments in Fig. 4. Another aspect of the cannabinoid–FBB reaction is illustrated by the CBN curves in Fig. 4: the rate of CBN–FBB formation is much slower than for CBD, THC (Fig. 2) and 11-OH-THC (not shown). The chemical basis for this slower reaction rate is not clear.

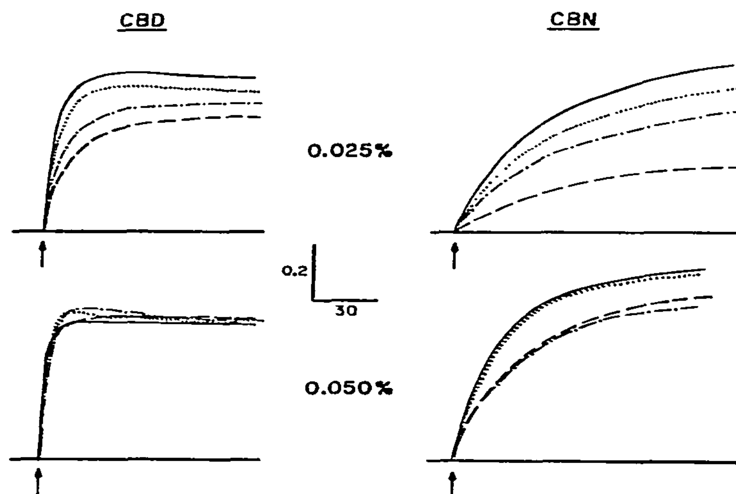


Fig. 4. Stability of FBB reagent as measured by the reaction rate of CBD and CBN with FBB. At different times after the preparation of a 0.025% or 0.05% FBB solution in 50% methanol containing 1% sodium nitrite, either CBD (15 μg) or CBN (14 μg) was added to a cuvette containing 2 ml of 85% methanol; the cuvette was placed in a spectrophotometer and the reaction of FBB with the cannabinoid was initiated by injecting 1 ml of a FBB solution into the cuvette (at the arrow). The calibration marks in the center of the figure represent time in seconds (horizontal) and absorbance units (vertical). Time after preparation of FBB reagent: 1 h (——), 4 h (· · ·), 6 h (–·–·) and 8 h (– – –). Wavelength = 490 nm.

The HPLC–FBB procedure for quantitating CBD and THC

Two methods for measuring the CBD or THC concentrations in tissue by integrating HPLC techniques and the reaction of FBB with cannabinoids were suggested initially by the results of the experiments described in the preceding sections. In the first method, a FBB solution would be added to an alcoholic solution of a tissue extract containing either CBD or THC and their respective metabolites. The cannabinoid–FBB products synthesized in the reaction could be extracted with chloroform, and the CBD– or THC–FBB product separated from its corresponding metabolite–FBB compounds on a normal-phase HPLC column. With this approach however, there is an inherent problem which was alluded to previously and is revealed in an experiment in which cannabinoid–FBB compounds were prepared from standards, isolated according to the methods outlined for the experiments in Fig. 3 and then chromatographed on a normal-phase μPorasil column. Example chromatograms for THC–, 11-OH-THC– and CBD–FBB compounds obtained in this experiment are shown in Fig. 5. Each of the cannabinoids examined produced a complex pattern of multiple dye derivatives consisting of at least four prominent peaks. The CBD peaks were not resolved under the conditions used in Fig. 5, but they were clearly seen when the acetone content in the eluting solvent was decreased; the particular chromatogram for CBD in Fig. 5 was included for the purpose of comparing the elution profiles of CBD and 11-OH-THC under the same chromatographic conditions. Alteration of the reaction conditions involving changes in solvents, temperature and pH might affect the pattern of the multiple FBB derivatives formed; however, this line of

