Constituents of *Cannabis sativa* L. VIII: Possible Biological Application of a New Method to Separate Cannabidiol and Cannabichromene

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Abstract □ Synthetic and naturally occurring cannabidiol and cannabichromene were distinctly separated without derivation by GLC using a 6% OV-1 column; an artifact of cannabichromene, cannabicyclol, was separated from (−)-Δ^9-*trans*-tetrahydrocannabivarin. This procedure is versatile and applicable for the quantitation of *Cannabis* containing both cannabidiol and cannabichromene. Biological interaction among (−)-Δ^9-*trans*-tetrahydrocannabinol, cannabichromene, and other cannabinoids in natural *Cannabis* preparations can now be studied. In the phenyl methyl silicone polymer series, cannabidiol precedes cannabichromene on columns containing below a 50% phenyl-to-methyl ratio. Columns containing a 50:50 or greater ratio of phenyl to methyl reverse the separation order with cannabichromene preceding cannabidiol.

Keyphrases □ *Cannabis sativa* L.—constituents, method to separate cannabidiol and cannabichromene, GLC conditions discussed □ Cannabinoids—separation of cannabidiol and cannabichromene, GLC □ GLC—separation of cannabidiol and cannabichromene

Recently, numerous data supporting pharmacological interactions between cannabinoids have been published. Carlini *et al.* (1) suggested that cannabidiol (I) could possibly block some effects of (−)-Δ^9-*trans*-tetrahydrocannabinol (II). Karniol and Carlini (2) postulated that both I and cannabinol (III) may alter the action of II. More recently, Karniol and Carlini (3) reported that I blocked several effects of II in animal models and that I potentiated the analgesic effects of II. Borgen and Davis (4) found that I induced reduction of the hypothermic response to II in rats and rabbits and that I can attentuate other effects of II. Furthermore, Jones and Pertwee (5) reported that I pretreatment altered the metabolism of II. Therefore, quantitation of cannabinoids other than II must be accomplished to describe pharmacological parameters and to correlate these parameters with the cannabinoid(s) responsible for the activity.

This laboratory has recommended that each sample of *Cannabis sativa* L. be quantitated routinely for all possible cannabinoids so that pharmacologists may study the potentiation or antagonism of II activity by other cannabinoids (6, 7). To accomplish this task, much time and effort have been assigned to the development of techniques. Results have shown that when proper analytical techniques are used, quantitation of II in plant material is relatively simple (8, 9).

Cannabinol (III) can be quantitated, but care must be observed since a C_{29}-hydrocarbon has nearly the same relative retention time and can cause error in quantitating III (9). However, I is not easily quantitated in plant material.

Vree *et al.* (10), using GC–mass spectrometry, showed that a Brazilian sample of *Cannabis* contained no I, although a strong GC peak existed where I is normally found. The compound yielding the peak normally assigned to I was cannabichromene (IV), which supported de Faubert Maunder's (11) statement that I was definitely absent in some samples of *Cannabis*. This researcher used a TLC system. Moreover, Turner and Hadley (8) investigated a South African sample and found an absence of I but the presence of IV; this finding was confirmed by GC–mass spectrometry, TLC, and GC.

Although the OV-17 column[1] used in routine quantitation of cannabinoids has been reported as separating I and IV (12), this separation was based on a 5-sec time interval between peaks of pure I and IV and not a mixture of the two.

A clear and concise separation of synthetic I and IV, using a trimethylsilyl procedure, was reported (13); this procedure also afforded separation of I and IV in plant material (14). The silyl procedure in these laboratories provides excellent reproducibility in quantitating all cannabinoids when responsible analytical procedures are followed. However, when some silyl reaction mixtures are allowed to stand at ambient temperature for more than 1 hr, reproducibility

Table I—GC Columns and Conditions for the Separation of a Mixture of Cannabichromene and Cannabidiol[a]

Liquid Phase	Percent	Oven Temperature	Separation		
			No	Yes[b]	Yes[c]
OV-1	3	180°		x	
OV-1	3	200°	x		
OV-1	6	180°			x
OV-1	6	200°	x		
OV-1	8	180°			x
OV-1	8	190°		x	
OV-3	3	180°			x
OV-7	3	180°		x	
OV-7	3	200°	x		
OV-7	6	180°		x	
OV-11	3	200°	x		
OV-17	2	180°	x		
OV-17	2	210°	x		
OV-17	3	180°	x		
OV-17	3	210°	x		
OV-17	5	250°[d]	x		
OV-17	5	210°	x		
OV-25	2	210°	x		
OV-25	2	210°	x		
QF-1	3	210°	x		

[a] The mixture can be natural and/or synthetic. [b] Quantitation is possible using a GC–computer system. [c] Quantitative without computer. [d] Using 80–100 mesh and 4-mm i.d., 1.8-m (6-ft) column.

