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Enantiomeric separation of substituted flavanoids by LC–DAD*

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Abstract: A variety of racemic flavanoids with anti-rhinovirus activity have been resolved for the first time by HPLC, using a chiral stationary phase. Baseline separation was easily obtained for racemic 4',6-dichloroisoflavan (**V**). The absolute configurations of two enantiomers (**Va** and **Vb**) were established by comparing their circular dichroism curves with those of other known isoflavans. Both the isomers were tested against human rhinovirus serotipe 1B infection *in vitro*; the *S* form was approximately four times more effective than the *R* form.

Keywords: *Chiral HPLC; enantiomeric separation; diode array detector; flavanoids; anti-rhinovirus activity.*

Introduction

Chromatographic separation of enantiomers of chiral drugs has become increasingly important in the verification of whether their pharmacological activities and toxicities are different. Chiral discrimination of some flavanoids was made to study the relationship between their stereochemistry and pharmacological activity.

It is known that 4',6-dichloroflavan (BW683C) (**I**) is a potent inhibitor of the replication of several rhinovirus serotypes *in vitro* [1]. It acts on the early stages of viral replication and it inhibits replication by binding to the protein coat of sensitive serotypes [2]. The compound **I** has a single chiral carbon atom in the 3,4-dihydro-2H-1-benzopyran ring, but only the activity of the racemic mixture has been tested [1]. Therefore the enantiomers were separated by LC–DAD and tested against the most sensitive human 1B rhinovirus serotype (HRV 1B) [3].

In searching for antiviral compounds, different series of (*R*,*S*)-flavans, (*R*,*S*)-isoflavans and 3(2H)-isoflavenes were synthesized in this laboratory; these flavanoids exhibited a broad antipicornavirus activity *in vitro* [4–10]. Among these (*R*,*S*)-4'-cyano,6-chloroflavan (**II**), (*R*,*S*)-4'-chloro,6-cyanoflavan (**III**), (*R*,*S*)-4',6-dicyanoflavan (**IV**) and (*R*,*S*)-4',6-dichloroisoflavan (**V**) showed the most interesting *in vitro* anti-rhinovirus activity [4, 8, 10].

All these flavanoids (**II–V**) have a single chiral carbon atom on the 3,4-dihydro-2H-1-benzopyran ring, but until now only the racemic mixture was tested [4–10] (Fig. 1).

Therefore a chromatographic method for the enantiomeric separation of compounds **II–V**, using a chiral stationary phase (CSP), is presented in this paper with the aim of assaying the anti-viral activity of each enantiomer.

Experimental

Apparatus

A high-performance chromatograph Waters Model 6000 A (Waters Assoc., Milford, MA, USA), equipped with the HP 1040 M linear photodiode array detector controlled by a computer HP 9000 Model 310 (Hewlett–Packard, WA, USA), was used. Standard and sample solutions were injected via a U6K loop valve injector (Waters Assoc.) using a manual method.

The circular dichroism (CD) was carried out with a Jasco 500 dichrograph.

Reagents and chemicals

The solvents used in this study were of HPLC grade and were purchased from Merck (Darmstadt, Germany). The compounds **II–V** were synthesized in the Dipartimento di Studi Farmaceutici of Rome University "La Sapienza", as previously reported [4, 8, 10].

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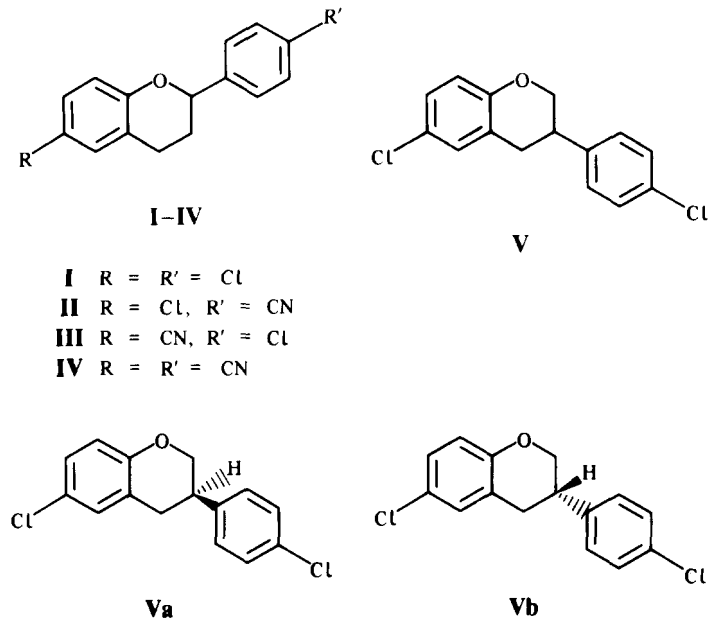

Figure 1
Structures of various flavanoids.

