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CANNABIS SATIVA L. (MARIJUANA)

III. THE RIM TEST: A RELIABLE AND USEFUL PROCEDURE FOR THE DETECTION AND IDENTIFICATION OF MARIJUANA UTILIZING COMBINED MICROSCOPY AND THIN-LAYER CHROMATOGRAPHY

ALVIN B. SEGELMAN

Department of Pharmacognosy, College of Pharmacy, Rutgers-The State University, New Brunswick, N.J. (U.S.A.)

SUMMARY

A simple, reliable and easily performed method for detecting and identifying marijuana in suspect material is presented. The method, designated as the RIM test (Rutgers Identification for Marijuana test), utilizes combined histochemical and thin-layer chromatography techniques and thus eliminates the need for a separate extraction step to obtain a suitable sample for thin-layer chromatographic study.

INTRODUCTION

It is well known that a variety of preparations derived from the plant *Cannabis sativa* L. have long been used for their intoxicating properties[1,2]. Perhaps the most widely used and abused of these preparations is marijuana, consisting of the dried and crushed leaves and tops of both male and female plants. This widespread misuse of marijuana has in recent years led to the development of many physico-chemical methods for detecting and identifying this important drug. In addition to the older and still used Beam test[3] and Duquenois–Levine test[4], the more recently developed detection methods and qualitative analytical techniques dealing with marijuana have been reported in several current papers[5–9] and reviews[10–13] and need not be considered here in detail. In general, these newer methods first involve the extraction of suspect material, using a suitable organic solvent such as petroleum ether, chloroform or alcohol, followed by thin-layer chromatographic (TLC) and/or gas–liquid chromatographic (GLC) examination of the concentrated organic extracts. If cannabinoid compounds are present in the organic extracts (*viz.*, if the suspect material contains marijuana), they can be revealed on the developed thin-layer chromatograms by means of suitable chromogenic reagents and in the cases of the GLC methods, the cannabinoids (as well as their easily prepared silyl derivatives) can be characterized by their retention times and compared with standards analyzed under similar conditions.

Some investigators[14,15] consider it desirable to utilize one or more of the de-

scribed physico-chemical methods together with microscopic methods for confirming the presence or absence of marijuana in suspect samples. The positive microscopic identification of marijuana in any particular sample is based on observing the presence of certain histological features such as cystolith hairs, glandular hairs, non-glandular hairs as well as other morphological structures which are characteristic and diagnostic for *C. sativa*[16]. However, certain of these diagnostic criteria may be obscured by the fact that marijuana is frequently adulterated with a variety of non-cannabis plant substances, including tobacco (*Nicotiana tabacum*), lavender (*Lavendula officinalis*), catnip (*Nepeta cataria*) and oregano (*Origanum vulgare*)[17]. The latter three plant species, as is the case with certain other members of the mint family (*Labiatae*), contain glandular hairs[18] which, especially when crushed and fragmented, may be confused with the typical marijuana glandular hairs. Furthermore, Nakamura[14] has recently pointed out more than eighty different plant species which contain cystolith hairs similar in many respects to those observed in marijuana. Thus, it is conceivable that a suspect sample of plant material could be erroneously identified as containing marijuana, whereas in effect the former would comprise a mixture of one or more plant substances having diagnostic morphological features similar to authentic marijuana material. Were this situation to occur, the legal implications would be of serious concern. Unfortunately, detailed methods which deal specifically with the microscopic analysis of marijuana have been lacking or fragmentary in the scientific literature.

For these reasons, we have recently developed a highly reliable microscopic method based on histochemical staining reactions for identifying and detecting marijuana[17]. As part of our continuing studies in this area, it was considered worthwhile to explore the possibility of combining this new microscopic method with TLC techniques in order to develop a simple, reliable and easily performed qualitative procedure that could be carried out by the majority of laboratories engaged in this type of work. Our investigations along these lines have resulted in a procedure now designated as the RIM test (Rutgers Identification for Marijuana test), which is outlined and discussed below.

EXPERIMENTAL

(1) Thoroughly mix a small amount (1–5 mg) of suspect material with two to four drops of clearing solution —chloral hydrate (75 g), propylene glycol or glycerin (10 ml), distilled water (sufficient quantity to make 100 ml)— on a clean microscope slide. It is preferable to remove larger particles of material (*viz.*, seeds and large stem fragments) from the sample prior to treatment with clearing solution. Alternately, the entire suspect sample, including seeds, may be ground to a powder, using a mortar or other suitable means for reducing particle size.