[1] High purity polar methyl silicone; approximately 30,000 mol. wt. used by National Institute on Drug Abuse for analyses of *Cannabis*.

Table II—Mass Spectra Data for Cannabichromene and Cannabidiol[a]

Compound	m/e														
I	314	299	286	271	—	260	259	258	—	246	—	232	231	230	229
IV	314	299	—	271	267	—	—	258	257	—	243	232	231	—	—

[a] Data obtained from combined GC–mass spectrometry as described under *Experimental*. Data were obtained at 70 ev.

diminishes. Also, silylation requires additional time and response factors. Therefore, the purpose of this present investigation was twofold: (*a*) to develop a method for the separation of I and IV without necessitating derivative formation, and (*b*) to eliminate the requirement for additional response factors.

EXPERIMENTAL

Analyses were performed using a gas chromatograph[2] equipped with hydrogen flame-ionization detectors and operated isothermally at 180°. The detector and inlet temperatures were 260 and 240°, respectively. Glass columns, 0.63 cm (0.25 in) o.d. and 2 mm i.d. × 2.43 m (8 ft), were packed with 6% OV-1 on 100–120-mesh Gas Chrom Q. Nitrogen was used as the carrier gas at a flow rate between 10 and 30 ml/min, depending upon instrument and column requirements.

The *Cannabis* samples used were from known seed stock[3] and prepared according to the modified Lerner extraction method (8). The synthetic standard solution was prepared by dissolving 5 mg of each standard [cannabichromene, cannabidiol, and androst-4-ene-3,17-dione (V)] in 1.5 ml of absolute ethanol. The resulting standard solution (1:1:1) was ultrasonically shaken for 30 sec, after which time 0.2–0.4 μl was injected. In these laboratories, quantitation reliability diminishes due to detector overload when any volume over 1 μl is injected. For additional analytical data on the analysis of *Cannabis*, see Refs. 6, 7, 9, and 14. Response factors were calculated using synthetic cannabinoids[4] and naturally occurring cannabinoids isolated in these laboratories.

RESULTS AND DISCUSSION

Gaoni and Mechoulam (12) reported pure I and IV to have retention times of 5 min 40 sec and 5 min 35 sec, respectively, using 2% OV-17 at 253°. This 5-sec time differentiation is insufficient for quantitating a mixture of I and IV. Additionally, de Zeeuw *et al.* (15) reported the separation of I and IV in a *Cannabis* sample using 5% OV-17, 5% OV-25, 3% QF-1, and 5% SE-30. The best separation was obtained on the 5% OV-17 column. Retention times reported, with the exception of SE-30, indicated that IV preceded I (15). However, these chromatograms from a 5-μl injection of a hex-

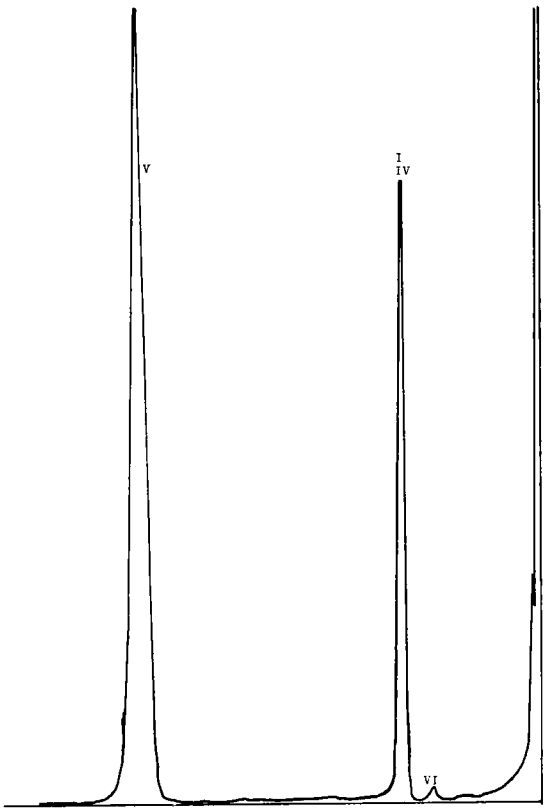

Figure 1—*Chromatogram showing the nonseparation of synthetic cannabidiol and cannabichromene on 2% OV-17. Key: VI, cannabicyclol; I, cannabidiol; IV, cannabichromene; and V, androst-4-ene-3,17-dione, the internal standard.*

