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Journal of Chromatography A, 679 (1994) 47–58

JOURNAL OF CHROMATOGRAPHY A

Role of hydroxyl groups in chiral recognition of cannabinoids by carbamated amylose

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First received 18 March 1994; revised manuscript received 1 June 1994

Abstract

The enantioselective retention of four pairs of enantiomeric cannabinoids that have hydroxyl groups was compared with that for the corresponding acetylated compounds, using amylose tris(3,5-dimethylphenylcarbamate) in the stationary phase. According to this study the hydroxyl groups were essential to the chiral discrimination by the amylose stationary phase, since blocking them by acetylation was detrimental to the enantioselective separation. Three of the four enantiomeric pairs had a relatively rigid tricyclic backbone, whereas the fourth, the cannabidiol, was a flexible compound. In contrast to the other three enantiomeric pairs, the resolution of the acetylated cannabidiol was not completely lost as a result of the acetylation, but it was decreased and the elution order was reversed. Conformational analysis of the acetylated and non-acetylated enantiomeric pairs was systematically performed, using molecular mechanics, in order to examine the effect of acetylation on the conformation of the molecules. The results indicated that acetylation did not change the conformations substantially and therefore the loss of resolution was attributed to the blockage of the hydroxyl groups. The molecular mechanics approach was validated by comparing the energy-minimized structure of cannabidiol with its X-ray crystallographic structure taken from the literature.

1. Introduction

With the increasing evidence of problems related to stereoselectivity in drug action, enantioselective analysis by liquid chromatography, using chiral stationary phases, has become a focus of intensive research [1]. One type of chiral stationary phase is an immobilized carbamated amylose, composed of D-glucose units that form a simple helical backbone [2]. This type of stationary phase has shown high capability of chiral discrimination [3–6]; however, it is not known whether the chiral information is conveyed on the molecular level (the D-glucose units) and/or the supra-molecular level (helical backbone). The rationalization of the mechanism of chiral discrimination needs a structure–enantioselective retention relationship (SERR) approach, i.e., a comparative investigation of the chromatographic behaviour of solutes. The best strategy would be to use families of chiral compounds with diverse structural features.

The resolution of two such families of enantiomeric terpenoids and cannabinoids was reported previously [5,6]. A comparative study of the structural and chromatographic behaviour of the

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0021-9673/94/$07.00
SSDI 0021-9673(94)00520-J

series of cannabinoids showed that enantiomeric pairs with very similar structures can be discriminated very differently, whereas very different enantiomeric pairs can be discriminated similarly [5]. The role of hydrogen bonding in the chiral discrimination of terpenoids was also investigated and it appeared as a major interaction [6]. The phenylcarbamated stationary phase resolved ketonic and alcoholic terpenoid enantiomers, although they had no aromatic moiety. Nevertheless, the presence of a hydroxyl group did not necessarily induced enantiomeric resolution, it had to be located in the right position on the molecular structure. The SERR studies were accompanied by a conformational analysis of the enantiomers, using molecular mechanics to verify that conformation was not different with derivatization of the solutes.

Some of the cannabinoids that were studied here had already been analysed by computer modeling as part of their structure–activity relationship (SAR) during the search for non-psychotropic therapeutic derivatives [7]. A major advancement in this field was achieved when this search brought about a therapeutic cannabinoid, devoid of tetrahydrocannabinol (THC)-like psychotropic effects [8]. This enantiomer was studied here, in addition to a natural enantiomer, (−)-cannabidiol (CBD), a non-psychotropic natural cannabinoid. The natural enantiomer has shown antiepileptic, antianxiety and antidystonia effects in man [9–11].

Comparisons between hydroxylated enantiomers and their alkyl analogues have been made in a few studies using chiral chromatography [12–14]. Enantiomers of *trans*-dihydrodiol derivatives of phenanthrene and benzopyrene, chrysene, and anthracene and their O-methyl ethers were separated by Yang et al. [12] using Pirkle chiral stationary phases. In contrast to the derivatized cannabinoids, the O-methyl ethers eluted with shorter retention times but were separated more efficiently than the enantiomers of underivatized dihydrodiols. Similarly, during the separation of bi-β-naphthols using a Pirkle-type stationary phase, the alkyl derivatives were less retained but were better resolved [13]. In this example it seems that the position of the bulkier alkyl groups must have changed the overall conformation of the enantiomers, hence their resolution. Another example is the separation of alcoholic γ- and δ-lactones and their corresponding alkyl derivatives, using cellulose triacetate. Retention was increased and separation was improved when the OH group was blocked by an alkyl group [14].

2. Experimental

2.1. Instrumentation

HPLC analysis was performed using a HP1050 instrument (Hewlett-Packard, Palo Alto, CA, USA) equipped with a diode-array UV detector, an HPCHEM data station and a ThinkJet printer. A Rheodyne (Cotati, CA, USA) injection valve was used, equipped with a 20-μl loop. The chiral column was a ChiralPak AD column (Daicel Chemical Industries, Tokyo, Japan) (250 mm × 4.6 mm I.D., 10 μm film thickness).

