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LC determination of the enantiomeric purity of levamisole using stationary phase with bonded naphthylethylcarbamoylated-β-cyclodextrin

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Abstract

A direct enantioselective high-performance liquid chromatography was employed successfully for determination of the enantiomeric purity of levamisole. The elaborated method used *S*-naphthylethylcarbamoylated β-cyclodextrin stationary phase in reversed-phase mode. The optimized mobile phase composition was acetonitrile-0.5% triethylammonium acetate buffer, pH 5.0 (2:8, v/v). Linearity, precision, accuracy, and the quantitation limit were determined. The method proved to be capable of determining 0.05% (w/w) of dexamisole (the enantiomeric impurity) contrary to the pharmacopoeial optical rotation measurement, in which only amounts of dexamisole higher than 2.2% (w/w) caused the test to fail. The enantiomeric purity of three different levamisole substances and levamisole tablets was assessed with the use of the method. The content of dexamisole impurity was found to be in the range 0.66–1.60% (w/w).

Keywords: Enantiomeric purity; Enantioselective; Liquid chromatography; Levamisole; Dexamisole; Chiral; Naphthylethylcarbamoylated-β-cyclodextrin stationary phase

1. Introduction

Levamisole (Fig. 1) is the *S*-enantiomer of tetramisole-a synthetic imidazo-thiazole derivative-acting as an anthelmintic [1]. The single enantiomer was introduced in 1969 since the other enantiomer (dexamisole) showed more adverse effects [1,2]. Levamisole proved to be also effective in combination with 5-fluorouracil as adjuvant therapy in patients with colon carcinoma and current investigations of levamisole are focused on its immunomodulatory effects [3].

In the case of single enantiomer drugs, all other stereoisomers should be treated as any other organic impurities and the enantiomeric purity of such drugs should be controlled. The pharmacopeias rely traditionally on polarimetric methods. For control of optical purity of levamisole,

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measurements of optical rotation of aqueous solutions of levamisole are used [4,5]. However, polar-imetric methods are known to be non-selective and often not sufficiently sensitive [6–8].

During the last decade, enantioselective separation methods have become a powerful and widely applicable analytical tool for separation and determination of enantiomers, even at trace levels. Among these methods, HPLC and capillary electrophoresis (CE) have acquired the dominant position.

There is a broad variety of chiral stationary phases (CSP) for enantioselective HPLC and chiral buffer additives for enantioselective CE available and the choice of the suitable one is a key point of development of an enantioselective method.

Separation of the enantiomers of tetramisole — levamisole and dexamisole — was achieved by HPLC using naphthylethylcarbamoylated-β-cyclodextrin (NEC-CD) [9] or silica-immobilized cellobiohydrolase [10] as CSP or by CE using heparin as a chiral additive to running buffers [11].

However, the above papers did not deal with the assessment of traces of one enantiomer in the presence of excess of the other enantiomer. To date, there has been only one published method for determining the enantiomeric purity of levamisole and dexamisole, namely NMR using a lanthanide shift reagent [12]. Unfortunately, the NMR method was not able to detect the enantiomeric impurities at levels lower than 8%.

In this work, an enatioselective HPLC method for determining the enantiomeric purity of levamisole was elaborated, validated and compared with the pharmacopoeial optical rotation test [5]. The HPLC method was based on enantioseparation of tetramisole achieved with the use of NEC-CD CSP.

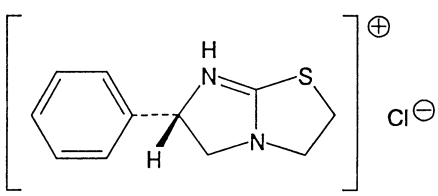

Fig. 1. Chemical structure of levamisole.

NEC-substituted β-cyclodextrin stationary phase belongs to the most versatile cyclodextrin based CSPs. It represents a multimodal CSP functioning by different chiral recognition mechanisms in three different modes (normal phase, polar organic or reversed-phase mode) of LC [9,13,14]. The incorporation of the NEC-substituents onto the cyclodextrin (CD) introduces additional stereogenic centers, the configuration of which plays an important role in the enantioselectivity of the CSP. Consequently, the NEC-CD CSP is available commercially in the R- or S-form, the latter being more successful in the reversed-phase mode [9].

