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Analysis of optically active compounds using conventional chromatography with a circular dichroism detector

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Abstract

Analysis of optically active compounds in complex samples is often based on chiral chromatography or capillary electrophoresis in order to separate the enantiomers. This requires a chiral reagent, when using conventional chromatography, or an expensive chiral column, or a chiral selector, when using capillary electrophoresis. The type of column, reagent, or additive depends highly on the compound to be analysed. A simple and generally applicable method is using a conventional HPLC column coupled to a CD detector. Separation of enantiomers is not required, as they can be identified by a positive or negative peak. A racemate does not produce a peak; neither does an optically inactive compound. The application of HPLC-CD for the identification of pharmacologically active compounds, such as dexamphetamine, 5-hydroxytryptophan, (−)-huperzine A, and interferon, as standards, in registered drugs, in falsifications, and in food supplements is described.

Keywords: Reversed-phase chromatography; Circular dichroism; Optically active compounds; Pharmacologically active compounds

1. Introduction

For the analysis of enantiomers chiroptical methods are required. In the European Pharmacopoeia [1] four methods are described for the analysis of raw materials: optical rotation, chiral chromatography, circular dichroism, and capillary electrophoresis. For the identification in complex samples separation of the enantiomers is often required and can be achieved by using chiral chromatography or capillary electrophoresis [2]. When using conventional columns derivatisation with chiral reagents preceding chromatography, or addition of a chiral substance to the mobile phase is required. Instead of conventional columns expensive chiral columns can be used. The application of capillary electrophoresis requires a chiral selector. These methods are not generally applicable, because the type of chiral column, the reagent, or the additive to be used depends highly on the compound to be analysed. This is a major

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disadvantage, besides the fact that derivatisation is labour and time intensive and chiral columns are expensive.

A simple and generally applicable method for compounds with an UV chromophore is using a conventional HPLC column coupled to a CD detector [3–7]. CD is based on absorption differences between right and left circularly polarised lights by the individual enantiomers [1]. Therefore, separation of the enantiomers is not required, as they can be identified by a positive or negative signal. A racemate does not produce a signal, neither does an optically inactive compound. The CD spectrum is a function of $\Delta\varepsilon$ (ε = molar extinction coefficient) or ΔA (extinction) versus λ (wavelength) and can be dependent of the medium used, just as UV absorption can be influenced by the solvent. However, an UV signal at a given wavelength is always positive, while a CD signal can be positive or negative, depending on the wavelength and the medium, a phenomenon that is also observed for optical rotation.

After the introduction of the first commercially available CD detector for online HPLC-CD [4] some articles were published describing the application of achiral HPLC-CD for the analysis of pharmacologically active compounds, as standard [5], in plasma [6], and in a plant extract [7]. In other articles the application of chiral HPLC-CD is described [8–10]. In our laboratory we used achiral HPLC-CD for the analysis of L-5-hydroxytryptophan (L-5-HTP), D-5-hydroxytryptophan (D-5-HTP), and D,L-5-hydroxytryptophan (D,L-5-HTP) standards, for analysis of interferon α-2a (INF-α-2a) and α-2b (INF-α-2b) in solutions for injection, for identification of amphetamine/dexamphetamine in a falsification of a registered drug, and for the identification of huperzine A in a herbal medicine.

2. Experimental

2.1. Chemicals

Solvents for chromatography were HPLC grade, except for water, which was demineralised. Salts and acids for preparing buffers were analytical grade chemicals.

Phosphate buffered saline, pH 7.4 (PBS), consisted of sodium chloride (final concentration 0.14 M), potassium chloride (2.9 mM), potassium dihydrogen phosphate (1.5 mM), and disodium hydrogen phosphate dihydrate (8.1 mM).

Phosphate buffer (pH 2.3) consisted of sodium dihydrogen phosphate dihydrate and 85% *ortho*-phosphoric acid, final concentrations of 14.7 and 7.5 mM, respectively.