2.2. Materials and Methods

HPLC-grade solvents (*n*-hexane and 2-propanol) were purchased from LabScan (Dublin, Ireland) and ethanol from Merck (Darmstadt, Germany). The four pairs of cannabinoids were prepared as described previously [5]. Acetylation of all the (+)- and (−)-enantiomers was performed using acetic anhydride in an excess amount of freshly distilled pyridine as a catalyst. The mixture was stirred at room temperature overnight and the resulting mixture was then washed with ethanol. Evaporation under vacuum left a viscous oil that was checked by TLC. The synthesized cannabinoids obtained were submitted to further purification procedures using a silica HPLC column (Adsorbosphere, Alltech, Deerfield, IL, USA) before the chiral separations (whenever needed) for final chemical purification, using 2-propanol–*n*-hexane (5:95) in most instances.

2.3. Procedure for analysis

A flow-rate of 1 ml/min was used in all the experiments at room temperature. The mobile phase consisted of mixtures of *n*-hexane with ethanol or 2-propanol (5%, v/v). Each run was monitored at two wavelengths simultaneously, 260 and 240 nm. In each instance, approximately 0.1 mg of analyte was dissolved in 1 ml of the solvent and injected both individually and as a mixture.

2.4. Computational method

Construction and treatment of the cannabinoid structures were performed with the Insight II/ Discover 2.0.0 software package from BIOSYM Technologies (San Diego, CA, USA). All calculations were made on a Silicon Graphics 4D/ 310VGX workstation. Molecular mechanics methods are based on a view of the molecular structures as set of balls and springs. The molecular force field is a sum of the potential energy functions in these sets. The following molecular mechanics (MM) potential energy function was used:

$$E_{\text{Tot.}} = F_s + E_q + E_f + E_{\text{VWD}} + E_{\text{elec}} \quad (1)$$

where E_s is the stretching energy, E_q is the bending energy, E_f is the dihedral (torsion) energy, E_{VWD} is the Van der Waals energy and E_{elec} is the electrostatic energy. The force field used in the calculations was CVFF (consistent valence force field). All parameters defining the geometry of the molecules were modified by small increments until the overall structural energy reached a local minimum. First, 1000 iterations were made in the steepest descent's algorithm, then it was swapped to the conjugate gradient minimizer, until a convergence criterion was reached. For CBD, a flexible compound that undergoes free rotation, the dihedral rotor was used to define a torsion angle in the segment of the free rotation and to determine the energy resulting from this rotation. The structure was minimized using 10° increments of the torsion angle over the entire range of 360°.

The fractional X-ray coordinates of CBD from the literature [15] were fed manually into personal CAChe software on a Macintosh IIC computer, and the resulting output file was transferred and rebuilt in the Silicon Graphics workstation to be used for the comparison.

The *RMS* value is a quantitative criterion for the difference between two structures or their portions when superimposed on each other. It is the least-squares fit between the two sets of *xyz* coordinates (Å units) of the two superimposed structures, and is calculated according to the following equation:

$$RMS = \sqrt{\sum_{i=1}^{N} \frac{(x - x_0)^2 + (y - y_0)^2 + (z - z_0)^2}{N}} \quad (2)$$

where N is the number of atoms compared.

3. Results and discussion

3.1. Structure–enantioselective retention relationship (SERR)

The structure–activity relationship (SAR) of cannabinoids has been extensively studied in the past, mainly to understand the structural features that are responsible for the therapeutic activity [16]. A similar approach can be adopted in order to investigate structural features of enantiomeric pairs that facilitate chiral discrimination. Chiral discrimination is measured chromatographically from the enantioselective retention or the selectivity and the resolution factors. This approach originates from the term "quantitative structure–enantioselective retention relation" (QSERR) that was pointed out by Kaliszan et al. [17].

A previous study using carbamated amylose as the stationary phase showed that the combination of hydrogen bonding by hydroxyl and ketonic groups in the appropriate positions played a key role in the chiral discrimination of enantiomeric terpenoids [6]. The role of the hydroxyl group in the enantioselective retention

of cannabinoids was therefore studied here. The hydroxyl groups were blocked by acetylation and the enantioselective retention was measured and compared with that of the non-acetylated compounds.

3.2. Chromatographic resolution of the enantiomeric cannabinoids

The structures of the four cannabinoids and their derivatives are presented in Fig. 1. The non-acetylated cannabinoids have already been resolved using amylose 3,5-dimethylphenyl carbamate. The enantioselectivity of the carbamated amylose towards all four non-acetylated cannabinoid enantiomers in this study was excellent, using any percentage of modifier [5]. A selectivity factor $\alpha > 1.2$ was obtained for all four using 2-propanol. The capacity factors k', selectivity α and the resolution R_s of the non-acetylated cannabinoids were determined previously using the same type of stationary phase [5]. The values of k' and α in the present study were slightly higher than in the previous study, because the stationary phase originated from a different batch. In spite of the inconsistency in performance between two different batches, the reproducibility of k' and α was relatively good when the same column was used throughout the study.

