Marihuana: Identification of Cannabinoids by Centrifugal Chromatography

Abstract. *Components in extracts of marihuana and hashish have been identified by a chromatographic technique in which centrifugal force is used to accelerate the migration of samples through columns of densely packed microparticulate gel. Rapid qualitative analysis and an estimate of the amounts of cannabinoids present was achieved.*

Procedures for the rapid separation and identification of cannabinoids in marihuana and hashish are, for one reason or another, somewhat unsatisfactory (*1*). A chromatographic technique, by which complex mixtures of bacterial lipids (*2*) and steroids (*3*) were readily separated, was successfully applied for this purpose. Centrifugal force was used to accelerate the migration of the sample and to achieve improved resolution through gels prepared with ultrafine silica.

A homogeneous gel for the adsorbent bed was prepared by thoroughly mixing 40 ml of a mixture of petroleum ether and diethylamine (99 : 1, by volume) with 1.5 g of ultrafine silica (Sorvall). Several other solvent systems used for paper and thin-layer chromatography of cannabinoids (*1*, *4*, *5*) were less suitable for the procedure described. The gel was packed into the tubing of chromatographic apparatus (*3*) by centrifugation at 1500*g* for 7 minutes. Samples were dissolved in cyclohexane to the stated concentrations, applied in 5-μl quantity to the top of the gel column, overlaid with petroleum ether–diethylamine (99 : 1), and developed by centrifugation at 1500*g* for 13 or 15 minutes. The chromatograms were then extruded from the tubing. Bands formed by the components were detected by spraying the chromatograms with a fresh aqueous solution (0.4 percent, weight to volume) of di-*o*-anisidine tetrazolium chloride (Echtblausalz B, Roboz, Washington, D.C.) according to the method of Korte and Sieper (*6*). Of nine color reactions studied by Korte and Sieper, that provided by Echtblausalz B was the most sensitive. The entire procedure took about 30 minutes.

Six synthetic cannabinoids [cannabichromene (CBC), cannabinol (CBN), Δ^9-tetrahydrocannabinol (Δ^9THC), Δ^8-tetrahydrocannabinol (Δ^8THC), cannabidiol (CBD), and cannabicyclol (CBL)] were chromatographed individually and as a mixture. They were well separated after 13 minutes of development (Fig. 1a), and the results were reproducible. The migration distances under the conditions stated, measured at the leading edge of each band, are listed in Table 1. They are also compared with retention times in gas-liquid chromatography and R_F values in thin-layer chromatography. It is evident from these data that the components of *Cannabis* are not sufficiently well separated for identification purposes or for isolation by gas-liquid or thin-layer chromatography.

Graded increments of synthetic standards of the three major *Cannabis* constituents (CBN, Δ^9THC, and CBD) were chromatographed to provide bases for estimation of their concentration in extracts of samples. The width and color intensity of bands varied directly with the amount of cannabinoid applied within the range of 0.5 to 10 μg (Fig. 1b). In comparison, Korte and Sieper found the useful range for colorimetric determination of cannabinoid-Echtblausalz B compounds to be between 2 and 8 μg/ml (*7*). An additional feature, not shown in the black and white figure, is that the different components produce different and characteristic colors which assist in qualitatively identifying the compounds. The bands did not fade noticeably during storage for 5 months in a light-tight container.

Extracts of marihuana (A, B, C) and hashish (D) were prepared from 0.1 to 0.5 g of finely ground air-dried samples by successive extraction with three 15-ml portions of petroleum ether while stirring under N_2 at room temperature for 10 minutes. Extracts from each sample were pooled, filtered, and evaporated to dryness under reduced pressure in a stream of N_2. They were then dissolved in cyclohexane to a concentration of 30 to 40 mg of solids per milliliter and stored at 0°C until used. Chromatograms prepared with 5-μl quantities of the extracts of marihuana and of hashish are illustrated in Fig. 1c. All extracts contained CBC, CBN, and Δ^9THC (*8*). CBD was a major constituent only in the hashish extract (D), CBL was a minor component, if present at all, and Δ^8THC was detected in none. This finding was to be expected since the concentration of Δ^8THC has been reported to be only about 1 percent that of Δ^9THC in the plant (*1*). Three components with migration distances of 2.5, 5, and 11 mm, respectively, were not identified.

To estimate the concentration of the major constituents (CBN, Δ^9THC, and CBD) in these extracts, they were usually diluted to 5 to 10 mg of solids per milliliter to obtain the chromatograms with less intensely colored bands (Fig. 1d) which were visually compared with the colored bands produced

Table 1. Retention times on gas-liquid chromatography, R_F values on thin-layer chromatography, and migration distances on centrifugal chromatography of neutral natural cannabinoids.