(2) Carefully heat the slide from below, using a microburner, until the mixture has boiled for a total time of 3 sec. Cool momentarily, add two additional drops of clearing solution and mix well. The mixture should have the appearance of a rather thick slurry. The chloral hydrate clearing solution serves to dissolve starch, plant pigments and other substances which tend to obscure easy microscopic observation of the diagnostic histological elements. Also, the clearing solution partially extracts the marijuana cannabinoids if marijuana is present in the specimen being studied. The

heating step accelerates the clearing process and also leads to the decarboxylation of any cannabinoid acids present to furnish the corresponding neutral phenolic cannabinoids. It is known[19,20] that certain cannabinoid acids may comprise a large percentage of the total cannabinoid content in various marijuana samples. Hence, it is important to ensure decarboxylation of these acids when using the RIM test. Otherwise, the cannabinoid acids tend to remain near or at[20] the origins of application during the TLC step of the RIM test and their identity may be obscured by pigments that also remain near the origins on the TLC plates.

(3) By means of a capillary spotting pipette, remove approximately 5–10 μl of liquid from the mixture from step (2) above. If care is taken to fill the pipette from the peripheral areas of the mixture, essentially clear, particulate-free liquid will be drawn into the pipette while leaving behind unwanted solids. Carefully spot 5 μl on a Silica Gel G coated (0.25-mm-thick layer previously activated at 100° for 20–30 min) glass plate. Because it will normally be found that the liquid spotted on the plate is somewhat viscous, drying of the spot can be accelerated by means of a hot-air gun. We have found that either hand-coated or pre-coated glass plates spread with Silica Gel G (Merck) give consistently superior results than do the various commercially available silica gel coated flexible films and sheets. Serious difficulties may arise in the latter instances due to spot broadening and overloading, presumably because of the low loading capabilities of the flexible products. For comparison purposes, authentic cannabinoids should be spotted on the same plate. The spotted plate is next allowed to develop in a closed chamber under "saturated conditions" with benzene as eluant for a total time of 25 min or until the solvent front has advanced to a point 15 cm from the origin of spot application. While the plate is developing, proceed to step (4).

(4) Treat the wet mount from step (2) with two drops of *freshly prepared* chromogenic reagent—Fast Blue B salt (0.3 g), clearing solution (sufficient quantity to make 100 ml)—, mix well using a microspatula, cover the preparation with a glass (not plastic) cover glass and examine microscopically (100–400× magnification). It has previously been shown that Fast Blue B salt (*o*-dianisidine diazotate) reacts with cannabinoids to give characteristic red- to purple-colored compounds[21]. Hence, upon microscopic examination, those plant tissues rich in cannabinoids will be observed to have acquired these red to purple colors. This phenomenon is particularly evident in the case of the marijuana glandular hairs, which contain relatively large amounts of cannabinoids. An additional feature helpful in detecting the presence of marijuana is the fact that when one is dealing with a marijuana-containing suspect sample, the entire wet mount rapidly acquires the characteristic red-purple (plum-cherry) color which is easily observed with the naked eye.

(5) Finally, place one drop of glacial acetic acid along one edge of the cover glass, draw the acid into the mount by touching the opposite edge of the cover glass with a piece of absorbent paper and observe microscopically. The presence of cystolith hairs is evidenced by the liberation of gas bubbles (carbon dioxide) from these calcium carbonate-containing hairs. The completion of step (5) finalizes the microscopic aspects of the RIM test.

(6) Attention is now directed towards the TLC plate. When the solvent front has advanced approximately 15 cm as previously described, the plate is removed from the developing chamber, air-dried and subsequently sprayed with Fast Blue B

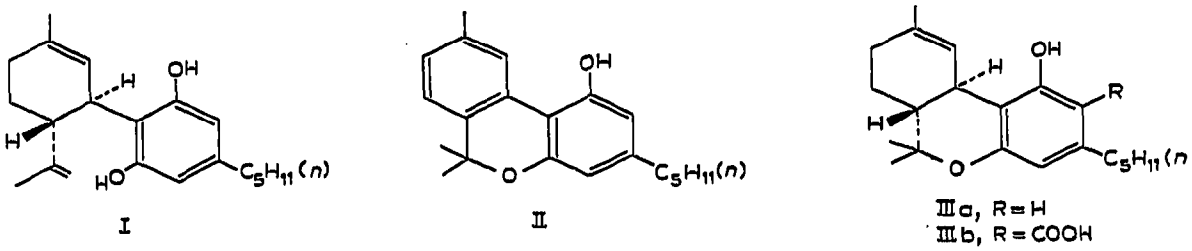

Fig. 1. Structures of selected cannabinoids. I = (—)-*trans*-Cannabidiol; II = cannabinol; IIIa = (—)-*trans*-Δ^9-tetrahydrocannabinol; IIIb = (—)-*trans*-Δ^9-tetrahydrocannabinol acid A.

