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Chromatographic studies of the enantiomeric composition of some therapeutic compositions applied in the treatment of liver and kidney diseases

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Abstract

A gas–liquid chromatographic system with α-cyclodextrin in formamide medium (coated on Chromosorb) was used for the separation of enantiomers of α-pinene, β-pinene, limonene and camphene in medicines applied in the therapy of liver and kidney diseases. The drugs under investigation were produced in Poland (Terpichol and Terpinex), in Germany (Rowachol and Rowatinex) and in Slovenia (Uroterp). It was found that, depending on the manufacturer, medicines possessing similar chemical compositions differ considerably from one another regarding the content of enantiomers, mainly those of α-pinene.

1. Introduction

Recently, it has been found that a large group of chiral macrocyclic compounds including cyclodextrins constitute a powerful tool for the enantiomeric separation of many compounds of various chemical natures, *i.e.*, acidic, basic and neutral, including hydrocarbons, which are very resistive to diastereoisomer formation.

There are two ways to apply cyclodextrins in gas chromatography for analytical purposes. In the early 1980s we initiated the first approach using cyclodextrins in the dissolved state (in a convenient matrix solvent) as stationary phases. This method is characterized by high enantioselectivity and a relatively low efficiency of the columns. For example, using α-cyclodextrin (α-CD) under appropriate conditions of partition gas chromatography (GC), very efficient separations of α-pinene, β-pinene and camphene into enantiomers can be achieved [1–3]. A few years later König and co-workers [4,5] and subsequently Armstrong *et al.* [6] initiated another approach, successfully using some cyclodextrin derivatives in the molten state as stationary phases in capillary columns. This important method is characterized by a relatively poor enantioselectivity but a high efficiency of the columns [7].

These two approaches are still being developed. They have their own advantages and limitations, but it would be a mistake to treat them as competitive techniques; rather, they are supplementary, and both merit attention.

This paper describes an attempt to apply inclusion processes in α-CD molecules to the study of the enantiomeric composition of ter-

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penic hydrocarbons in some medicines used in the therapy of liver and kidney diseases.

It is well known that the semi-natural mixture of several terpenes (menthol, menthone, pinenes, borneol, camphene and cineol) in olive oil has choleretic, spasmolytic and bacteriostatic properties, and it has been used as an inexpensive preparation to treat cholesterol stones in the gall bladder and the bile ducts [8–10] and in the treatment of patients with ureteric stones [11,12]. Although, the mechanism by which the terpene mixture inhibits the formation of cholesterol crystals in bile is not fully recognized, it has been found that menthol and other terpenes inhibits the lecitin–cholesterol acyltransferase activity of human plasma [13] and also (*S*)-3-hydroxy-3-methylglutaryl-CoA reductase which leads to the physiological inhibition of hepatic sterol synthesis [14].

The enzymatic mechanism of terpene action strongly suggests that the enantiomeric composition of the drug might be a very important factor determining its therapeutic properties. Many of the terpenes used for medical purposes are chiral compounds and they can exist in natural mixtures in one or two enantiomeric forms and in various proportions. Despite this fact, commercially available terpene drugs (*e.g.*, Rowachol) are usually described as a mixture of six terpenes in which even the two pinenes (α- and β-pinene) are not discriminated and the enantiomeric composition is virtually unknown [15,16].

2. Experimental

2.1. Reagents

α-CD was supplied by Chinoin (Budapest, Hungary). Chromosorb W NAW (0.18–0.25 mm) for GC was a product of Johns-Manville (Litho, USA). Commercially available drugs investigated were as follows: Terpinex and Terpichol from Herbapol (Wroclaw, Poland), Rowachol and Rowatinex from Rowa-Wagner, (Bergish, Germany) and Uroterp donated by KRKA (Novo Mesto, Slovenia). Terpene standards were as follows: (+)- and (−)-α-pinene from Aldrich (Milwaukee, WI, USA), (+)- and (−)-limonene from Merck–Schuchardt (Hohenbrunn, Germany) and (+)- and (−)-β-pinene from Fluka (Buchs, Switzerland); (+)- and (−)-camphene were prepared by ourselves [3].