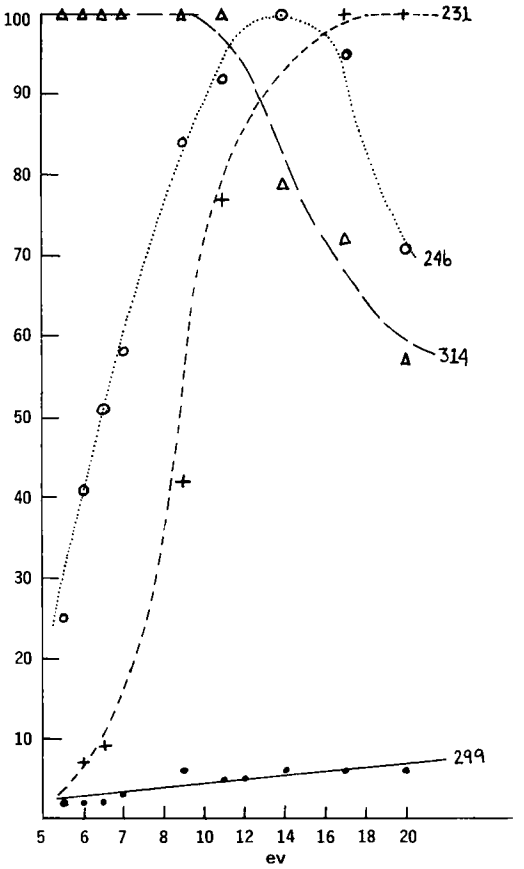

Figure 2—*Electron voltage–mass fragment intensity graph of cannabidiol. The point of intercept for mass fragments 231 and 314 is approximately 12 ev; in the system of Vree* et al. *(10), the intercept is approximately 13 ev. Although mass fragment intercepts are not identical, the patterns are identical.*

[2] Beckman GC-45, GC-65, interfaced to digital PDP-8 computer for data retrieval and determination of response factors.

[3] Grown at the University of Mississippi. *Cannabis* herbarium specimens are stored in the Herbarium, Department of Pharmacognosy, School of Pharmacy, University of Mississippi, University, MS 38677

[4] Obtained through the National Institute on Drug Abuse.

Table III—Relative Retention Times of Underivatized Cannabinoids and Other Components Found in *Cannabis*

Olivetol	0.04
Cannabidivarin (CBDV; VIII)	0.18
Tetrahydrocannabivarin (THC; VII)	0.26
Cannabicyclol (CBL; VI)	0.26
Cannabichromene (CBC; IV)	0.34
Cannabivarin (CBV; IX)	0.34
Cannabidiol (CBD; I)	0.34
Hexahydrocannabinol (HHC; X)	0.37
Cannabigerol monomethyl ether (CBGM; XI)	0.38
$\Delta^{9,11}$-Tetrahydrocannabinol (ETHC; XII)	0.41
Δ^{8}-Tetrahydrocannabinol (Δ^{8}-THC; XIII)	0.44
Cannabielsoin (CBE; XIV)	0.48
Δ^{9}-Tetrahydrocannabinol (Δ^{9}-THC; II)	0.49
Cannabigerol (CBG; XV)	0.57
Cannabinol (CBN; III)	0.63
C_{29}-Hydrocarbon (XVI)	0.67
Androst-4-ene-3,17-dione (Δ^{4}-dione; V)	1.00

ane extract of *Cannabis* contained only a trace amount of I and IV. Thus, it was difficult to ascertain if the separation reported allows for quantitation.

Duplication of the reported experimental conditions in these laboratories using synthetic cannabinoids did not separate a mixture of I and IV; however, cannabicyclol (VI), an artifact from IV, was separated and did procede the single peak observed for the mixture of IV and I. De Zeeuw *et al.* (15) did not mention the presence of VI, which is routinely observed when synthetic or naturally occurring IV is subjected to GC analyses (9). Therefore, the relative retention times reported by de Zeeuw *et al.* are indicative that VI is mislabeled and misidentified as IV.

