SEMMELWEIS UNIVERSITY

DOCTORAL SCHOOL OF PHARMACEUTICAL AND PHARMACOLOGICAL SCIENCES

Doctoral (Ph.D.) theses

Application of circular dichroism (CD) spectroscopy for determination of pharmaceutical compounds. Analysis of stereoisomers by the HPLC-CD/UV method

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Budapest, 2002

Application of difference circular dichroism (CD) spectroscopy for determination of pharmaceutical compounds. Analysis of stereoisomers by the HPLC-CD/UV method

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Budapest, 2002
Programme: Modern trends in pharmaceutical scientific research

SUMMARY

Circular dichroism (CD) spectroscopy is gaining an increasing importance in pharmaceutical analysis. One of the main advantages of the CD spectroscopic method is its inherent selectivity, which provides the selective analysis of chiral molecules possessing optically active absorbance bands.

In addition to direct CD spectroscopic methods, several new procedures have been developed based on CD spectroscopy for the analysis and determination of pharmaceutical compounds in the past few years. One of the most progressive fields of chiroptical analysis is the combination of high performance liquid chromatographic separation and CD spectroscopy and the application of simultaneous dual CD/UV detection.

The aim of our research work was to work out new procedures based on CD and CD related techniques and to investigate the application of these methods in pharmaceutical analysis.

This work presents a new, selective and sensitive difference CD spectroscopic method based on oxime formation of the keto group for the determination of Δ^4-3-ketosteroids and 6-keto-morphinans.

Molar CD and UV spectral parameters of E and Z oxime isomers, produced in the course of oxime formation reaction, have been determined by HPLC separation and simultaneous dual CD and UV detection, without isolation of the isomers.

The stereochemistry of spirocyclic pyridopyridazine compounds has been investigated by simultaneous dual CD and UV spectroscopy following HPLC separation. The enantiomeric relationship of the pyridopyridazine stereoisomers has been proved by the application of ellipticity-absorbance ratio spectra in a novel way.

1. INTRODUCTION

One of the main characteristics of the biochemical reactions of the human body is their high degree of stereoselectivity. Therefore, the effects of exogenous substances depend greatly on their stereochemical structure.

The investigation of stereoselective effects of pharmaceutical compounds, that can be applied as therapeutics or diagnostics, is especially important. One part of the pharmaceutical studies deals with the mechanism of drug effects (pharmacodynamics) and the other investigates the fate of drugs in the human body (pharmacokinetics).

The stereochemical structure of the molecules may differ in their configuration or conformation, therefore the analysis of the effect of pharmaceutical compounds possessing contrasting configuration is an essential task in the majority of pharmaceutical research laboratories. One of the most important and progressive fields of pharmaceutical analysis is the determination of the quantity and ratio of enantiomers in bulk drug materials, pharmaceutical formulations and biological samples. Several publications deal with the separation of enantiomers and the determination of enantiomeric impurity.

Nowadays cis-trans or geometric isomers of pharmaceutical molecules are not as widely investigated as enantiomers, despite the fact, that there are a lot of examples proving the different biological actions of geometric isomers.

The present Ph.D. work deals with the application of CD spectroscopy and the simultaneous dual CD/UV detection following high performance liquid chromatographic separation, for the selective determination of pharmaceutical compounds as well as for the analysis of enantiomers and geometric isomers.

2. AIMS

The application of circular dichroism (CD) spectroscopy enables the selective analysis of chiral molecules possessing optically active absorbance bands. In addition to direct CD spectroscopic methods, a number of new procedures have been developed based on CD spectroscopy for the analysis and determination of pharmaceutical compounds in the past few years.

The aim of this Ph.D. work was to work out new procedures based on CD and CD related techniques and to investigate the application of these methods in pharmaceutical analysis.

The application of the difference CD spectroscopic method has been studied for two therapeutically important groups of drugs, Δ^4-3-ketosteroids and 6-keto-morphinans. The purpose of the application of the difference CD spectroscopic method based on oxime formation of keto groups was the elimination of the effects caused by other chiral compounds, which disturb the determination of the above mentioned drug compounds.