All the chromatographic runs in this study were made using ethanol–or 2-propanol–*n*-hexane (5:95). Fig. 2 shows typical comparisons of the chromatographic resolution of three mixtures of cannabinoid enantiomeric pairs (1a–3a) and the corresponding acetylated compounds (pairs 1b–3b), using 2-propanol as the mobile phase modifier. Fig. 3 shows a mixture of the (+)- and (−)-enantiomers of CBD (pair 4a) vs. the corresponding mixture of the acetylated (+)- and (−)-enantiomers (pair 4), using (I) ethanol and (II) 2-propanol as modifier. As can be seen in Fig. 2, the separation of the acetylated enantiomeric pairs was lost owing to blocking of the hydroxyl groups. The loss of separation was especially dramatic for the two acetylated enantiomers HU-249 and HU-250 (pair 3b). The enantioselectivity of the phenylcarbamated amylose towards this enantiomer pair was extraordinary [$\alpha = 4.8$ and $R_s = 19.9$ with 2-propanol–*n*-hexane (5:95)]; nevertheless, it disappeared completely when the hydroxyl groups of the two enantiomers were acetylated [Fig. 2(III)].

In contrast to pairs 1b–3b, the acetylated enantiomers of CBD (pair 4b) were still partially separated. Acetylation of the CBD resulted in a large decrease in the capacity factor (93% using ethanol and 80% using 2-propanol) and resolution (70% using ethanol and 50% using 2-propanol). Moreover, when the modifier was 2-propanol, inversion of the elution order was observed, i.e., the (−)-isomer eluted first. A similar inversion of elution order was observed in the separation of acetylated terpenoid enantiomers using the same stationary phase [6].

The loss of chromatographic resolution of the acetylated enantiomers (pairs 1b–3b) indicates that hydrogen bonding dominates the recognition process by the stationary phase. A similar loss of resolution was observed in the case of terpenoid chiral separations when acetylated [6]. The decrease in retention and the loss of separation could be explained also by a steric repulsion of the solutes from the chiral sites due to the bulkier acetoxy derivatives.

The chromatographic behaviour of the two acetylated CBD enantiomers was different from that of the other three enantiomeric pairs, probably owing to the difference is structure. Rings A and C in CBD are perpendicular to each other and can rotate freely. According to ^{1}H NMR measurements, acetylation of CBD did not restrict the free rotation of the two rings. The flexibility of these enantiomers facilitated a different fit to the stationary phase in spite of the loss of OH functionality. The acetoxy groups on the CBD could probably rearrange themselves so that weak hydrogen bonding between their two CO ester groups (hydrogen acceptors) and the NH of the carbamated residue (hydrogen donor) was still effective.

The loss of separation of pairs 1b–3b and the intriguing behaviour of pair 4b raised the question of whether the acetylation caused conformational changes in the enantiomeric pairs and