Stationary phase

A commercially available Chiracel OD (250 × 4.6 mm i.d., 10 mm) was purchased as CSP (Daicel Chem. Ind.). CSP Chiracel OD is cellulose tris(3,5-dimethylphenylcarbamate) coated onto silica.

Chromatographic analysis

The chromatographic separation of enantiomers was made by normal-phase HPLC on CSP cellulose tris(3,5-dimethylphenylcarbamate) coated onto silica. The used eluents for each compound were: hexane:ethanol (4:6, v/v) at flow-rate of 0.7 ml min^{-1} for **II**; hexane:ethanol (4:6, v/v) at flow-rate of 0.8 ml min^{-1} for **III** and **IV**; and hexane–2-propanol (9:1, v/v) at a flow rate of 0.9 ml min^{-1} for **V**. The photodiode array detector conditions were: λ values 233, 253 and 495 nm; acquisition rate of spectra 1280 msec; bandwidth for each channel 4; sensitivity range 50; reference wavelength 600 nm; and reference bandwidth 4. The identity of the UV spectra of the separated enantiomers was verified in all cases.

Circular dichroism curves

The CD curves were used to confirm the enantiomeric separation. The curves were obtained by ethanolic solutions (0.40 mg ml^{-1}), using 0.1-mm cells.

Biological assay

Cells and virus. HeLa (Ohio) cells were grown as monolayers in Eagle's minimum essential medium supplemented with 10% foetal bovine serum, antibiotics and glutamine. For cell maintenance the serum concentration was lowered to 2% (maintenance medium). Rhinovirus 1B was propagated in HeLa cells which were infected at 0.5–1 plaque forming units (PFU)/cell and incubated at 33°C until an extensive cytopathic effect (CPE) was recorded. After centrifugation at low speed to remove cellular debris, the infectious supernatant was stored at −80°C.

Toxicity of compounds for uninfected cells. The compounds were dissolved in absolute ethanol to make stock solutions (1 mg ml^{-1}) and subsequently diluted in the cell culture medium before use.

To determine the cytotoxic effect, confluent monolayers of HeLa cells in 96-well plates were exposed to serial five-fold dilutions of each drug in maintenance medium for 72 h at 33°C. Triplicate monolayers were utilized for each drug concentration. Cells were inspected daily to detect changes in cell morphology such as swelling, granularity, rounding or floating. The viability of the cells was determined by

neutral red uptake at the third day of incubation.

Plaque assay. The plaque assay technique was essentially that of Fiala and Kenny [11]. Briefly, monolayers of HeLa cells in six-well plates were infected for 1 h at 33°C with about 100 PFU of virus in the presence or absence of five-fold dilutions of drugs, starting from the maximal non-cytotoxic concentration. After washing twice with PBS to remove unadsorbed virus, the monolayers were overlayed with medium with or without the corresponding concentration of compounds. After incubation for 72 h at 33°C in 5% CO_2, the cells were stained with neutral red (2 h, 33°C) and the plaques counted. Three wells were utilized for each drug concentration and the mean value of plaque number was calculated. The IC_{50} value was the amount of drug reducing the plaque number by 50% and was calculated by plotting the percentage of plaque reduction with respect to the control plaque count against the logarithm of compound dose to yield a dose–response line.

Results and Discussion

For all compounds examined, baseline separation can easily be achieved only for isoflavan **V** (Fig. 2). For compounds **II**, **III** and **IV** (Figs 3–5), using the measure of the resolution by the chromatographic resolution factor as suggested by Clark and Mama [12], it was possible to assess the effect of changes in mobile phase composition on separation during mobile phase optimization. It was noted that the resolution was improved by replacing 2-propanol with ethanol. Each figure (Figs 2–5) shows the perfect superimposition of the enantiomers UV spectra.