2.2. Apparatus and procedure

Chromatographic studies were performed using a Hewlett-Packard (Avondale, PA, USA) Model 5890 gas chromatograph equipped with dual flame ionization detectors. The peak areas and retention times were measured by means of a Hewlett-Packard Model 3396 integrator. The compounds (0.02–0.15 μl) were injected with Hamilton microsyringes separately or as a mixture. Two types of columns were used: a packed glass column (4 m × 4 mm I.D.) containing Chromosorb W NAW (0.18–0.25 mm), coated with a formamide solution of α-CD (0.18 molal) and an HP-1 cross-linked methylsilicone fused-silica capillary column (30 m × 0.53 mm I.D.). The details for the preparation of the α-CD column have been described previously [17,18]. A constant inlet pressure (100 ± 5 kPa) and a constant argon flow-rate (40 ± 0.5 ml/min) were maintained.

The operating temperature of the α-CD column was isocratic (35°C), whereas the capillary column temperature was programmed (70°C held for 35 min, then increased from 70 to 200°C at 5°C/min and held at 200°C for 10 min).

2.3. Validation of the analytical method

In order to evaluate the repeatability of the method, an artificial mixture of (+)-α-pinene, (−)-α-pinene, (−)-β-pinene, (+)-limonene, (−)-limonene, (+)-camphene and (−)-camphene was injected. The results and the statistical evaluation are given in Table 1. The relative standard deviation (R.S.D.) in each instance is lower than 10%. The only one exception is (+)-β-pinene, and therefore, only small amount of this compound is present in the mixture. In Table 2 the separation factors (α) are presented. As can be seen, each value is higher than 1.1 and the R.S.D.s are lower than 4%. Therefore, it can be

Table 1
Repeatability of the analytical method

Run No.	Concentration (%)							
	(+)-α-Pinene	(−)-α-Pinene	(+)-β-Pinene	(−)-β-Pinene	(+)-Limonene	(−)-Limonene	(+)-Camphene	(−)-Camphene
1	14.20	13.37	1.16	12.78	15.04	11.72	15.88	15.51
2	14.87	13.25	1.13	12.30	13.35	10.73	16.60	17.77
3	14.58	13.04	1.11	12.19	15.93	12.69	14.04	15.38
4	15.21	13.26	1.78	13.24	14.39	10.94	15.03	15.80
5	14.60	13.26	1.44	12.96	14.25	10.57	16.93	17.33
6	14.97	13.12	1.52	13.02	14.75	10.60	15.12	16.80
Mean	14.74	13.22	1.36	12.75	14.62	11.21	15.60	16.43
S.D.	0.323	0.107	0.246	0.382	0.787	0.768	0.986	0.921
R.S.D. (%)	2.2	0.8	18.1	3.0	5.4	6.9	6.3	5.6

concluded that the analytical method is suitable for the determination of terpenes in therapeutic compositions. The data quoted in Tables 5 and 6 are the mean values from at least three measurements.

3. Results and discussion

3.1. Chemical composition

Terpenic mixtures are used as drugs in two different compositions. For the treatment of cholesterol stones in the gall bladder and the bile ducts, mixtures rich in menthol and pinenes are sold under the trade names Rowachol and Terpichol, whereas in compositions used for the treatment of ureteric stones (Terpinex, Rowatinex and Uroterp) the predominant compounds are pinenes and camphene. For the evaluation of the chemical composition an achiral capillary column was used.