Using another support phase, 3% OV-7 at 210°, Small and Beckstead (16) reported the separation of I and IV. Although no retention times were reported, a representative chromatogram showed

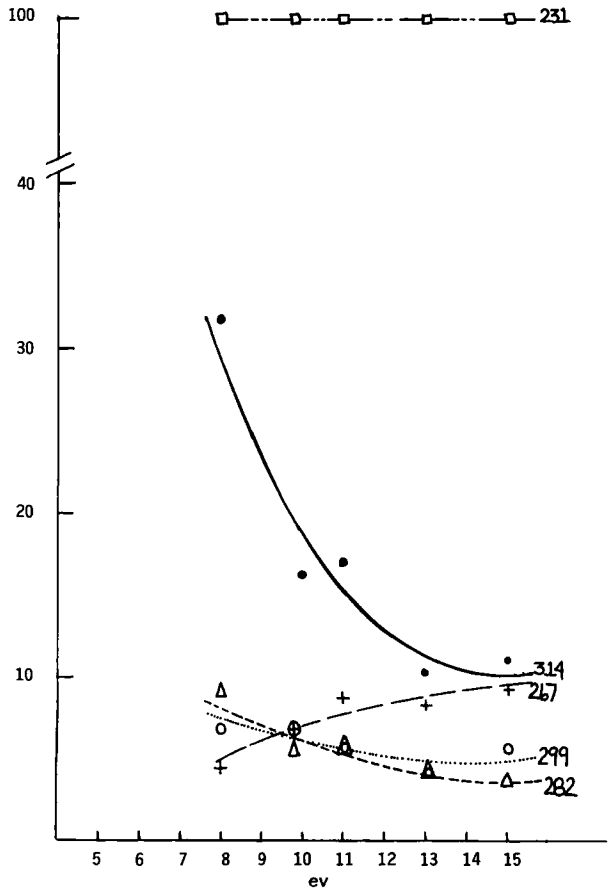

Figure 3—*Electron voltage–mass fragment intensity graph of cannabichromene.*

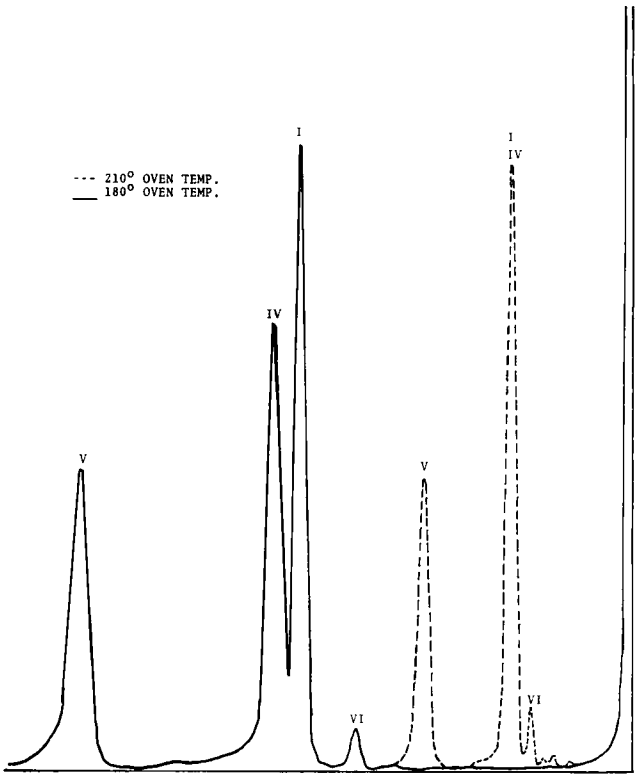

Figure 4—*Overlay of cannabidiol and cannabichromene on 6% OV-1 at oven temperatures of 210° (dotted line) and 180° (solid line). Key: VI, cannabicyclol; I, cannabidiol; IV, cannabichromene; and V, androst-4-ene-3,17-dione, the internal standard.*

IV preceding I. Investigation of the 3% OV-7 as a potential replacement for the 2% OV-17 column, which does not separate a mixture of IV and I, indicates that 3% OV-7 does not adequately separate I and IV for routine analyses.