spray reagent—Fast Blue B salt (0.2 g) in 80% ethanol. If the sample under study contains marijuana, the major cannabinoids usually found in marijuana (*viz.*, Δ^9-tetrahydrocannabinol, cannabidiol and cannabinol; see Fig. 1) can be revealed and characterized by their colors and R_F values relative to reference standards run on the same plate.

RESULTS AND DISCUSSION

Microscopic aspects

It was found that the Fast Blue B chromogenic reagent appears to stain selectively marijuana glandular hairs as well as other cannabinoid-rich tissues (Figs. 2–4).

Fig. 2. Microscopic appearance of a chloral hydrate-cleared mount (unstained) prepared from female marijuana plant material collected in 1890 (herbarium specimen).

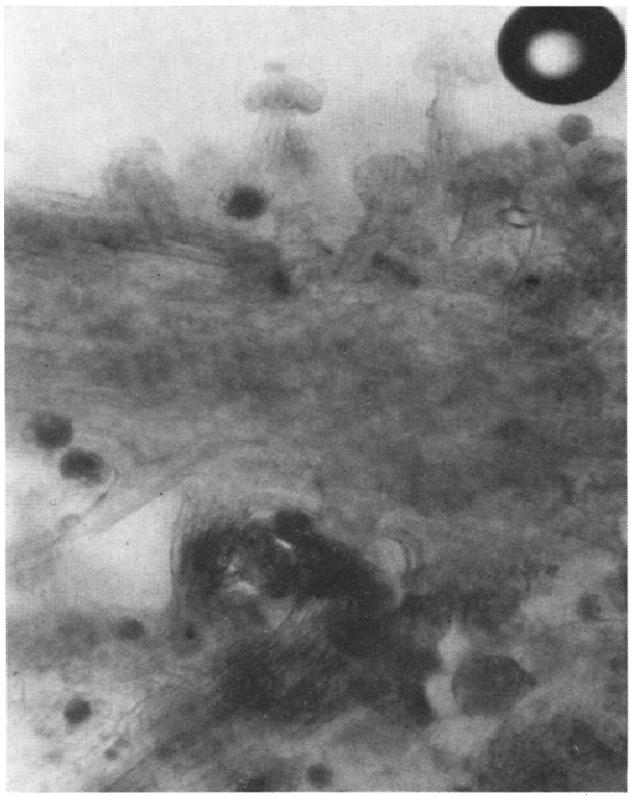

Fig. 3. Microscopic appearance of plant material shown in Fig. 2, 20 sec after treatment with Fast Blue B chromogenic reagent. Note that the cannabinoid-containing tissues have rapidly acquired pink to red colors (shown as darker shades in black and white).

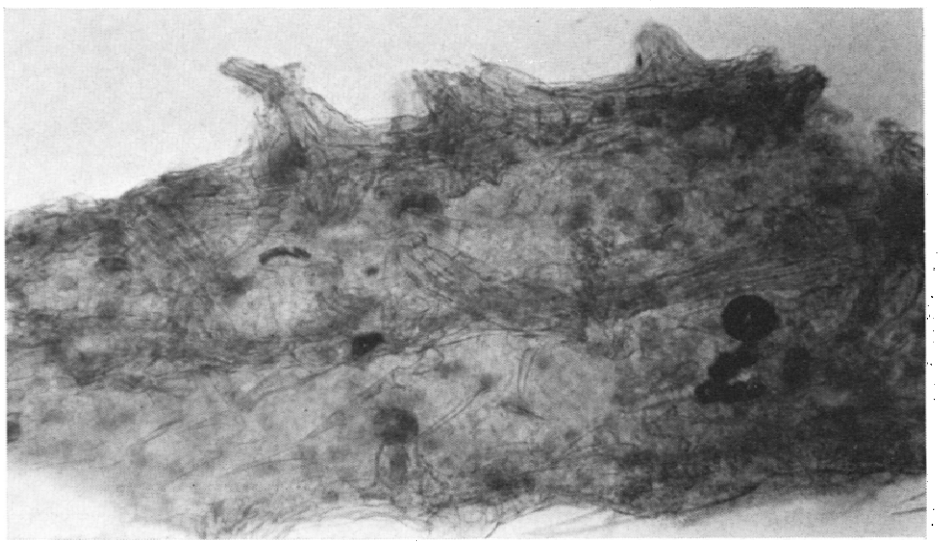

Fig. 4. Microscopic appearance of chloral hydrate-cleared female plant material 5 min after treatment with Fast Blue B chromogenic reagent. The cannabinoid-rich glandular hairs have acquired characteristic red colors and are clearly visible.