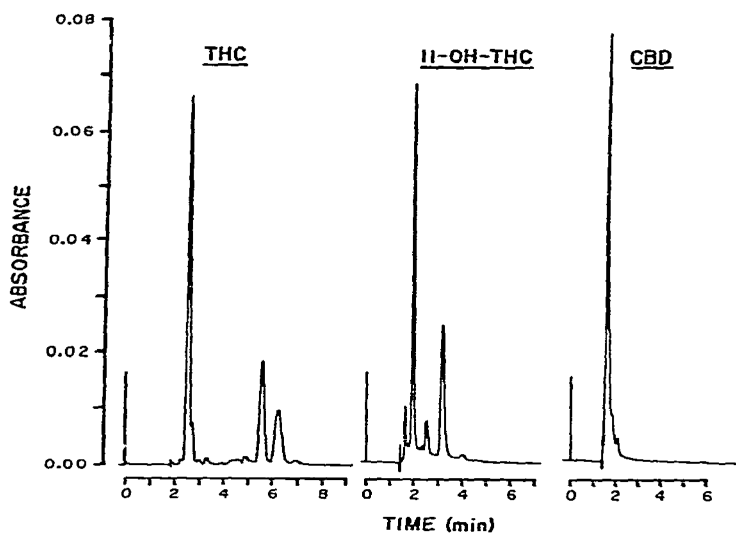


Fig. 5. Normal-phase chromatography of cannabinoid–FBB compounds. THC, 11-OH-THC and CBD were reacted with FBB, the products extracted with chloroform, the chloroform extract dried, the residue redissolved in acetone–isooctane (1:4), and a 10-μl aliquot representing approx. 1 μg of each cannabinoid was applied to a μPorasil column. A 10% acetone in isooctane solvent was used to chromatograph the THC products and a 50% acetone in isooctane solvent was used to chromatograph the CBD and 11-OH-THC products. The flow-rate was 2 ml/min; peaks were detected at 436 nm with a Waters Assoc. 440 absorbance monitor.

investigation was not pursued, and the approach of chromatographing pre-formed CBD- and THC-FBB compounds was abandoned in favor of the second method.

In the second method, the HPLC-FBB method, an alcoholic solution of a tissue extract was applied directly to a reversed-phase μBondapak C_{18} column in order to separate the CBD or THC in the extract from its metabolites. The CBD- or THC-FBB compound is synthesized by mixing a FBB solution with the column eluate prior to the entry of the eluate into the flow cell of a spectrophotometer; this method takes advantage of the rapidity with which FBB can react with CBD and THC (see Fig. 2). The operational details of the HPLC-FBB procedure have been described in the Experimental section, and the performance characteristics of the method are demonstrated in Figs. 6 and 7. Examples of recordings obtained by chromatographing