When pure synthetic standards were used, IV did, indeed, precede I on OV-17 and OV-25 columns by seconds. However, these separations were not sufficient to provide separation of a synthetic mixture of I and IV (Fig. 1) in equal or unequal ratios. This finding was confirmed using GC–mass spectrometry[5] and, subsequently, "mass-fragmentograms" as described by Vree *et al.* (10) (Figs. 2 and 3). Additionally, no separation was obtained when chromatograms were produced at different temperatures (Table I).

Cannabidiol has been reported as preceding IV with a 5% SE-30 column (15), a methyl silicone as is OV-1. In the phenyl methyl silicone polymer series in these laboratories, I preceded IV when the percent phenyl-to-methyl ratio of the polymer was below 50% (*e.g.*, OV-1, 3, 7, and 11). However, VI precedes both I and IV (Fig. 4). This separation is based on pure synthetic samples. Mixtures were not separated if they are not reported in Table I. Thus, when the phenyl-to-methyl ratio was below 50%, the data from this study support de Zeeuw *et al.* (15) but are in direct conflict with data published by Small and Beckstead (16). When the phenyl-to-methyl ratio was 50:50 or greater, the separation of pure I and IV was reversed with IV preceding I. Cannabicyclol (VI) also precedes IV and I and is mislabeled IV in some publications (15–17)[6]. The OV-17 polymer is 50:50 phenyl to methyl, whereas OV-25 is composed of a higher percent phenyl-to-methyl ratio. As previously stated, this separation was based on pure synthetic samples, with no clear separation being obtained when a mixture of I and IV was analyzed (see Table III for relative retention times on OV-17).

Methyl silicone (6% OV-1) in these laboratories affords quanti-

[5] Varian Series 1400 interfaced to DuPont 21-492 high resolution interfaced with digital PDP-12.

[6] Since this manuscript was prepared, personal communications from Dr. Harry Beckstead revealed that Dr. Beckstead is aware of this problem. He agrees with our results: cannabicyclol is usually misidentified and labeled cannabichromene.

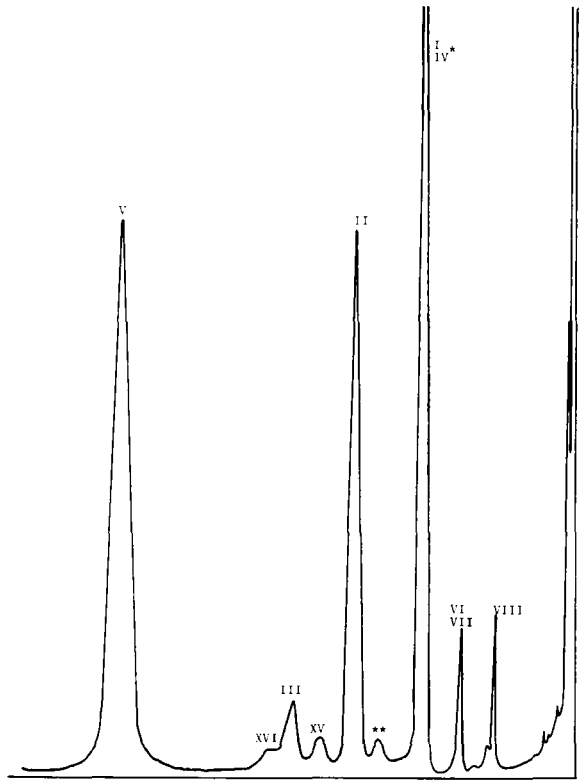

Figure 5—*Chromatogram of Indian* C. sativa *L., coded IN-B(2)/C-72, run on 2% OV-17. Key: VIII, cannabidivarin; VII,* (−)-Δ^9-trans-*tetrahydrocannabivarin; VI, cannabicyclol; I, cannabidiol; IV, cannabichromene; II,* (−)-Δ^9-trans-*tetrahydrocannabinol; XV, cannabigerol; III, cannabinol; XVI, C_{29}-hydrocarbon; and V, androst-4-ene-3,17-dione, the internal standard. *In certain samples, cannabivarin (IX) occurs under this peak. **This peak is normally labeled* (−)-Δ^8-trans-*tetrahydrocannabinol (XIII), but spectral data in these laboratories indicate that this particular peak is not XIII.*