2. Experimental

2.1. Chemicals

Levamisole hydrochloride and tetramisole hydrochloride were the products of Sigma (St. Louis, MO, USA), levamisole hydrochloride was also obtained from Janssen Pharmaceutica (Beerse, Belgie), and reference standard of levamisole hydrochloride (CRS) was purchased from European Pharmacopoeia (Strasbourg, France). Levamisole was dried at 105°C for 4 h [5] prior to measurements. Methanol (gradient grade), acetonitrile (gradient grade) and glacial acetic acid (analytical-reagent grade) were obtained from Merck (Darmstadt, Germany). Triethylamine (super purity solvent) was purchased from Romil (Loughborough, UK). Water was passed through an Elgastat UHQ-PS water purification unit (USF Elga, Lane End High Wycombe Bucks, UK). All other chemicals used were of analytical-reagent grade.

2.2. Chromatography

HPLC measurements were performed with two chromatographic systems,

1. a Shimadzu Model LC-6A liquid chromatograph (Kyoto, Japan) equipped with a variable-wavelength detector, a Rheodyne 7125 injector and a datastation CSW Data Apex (Prague, Czech Republic);
2. an HPLC apparatus consisting of a Constametric 3500 pump, a Rheodyne 7725i injector, a Spectromonitor 4100 variable-wavelength detector (Thermo Separation Products, Riviera Beach, FL, USA), and a datastation CSW Data Apex (Prague, Czech Republic).

The HPLC column used was Cyclobond I 2000 SN (5 μm), 250 × 4.6 mm with a guard column Cyclobond I 2000 SN (5 μm), 10 × 3.2 mm, (Astec, Whippany, NJ, USA).

A 0.5% triethylammonium acetate (TEAA) buffer for mobile phases was prepared by adjusting a 0.5% (v/v) solution of triethylamine with glacial acetic acid to the appropriate pH. Mobile phases were prepared by mixing acetonitrile or methanol and a TEAA buffer and passing the mixture through a 0.45-μm Millipore filter before use. The optimum mobile phase consisted of acetonitrile and the 0.5% TEAA buffer at pH 5.0 (2:8, v/v). The flow rate was kept at 0.8 ml/min and the detection wavelength was 254 nm. The column temperature was ambient.

Standard solutions were obtained by appropriate dilution of stock solutions of levamisole hydrochloride (1.0 mg/ml) and tetramisole hydrochloride (0.1 mg/ml), that were prepared in the mobile phase. Samples of levamisole and tetramisole to be analyzed were dissolved in the mobile phase to give a concentration of 0.5 mg/ml. Tablets containing levamisole were ground to fine powder, extracted with water under sonication for 15 min and filtered through a 0.45-μm Millipore filter. The amount injected was 10 μl.

2.3. Polarimetry

Measurements of optical rotation were performed on a Perkin–Elmer Model 241 polarimeter (Norwalk, CT, USA) equipped with a jacketed microcell and a thermostat kept at 20°C. Levamisole solutions (1.0 mg/ml) were prepared in water.

3. Results and discussion

3.1. Optimization of mobile phase composition

Using *S*-NEC-CD CSP, we started experiments with the mobile phase composed of acetonitrile and 1.0% TEAA buffer at pH 4.5 (3:7, v/v) [9]. Under these conditions, the enantiomers of tetramisole were separated to the base-line, the elution order being dexamisole-levamisole. This elution order was favorable for the determination of dexamisole in the presence of excess of levamisole as the minor peak of this impurity was eluted prior to the major peak of levamisole. However, the achieved enantioresolution $R = 1.6$ was not sufficient for sensitive determination of the enantiomeric purity. Generally, resolution greater than 2 for the racemate is needed for quantitative trace analyses [15].

Under reversed-phase conditions, chiral recognition of CDs and their derivatives as well is based on inclusion complexation [14,16]. As native CDs cannot resolve tetramisole [9], the important contribution to the enantioselectivity of *S*-NEC-CD CSP is thought to be due to additional interactions between the analyte and the CD substituents. In optimizing such enantioseparations, several important parameters such as the pH and concentration of buffer, nature and content of organic modifier and flow-rate must be investigated [16].

