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Chromophoric Labeling of Cannabinoids with 4-Dimethylaminoazobenzene-4′-Sulfonyl Chloride

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ABSTRACT: A simple and sensitive assay for the cannabinoids is presented using a dabsylation procedure. Dabsyl derivatives of Δ^9-tetrahydrocannabinol (Δ^9-THC) and cannabinol (CBN) were prepared by reacting with 4-dimethylaminoazobenzene-4′-sulfonyl chloride (dabsyl chloride) in acetone in the presence of sodium carbonate-sodium bicarbonate buffer (pH 10). Crystalline dabsylcannabinoids gave intense absorption in the visible region. With these derivatives, analysis by thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC) were tested. These techniques gave good separation and nanogram detection of dabsyl-THC and -CBN by using *n*-hexane-ethyl acetate-diethylamine (20:5:1) for TLC and MeOH—H_2O (95:5) at 450 nm for HPLC.

KEYWORDS: toxicology, marihuana, chromatographic analysis, dabsylation, cannabinoids, dabsyl-THC, dabsyl-CBN

Cannabinoid-containing samples are commonly encountered in forensic science laboratories. Identification of cannabis has been made generally by color test, thin-layer chromatography (TLC), or gas chromatography (GC) of Δ^9-tetrahydrocannabinol (THC), a major active component of *Cannabis*, as well as by its morphological characteristics [*1*]. On the other hand, for the detection and analysis of THC in biological fluids, there are several methods currently available as will be discussed later.

Recently, fluorescent analysis by means of TLC has been developed as a suitable method for routine analysis [*2,3*]. The dansyl derivative of THC gives a fluorescent spot detectable at 0.5 ng on a thin-layer plate [*2*], and this method has also been applied to HPLC analysis [*4*]. But as its weak point, the derivative has been reported to be very unstable to light.

In this paper, we describe TLC and high performance liquid chromatography (HPLC) methods suitable for routine use for the detection of cannabinoids using a new chromophoric labeling reagent, 4-dimethylaminoazobenzene-4′-sulfonyl chloride (dabsyl chloride).

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Experimental Procedure

Materials

Cannabis grown in the Shimane prefecture of Japan was used. Dabsyl chloride was purchased from Dojindo Laboratories, Kumamoto, Japan. Silica gels used were as follows: Wakogel B-5F® (325 mesh for preparative use, Wako Pure Chemical Industries, Japan), Wakogel Q-23® (100–200 mesh for column chromatography) and Silica gel 60 (for analytical use). Florisil (60–80 mesh, subdivided from Florisin Co.) was furnished by Wako Pure Chemical Industries, Japan. For HPLC a Shimadzu LC 3A liquid chromatograph equipped with a Model SPD-1 variable wavelength photometer was used and the column was of stainless steel packed with Zorbax BP-ODS (octadesyl silanebonded silica, Du Pont).

Sample Collection of Cannabinoids

Samples of cannabinoids were obtained from *Cannabis* by the method of Aramaki et al [5]. The details are as follows. The *Cannabis* was dried in a desiccator and powdered. The powdered sample was allowed to stand in methanol in a covered vessel at room temperature and shaken at intervals. After filtration, the extract was evaporated to dryness. The crude extract was chromatographed through a Florisil column using benzene as eluting solvent. The eluate was examined by a spot test with Gibbs reagent (40 mg of 2,6-dichloroquinone-4-chlorimide in 10 mL of ethanol), and the positive fractions were combined and evaporated under reduced pressure. The residue was very viscous and slightly brown colored. This was then applied to preparative TLC (plate: Wakogel B-5F, 0.75 mm in thickness, homemade) using two solvent systems: (1) benzene-*n*-hexane-diethylamine (25:10:1) and (2) *n*-hexane-ethyl ether (3:1). THC and cannabinol (CBN) were found at R_f 0.43 and 0.33, respectively, by Solvent 1, and at R_f 0.41 and 0.36, respectively, by Solvent 2. When the components from preparative TLC required still more purification, they were applied to preparative HPLC (column: Zorbax BP-ODS, 25 cm by 7.6 mm inner diameter; mobile phase: acetonitrile-water at 75:25; flow rate: 2.0 mL/min; and detector: ultraviolet at 280 nm).