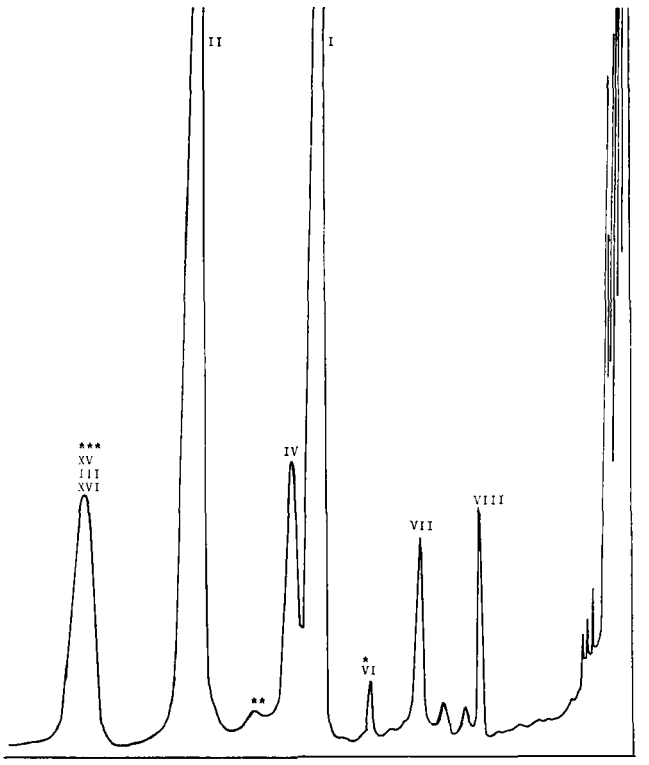

Figure 6—*Chromatogram of Indian* C. sativa *L., coded IN-B(2)/C-72, run on 6% OV-1. Key: VIII, cannabidivarin; VII,* (−)-Δ^9-trans-*tetrahydrocannabivarin; VI, cannabicyclol; I, cannabidiol; IV, cannabichromene; II,* (−)-Δ^9-trans-*tetrahydrocannabinol; XV, cannabigerol; III, cannabinol; and XVI, C_{29}-hydrocarbon. *When cannabivarin (IX) is present in the sample, it is under this peak. **Data indicate that this peak is probably the peak in question in Fig. 5 and is currently being investigated. ***Internal standard appears under this peak when present. This chromatogram is of a normalization analysis to determine the ratio of IV to I.*

tative separation of I and IV (Table II and Figs. 2 and 3). Figure 4 shows the separation of synthetic I and IV obtained on the 6% methyl silicone column at 180°. Preceding I is a small peak representing VI. Cannabicyclol (VI), an artifact, is routinely observed in chromatograms when IV is present (9).

Temperature was most critical in the separation of I and IV. When methyl silicone is used, 180° gives excellent separation; at 190°, the separation is less distinct; and above 200°, no separation occurs (Table I and Fig. 4). Synthetic I on methyl silicone has a retention time of 19 min 30 sec, and synthetic IV elutes at 21 min 15 sec. This separation is more than sufficient for quantitating I and IV. The separation and quantitation of (−)-Δ^9-*trans*-tetrahydrocannabivarin (VII) and VI are possible. When using a 2% OV-17 column, VI and VII occur under the same peak (Fig. 5 and Table III). Thus, for the separation and quantitation of I and IV and VI and VII without preparing derivatives (14), the methyl silicone is superior to those columns investigated in this experiment (Table I and Fig. 6). Eight percent methyl silicone and 3% OV-3 will separate I and IV, as does methyl silicone, but extended retention times diminish the separation advantages.

Fentiman[7], using 10% OV-101 at 190°, obtained a separation comparable to the separation on 6% OV-1, with cannabidiol and cannabichromene giving retention times of 1 hr 42 min and 1 hr 50 min, respectively.

Thus, in view of these findings, it appears that many reported procedures (12, 15–17) have not separated I and IV but have mistakenly identified VI and IV. Also, these findings and others (18) make the validity of a classification system for *Cannabis* using I, II, or I–III questionable. However, it is now possible to quantitate I and IV in any preparation of *Cannabis* without preparing derivatives. Additionally, in these laboratories, response factors from OV-1 and OV-17 columns are within experimental error.