Our new difference CD spectroscopic method can be applied in the determination of Δ^4-3-ketosteroid contamination in steroid compounds which contain no keto groups.

The determination of 6-keto-morphinans is of great importance in the field of pharmaceutical and forensic drug analysis. Our purpose was the study of the application of the difference CD spectroscopic method for the determination of these compounds.

The simultaneous dual CD and UV detection following HPLC separation provided a possibility for the study of the different CD and UV spectral parameters of *E* and *Z* geometric isomers of oximes produced in the course of the oxime formation reaction. The characteristic molar CD and UV spectra of the oxime isomers were calculated in a novel way, without the isolation of the isomers.

The enantiomeric relationship of spirocyclic compounds has been proved by the HPLC-CD/UV technique. We have applied a new method based on the analysis of ellipticity-absorbance ratio spectra. The use of this method is very advantageous because the ellipticity-absorbance ratio value is independent of the concentration of the enantiomers.

3. METHODS AND INSTRUMENTS

3.1. CD SPECTROSCOPY

Chiroptical measurements were carried out on a Jasco J-720 type spectropolarimeter (Jasco Ltd., Japan).

CD spectra of the chiral compounds studied were recorded in wavelength mode. The ellipticities were measured in time mode for the monitoring of the oxime formation reaction of keto compounds. The discrete values of ellipticities were measured in time mode for the linearity studies.

Calculations were aided by the program package of MS Excel 97 and STATISTICA 5.0 for Windows.

3.2. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

The separation of the compounds examined were carried out in normal and reversed phase achiral HPLC system, as well as in normal phase chiral HPLC system. Chromatogram evaluation and data handling were done using the BORWIN 1.2 for Windows chromatographic software.

3.3. SIMULTANEOUS DUAL CD/UV SPECTROSCOPY

♦ HPLC-CD/UV METHOD

The Jasco J-720 type spectropolarimeter was connected with the HPLC unit. Ellipticities and absorbances were simultaneously detected in time mode following the high performance liquid chromatographic separations.

♦ RECORDING OF CD AND UV SPECTRA

CD and UV spectra were simultaneously recorded in the chromatogram peaks with stop-flow method in wavelength mode following HPLC separation (on-line detection). In some case the ellipticity and absorbance spectra were recorded following the semipreparative HPLC separation of the compounds examined (off-line detection).

3.4. ^{1}H-NMR SPECTROSCOPY

The ^{1}H-NMR spectra were recorded on a Bruker AM 200 spectrometer in the Department of Organic Chemistry of Semmelweis University.

4. SCIENTIFIC RESULTS

4.1. DETERMINATION OF Δ^4-3-KETOSTEROIDS AND 6-KETO-MORPHINANS BY DIFFERENCE CD SPECTROSCOPY

A new difference CD spectroscopic method have been worked out, based on oxime formation, for the selective determination of Δ^4-3-ketosteroids (levonorgestrel, levonorgestrel acetate, norethisterone, methyltestosterone, testosterone phenylpropionate, nortestosterone phenylpropionate) [1] and 6-keto-morphinans (oxycodone, hydrocodone, 14-hydroxy-codeinone) [4]. The method is based on the fact, that CD spectra of the keto compounds examined and their oxime derivatives are quite different. Optimum conditions of the oxime formation reaction have been determined, the oxime formation was monitored via CD spectroscopic as well as reversed phase HPLC method. Furthermore, we have studied the linearity of the difference ellipticities, measured before and after the oxime formation, with the concentration of keto compounds. The applicability of the difference CD spectroscopic method was described by statistical parameters.

The difference CD spectroscopic method has been applied for the determination of levonorgestrel acetate contamination of norgestimate (the oxime derivatives of levonorgestrel acetate) samples. The results demonstrates that 0,02-0,1 % Δ^4-3-ketosteroid contamination in norgestimate can be determined with required accuracy by difference CD spectroscopic method described above [1].