The separation of the enantiomeric compounds of interest was probably based on a multi-mode mechanism of hydrogen bonding π-π electron interactions, and possibly inclusion complexes. Using this CSP the presence of chlorine atoms in this series of compounds played an important role in the separation mechanism. In fact not only 4′,6-dichloroisoflavan **V**, but also 4′,6-dichloroflavan **I** [3], were better resolved than the two chlorocyanoflavans **II** and **III**. The resolution was reduced when chlorine atom is not present (4′,6-dicyanoflavan **IV**). The baseline separation obtained from racemic isoflavan **V**

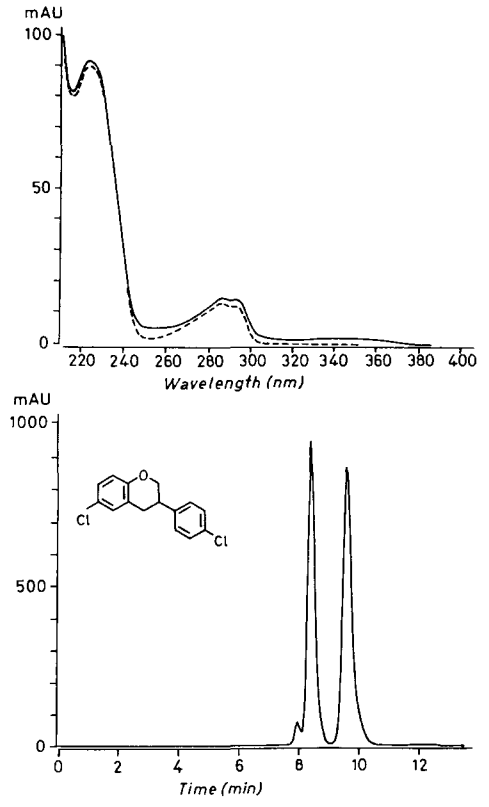

Figure 2
Chiral discrimination of compound **V** and relative UV spectra of two separated enantiomers. The first enantiomers eluted (**Va**) has *R*-configuration and the second one (**Vb**) *S*.

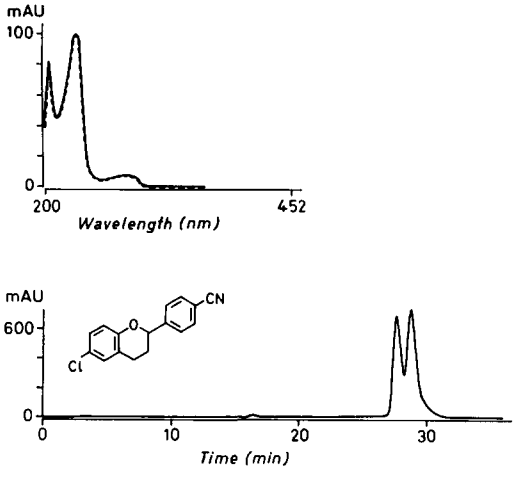

Figure 3
Enantiomeric separation of compound **II** and relative UV spectra of two separated enantiomers.

allowed us to separately collect the enantiomers. The CD measurement of two enantiomers was carried out in order to determine the absolute configuration of each one. The

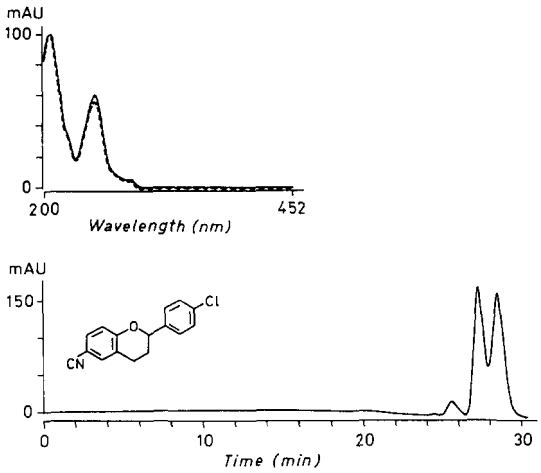

Figure 4
Enantiomeric separation of compound **III** and relative UV spectra of two separated enantiomers.