The most significant parameter is pH [9]. The effect of pH was investigated with 1.0% TEAA buffer in the range 4.0–7.0, which is the stability range for NEC-CD CSP [16]. As indicated in Fig. 2, capacity factors of dexamisole and levamisole increased significantly for pH values above 6, i.e. with decreasing ionization ($pK = 8.0$ [17]). The enhanced retention did not produce an increase of the enantioresolution (see Fig. 2). On the basis of these experiments, pH 5.0, which corresponded to a flat maximum of the enantioresolution dependence, was chosen for further experiments.

Two organic modifiers, acetonitrile and methanol, were examined in hydroorganic mobile phases that contained the above buffer as the aqueous component. As can be seen from Fig. 3, enantioseparation was achieved with both organic

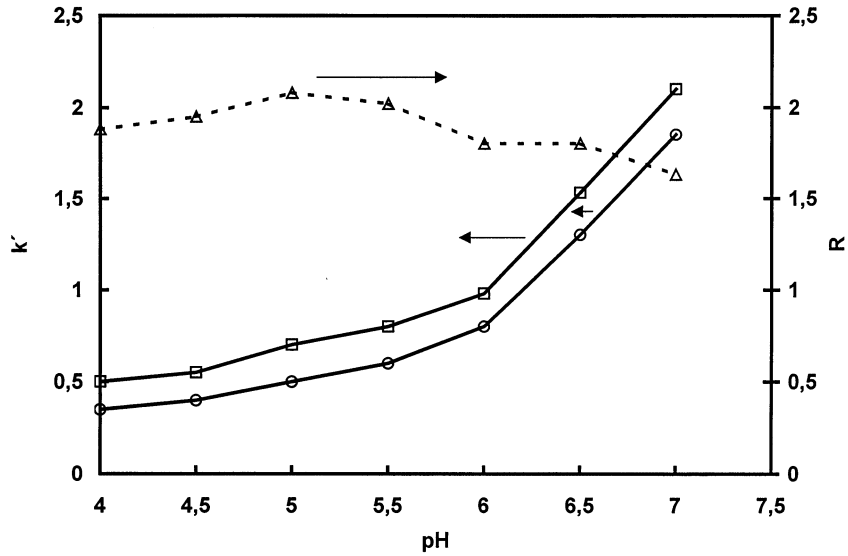

Fig. 2. Dependence of capacity factors k' of dexamisole (○), levamisole (□) and enantioresolution R of tetramisole (∇ and dashed line) on the pH. Column — Cyclobond I 2000 SN (5 μm), 250 × 4.6 mm with a precolumn Cyclobond I 2000 SN (5 μm), 10 × 3.2 mm. Mobile phase — acetonitrile, 1.0% TEAA buffer (3:7, v/v); flow rate, 0.8 ml/min.