Preparation of Dabsylcannabinoids

Dabsyl derivatives of THC and CBN were prepared at 70°C for 30 min with dabsyl chloride (60 mg in 70 mL of acetone) and the *Cannabis* oil (50 mg) obtained from column chromatography as described above in the presence of sodium carbonate (200 mg in 20 mL of water). After removal of acetone under reduced pressure and addition of chipped ice, the precipitate was filtered, washed thoroughly with water, and dried over solid potassium hydroxide in a desiccator. The dried precipitate was dissolved in a small amount of ethyl ether and was applied to a column packed with Wakogel Q-23. As eluting solvent, *n*-hexane-ethylether (3:1) was used. The red-orange fractions were collected and evaporated. The residue was purified using preparative TLC (plate: silica gel 60, 0.25 mm in thickness, homemade, solvent system: *n*-hexane-ethylacetate-diethylamine at 20:5:1). Dabsyl derivatives of THC and CBN were observed at R_f 0.35 and 0.31, respectively, on the plate. The melting points of these compounds were measured. Electronic spectra were determined on a Hitachi 220A spectrophotometer. Infrared (IR) spectra were recorded with a JASCO IRA-1 spectrophotometer. Chemical ionization (CI) mass spectra were obtained on a JEOL JMS-D 300 equipped with a computer-controlled data analysis system (JMA 2000E) using a direct introduction method under the following conditions: electron energy: 180 eV, ion source temperature: 250°C, acceleration voltage: 3 kV, ionization current: 300 μA, and reacting gas: ammonia. Nuclear magnetic resonance (NMR) spectra were measured with a JEOL JNM-PMX 60 spectrometer in chloroform-d_1 using tetramethylsilane as an internal standard.

Analytical Procedure

A small amount of *Cannabis* or an extract from a cannabinoid-containing sample was mixed with 0.2 mL of dabsyl chloride solution (0.1% in acetone) and 0.2 mL of sodium carbonate-sodium bicarbonate buffer (pH 10). The mixture was tightly stoppered with a glass stopper and allowed to react at 70°C in a water bath with constant shaking for 10 min. Then 0.2 mL of dabsyl chloride solution and 0.2 mL of buffer were again added to the mixture, and allowed to react under the same condition. The acetone of the resultant mixture was partially evaporated by a nitrogen stream. The residual mixture was then extracted with 2 mL each of ethyl ether three times. After centrifuging, the organic layers were combined and evaporated. The residue was dissolved in methanol and examined by TLC and HPLC.

TLC Analysis of Dabsylcannabinoids

Silica gel plates (precoated TLC plate of silica gel 60, 0.25 mm in thickness) were used for the TLC analysis of dabsylcannabinoids using *n*-hexane-ethylacetate-diethylamine (20:5:1) as developing solvent.

HPLC Analysis of Dabsylcannabinoids

HPLC of dabsylcannabinoids was accomplished under the following conditions: stationary phase: Zorbax BP-ODS, mobile phase: MeOH—H_2O (95:5), column: 25 cm by 4 mm inner diameter, flow rate: 1.2 mL/min, column temperature: 50°C, and detector wavelength: 450 nm. The solvent was filtered through a 0.45 μm filter to remove suspended particles and degassed in an ultrasonic bath just before use.

Results

Identification of Dabsylcannabinoids

The structures of dabsylated THC and CBN, prepared as described above, were confirmed by elemental analysis (Table 1), IR spectra, CI mass spectra, and NMR spectra. IR spectra are given in Fig. 1. Presence of sulfonic ester groups ($-SO_2-O-$) in these dabsylated cannabinoids were revealed by their IR absorptions around 1140 cm^{-1} and 1380 cm^{-1}. In the CI mass spectra of these dabsylated cannabinoids (Fig. 2), the m/z 602 for dabsylated THC and the m/z 598 for dabsylated CBN corresponded respectively to the protonated molecular ion of these dabsylcannabinoids.

The electronic absorption spectra of dabsylated THC in ethanol and in 0.2*N* hydrochloric acid/ethanol solution are illustrated in Fig. 3.