Compound	Retention time*	R_F†	Migration‡ (mm)
Cannabicyclol	4′33″	0.62	51
Cannabidiol	5′40″	0.58	43
Δ^8-Tetrahydrocannabinol	7′10″	0.57	34
Δ^9-Tetrahydrocannabinol	7′52″	0.51	30
Cannabinol	10′12″	0.47	16
Cannabichromene	5′35″	0.43	7

* Analytical data from gas-liquid chromatography by Mechoulam (*1*). † Thin-layer chromatographic data by Mechoulam (*1*). ‡ Distance of migration of component.

Table 2. Concentrations of major components of air-dried marihuana and hashish as estimated from centrifugal chromatography.

Source	Yield of extract (%)	CBD (%) in*		Δ^9THC (%) in		CBN (%) in	
		Extract	Sample	Extract	Sample	Extract	Sample
Marihuana							
A. Midwestern U.S.	4.5	None	None	6.9	0.31	2.4	0.11
B. Southwestern U.S.	3.7	0.58	0.02	17	0.64	2.4	0.09
C. Southeastern Asia	5.0	None	None	8.8	0.44	4.1	0.21
Hashish							
D. Unknown	10	6.2	0.62	12	1.2	2.6	0.26

* Average values based on visual reading of chromatograms (at least two readings of two separate analyses) are rounded to two significant figures. No such accuracy is claimed, but see text for evidence of reproducibility.

on chromatograms by known quantities of synthetic standards (Fig. 1b). On occasion, as with sample B (Fig. 1c), it was necessary to adjust dilutions of extracts to obtain appropriate concentrations of individual components.

For these estimates of quantity, the chromatograms were read by two independent observers. Agreement was such that there never has been any doubt whether the width and intensity of a band were greater or less than those of the nearest standard band, but often they were somewhere between two concentrations of the standard. In such cases we have assigned a value halfway between. From the concentrations so estimated and the determined yields of extracts, it was possible to calculate the proportions of given components both in extracts and in the original samples (Table 2). Although improvement is possible (for example, by better statistical methods for evaluating the visual scores, by mechanical scanning of the chromatograms, or by colorimetric measurement of the eluted components), the estimates of CBN, Δ^9THC, and CBD in the samples tested were within the general range reported by others (*1, 8, 9*). In more recent tests, "street" samples of hashish have been encountered varying from 0.5 to 4 percent of Δ^9THC, the physiologically active component.

As an example of reproducibility, ten chromatograms of sample D (Fig. 1d) were prepared and read independently by two observers. There was no variation among the readings. Eight of the chromatograms were identical. In two, the bands were shifted down, one dilution in one case and one-half dilution in the other.

A petroleum ether extract of smoke condensate from a machine-smoked sample of synthetic Δ^9THC was estimated by our chromatographic technique and contained 60 percent unaltered Δ^9THC and 40 percent CBN. Thus, a substantial part of the original Δ^9THC was converted to CBN, in addition to what was undoubtedly destroyed in other ways by burning. A similar increase in CBN content at the expense of Δ^9THC was observed when sample C (Fig. 1d) was machine-smoked. These results confirmed the findings of Claussen and Korte (*10*) that some Δ^9THC is converted to CBN during the smoking process. These authors also reported that heating in the absence of oxygen brought about such conversion, and we noted that it