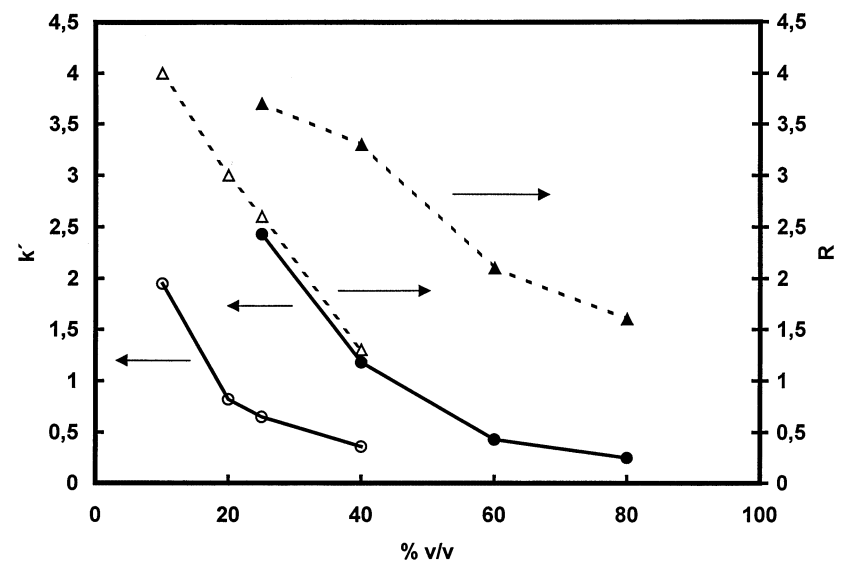

Fig. 3. Dependence of capacity factors k' of dexamisole and enantioresolution R (dashed lines) of tetramisole on the content of the following organic modifiers — acetonitrile (○ (k') and ∇ (R)) and methanol (● (k') and ▲ (R)). Column — as in Fig. 2 Mobile phase—organic modifier, 1.0% TEAA buffer, pH 5.0; flow rate, 0.8 ml/min.

modifiers in the whole concentration range examined. As expected, the capacity factors decreased with increasing content of organic modifiers, since the organic modifiers compete with the analytes for the CD cavity. At the same time, a decrease of enantioresolution was observed. These effects

Table 1
Effect of acetonitrile concentration on capacity factors of dexamisole (k'_D) and levamisole (k'_L), separation factors α and enantioresolution R[a]

Acetonitrile (%)	k'_D	k'_L	R	α
10	1.39	2.48	3.5	1.78
20	0.56	0.88	2.8	1.57
25	0.34	0.53	2.2	1.56
30	0.27	0.41	1.9	1.52
40	0.24	0.32	1.1	1.33

[a] Mobile phase — acetonitrile-0.5% TEAA buffer, pH 5.0.

were more significant with acetonitrile which is known to be a stronger eluting solvent. Thus, acetonitrile gave sufficient enantioresolution in shorter time than methanol. Moreover, it afforded better separation efficiency.

The effect of buffer concentration, which was studied in the range 0.25–1.0%, was not pronounced. In Table 1, retention and separation data for the mobile phase composed of acetonitrile and 0.5% TEAA buffer are presented. When comparing these data with the data shown in Fig. 3, that were measured with the use of higher buffer concentration (1.0%), only a slight increase of retention and enantioresolution for the enhanced buffer concentration appeared. At buffer concentration lower than 0.2%, bad peak shapes were observed. The buffer concentration of 0.5% (v/v) was chosen as an optimum concentration.

At first, analyses were performed out at a flow-rate of 1.0 ml/min. Decreasing the flow-rate to 0.8 ml/min slightly enhanced the enantioresolution as a consequence of improved separation efficiency.

The mobile phase containing acetonitrile (20% (v/v)) and 0.5% TEAA buffer, pH 5.0 was chosen as optimum for the enantiomeric purity determination. As Fig. 4 shows, sufficient enantioresolution ($R = 2.8$) in a short analysis time was achieved under these conditions. The retention and resolution of dexamisole and levamisole were controlled by the content of acetonitrile and slightly affected by the buffer concentration. A higher buffer concentration required somewhat