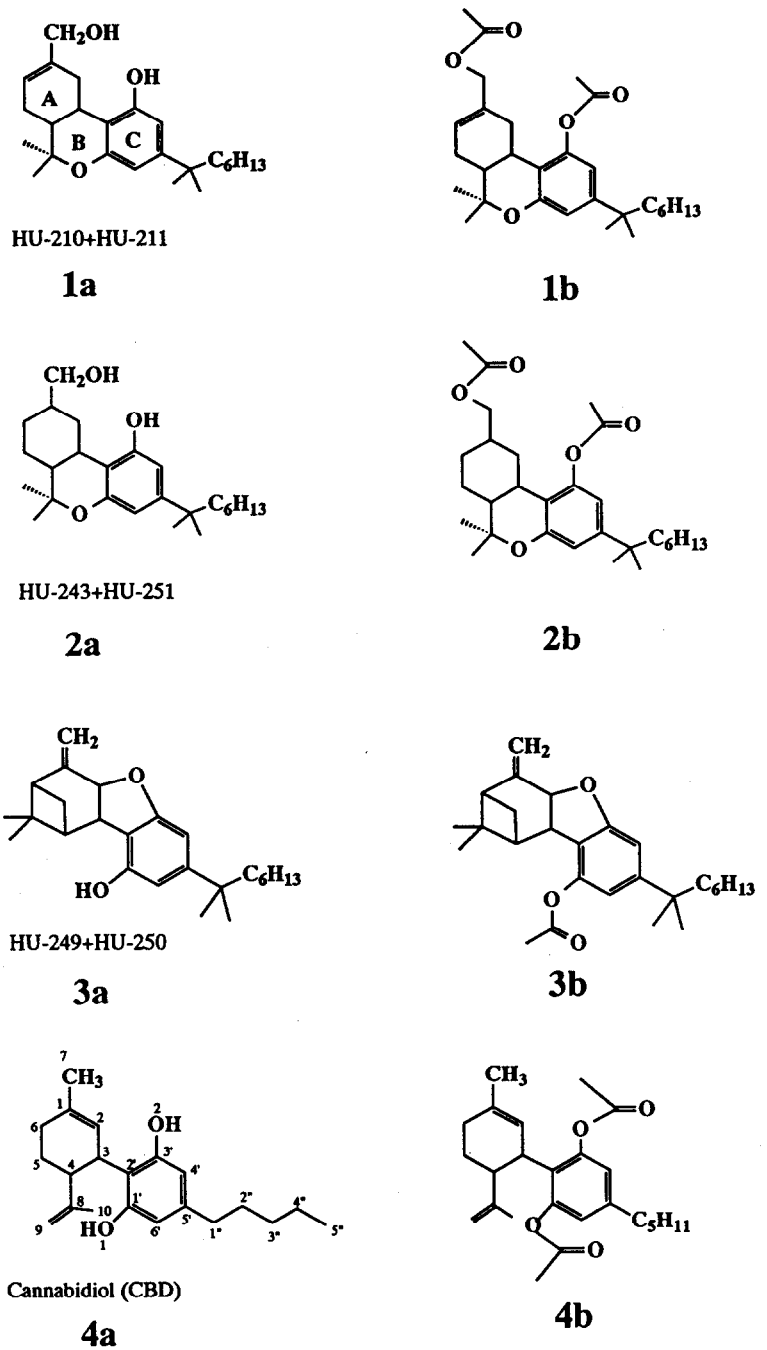


Fig. 1. Structures of the enantiomeric cannabinoids used in this study and their acetoxy derivatives.

these changed affected the discrimination. Conformational analysis of the four cannabinoid and their