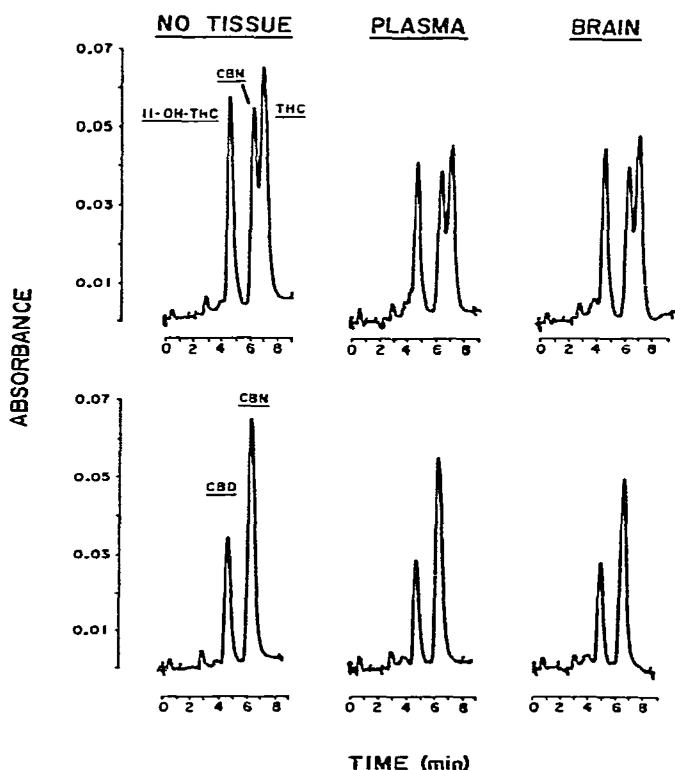


Fig. 6. Chromatograms of cannabinoid standards using the HPLC-FBB procedure. A mixture of cannabinoid standards was added to tissue grinders before preparing homogenates of control mouse brain or to 1 ml of plasma. The brain homogenates and the plasmas were extracted, the resulting residues containing the cannabinoids were dissolved in 100 μl of methanol and a 75-μl aliquot chromatographed on a μBondapak C_{18} column. In addition, a 100-μl methanol solution was prepared which contained the mixture of cannabinoid standards added to brain and plasma. This solution was not extracted; 75 μl were chromatographed and the results are illustrated by the "no tissue" recordings in the figure. Cannabinoid mixtures: top traces = 11-OH-THC (1.0 μg), CBN (0.8 μg) and THC (1.0 μg); bottom traces = CBD (0.5 μg) and CBN (1.1 μg). FBB reagent: 0.25% FBB in a 50% methanol solution containing 1% sodium nitrite. Flow-rates: column = 1.5 ml/min (85% methanol); FBB reagent = 0.5 ml/min. Wavelength = 490 nm.

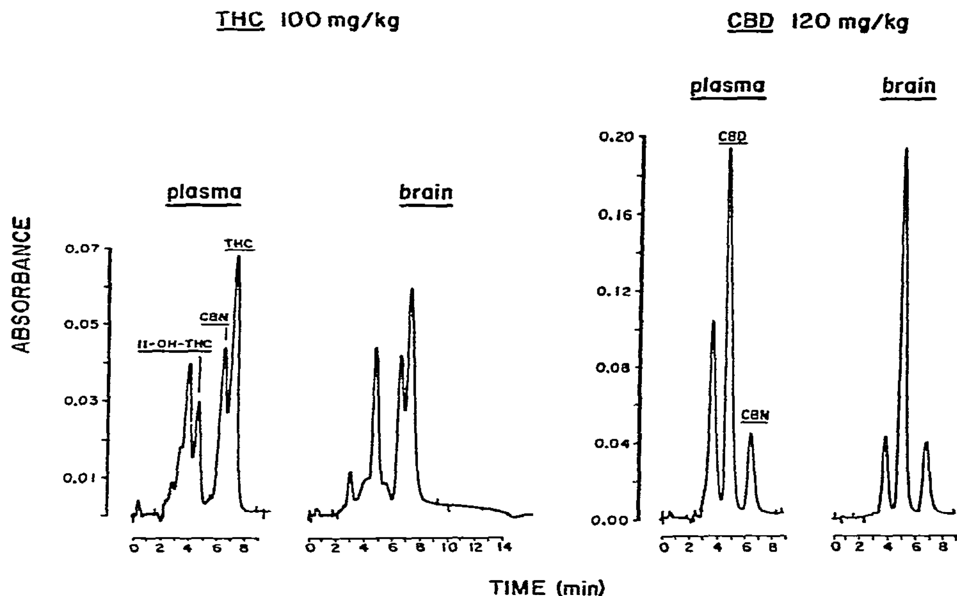


Fig. 7. Chromatograms of plasma and brain extracts from mice treated with THC or CBD. Mouse plasma and brain samples were obtained 1 h after the intraperitoneal injection of either THC or CBD; an aliquot of CBN standard was added to the brain samples prior to homogenization with methanol and to a 1-ml aliquot of plasma obtained by pooling the blood of eight animals. The amount of CBN added to the THC samples was 0.8 μg; the amount of CBN added to the CBD samples was 1.1 μg. The brains and plasmas were extracted according to the procedure described in the Experimental section. The chromatographic conditions were identical to those outlined in Fig. 6.

a mixture of THC, 11-OH-THC and CBN standards and a mixture of CBD and CBN standards are presented in Fig. 6, which also includes chromatograms of the same two mixtures extracted from plasma and brain samples taken from control animals. A comparison of these tissue chromatograms with those of the non-extracted standards illustrates that there are no significant quantities of potentially interfering non-cannabinoid, FBB-reacting substances in extracts prepared from either brain or plasma. In contrast, chromatograms of extracts from plasma and brain samples taken from animals injected with either CBD or THC reveal the presence of polar metabolites in significant amounts (Fig. 7). These metabolites do not interfere with the quantitation of CBD or THC, but, in the case of extracts derived from THC-treated animals, the quantitation of 11-OH-THC is difficult, and in some instances, impossible. Occasionally, when a group of brain extracts are chromatographed, a very broad peak, over which the cannabinoid peaks are superimposed, may be seen in the chromatograms. A recording that exemplifies this broad peak is presented as the chromatogram of a brain sample from a THC-treated mouse in Fig. 7. There is no obvious explanation for the sporadic appearance of this broad peak, but its presence may be related to subtle variations in the composition of the final extract injected onto the column; in fact, the peak may reflect differences in the amount and type of lipid in the extract.