4.2. STUDY OF OXIME-ISOMERS BY THE HPLC-CD/UV METHOD

The reaction of keto compounds examined with hydroxylamine leads to the formation of *Z* and *E* oxime isomers (***Figure 1***.)

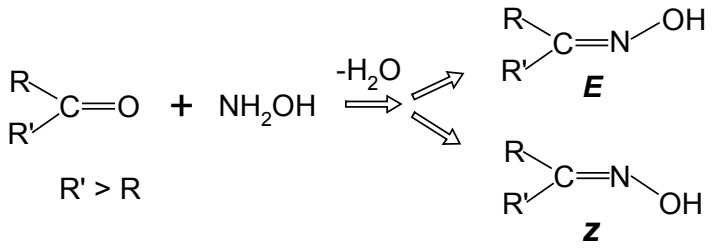

Figure 1.: *The formation of E and Z oxime isomers*

The presence of both isomers has been proved in the case of oxime derivatives of Δ^4-3-ketosteroids. The oxime isomers have been separated in achiral, normal phase HPLC system applying simultaneous dual CD and UV spectroscopic detection. CD and UV spectra of the oxime isomers were recorded in the chromatographic peaks by the HPLC-CD/UV method, without the preparative separation of the isomers. The identification (elution order assignation) and determination of the formation ratio of the isomers have been performed by ^{1}H NMR spectroscopy on the basis of the chemical shift differences of C_4-H signals. Characteristic CD and UV spectral properties of *Z* and *E* oxime isomers were calculated from the spectra of the mixed and the pure isomers, as well as the isomeric ratio, by parameter estimation method. The above calculation is based on the fact that ellipticities and absorbances measured for the mixed isomers (Θ_m, A_m) must be the sum of those of the two pure isomers:

$$\Theta_v = n \times \Theta_E + m \times \Theta_Z$$

$$A_v = n \times A_E + m \times A_Z$$

where Θ_E, Θ_Z and A_E, A_Z are the ellipticitiy and absorbance values of the on-line HPLC-CD and UV spectra of the isomers at the same wavelength, n and m are fitted parameters. Molar CD and UV spectra of standard levonorgestrel oxime isomers was in good agreement with the spectra calculated by the parameter estimation method [2].

The oxime derivatives of 6-keto-morphinans have been investigated by ^{1}H NMR spectroscopy and HPLC method. On the basis of the HPLC analysis of oximes, in the case of saturated derivatives only one of the isomers has been formed, while for 14-hydroxy-codeinone two chromatographic peaks have been detected. The identification of the oxime isomers has been performed by ^{1}H NMR spectroscopy on the basis of the chemical shift differences of C_5-H signals. On the results of the ^{1}H-NMR spectroscopic analysis, *E* isomer of oxycodone and hydrocodone oxime has been formed, while this method also proved the presence of both 14-hydroxy-codeinone oxime isomers.

4.3. ANALYSIS OF SPIROCYCLIC VINYL- AND PYRIDOPYRIDAZINE STEREOISOMERS

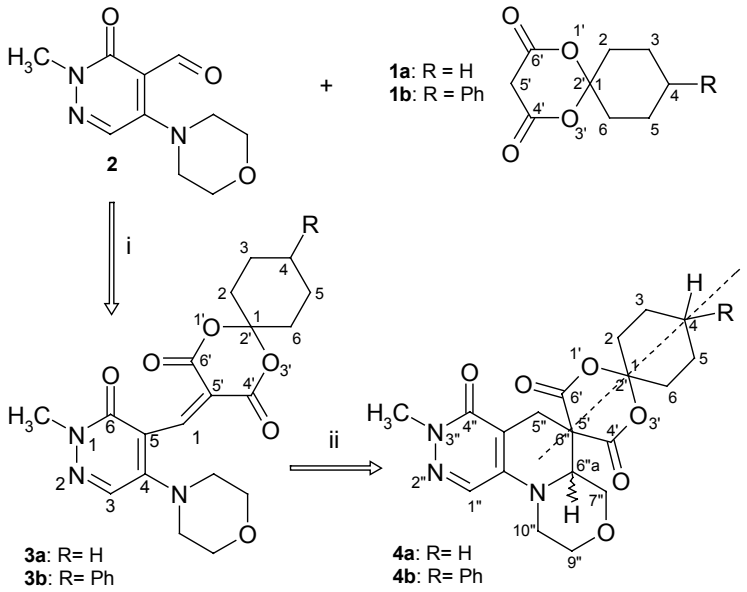

Figure 2.: Synthesis of pyridopyiridazines; i: ethanol, piperidine, 25 °C, 2.5 h, ii.: DMF, 100 °C, 6 h