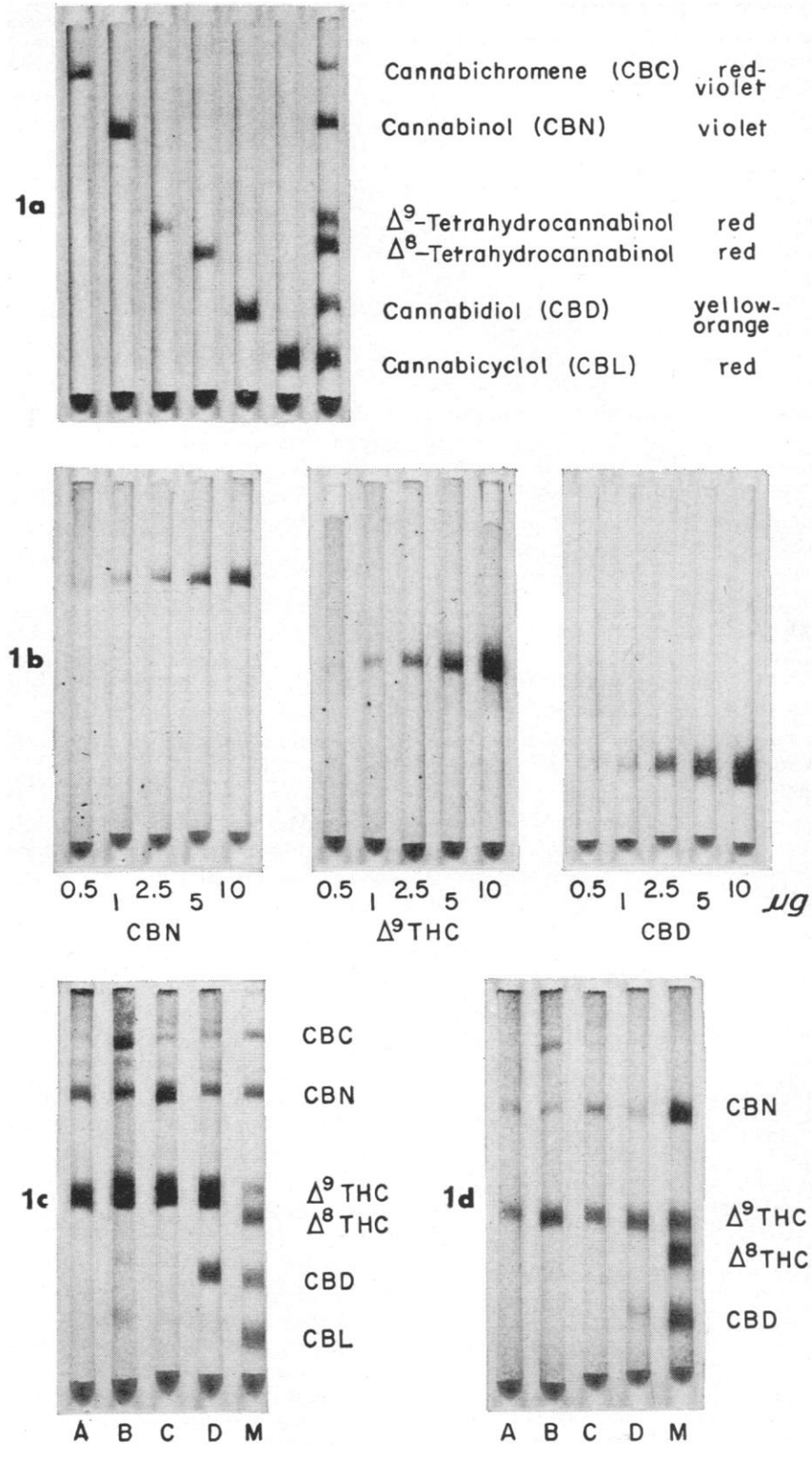

Fig. 1. Chromatograms of cannabinoids. (1a) Individual and mixed synthetic standards. (1b) Bands of synthetic standards produced by quantities indicated. Width and intensity of colored bands parallel quantities of cannabinoids in samples chromatographed. (1c) Petroleum ether extracts of marihuana (samples A, B, and C) and hashish (sample D) and a mixture of synthetic standards (M). Five microliters of solutions (30 to 40 mg/ml) applied except for M where the concentration was 0.7 mg of each component per milliliter. (1d) Bands formed by sixfold dilutions of the same extracts used to prepare chromatograms shown in Fig. 1c. Visual comparison of these colored bands with those produced by known quantities of synthetic standards, shown in Fig. 1b, permitted estimation of the concentration of CBN, Δ^9THC, and CBD in these extracts (Table 2). All chromatograms were developed with a mixture of petroleum ether and diethylamine (99 : 1) at 1500*g* for 13 minutes, except those shown in Fig. 1d, which were developed for 15 minutes. Chromatograms are shown actual size.

occurred by mere storage of synthetic Δ^9THC, dissolved in cyclohexane, for several months in the frozen state. The absence of the Δ^8THC band in our chromatograms of smoke condensates supports the finding of Claussen and Korte (*10*) and of Lerner and Zeffert (*11*) that isomerization of Δ^9THC into Δ^8THC occurs only to a very small extent under smoking conditions.

Extracts of thyme, oregano, sage, rosemary, clove, caraway seeds, alfalfa, tobacco, menthol, green tea, and rhubarb leaves were chromatographed because these substances reportedly have been used to dilute or substitute for marihuana (*4*). Thyme, clove, oregano, and thymol each formed a single band which migrated about 20 mm. These bands did not mask those of cannabinoids, alter migration, or interfere with identification when mixed with the synthetic standards. The other extracts in this group did not produce detectable bands.