acetylated analogues was therefore performed, and the energy-minimized structures of the original and the acetylated compounds were superimposed of each other.

I

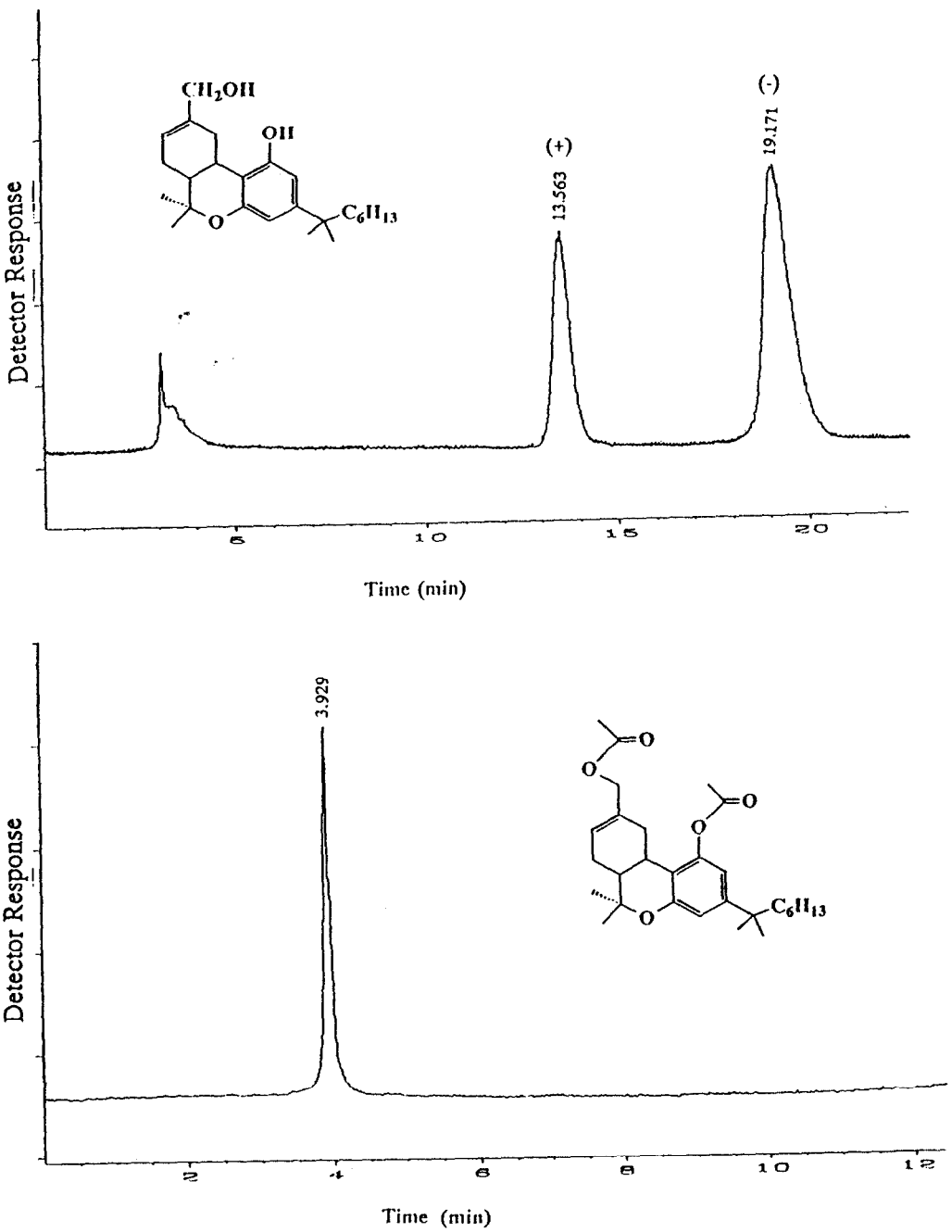
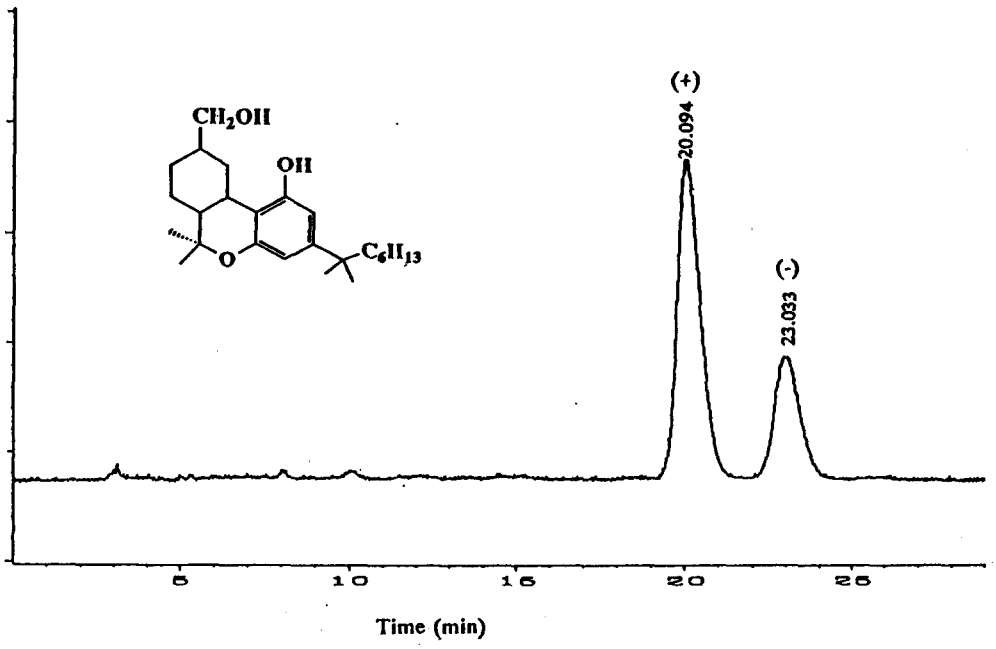