Fig. 1 shows a typical chromatogram of a Terpichol sample. The concentrations (peak areas) found in Terpichol and Rowachol samples together with the values declared by the producer are given in Table 3. From the data in Table 3, menthol and α-pinene appear to be the predominant components of both drugs. However, in the Rowachol sample larger differences

Table 2
Statistical evaluation of enantioselectivity factors (α)

Run No.	$\alpha_{(+)/(-)}$			
	α-Pinene	β-Pinene	Limonene	Camphene
1	2.16	1.49	1.11	2.73
2	2.04	1.41	1.09	2.46
3	2.31	1.46	1.10	2.50
4	2.10	1.46	1.10	2.60
5	2.21	1.49	1.11	2.48
6	2.15	1.45	1.11	2.56
Mean	2.16	1.46	1.10	2.56
S.D.	0.085	0.027	0.007	0.089
R.S.D. (%)	3.9	1.8	0.6	3.5

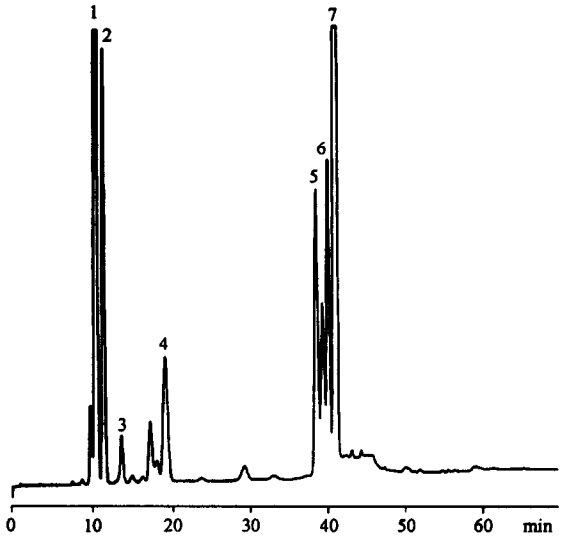

Fig. 1. Chromatogram of Terpichol obtained on an achiral capillary column. Conditions as under Experimental. Peaks: 1 = α-pinene; 2 = camphene; 3 = β-pinene; 4 = cineole; 5 = menthone; 6 = borneol; 7 = menthol.

from the declared values were found. Moreover, in the Terpichol sample undeclared limonene is also present.

Fig. 2 shows typical chromatograms of (a) Rowatinex and (b) Uroterp obtained on an achiral capillary column. In Table 4 are given the data on the chemical composition of Terpinex, Rowatinex and Uroterp obtained on the same column. The amount of limonene, eluted together with cineole, was elucidated using supplementary data from the chiral column. As can be seen, only the Rowatinex sample is in full accordance with the standard; Terpinex and Uroterp are strongly admixed with compounds not declared by the manufacturers and not identified by us (*ca.* 20%). The Uroterp sample has a high concentration of limonene and Terpinex contains large amounts of camphene and borneol. Nevertheless, α-pinene is the main constituent of the drugs used in kidney diseases.

3.2. Enantiomeric composition

The enantiomeric composition was studied using the chiral α-CD column. Fig. 3 shows chromatograms of (a) Terpichol and (b) Rowachol. Table 5 gives the enantiomeric composition of both the monoterpenic fractions obtained on the same column. It is seen that the main component, α-pinene, is mostly (81%) dextrarotatory in Terpichol whereas Rowachol contains 90% of its antipode. Similar large differences in enantiomeric composition can be observed for camphene. In Terpichol it is almost a racemic mixture consisting of 60% of the dextrarotatory component. In contrast, in Rowachol the dextrarotatory enantiomer dominates (85%). Although β-pinene is declared by the producers as a minor component, it is noteworthy that it is present as almost the

Table 3
Contents of terpenes in Terpichol and Rowachol determined on an achiral capillary column

Compound	Terpichol (%)		Rowachol (%)	
	Declared	Found	Declared	Found
α-Pinene	20.0	21.4	20.0	25.0
β-Pinene	5.0	1.0	5.0	6.5
Camphene	8.0	6.1	8.0	9.1
Cineole	3.0	3.7	3.0	2.8
Limonene	–	2.5	–	–
Menthone	9.0	6.5	9.0	7.1
Borneol	8.0	5.3	8.0	6.0
Menthol	48.0	44.7	48.0	37.0
Unidentified		8.8		12.4

Chromatographic conditions as described under Experimental.