Phosphate buffer (pH 3.7) consisted of 50 mM sodium dihydrogen phosphate monohydrate, set at pH 3.7 using 10% *ortho*-phosphoric acid.

Ammonium acetate buffer (pH 6.0) consisted of 80 mM ammonium acetate.

D-5-HTP, L-5-HTP, and D,L-5-HTP (for all: > 99% purity on TLC) were purchased from Sigma Chemical Co., St. Louis, USA.

INF-α-2a and INF-α-2b standard solutions (chemical reference substances) and samples (one sample 'powder and solvent for solution for injection' **1** and two samples 'solutions for injection' **2** and **3**) were supplied by the European Directorate for the Quality of Medicines (EDQM). The supplied standard solutions were approximately 2 mg/ml for INF-α-2a and approximately 7.5 mg/ml for INF-α-2b in PBS.

(d,l)-Amphetamine sulphate was purchased from Merck AG, Darmstadt, Germany; D-(+)-dexamphetamine sulphate was purchased from Interpharm B.V., Den Bosch, The Netherlands; yohimbine·HCl was purchased from OPG Groothandel B.V., Utrecht, The Netherlands (all of pharmacopeial quality). A Viagra falsification (**4**) was obtained from the Netherlands Forensic Institute.

(−)-Huperzine A (> 99% purity on TLC; water content 8.7%) was purchased from Sigma Chemical Co., St. Louis, USA. A commercially available food supplement (**5**), capsules, which according to the label contain *Huperzia serrata* extract, *Gingko biloba* extract, vitamins E, B1, B11, and B12, fish oil, lecithin, and coenzyme Q10, was sent in for analysis by the Inspectorate for Health Protection and Veterinary Public Health.

2.2. Standards, standard solutions and sample preparation

2.2.1. 5-Hydroxytryptophan

Standards of D-5-HTP and L-5-HTP were dissolved in phosphate buffer (pH 2.3) to concentrations of 71.5 and 73.3 μg/ml, respectively. From these solutions a series of mixtures of D- and L-5-HTP in different ratios varying from only L-5-HTP, via L-5-HTP and D-5-HTP (1:1), to only D-5-HTP was prepared; the overall concentration of 5-HTP in this series varies slightly from 73.3 to 71.5 μg/ml. D,L-5-HTP was dissolved in phosphate buffer (pH 2.3) to a concentration of 78.6 μg/ml.

2.2.2. Interferon

Standards INF-α-2a and 2b were diluted with PBS to concentrations of 5, 15, 30, 60, 80, and 100 μg/ml. Sample **1** was reconstituted according to the instructions for use. The obtained solution for injection and samples **2** and **3** were analysed without further sample handling.

2.2.3. Amphetamine

Two mixtures of standards in acetonitrile–water (25:75, v/v) were prepared, one with final concentrations of 2.0 mg/ml (d,l)-amphetamine sulphate and 0.08 mg/ml yohimbine·HCl, the other with final concentrations of 2.0 mg/ml D-(+)-dexamphetamine sulphate and 0.08 mg/ml yohimbine·HCl. Two tablets of sample **4** were powdered and a sample solution of approximately 15 mg/ml in acetonitrile–water (25:75, v/v) was prepared by shaking for 10 min.

2.2.4. Huperzine A

(−)-Huperzine A was dissolved in methanol and diluted to concentrations of approximately 8, 16, and 32 μg/ml. A standard solution of 13 μg/ml was used to determine the repeatability. The content of five capsules of **5** was dissolved in 10 ml of methanol using an ultrasonic bath for 5 min and shaking for 5 min.

2.3. Instrumentation

HPLC-CD experiments were performed using the following system: Jasco AS-950 Intelligent Sampler, Jasco PU-980 Intelligent HPLC Pump, Jasco LG-980-02 Ternary Gradient Unit, Alltech On-Line Degassing System, Spectra Physics SP8790 Column Oven, and Jasco CD-1595 detector (CD and UV signal). The system was controlled by BORWIN software version 1.5. In some cases a separate Jasco UV-975 Intelligent UV/Vis Detector was used in line with the CD detector. All samples and solutions were filtered before use over 0.45 μm filter.