It is expected that, in addition to its value for qualitative work, the technique described will be useful for purification of components for structural analysis by other methods.

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12. We thank Dr. J. A. Scigliano, Center for Studies of Narcotics and Drug Abuse, National Institute of Mental Health, Chevy Chase, Md., for the synthetic cannabinoid standards; the Montana Tuberculosis Association for support of S.M.S.; and D. E. Dye, Sheriff of Ravalli County, Montana, for confiscated marihuana and hashish.

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Ascorbate Sulfate: A Urinary Metabolite of Ascorbic Acid in Man

Abstract. *Ascorbate-3-sulfate is a significant metabolite of ascorbic acid excreted in human urine. The characteristics of this compound were determined in experiments in which labeling with carbon-14 and tritium was used coupled with cochromatography with synthetic ascorbate-3-sulfate (both labeled and not labeled with sulfur-35) in a variety of solvent and absorbent systems.*

Little information is available on urinary metabolites of ascorbic acid in man. Oxalate has been identified (*1*). The only other known excretion product is ascorbate itself. In this report, we describe the identification of ascorbate-3-sulfate in urine of human subjects with scurvy (*2*).

Male volunteers fed a diet deficient in ascorbic acid were given [1-^{14}C, 4-^{3}H]ascorbate (*2*). Urine from these subjects was fractionated into a lead acetate precipitate at *p*H 4 and *p*H 8, and a soluble (S) fraction. The presence of both ^{14}C and ^{3}H in each fraction indicated a variety of labeled metabolites. The percentage of ^{14}C in the S fraction was markedly lower at the 9th week for one subject with signs of severe scurvy at that time. A qualitative correlation between the degree of scurvy and the percentage of ^{14}C in the S fraction was observed in all five subjects in this study (*2*).

The S fractions were examined by electrophoresis in a 0.05*M* ammonium acetate buffer (*p*H 5.8). The major component in this fraction proved to have an electrophoretic mobility slightly less than picrate ($R_{picrate} = 0.78$) and a ratio of ^{14}C to ^{3}H the same as that of the administered labeled ascorbate. These properties show that this metabolite was a strong acid in which the carbon chain was almost certainly intact. Furthermore, the compound must have had an unusual stability since the urine samples, stored in frozen acidified solution, were almost 2 years old at the time of analysis.

Experiments were undertaken to show whether the metabolite was ascorbate sulfate, such as has been described by Mead and Finamore for brine shrimp cysts (*3*) and synthesized by Chu and Slaunwhite (*4*). Ascorbate-3-sulfate was synthesized by a variation of their method and purified by barium salt precipitation and chromatography on a diethylaminoethyl (DEAE) cellulose–sulfate ion exchange column eluted with a linear gradient of dilute sulfuric acid. This procedure permits separation of the several ascorbate monosulfates. The reference

Table 1. Cochromatography of synthetic and urinary ascorbate-3-sulfate. The composition of solvent systems was as follows; all proportions are by volume. Pabst III: 0.1*M* potassium phosphate buffer (*p*H 6.8), ammonium sulfate, and 1-propanol (10 : 6 : 2), ascending paper; BAW: 1-butanol, acetic acid, and water (5 : 2 : 3 or 6 : 4 : 3), descending paper; AFI: 0.04*M* ammonium formate buffer (*p*H 4.3) and 2-propanol (6 : 4), ascending thin-layer chromatography (TLC); EAW: ethylacetate, acetic acid, and water (6 : 3 : 2 or 5 : 2 : 3), ascending TLC; CMW: chloroform, methanol, and water (65 : 25 : 4), ascending TLC; ABW: acetonitrile, butyronitrile, and water (66 : 33 : 2), ascending TLC; EPW: ethylacetate, pyridine, and water (8 : 2 : 1), ascending TLC; BB: methylethylketone, acetic acid, and saturated boric acid (9 : 1 : 1), ascending TLC.

Absorbent	Solvent	R_F	
		Synthetic	Urinary
Whatman No. 1 paper	Pabst III	0.89	0.90
Whatman No. 1 paper	BAW	.52	.54
Bakerflex cellulose	AFI	.65	.64
Bakerflex cellulose	EAW	.39	.39
Silica gel H	ABW	.0	.0
Silica gel H	EPW	.0	.0
Silica gel H	BB	.0	.0
Silica gel H	BAW	.25	.28
Silica gel H	CMW	.0	.0