In addition to glandular hairs, for example, lactiferous vessels, fragments of resin and even pollen grains acquire characteristic red to purple colors. To determine the possibility of false-positive reactions to the Fast Blue B reagent, we assessed many different noncannabis plant species, including some having glandular hairs, using the described microscopic method. In these cases no staining of the glandular hairs was observed, although in certain instances we noted that various plant tissues or the entire wet mounts acquired colors ranging from pink to purple subsequent to treatment with Fast Blue B reagent. These colors, attributed to the reaction of Fast Blue B with plant phenolics, could usually be distinguished from the characteristic red-purple colors exhibited by the marijuana samples studied.

TLC aspects

It was found that an aliquot of the chloral hydrate solution used to clear plant tissues prior to microscopic examination could be directly chromatographed on Silica Gel G layers using standard TLC techniques (Fig. 5). The chloral hydrate solution effectively extracts the major cannabinoid compounds from marijuana plant material as well as numerous other phytoconstituents both from marijuana and non-marijuana plant material. However, it was found that by using benzene as the developing solvent, the major cannabinoids travel significantly faster than do the majority of the non-cannabinoid phytoconstituents, most of which remain at or near the origins on the plates. Thus, the cannabinoids are well separated and can be adequately revealed and characterized on the developed plates following treatment with Fast Blue B spray reagent. By spotting an aliquot of the chloral hydrate extract from the micro-

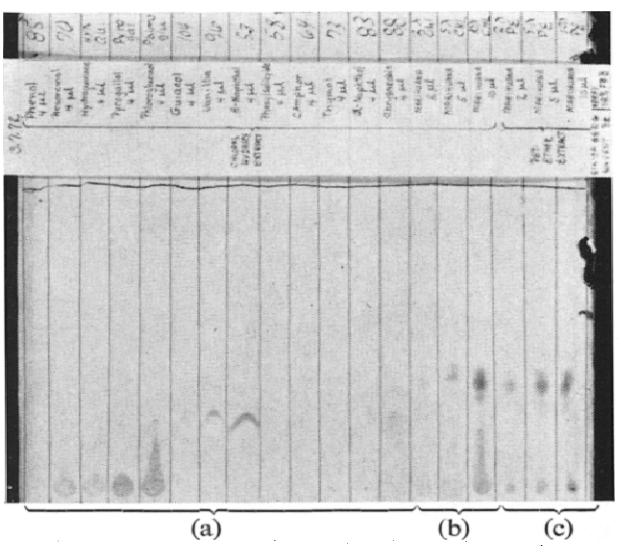

Fig. 5. TLC of: (a) Chloral hydrate clearing solution extracts (1 mg/1 ml) of selected natural products; from left to right (channels 1–13): phenol, resorcinol, hydroquinone, pyrogallol, phloroglucinol, guaiacol, vanillin, β-naphthol, phenylsalicylate, camphor, thymol, α-naphthol and chrysarobin; 4 μl of each extract was spotted. (b) Chloral hydrate clearing solution extracts (channels 14–16) from a marijuana sample examined according to the RIM test protocol. (c) Petroleum ether (b.p. 30–60°) extracts (channels 17–19) prepared from the identical marijuana material as the sample studied in (b) above. Conditions: Silica Gel G, 0.25-mm thick layer; benzene as eluant; 25-min running time; Fast Blue B salt spray reagent.

scopic clearing step, the investigator using the RIM test protocol eliminates the need for further extracting additional suspect material with an organic solvent to obtain a sample for TLC study. This is especially important when one has a limited quantity of available suspect material.

Authentic marijuana samples, ranging in age from four months to one hundred years, gave consistently positive results when examined by means of the RIM test. On this basis, and also considering the lack of observable false-positive reactions in the cases of the non-cannabis plant species studied, the RIM test appears to be a highly reliable and useful method for confirming the presence or absence of marijuana in suspect materials.

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