Conditions for Dabsylation

To establish a practical analysis of cannabinoids by dabsylation, conditions of the reaction were investigated as to pH, temperature, and reaction time. As illustrated in Fig. 4, formation of dabsyl-THC reached an optimal peak at about pH 10. As compared with the pH's effect, variation in temperature (25 to 70°C) or in time (5 to 30 min) did not give much difference in the yield of dabsyl-THC. However, in the dabsylation at a lower temperature, increased formation of by-product was noted. The optimum condition for dabsylation therefore proved to be at pH 10 and at 70°C for 10 min.

TLC and HPLC Analyses of Dabsylcannabinoids

Analysis of *Cannabis* was tested with the use of the TLC and HPLC methods. Figure 5 shows a thin-layer chromatogram of a reagent blank, standard samples of dabsyl-THC and

TABLE 1—*Physicochemical properties of dabsylcannabinoids.*

Compound	Formula (Mol. Wt.)	Color	Melting Point, °C	Analysis, % Calculated (Found)			Ultraviolet λ_{max}^{EtOH}, nm (log ϵ)
				Carbon	Hydrogen	Nitrogen	
Dabsyl-THC	$C_{35}H_{43}N_3O_4S_1$ (601.814)	red orange	127 to 131	69.85 (69.84)	7.21 (7.35)	6.99 (6.84)	276 (4.13) 450 (4.51)
Dabsyl-CBN	$C_{35}H_{39}N_3O_4S_1$ (597.782)	yellow orange	173 to 175	70.33 (69.50)	6.58 (6.57)	7.03 (6.82)	270 (4.39) 310 (4.16) 448 (4.49)

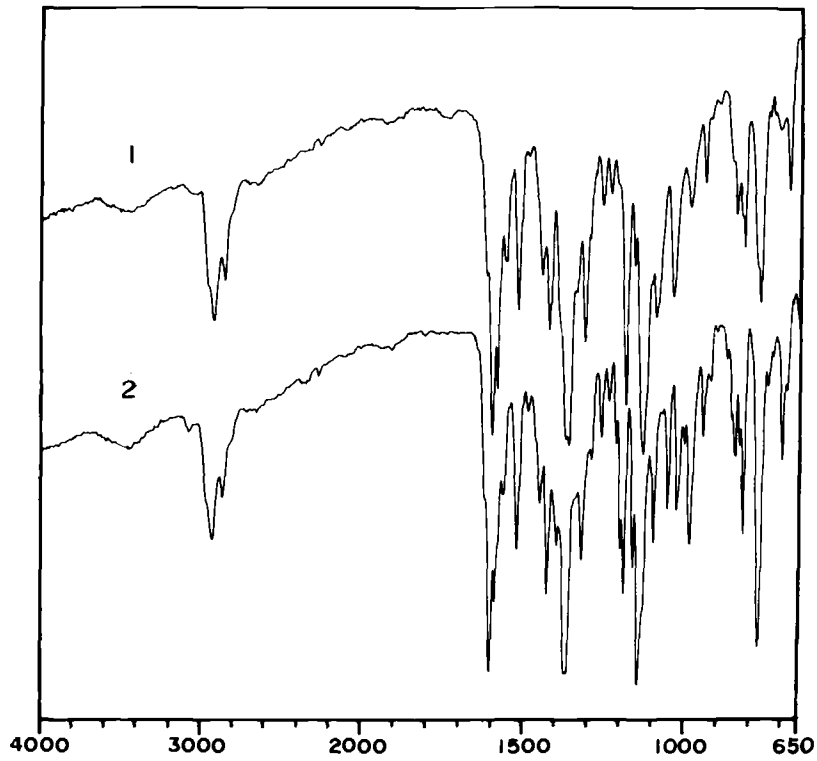

FIG. 1—*Infrared spectra of dabsyl-THC* (1) *and dabsyl-CBN* (2).

dabsyl-CBN, and an authentic sample. Dabsyl-THC and -CBN were observed as yellow-orange spots at R_f values of 0.35 and 0.31, respectively, and were well separated from naturally occurring compounds. Dabsylcannabinoids could be detected clearly to about 15 ng on the TLC plate. However, by spraying 10% hydrochloric acid solution in ethanol, the spot's color changed from yellow orange to wine red and a higher sensitivity of 5 ng was achieved.