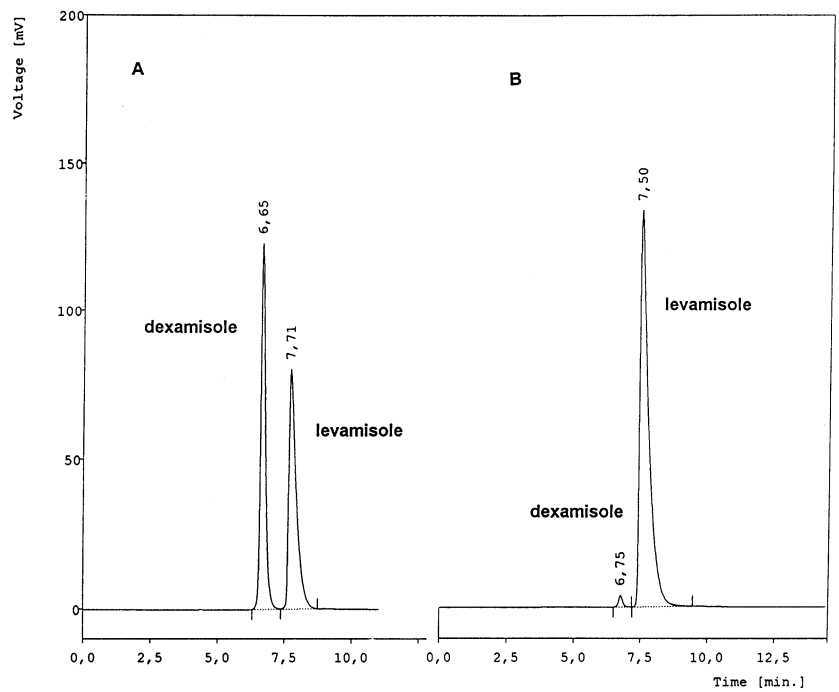

Fig. 4. Separation of dexamisole and levamisole in substances of — A, tetramisole; and B, levamisole. Column — as in Fig. 2 Mobile phase—acetonitrile, 0.5% TEAA buffer, pH 5.0 (2:8, v/v); flow rate, 0.8 ml/min.

Table 2
Method linearity

Calibration curves[a]	Slope (Area counts ml/μg)	Correlation coefficient r^2	Intercept	Range (μg/ml)
1	6.2493	0.9999	3.25	
2	6.4242	0.9999	0.79	
3	6.3721	0.9999	0.12	6.5–31.0
4	6.3191	0.9999	1.22	
Mean ± S.D.	6.3418 ± 0.0748		1.34 ± 1.34	

[a] Measured on 4 different days.

higher acetonitrile content — retention and enantioresolution comparable with those presented in Fig. 4A were obtained with the mobile phase composed of 25% (v/v) of acetonitrile and 1.0% TEAA buffer.

3.2. Method validation

In order to evaluate the suitability of the method for determining the enantiomeric purity of levamisole, validation studies were carried out.

Linearity of the method and the range studied are demonstrated in Table 2, precision and accuracy in Table 3. The spiked samples of levamisole that had composition shown in the second column of Table 3 were prepared from levamisole and tetramisole. The substance of levamisole employed in the experiments contained 1.34 ± 0.05% (w/w) of dexamisole. Hence we could not test quantitation down to lower content of the R-enantiomer. Precision of the method was expressed as relative standard deviation (R.S.D.).

The quantitation and detection limits were determined with dilute solutions of tetramisole. The quantitation limit was the level, which produced a relative standard deviation of about 10%, the detection limit was determined for a signal-to-noise ratio 3:1. 0.05 and 0.02% of dexamisole were considered to be the quantitation limit and the detection limit, respectively.

The method could be applied simultaneously to assessment of levamisole. The assay repeatability, expressed as the relative standard deviation of results of analyses of five parallelly prepared solutions ($n = 5$), was found to be R.S.D. = 0.8%.

The excellent results of the validation study showed that the method enabled reliable determination of the enantiomeric impurity dexamisole as well as of the active substance levamisole.

In order to verify applicability of the method for determining the enantiomeric purity to a dosage-form, we performed analogous validation experiments with levamisole tablets, matrix of which contained polyvidone, talc, starch, saccharin, magnesium stearate, an apricote flavor and a yellow color. Prior to analyses, tablets were extracted as described in the Section 2.2. A typical chromatogram of a sample prepared from levamisole tablets is shown in Fig. 5, the validation parameters obtained are presented in Table 4. The assessed recovery indicated that the accuracy of the method using the simple extraction procedure was excellent.

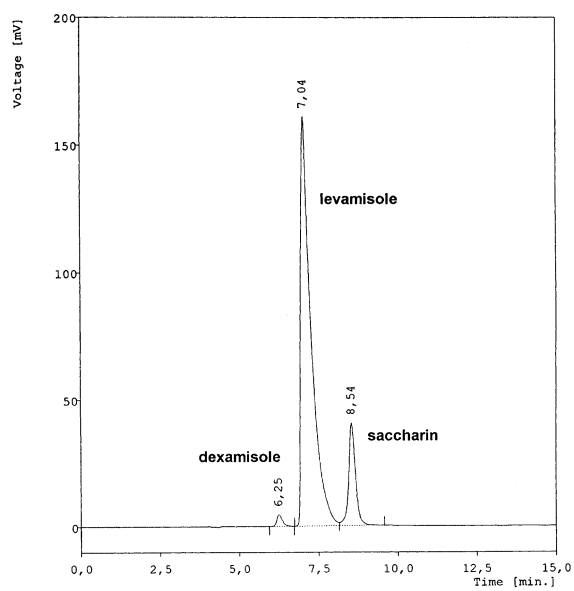

Fig. 5. Analysis of levamisole tablets. Chromatographic conditions as in Fig. 4.