For several cannabinoids, changes in the peak height, width and retention time, as a function of the water content in the eluting solvent, are summarized in Table IV. An inspection of the THC chromatograms in Fig. 6 and of the retention times for CBN and THC in Table IV raises the question of why a 15% water concentration in the eluting solvent was chosen for the HPLC–FBB procedure, since baseline separation of the internal standard (CBN) from THC was not achieved. The HPLC–FBB method described in this paper was originally developed to provide pharmacokinetic data for an investigation of the effects of chronic CBD and THC administration on the anticonvulsant properties of these cannabinoids (to be reported elsewhere). A considerable number of samples had to be analyzed in that study; consequently, a compromise in the resolution of the CBN and THC peaks was made in order to increase the throughput of the assay. Although baseline separation of THC and CBN was not achieved, the separation was more than adequate for the construction of reproducible internal standard calibration curves.

Tables V and VI represent a collection of internal standard calibration curves developed from cannabinoid standards added to control mouse brain; these curves were used in several of the analyses of samples from the chronic study mentioned above. The slopes and intercepts for the individual calibration curves were determined by a linear regression analysis of the peak-height ratios. In addition, the peak-height ratios were pooled and subjected to a linear regression analysis; the slope and intercept of a line fitting these points and the predicted peak-height ratios ($\hat{y}$) are included in each table. The coefficients of variation, calculated for the THC/CBN and CBD/CBN ratios in Tables V and VI, for which three or more observations were made, ranged from 3 to 13% and averaged 7%. In experiments designed to measure the mean brain concentrations of CBD or THC at different time intervals following the administration of these cannabinoids, the coefficient of variation of the mean has frequently approached 100%. In view of this large biological variability, the relatively small variability associated with the calibration curves (Tables V and VI) is acceptable for pharmacokinetic studies. In addition to illustrating the reproducibility of the internal standard calibration curves, the data in Tables V and VI illustrate the stability of the stock standard cannabinoid solutions. Curve 1 in Table V and curves 1 and 2 in Table VI were calculated from analyses performed approximately 3 months prior to those from which the remaining curves in the two tables were determined.

Stability of FBB solutions when used in HPLC–FBB procedure

During the initial developmental phase of the HPLC–FBB procedure, a 0.1% FBB solution, based on the findings from the preceding spectrophotometric experiments (Figs. 2 and 4; Table III), was used routinely. In these early HPLC investigations, a decrease with time in the peak height or area was observed for CBN at 3 or 4 h and for CBD at 6 h after preparation of the FBB solution. These changes in peak heights or area suggested that the reactivity of the FBB reagent was decreasing, a conclusion in direct conflict with the stability results obtained under different conditions and summarized in Table III. In order to obtain more definitive information on the apparent decrease in dye reactivity associated with the HPLC analysis, two experiments (analogous to those summarized in Table III) were undertaken and the results are presented in Table VII. The slope data in Table VII are calculated from the peak areas of standard calibration curves constructed at various times after the prepa-

TABLE IV

CHROMATOGRAPHIC CHARACTERISTICS OF VARIOUS CANNABINOIDS: EFFECTS OF CHANGING WATER CONTENT OF ELUTING SOLVENT

Ph = peak height (absorbance units); Pw = peak width at baseline (sec); t_R = retention time (sec); $k' = (t_R - t_m)/t_m$, where t_m = retention time of mobile phase. Flow-rate of eluting solvent was 1.5 ml/min; flow-rate of dye solution was 0.5 ml/min. Monitoring at 490 nm. Dye solution = 0.1 % FBB in 50 % methanol containing 1 % sodium nitrite.