CH2OH
OH
C6H13
O
(+)
13.563
(-)
19.171
Detector Response
5
10
15
20
Time (min)
3.929
O
O
O
O
C6H13
O
Detector Response
2
4
6
8
10
12
Time (min)

Fig. 2.

II



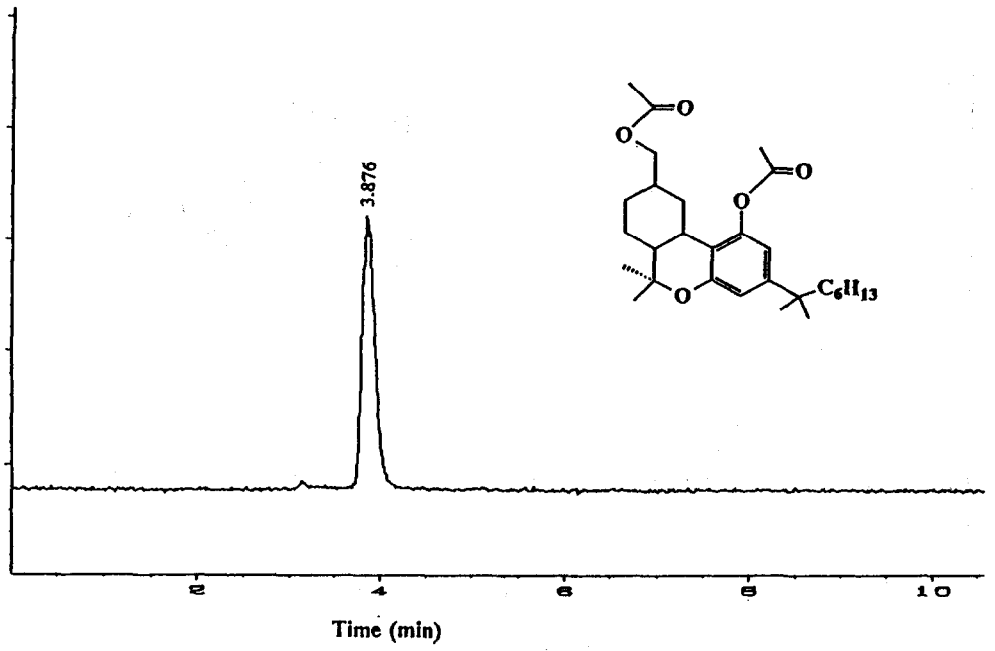


Fig. 2 *(Continued on p. 54)*

III

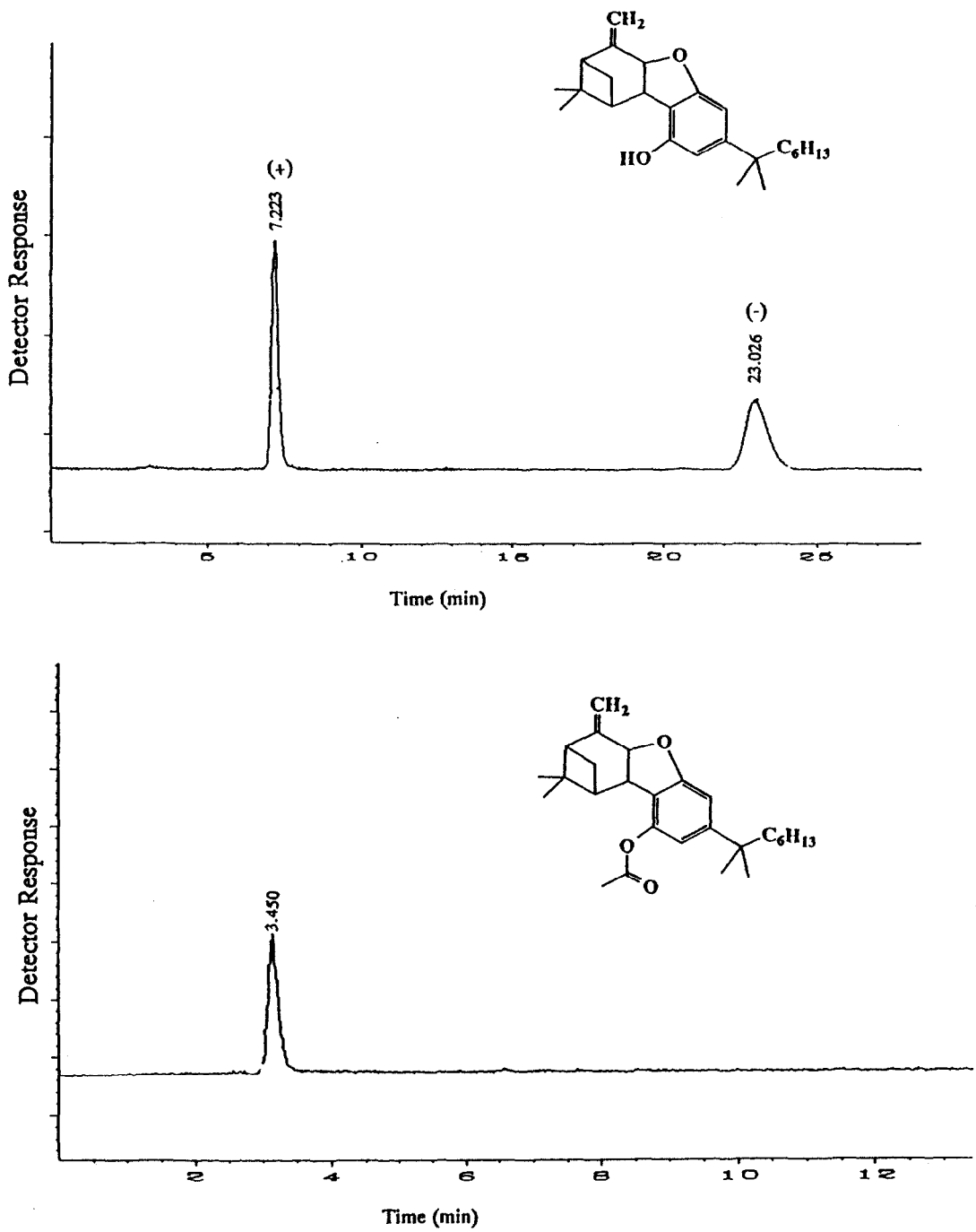


Fig. 2. Chromatograms showing the separation of mixtures of the enantiomeric pairs (I) 1a and 1b, (II) 2a and 2b and (III) 3a and 3b. Mobile phase, *n*-hexane–2-propanol (95:5, v/v); detection wavelength, 240 nm.