Table 3
Precision and accuracy of determining the enantiomeric purity of levamisole substance

Added amount of dexamisole % (w/w)	Content of dexamisole % (w/w)	R.S.D. (%)				Recovery (%)	
		Analyst A		Analyst B		Analyst A	Analyst B
		Intraday $n=5$	Interday $n=5$	Intraday $n=4$	Interday $n=3$		
0	1.34[a]	2.1	–	1.1	–	–	–
0.52	1.86	1.1	1.5	1.5	–	99.0	99.2
3.77	5.11	0.7	0.6	0.8	1.8	99.4	98.0

[a] Determined as area percentage.

Table 4
Precision and accuracy of determining the enantiomeric purity of levamisole tablets

Added amount of dexamisole% (w/w)	Content of dexamisole % (w/w)	R.S.D. (%)		Recovery (%)
		Intraday $n=5$	Interday $n=4$	
0	1.43	2.4	–	–
0.62	2.05	1.2	4.3	99.5
1.52	2.95	0.9	1.6	99.9
3.19	4.62	0.7	1.0	99.6

3.3. Optical rotation measurement

The optical purity of samples prepared as mixtures of levamisole and tetramisole was assessed using the pharmacopoeial method [5]. The results summarized in Table 5 show that the pharmacopoeial polarimetric method is not sufficiently sensitive. The limits set in the pharmacopoeia were not exceeded until more than 2.2% (w/w) of dexamisole was present in the samples tested. Thus, in contrast to the enantioselective HPLC method, the pharmacopoeial test of optical rotation measurements proved to be unable to detect traces of dexamisole.

3.4. Enantiomeric purity of substances and tablets

Table 6 summarizes results of the determination of the enantiomeric purity of levamisole substances obtained from various sources and levamisole tablets as a representative of a levamisole dosage-form. With the exception of levamisole CRS, all samples analyzed contained more than 1% (w/w) of dexamisole as the enantiomeric impurity.

4. Conclusion

An enantioselective HPLC method that enabled sensitive determination of the enantiomeric purity of levamisole was developed. The method was found to be precise, accurate, sensitive and reliable. As it is also simple, fast and convenient, it is well suited for routine control. The enantioresolution required for the sensitive determination of the enantiomeric purity could be set easily by tuning the acetonitrile content in the mobile phase. Small changes in the buffer concentration and pH did not influence the separation significantly.

The employed chiral column required only standard conditioning and proved to be stable. We used it for 1 year with pauses and no decrease of efficiency or retention was observed.

The pharmacopoeial optical rotation test for levamisole proved to be, not unexpectedly, insensitive to low levels of dexamisole present in levamisole.

In conclusion, assessment of the enantiomeric purity of drugs requires the use of state-of-the-art analytical methods. With the present broad range

Table 5
Results of the pharmacopoeial optical rotation test

Dexamisole added (% w/w)	Specific optical rotation $[\alpha]_D^{20}$	Pharmacopoeial limits (°)
1.30	-124.2 ± 0.2	-121 to -128
2.20	-121.9	
3.25	-119.4	
5.20	-115.8	

Table 6
Enantiomeric purity of levamisole substances and tablets

Sample	Content of dexamisole % (w/w) ± S.D.
Levamisole CRS (European Pharmacopoeia)	0.66 ± 0.01
Levamisole (Sigma)	1.34 ± 0.03
Levamisole (Janssen Pharmaceutica)	1.34 ± 0.04
Levamisole tablets	1.60 ± 0.03

of available CSPs and advances in column technology, enantioselective HPLC is the method of choice.

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