Figure 6 shows an HPLC analysis of *Cannabis* by dabsylation. The presence of CBN and THC in *Cannabis* is confirmed by Peaks 1 and 2, respectively, with retention times corresponding to that of dabsylated standards. Both peaks were well resolved and were not disturbed by naturally occurring compounds.

Figure 7 shows a calibration curve by HPLC for the quantitation of THC up to 30 ng. Good linearity with a coefficient of correlation of 0.999 was observed. The minimum sensitivity for THC detection was 1 ng.

HPLC Analysis of Cannabinoids in Human Urine and Plasma

The HPLC method was also applied to the routine analysis of human urine and blood plasma. The procedure of extraction from urine and blood plasma is as follows. After adding 1 mL of 0.1*N* hydrogen chloride to 5 mL of urine, the mixture was shaken with 10 mL of *n*-hexane and centrifuged (3000 rpm, 5 min) three times, respectively. The combined organic layer was washed with 1 mL of 0.1*N* sodium hydroxide and evaporated to dryness. For the analysis of THC in blood plasma, 1 mL of plasma was extracted with 3 mL of *n*-hexane as described above. The combined organic layer was evaporated to dryness. The

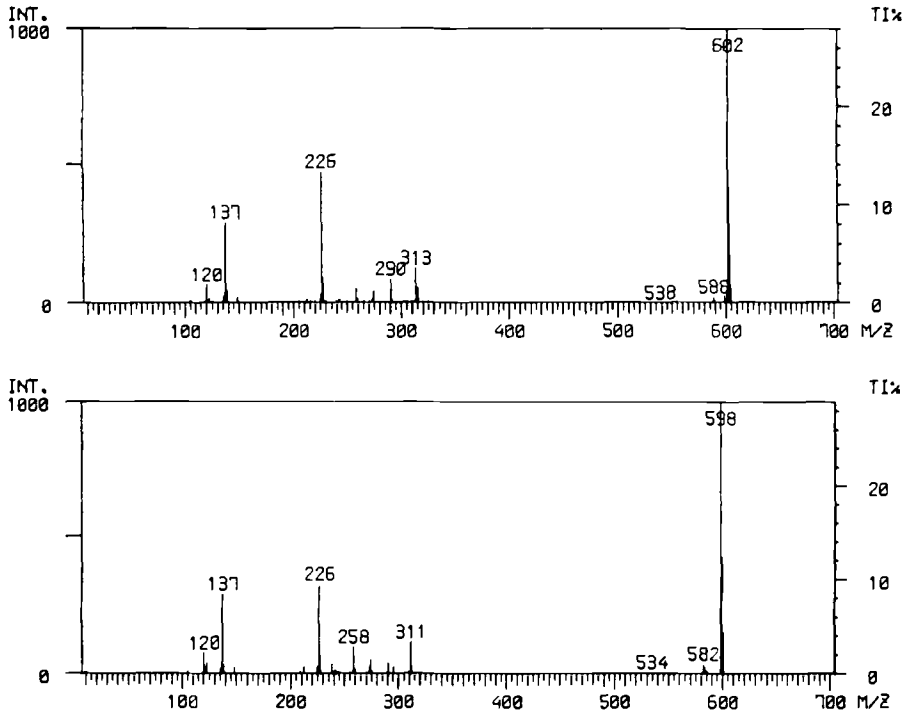

FIG. 2—*Mass spectra of dabsyl-THC* (top) *and dabsyl-CBN* (bottom) *by chemical ionization.*