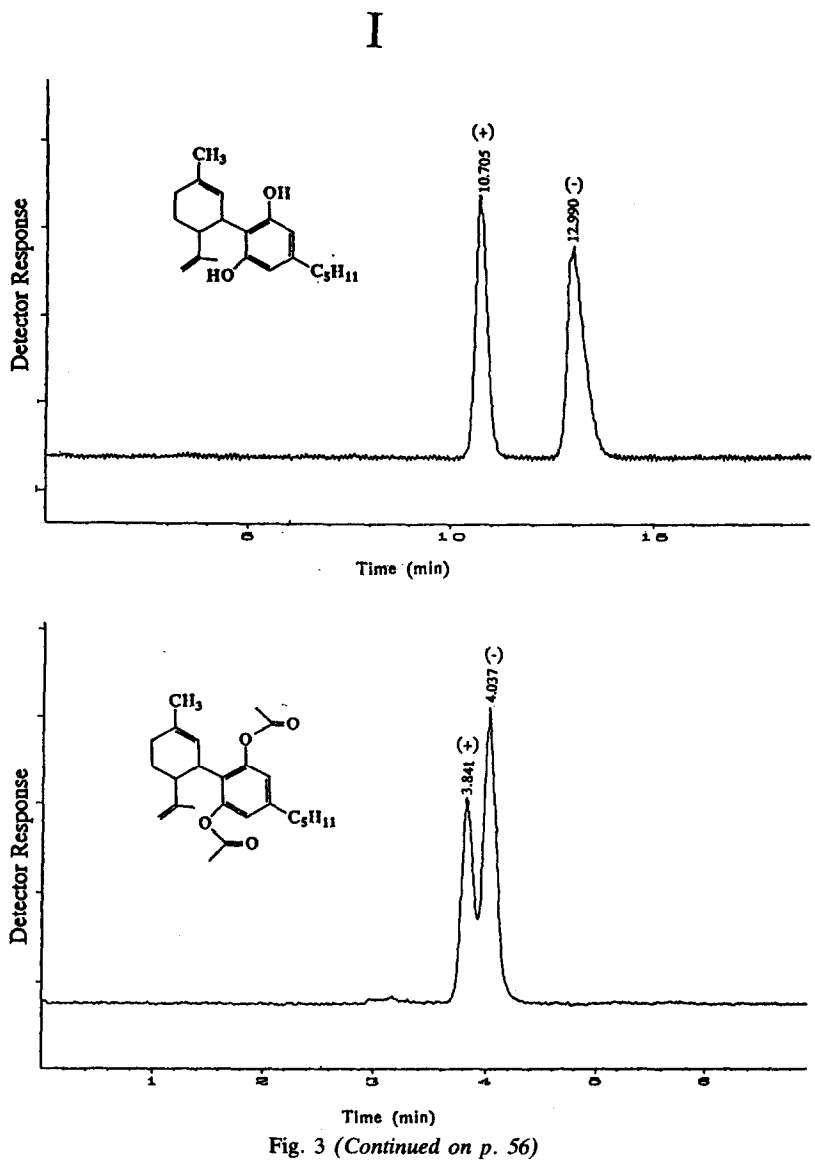


Fig. 3 *(Continued on p. 56)*

3.3. Conformational analysis

The tricyclic and tetracyclic fused rings of the three cannabinoids (see Fig. 1) provide considerable rigidity to these enantiomers, hence it was not surprising that according to molecular mechanics calculations, acetylation of the cannabinoids did not affect their conformational significantly. Superposition of each enantiomer with its corresponding acetylated form gave rise to relatively small *RMS* values ($RMS \leq 0.126$ Å), as shown in Table 1. In the case of cannabidiol, in spite of its flexibility, the overall conformation of its acetylated derivative was approximately the same as that of the cannabidiol itself, as shown in Fig. 4, where the two structures are superimposed.