Water (%)	*11-OH-THC*				*CBD*				*CBN*				*THC*			
	Ph	*Pw*	t_R	k'	*Ph*	*Pw*	t_R	k'	*Ph*	*Pw*	t_R	k'	*Ph*	*Pw*	t_R	k'
5	0.079	48	205	0.14	0.094	48	204	0.13	0.088	48	231	0.28	0.111	48	242	0.34
10	0.064	48	232	0.29	0.082	48	232	0.29	0.065	48	283	0.57	0.092	60	304	0.69
15	0.049	60	283	0.57	0.063	54	293	0.63	0.037	60	396	1.20	0.060	66	440	1.44
20	0.037	66	369	1.05	0.050	69	397	1.21	0.025	90	599	2.36	0.038	102	690	2.86
25	0.026	96	539	1.99	0.034	90	607	2.37	0.014	144	1026	4.70	0.022	168	1232	5.84
30	0.014	162	909	4.05	0.019	168	1104	5.13	—	—	—	—	—	—	—	—

TABLE V

THC CALIBRATION CURVES

Curve	Ratio: μg THC/μg CBN*						Slope	Intercept
	0.24	0.36	0.49	1.21	2.42	3.64		
1	0.33	—	0.58	1.20	2.56	—	1.02	0.06
2	0.17	—	0.46	—	2.19	3.63	0.99	−0.07
3	—	0.40	0.50	1.03	2.21	3.27	0.88	0.05
4	—	0.41	0.62	1.18	2.21	3.68	0.96	0.06
Mean	0.25	0.41	0.54	1.14	2.29	3.53		
Regression analysis on pooled observations: slope = 0.95; intercept = 0.04								
$\hat{y}$	0.27	0.38	0.50	1.18	2.33	3.49		

* CBN internal standard: 0.8 μg; values in the table represent THC peak height/CBN peak height.

TABLE VI

CBD CALIBRATION CURVES

Curve	Ratio: μg CBD/μg CBN*					Slope	Intercept
	0.22	0.44	1.11	2.22	3.33		
1	0.27	0.56	1.41	2.74	—	1.23	0.02
2	0.26	0.54	1.50	3.05	—	1.40	−0.06
3	0.23	0.48	1.31	2.54	3.64	1.10	0.03
4	0.23	0.51	1.30	2.74	3.80	1.17	0.01
5	0.25	0.55	1.31	2.80	3.82	1.17	0.04
Mean	0.25	0.53	1.37	2.77	3.75		
Regression analysis on pooled observations: slope = 1.17; intercept = 0.04							
$\hat{y}$	0.30	0.55	1.34	2.63	3.92		

* CBN internal standard: 1.1 μg; values in the table represent CBD peak height/CBN peak height.

TABLE VII

EFFECTS OF TIME ON REACTIVITY OF A FBB SOLUTION AS REFLECTED BY CHANGES IN THE SLOPES OF CANNABINOID STANDARD CURVES DETERMINED BY HPLC

Slopes are given in arbitrary units and are calculated from linear regression analysis of areas under the peaks formed from 5 different concentrations of each cannabinoid.

Time (h)	CBD				THC				CBN			
	0.1%*	%**	0.25%	%	0.1%	%	0.25%	%	0.1%	%	0.25%	%
1	429066	100	464959	100	467987	100	464109	100	392406	100	452624	100
3	395802	92	434178	93	467661	100	460671	99	360719	92	434323	96
5	386218	90	428566	92	458851	98	458157	99	315649	80	404853	89
7	346766	81	393765	85	428660	92	447434	96	251100	64	368836	82

* Concentrations of FBB were 0.1% and 0.25%, prepared in 50% methanol solution containing 1% sodium nitrite.

** Slope of standard curve as a percentage of 1-h value.

ration of a 0.1% and 0.25% FBB solution; the decrease in the slope of the CBN calibration curves, as determined with a 0.1% FBB solution, contrasts sharply with the lack of significant change in the slope of the CBN calibration curves in Table III. There are no striking differences between the change in slopes of the CBD or THC calibration curves in Table III and Table VII; however, the decrease in the CBD slope which occurs between 5 and 7 h did confirm the original observation of such a change. When the concentration of the FBB reagent was raised to 0.25% and the experiment repeated, the onset of the decrease in the slope of the CBN curve (Table VII) was delayed. One possible explanation is that the geometry involved in mixing the FBB reagent with the column effluent, coupled with the relatively slower rate of reaction of CBN (Fig. 4), creates a condition more sensitive to changes in dye reactivity. The importance of the particular method used to mix two solutions for on-line derivatization has been discussed by Deelder *et al.*[16]. In an attempt to counteract the apparent change in dye potency, the flow-rate of the dye solution was increased; however, as may be seen in Table VIII, such a procedure decreased the area of the various cannabinoid peaks to an unacceptable level. The problem of the decreased reactivity of the FBB reagent was circumvented by using a 0.25% solution of FBB and by changing to a fresh dye solution at 3 or 4 h into an analysis; age of the dye was measured from the time when the methanol and sodium nitrite were added to the initial water solution of the dye. Although the change of dye solution during an assay is not very difficult, it is inconvenient; and, although a better mixing technique might obviate the change in FBB reagent, the effects of different mixing-device designs based on the suggestions of Deelder *et al.*[16] have not been explored.