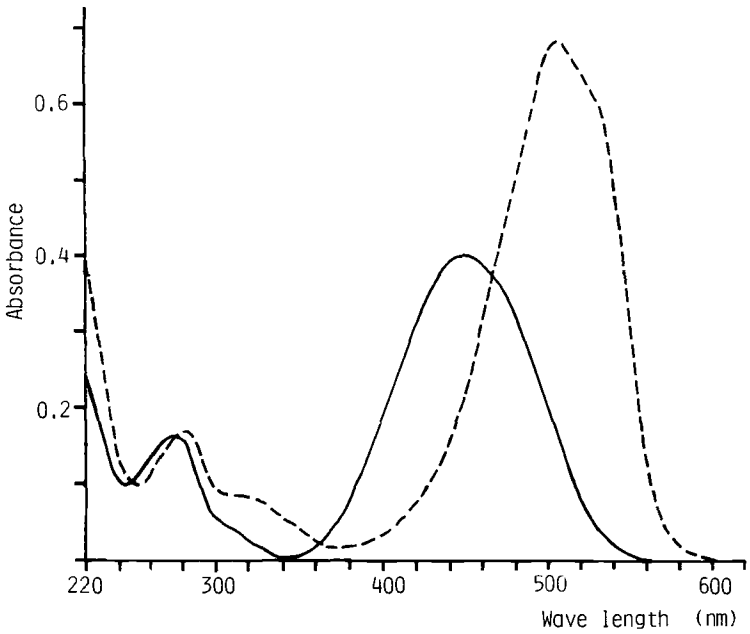

FIG. 3—*Electronic absorption spectra of* 1×10^{-5} *M dabsyl-THC in ethanol* (solid line) *and in 0.2*N *hydrochloric acid/ethanol* (broken line).

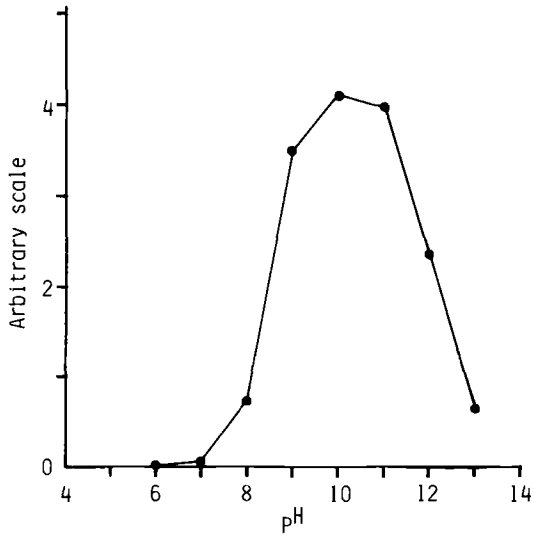

FIG. 4—*Effect of pH on amount of dabsyl-THC produced. Dabsylation of THC was carried out at 70°C for 10 min in 0.1M phosphate buffer (pH 6, 7, and 8), in carbonate-bicarbonate buffer (pH 9, 10, and 11), and in carbonate-sodium hydroxide (pH 12 and 13). The amount of dabsyl-THC was determined by HPLC. The other conditions are described in the text.*

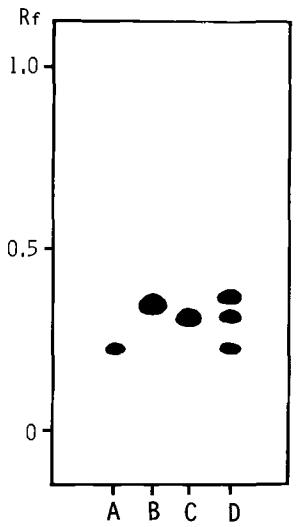

FIG. 5—*TLC separation of dabsyl-THC and dabsyl-CBN where* a = *blank*, b = *dabsyl-THC*, c = *dabsyl-CBN, and* d = Cannabis *leaf.*

extracts from urine and blood plasma were then dabsylated for analysis. The dabsylation procedure was as described in the experimental section.

Figure 8 shows the result of HPLC of urine and blood plasma after adding about 100 ng/mL of THC. As can be seen in Fig. 8, there were no interfering peaks caused by impurities around the peak of dabsyl-THC for both urine and blood plasma. Recoveries of THC added to urine and blood plasma were 63 and 54%, respectively.

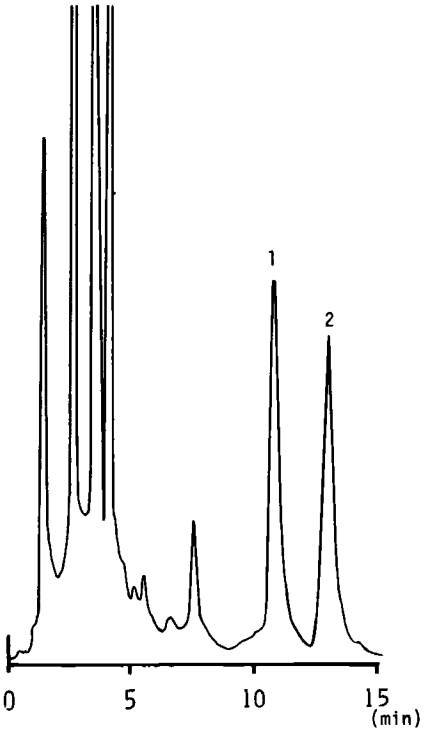

FIG. 6—*HPLC chromatogram of dabsylated* Cannabis *where* 1 = *dabsyl-CBN and* 2 = *dabsyl-THC.*