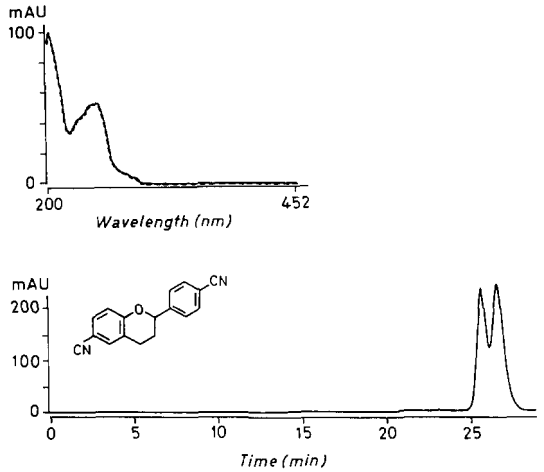

Figure 5
Enantiomeric separation of compound **IV** and relative UV spectra of two separated enantiomers.

first enantiomer eluted (**Va**) shows a band of positive sign in the 260–300 nm region ($\Delta\epsilon$ = +1.35, λ = 281 nm) (Fig. 6), the second one (**Vb**) a symmetric band of negative sign ($\Delta\epsilon$ = −1.35, λ = 281 nm).

(3*S*)-(−)-5,7,3′,4′-tetramethoxyflavan of known absolute configuration shows a negative Cotton Effect in the 260–300 nm region [13, 14]. This correlation was used to establish the configuration of other natural isoflavans [15]. On this basis the *R*-configuration was assigned to **Va** and the *S* to **Vb**.

The activity of two enantiomers was studied on HRV 1B multiplication in HeLa cell cultures by means of plaque reduction assays. The percentage of reduction of plaque formation, produced by the incorporation of various non-cytotoxic concentrations of each compound

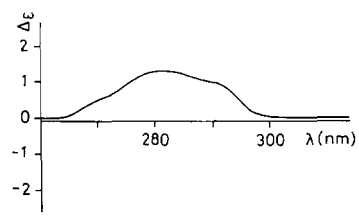

Figure 6
Circular dichroism spectrum relative to compound **Va**.

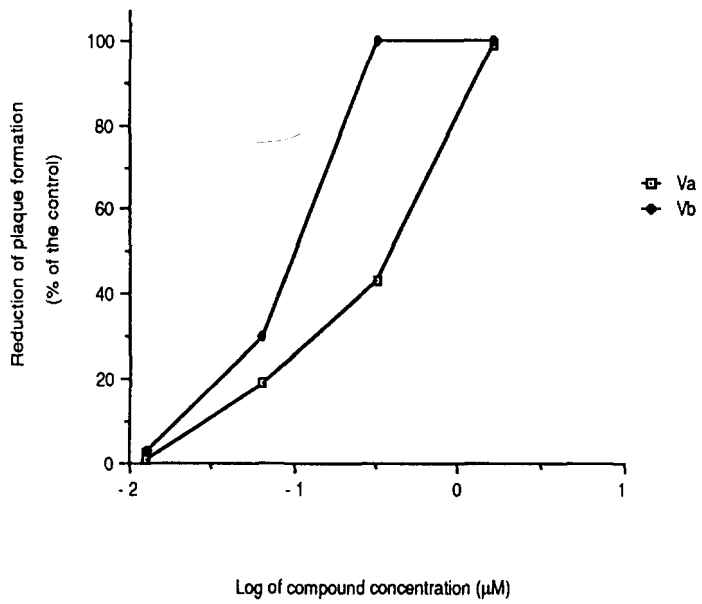

Figure 7
Dose–response line of HRV 1B plaque reduction by **Va** and **Vb**.

Table 1
Reduction of HRV 1B plaque formation by **Va** and **Vb** at different concentrations

Concentration (μM)	Per cent of inhibition of plaque formation*	
	Va	**Vb**
1.6	99	100
0.32	43	100
0.064	19	30
0.0128	1	3

* Data reported are the mean of two separate experiments.

into the agar overlay medium, is reported in Table 1. A dose–response line was obtained by plotting the per cent of plaque reduction against the logarithm of the compound dose (Fig. 7). Results show that both enantiomers exhibited an inhibitory effect on HRV multiplication, although with different extent, the *S* enantiomer being the most effective. By comparing the IC_{50} values (0.391 μM for **Va** and 0.101 μM for **Vb**) the *S* form was approximately four times more effective than the *R* form; the same result was observed for 4′,6-dichloroflavan [3].

Previous studies on the effect of the racemic compound (**V**) on HRV 1B multiplication indicated that the viral inactivation may be due to the binding of the compound to some specific site or sites of the capside [5]. The results obtained with the two enantiomers suggest that the half-chair conformation with an equatorial 3-aryl substituent is involved in the binding. If the conformation with an axial substituent were involved, a very low activity or the complete inactivity of one enantiomer would be expected, owing to the greater difference in the spatial disposition of the atoms probably bound to the active site or sites.

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