The similarity of the conformations of the

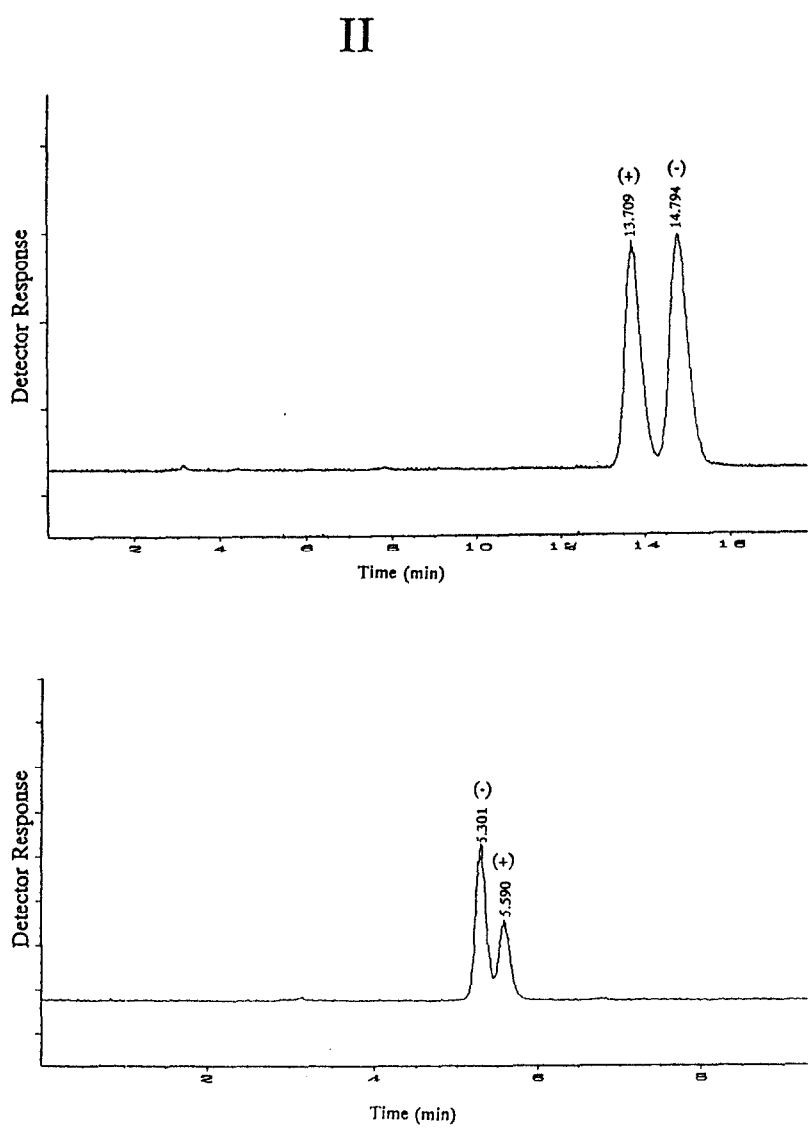


Fig. 3. Chromatograms showing the separation of a mixture of the enantiomeric pairs 4a and 4b. Mobile phase: (I) *n*-hexane–ethanol (95:5, v/v); (II) *n*-hexane–2-propanol (95:5, v/v). Detection wavelength, 240 nm.

acetylated and non-acetylated analogues supports earlier observations that the nature and position of the substituents is a determining factor in the chiral discrimination of cannabinoids rather than overall conformation [6].

3.4. Comparison with X-ray crystallography

The computational approach described above was validated by comparison with an X-ray crystallographic structure of cannabidiol from the literature [15]. As shown in Fig. 1, cannabidiol has no ring B and rings A and C are almost perpendicular to each other, with two phenolic groups on ring C. The coordinates of the X-ray structure of cannabidiol were introduced into the DISCOVER software manually and the structure was reconstructed. Superposition of the reconstructed X-ray structure and the energy minimized structure revealed a small *RMS* value of 0.095 between them.

In CBD, the two torsion angles ψ (C5–C4–

Table 1
Values of root mean square (*RMS*) of the differences in *xyz* coordinates of the ten heavy atoms common to all the (+)-cannabinoid structures compared with the corresponding (+)-acetate isomers

Pair No.	Solute	*RMS* (Å)
1	HU-211	0.126
2	HU-251	0.056
3	HU-250	0.036
4	(+)-CBD	0.086

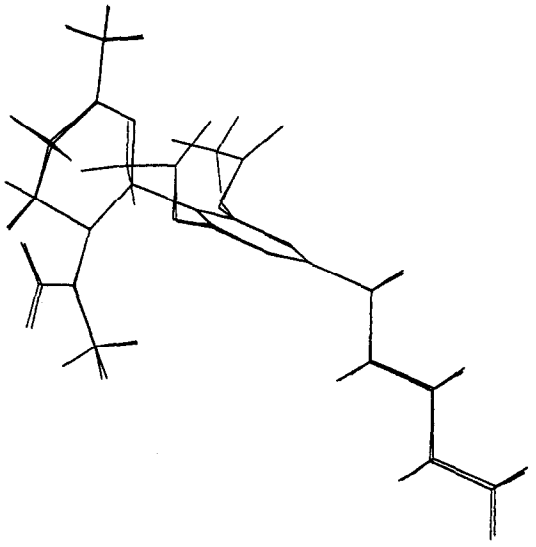


Fig. 4. Superposition of the common ten heavy atoms of the two structures of CBD and acetylated CBD.

C8–C10) and ϕ (C2–C3–C2′–C3′) (Fig. 5) were calculated to be 120.7° and −59.9°, respectively, at the global minima. X-ray crystallography gave these torsion angles as 127° and −59.4°, respectively.

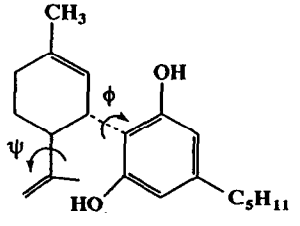


Fig. 5. Torsion angles ϕ and ψ of cannabidiol (CBD).

4. Conclusions

This systematic comparative study of four cannabinoids has highlighted the functionality of OH groups in chiral discrimination by phenylcarbamated amylose. When the OH groups of the cannabinoids were blocked, separation was completely lost for the three rigid enantiomeric pairs, and just partially lost for the flexible cannabidiol enantiomers. Molecular modelling, which was validated by comparison with an X-ray structure, indicated that the loss of separation was not caused by a change in conformation.

Acknowledgements

This work was financed by the Israel Ministry of Health. We thank Professor Raphael Mechoulam and Dr. Aviva Breuer of the Department of Natural Products, School of Pharmacy, for their guidance and assistance in preparing the acetylated cannabinoid enantiomers, and Galia Tawil for technical help.

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