TABLE VIII

EFFECTS OF DIFFERENT FLOW-RATES OF A FBB SOLUTION ON PEAK AREAS

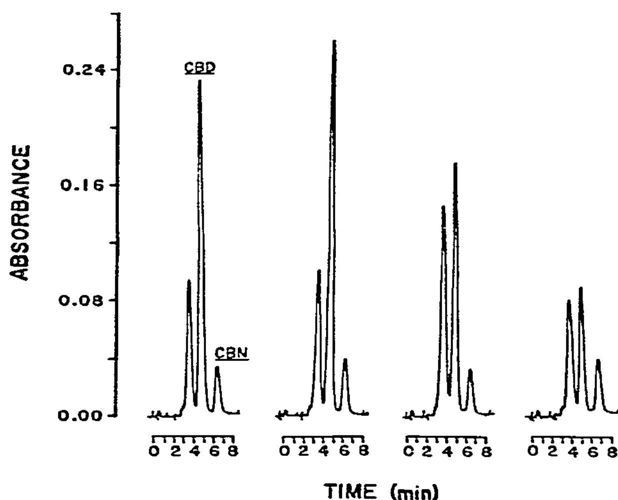
Flow-rate (ml/min)	*CBD*		*CBN*		*THC*	
	*Area**	%	*Area*	%	*Area*	%
0.5	871635	100	482193	100	799372	100
1.0	740473	85	307776	64	636050	80
1.5	605640	70	209162	43	496322	62
2.0	426303	49	165892	34	460493	58

* 0.1% FBB solution in 50% methanol containing 1% sodium nitrite. Eluting solvent was 85% methanol and flow-rate = 1.5 ml/min.

Application of the HPLC–FBB procedure for quantitating CBD and THC

The chromatograms in Figs. 8 and 9 were taken from an experiment in which the acute time courses of CBD and THC in plasma were determined. Mice were injected with either 120 mg/kg CBD or 100 mg/kg THC; they were decapitated and bled into a heparinized 12-ml culture tube. Each time point in Figs. 8 and 9 was determined by analyzing an aliquot of the plasma derived from the pooled plasmas of eight animals. The pooling of samples was necessary in order to measure the plasma levels at post-injection times equal to, or greater than, 6 h. An interesting feature of the THC time course is the persistence of the peak preceding the 11-OH-THC peak in Fig. 9. THC and 11-OH-THC levels decline quite rapidly and the more polar peak

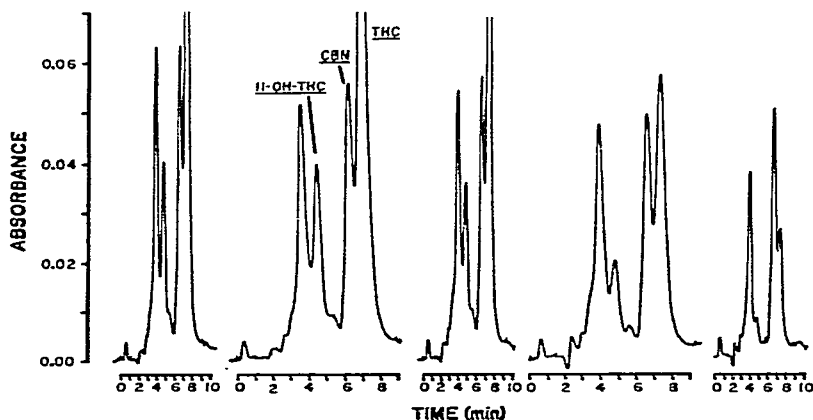
Plasma levels at different times after 120 mg/kg CBD



Time after injection (hr)	0.5	1.0	2.0	4.0
Volume of plasma (ml)	1.4	1.5	1.6	2.0

Fig. 8. Acute time course of CBD in plasma. Mice were injected intraperitoneally with CBD (120 mg/kg). At various times following the injection, plasma samples were prepared by pooling the plasma of eight animals. An aliquot was removed from each of the pooled samples, 1.1 μg of CBN was added and the samples were extracted; the volume of plasma extracted is given below each chromatogram. Chromatographic conditions were the same as in Fig. 6.