It is the recommendation of this research group that routine *Cannabis* preparations analyzed on 2% OV-17 be supported by an analysis using methyl silicone, thereby enabling pharmacologists to extend and validate their findings on the potentiation and/or antagonism of II by other cannabinoids. Moreover, a recent recommendation by an United Nations working group on the chemistry of *Cannabis* and its components[8] that accurate analyses of I–III be included in all scholarly reports on *Cannabis* can become a reality. The biological significance and implications of these findings are far reaching but cannot be adequately documented at this time; however, preliminary data obtained from the methyl silicone column indicate that IV may be more abundant in nature than I. Since IV has been reported previously as I, alteration of the classical biological activity of II attributed to I may have been due to IV or to a combination of I and IV or of other cannabinoids in experiments where natural plant material was used.

SUMMARY

Cannabidiol and cannabichromene were separated and quantitated in *C. sativa* L. Most previous literature reporting the separation of cannabidiol and cannabichromene have mislabeled cannabicyclol, an artifact from cannabichromene, as cannabichromene. Cannabicyclol and (−)-Δ^9-*trans*-tetrahydrocannabivarin can be separated and quantitated on OV-1 but cannot be separated and quantitated on OV-17. Cannabichromene, often erroneously re-

[7] Dr. Al Fentiman, Battelle, Columbus, Ohio, confirmed results from these laboratories.

[8] United Nations Document MNAR/9/1974.

ported as cannabidiol, is more abundant in nature than previously thought; any previous classification of *C. sativa* L. based on cannabidiol, cannabinol, and (−)-Δ^9-*trans*-tetrahydrocannabinol must be questioned. Since it is known that synthetic cannabidiol antagonizes certain effects of (−)-Δ^9-*trans*-tetrahydrocannabinol, much work is now needed on variants of *Cannabis* containing various ratios of cannabidiol and cannabichromene in relation to (−)-Δ^9-*trans*-tetrahydrocannabinol.

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Electron-Capture GLC Determination of a New Antiarrhythmic Agent, α,α-Dimethyl-4-($\alpha,\alpha,\beta,\beta$-tetrafluorophenethyl)benzylamine, in Biological Fluids

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Abstract □ A highly specific and sensitive GLC method was developed for the analysis of α,α-dimethyl-4-($\alpha,\alpha,\beta,\beta$-tetrafluorophenethyl)benzylamine, a new orally active antiarrhythmic drug, in biological fluids. The procedure involves the addition of an internal standard, 4-($\alpha,\alpha,\beta,\beta$-tetrafluorophenethyl)benzylamine, to the plasma or urine samples followed by extraction of the drugs into benzene at pH 8. The extracted amines are converted to the trifluoroacetyl derivatives (characterized by GLC–mass spectrometry), chromatographed, and detected with a ^{63}Ni electron-capture detector. The sensitivity of the method is such that 10 ng of α,α-dimethyl-4-($\alpha,\alpha,\beta,\beta$-tetrafluorophenethyl)benzylamine/ml of plasma can be analyzed. These levels are suitable for the analysis of samples obtained following a therapeutic dose.

Keyphrases □ α,α-Dimethyl-4-($\alpha,\alpha,\beta,\beta$-tetrafluorophenethyl)benzylamine—electron-capture GLC determination in biological fluids □ Antiarrhythmic agents—electron-capture GLC determination of α,α-dimethyl-4-($\alpha,\alpha,\beta,\beta$-tetrafluorophenethyl)benzylamine in biological fluids □ GLC, electron capture—analysis, α,α-dimethyl-4-($\alpha,\alpha,\beta,\beta$-tetrafluorophenethyl)benzylamine in biological fluids

α,α-Dimethyl-4-($\alpha,\alpha,\beta,\beta$-tetrafluorophenethyl)benzylamine (I) is a new orally effective agent for the treatment of ventricular arrhythmias resulting from myocardial infarction (1). The compound is one of the more potent agents in preventing or modifying the ventricular arrhythmia produced in anesthetized dogs by the intracoronary injection of a sclerosing agent (2, 3). The drug was designed as a prophylactic agent and has advantages over other drugs currently employed. Studies on the physiological disposition of the compound (4) have not included a specific method of assay.

Preliminary quantitative experiments in this laboratory using colorimetric, fluorometric, and GLC (flame-ionization detection) methods lacked the sensitivity required to detect clinical levels of the drug. The use of electron-capture techniques for the assay of low levels of drugs, has been reported (5–15). How-