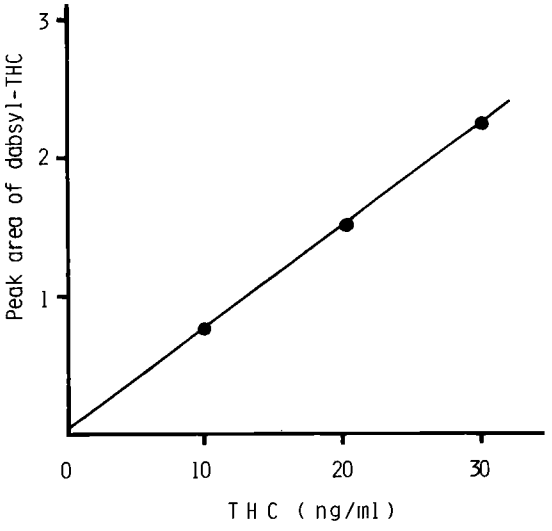

FIG. 7—*Calibration curve for THC by HPLC. Sample size is 10 μL. For other conditions, see text.*

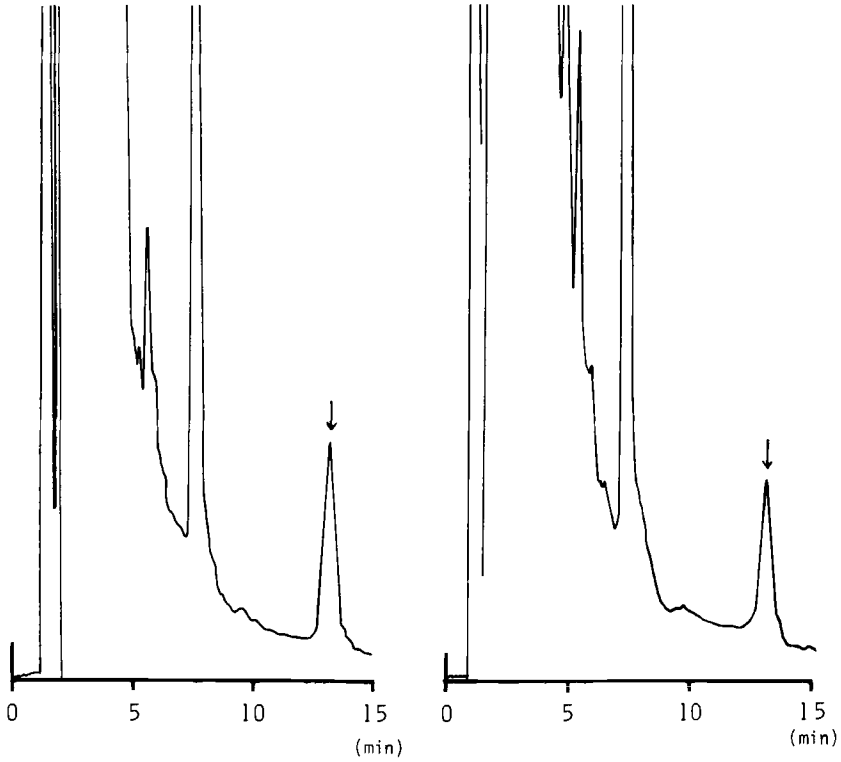

FIG. 8—*HPLC detection of THC* (arrow) *in biological samples where* left = *urine and* right = *blood plasma.*