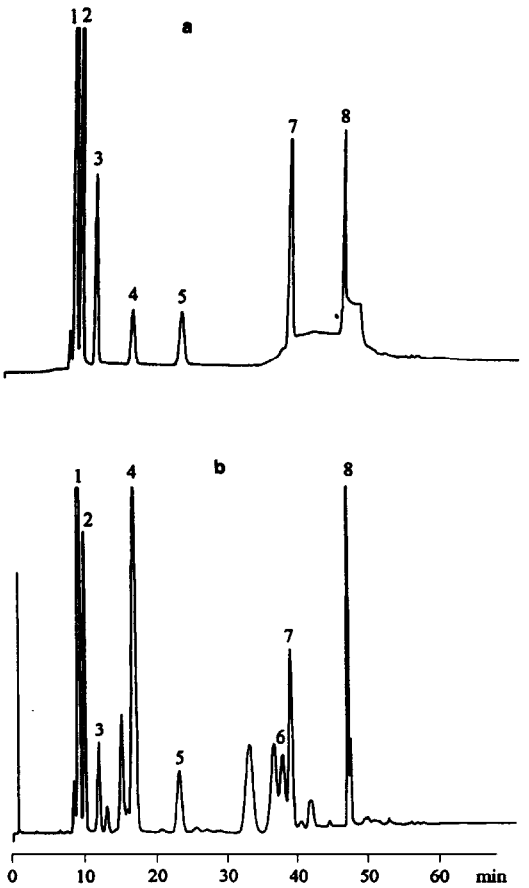

Fig. 2. Chromatograms of (a) Rowatinex and (b) Uroterp obtained on an achiral capillary column. Conditions as under Experimental. Peaks: 1 = α-pinene; 2 = camphene; 3 = β-pinene; 4 = cineole and limonene; 5 = fenchone; 6 = menthone; 7 = borneol; 8 = *trans*-anethole.

optically pure (−)-enantiomer in both preparations.

Fig. 4 shows chromatograms of (a) Terpinex, (b) Rowatinex and (c) Uroterp obtained on the chiral α-CD column. Table 6 gives the enantiomeric composition of monoterpenic fraction of the drugs. The main component (α-pinene) is mostly dextrarotatory (91%) in the Terpinex preparation and laevorotatory (89%) in Rowatinex. In contrast, Uroterp has an intermediate composition of α-pinene, consisting of 64% of the dextrarotatory isomer. Camphene exists as a nearly racemic mixture in all the drugs. Moreover, large amounts of limonene

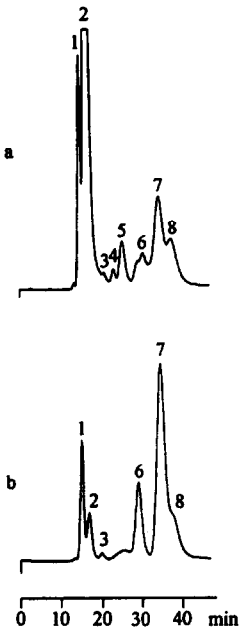

Fig. 3. Chromatograms of (a) Terpichol and (b) Rowachol obtained on a chiral α-CD column at 35°C. Peaks: 1 = (+)-camphene; 2 = (+)-α-pinene; 3 = (+)-β-pinene; 4 = (−)-limonene; 5 = (+)-limonene; 6 = (−)-β-pinene; 7 = (−)-α-pinene; 8 = (−)-camphene.

were found in Uroterp, with 70% of the (+)-enantiomer prevailing and also a trace of limonene in the Terpinex preparation with 83% of the (+)-enantiomer. Finally, it is noteworthy that β-pinene in Rowatinex and Uroterp is present as the almost optically pure (−)-enantiomer.