The stereochemistry of spirocyclic pyridopyridazine compounds, prepared in the Department of Organic Chemistry of Semmelweis University, have been investigated by the HPLV-CD/UV method [3]. The synthesis of pyridopyridazines (***Figure 2.***) could be achieved in two steps, starting from pyridazinecarbaldehyde (**2**) and cyclic malonates (**1a, 1b**). Thus, in the first step, Knoevenagel condensation reaction of the aldehyde with the malonate afforded the corresponding vinylic derivatives **3a and 3b**, which underwent rearrangement smoothly upon heating to give pyridopyridazine derivatives **4a** and **4b**, respectively.

In the case of **3b** vinylpyridazine derivatives, the cyclohexane carbon atom bearing the phenyl group (*i.e.* the C-4 atom) together with the vinylic C-1 atom serve as 'olefinic' bridgehead atoms, thereby, due to their substitution pattern, rendering the existence of two geometric isomers possible (*cis*- or *trans* arrangement of the phenyl and pyridazinyl groups). In the ^{1}H NMR spectrum of **3b** several signals appear in two sets with the intensity ratio of 55:45 indicating that two isomers are present. The normal phase HPLC analysis of **5b** did also prove the presence of two isomers. In an achiral system, two peaks with a ratio of 54:46 could be observed.

Compound **4a** has central chirality (C-6"a) and accordingly, two enantiomers. On the other hand, compound **4b** has two stereogenic elements, an asymmetric carbon (C-6"a), and a chiral axis passing through the two spiro centers C-1,2' and C-5',6". Accordingly, **4b** may possess four stereoisomers which form two diasteromers, *i.e.* two enantiomeric pairs designated as 6"a**R**,**M**; 6"a**S**,**P**; and 6"a**R**,**P**; 6"a**S**,**M**, respectively. All four stereoisomers of **4b** could be detected by HPLC analysis on a chiral normal phase system.

Since in situ CD spectra of the peaks detected by chiral HPLC, were unsuitable for further evaluation, a semipreparative HPLC was next carried out, than CD and UV spectra of the pure isomers were recorded. The maximum and minimum values of the Cotton effect and the wavelength of zero ellipticity suggested the presence of two enantiomeric pairs.

The stereochemical relation of the components could unambiguously be proved by the application of our new method, on the basis of the analysis of ellipticity-absorbance ratio spectra (shorted ratio spectra). For a pair of enantiomers, the absolute value of the ratio, usually measured at one wavelength, is equal, thereby it may be used for identification of their relationship. For providing an even more reliable assignment of enantiomeric relationship, in our new strategy, we compared the whole ratio spectra of two isomers. The extreme values of ratio spectra of all four isomers fully proved their stereochemical relationships, and allowed us to come to the conclusion, that isomers eluting from peaks 1, 4 and peaks 2, 3, respectively, must be enantiomeric pairs. The application of this method is very advantageous because the ellipticity-absorbance ratio value is independent from the concentration of the enantiomers.

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ACKNOWLEDGEMENTS

Thanks are due to:

Dr. András Gergely, C.Sc.,

Professor Béla Noszál, D.Sc., director of Department of Pharmaceutical Chemistry

Professor György Szász, D.Sc., former director of Department of Pharmaceutical Chemistry

Dr. Péter Horváth, Ph.D.,

Professor Péter Mátyus, D.Sc., director of Department of Organic Chemistry

Dr. Gyula Beke, Ph.D.,

Ilona Matalin-Foór,

Ilona Kovács-Derzsi