Plasma levels at different times after 100 mg/kg THC



Time after injection (hr)	0.5	1.0	2.0	4.0	6.0
Volume of plasma (ml)	1.3	1.6	1.6	2.0	2.0

Fig. 9. Acute time course of THC in plasma. The experimental protocol is similar to the one described in the legend of Fig. 8; CBN (0.8 μg) was added to each plasma before extraction. Chromatographic conditions were the same as in Fig. 6.

eventually predominates. The composition of this early peak has not been investigated: whether it is a single THC metabolite or a complex mixture would have to be determined with an eluting solvent of higher water content. An analogous peak was not seen in the CBD samples (Fig. 8): only one peak precedes the CBD peak and, presumably, it is 11-OH-CBD; the actual composition of the peak has not, however, been determined.

COMMENTS AND CONCLUSIONS

The HPLC–FBB procedure for quantitating CBD and THC was developed out of necessity, since none of the methods currently available for THC analysis were particularly suitable for investigating the pharmacokinetics of CBD in mice in our laboratory. Although several of the techniques used in other laboratories are more sensitive, all are subject to certain limitations that preclude their application to our problem. For example, a GC–MS approach was not feasible because the necessary apparatus was not available; there is no suitable radioimmunoassay for CBD, the fluorometric analysis of dansylated cannabinoids was of no value because of the formation of at least two fluorescent CBD derivatives and, finally, the monitoring of a column eluate at 220 nm is plagued by the presence of interfering substances in a tissue extract. In fact, the sensitivity of the HPLC–FBB procedure appears to be equal to, or greater than, that of the UV technique.

There are certain features of the HPLC–FBB procedure which may be of value to other investigators. For example, the assay can be run with just two, relatively inexpensive, pumps similar to the Milton-Roy used in this study. Since the cannabinoids are eluted isocratically, one pump, a suitable reversed-phase column and an injector are the basic requirements for separating either CBD or THC from their respective metabolites; the FBB dye reagent is added to the column eluate with the second pump. Detection of the peaks does not require a dedicated instrument; during the initial development of the assay, an older spectrophotometer (Gilford Model 222) and a cuvette-type flow cell were used quite successfully to detect cannabinoid–FBB compounds in the column eluate. One of the major factors limiting the sensitivity of the HPLC–FBB assay is the baseline noise inherent in the detector and flow cell combination. With the Perkin-Elmer LC-55B spectrophotometer, the HPLC–FBB procedure can measure CBD, THC and CBN standards in amounts less than 50 ng.

The HPLC–FBB assay is not significantly affected by the unavoidable presence of endogenous tissue substances remaining in the extract prepared by the method outlined in the Experimental section. Attempts to introduce additional purification steps to the extraction procedure resulted in a precipitous decline in the recovery values for CBD, THC and CBN. The assay is also a potentially useful method for quantitating CBD and THC metabolites; however, the chromatographic conditions would have to be adjusted to increase the resolution of these compounds, since they tend to elute very early with 85% methanol.

There are two principal technical problems associated with the HPLC–FBB method, both of which are related to the choice of CBN as an internal standard. The first problem is the sensitivity of CBN to changes in the potency of the FBB reagent and, consequently, an analysis lasting several hours must be interrupted in order to replace the FBB reagent with a fresh dye solution. This difficulty might be eliminated

by incorporating a better mixing device into the system. The second problem is that CBN and THC peaks are not resolved at baseline, and an alteration of the eluting solvent in order to achieve this resolution would result in a marked increase in the analysis time required for each sample. A gradient could be employed for each sample, thereby producing a better separation of the metabolites, but this measure, too, would significantly increase the analysis time and severely restrict the number of samples that could be processed in one day. In spite of these problems, the HPLC–FBB assay in its present form has been used successfully in our laboratory, and it may be of value to other investigators who seek to measure tissue concentrations of CBD, THC or other cannabinoids, but who do not have access to GC–MS equipment or a radio-immunoassay. To this extent, the HPLC–FBB procedure may prove to be a helpful alternative to the methods now available.

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