The results indicate large differences of the enantiomeric compositions of all the drugs considered, suggesting that the enantiomeric composition is not standardized by the manufacturers. On the other hand, biological systems are largely constructed from chiral compounds. Therefore, in such a highly chiral environment it should not be surprising that some drugs, which possess an asymmetric centre, exhibit a high degree of stereoselectivity in their interactions with macromolecules. Particularly, growing evidence of an enzymatic mechanism of terpene action on living organisms [13,14] strongly suggests enantioselectivity of this process. Moreover, clinical observation does not univocally confirm the usefulness

Table 4
Contents of terpenes in Terpinex, Rowatinex and Uroterp determined on an achiral capillary column

Compound	Terpinex (%)		Rowatinex (%)		Uroterp (%)	
	Declared	Found	Declared	Found	Declared	Found
α-Pinene	46.2	23.9	37.0	37.9	44.0	23.7
β-Pinene		1.9	9.0	9.6		2.6
Camphene	22.4	23.7	22.0	22.0	10.0	7.9
Cineole	4.5	3.7	4.0	4.0	14.0	17.6
Limonene	–		–	–	–	
Fenchone	6.0	4.6	6.0	5.0	5.1	3.3
Borneol and	15.0	9.3	15.0	13.4	14.0	8.2
isoborneol		2.6		–		4.3
trans-Anethole	6.0	4.2	6.0	5.7	12.1	9.5
Unidentified		24.0		2.0		23.0

Conditions as described under Experimental.

Table 5
Enantiomeric composition of terpenes in Terpichol and Rowachol determined on a chiral α-CD column at 35°C

Compound	Terpichol (%)	Optical purity (%)	Rowachol (%)	Optical purity (%)
(+)-α-Pinene	59.6	81	7.0	10
(−)-α-Pinene	13.6	19	60.0	90
(+)-β-Pinene	0.1	2	1.0	6
(−)-β-Pinene	4.7	98	14.8	94
(−)-Limonene	0.7	16	–	–
(+)-Limonene	3.7	84	–	–
(+)-Camphene	10.5	60	12.8	85
(−)-Camphene	7.1	40	2.2	15

Table 6
Enantiomeric composition of terpenes in Terpinex, Rowatinex and Uroterp determined on a chiral α-CD column at 35°C

Compound	Terpinex (%)	Optical purity (%)	Rowatinex (%)	Optical purity (%)	Uroterp (%)	Optical purity (%)
(+)-α-Pinene	38.3	91	6.0	11	33.3	64
(−)-α-Pinene	4.0	9	47.6	89	18.8	36
(+)-β-Pinene	1.5	50	1.0	7	–	0
(−)-β-Pinene	1.5	50	12.6	93	5.3	100
(+)-Limonene	2.9	83	–	0	13.2	70
(−)-Limonene	0.6	17	–	0	5.6	30
(+)-Camphene	25.6	55	17.7	54	7.3	46
(−)-Camphene	20.7	45	14.8	46	8.6	54

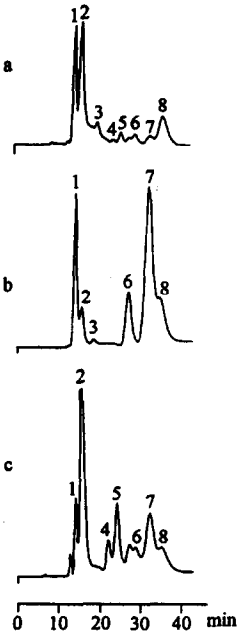

Fig. 4. Chromatograms of (a) Terpinex, (b) Rowatinex and (c) Uroterp obtained on a chiral α-CD column at 35°C. Peaks: 1 = (+)-camphene; 2 = (+)-α-pinene; 3 = (+)-β-pinene; 4 = (−)-limonene; 5 = (+)-limonene; 6 = (−)-β-pinene; 7 = (−)-α-pinene; 8 = (−)-camphene.

of terpene drugs in the treatment of cholesterol gall stones [10]. Presumably, a lack of general acceptance of these drugs by clinicians is due to the large differences in enantiomeric composition that we have observed. On the other hand, the quality and amounts of terpenes in plant species differ greatly, not only from species to species but also within species [19]. In such cases, in addition to genetic factors, both the macro- and micro-environments may be responsible for the variations in the enantiomeric compositions of terpenes. This could probably explain why the terpenic drugs produced in different countries differ so substantially.

The results presented here suggest that the difficult problem of the evaluation of terpenic drugs could be completely resolved on the basis of α-CD complexation. To achieve this goal very detailed optimization studies should be performed.

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