Discussion

The active physiological level of THC in biological fluids is usually quite low, rarely exceeding 100 ng/mL of blood [*6*], and what is more unfavorable for analysis is the rapid metabolism and excretion of THC [*6-10*]. Accordingly, the detection method of cannabinoids in biological materials must have sufficient sensitivity. GC has been shown to have requisite sensitivity when a suitable derivative is made for electron capture or flame photometric detection [*11-13*]. But these GC methods require an extensive cleanup in order to remove naturally occurring interferences. Mass fragmentography used in combination with GC offers excellent sensitivity and selectivity [*7,14-16*]. However, this method is too expensive for routine monitoring and requires considerable expertise. Immunoassay technique has been developed in recent years, and the rapidity and sensitivity of the technique have been emphasized [*8,16-18*]. TLC is the simplest and least expensive technique and thus suitable for routine use. However, as for the TLC with color forming reagents, sensitivity is much lower than the other methods.

Dabsyl chloride used in this work is a sensitive chromophoric labeling reagent whose sulfonyl group reacts easily with primary and secondary amino groups, thiols, phenols, and so on. Recently, Lin, Chang, and their respective co-workers have investigated on effective detection of amines and amino acids by dabsylation and have found that the dabsyl derivatives are easily crystallized, having high sensitivity and high photo-stability [*19-21*].

In the present study, we successfully obtained the crystallized dabsyl derivatives of THC and CBN, the major components of *Cannabis*, with strong absorption at visible region. The

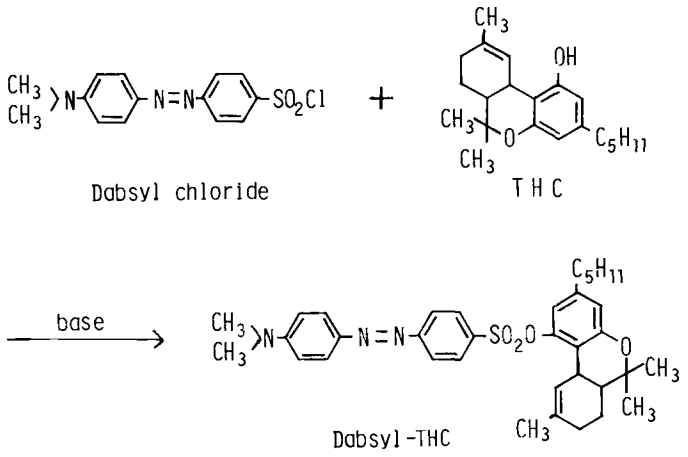

FIG. 9—*Formation of dabsyl-THC.*

reaction of dabsyl chloride with the phenolic group of THC used in the present study is assumed as in Fig. 9. This reaction was confirmed by disappearance of the phenolic proton signal of THC from the NMR spectrum and by the presence of a sulfonic ester group revealed by IR absorption at 1140 and 1380 cm^{-1}.

For analysis of cannabinoids by dabsylation, TLC and HPLC techniques were tested and found to be suitable for routine use. The TLC gave good separation of dabsyl-THC and dabsyl-CBN from other components by employing *n*-hexane-ethylacetate-diethylamine (20:5:1) as developing solvent. The intense chromophoric character of dabsylcannabinoids permitted their direct detection as colored spots on thin-layer plate in the range of 10^{-10} to 10^{-11} mol. Since dabsylcannabinoids have the methyl orange structure, their spots' color turned from yellow orange to bright red by using an acidifying reagent which resulted in a significant increase of the detection limit. The HPLC technique also gave good separation and higher sensitivity of 1-ng order of THC and proved to be favorable for quantitative analysis of cannabinoids. Good linearity in the calibration curve was obtained for the quantitation of THC. The HPLC was then applied to the analysis of urine and plasma with added THC, in order to approach to the detection with dabsylation technique of THC and its metabolites in biological materials. There were no interferences from endogenous compounds of these samples. Since 11-nor-9-COOH-Δ^9-THC is the major metabolite after taking *Cannabis*, the chromophoric labeling of the substance would also be expected to be useful for analysis of biological materials from *Cannabis* users.

It is well known that the storage of THC as a standard material is very difficult because of its unstability. Our previous study has proved, on the other hand, that the THC and CBN when crystallized by dabsylation are unchanged for at least one year.[3] The stability after dabsylation would therefore be favorable as a standard sample for analysis. The crystallization of these substances has the further advantage of easy treatment during analytical work.

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