2.4. Chromatographic conditions

2.4.1. 5-Hydroxytryptophan

The HPLC-CD system described above was used, without the separate UV/Vis detector. Chromatographic conditions were: a Lichrospher RP 18e column (250 × 4.0 mm, 5 μm); isocratic mobile phase of methanol–phosphate buffer (pH 2.3) (15:85, v/v); flow rate of 1.0 ml/min; column temperature at 25 °C; injection volume of 20 μl; CD and UV detection at 234 nm. CD and UV spectra were recorded using stop-flow scanning from 220 to 420 nm.

2.4.2. Interferon

The HPLC-CD system described above was used, including the separate UV/Vis detector. Chromatographic conditions were based on the monograph '*Interferon Alfa-2 Concentrated Solution*' [11], modified as described by Buchheit et al. [12]. A Vydac 218TP54 C_{18} column (250 × 4.6 mm, 5 μm, 300 Å) was used; UV detection was carried out at 214 nm, CD detection at 220 nm.

2.4.3. Amphetamine

The HPLC-CD system described above was used, without the separate UV/Vis detector. Chromatographic conditions were: a Symmetry C_8 column (250 × 4.6 mm, 3 μm); mobile phases acetonitrile and phosphate buffer (pH 3.7); gradient elution, starting at 10% acetonitrile, ramping in 10 min to 30% acetonitrile, and holding for 20 min; flow rate of 1.0 ml/min; column temperature at 25 °C; injection volume of 10 μl; UV and CD detection at 258 nm.

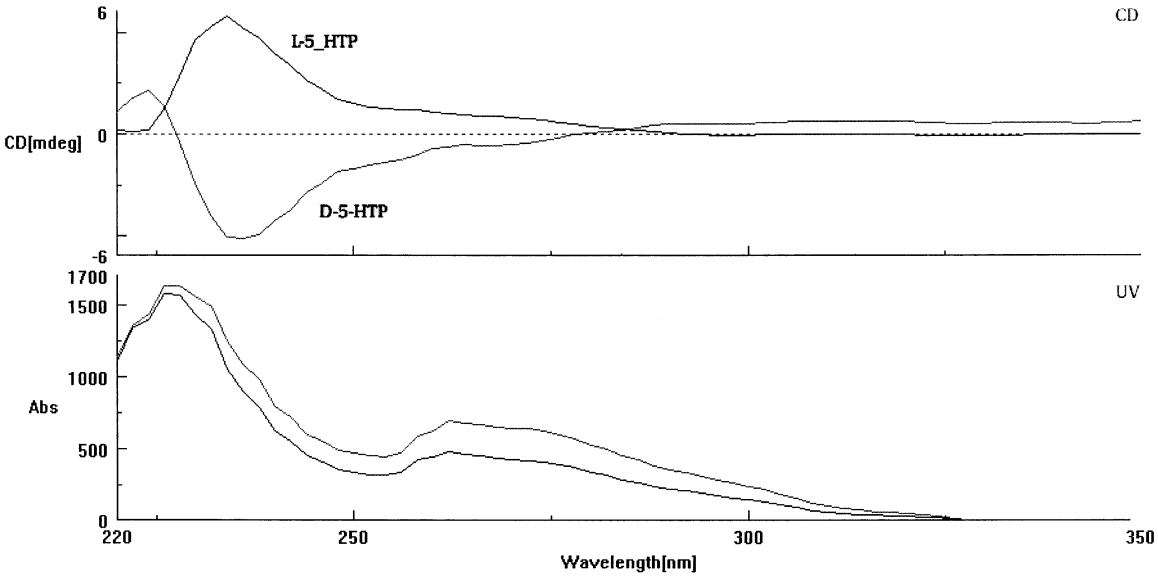

Fig. 1. CD and UV spectra of L-5-HTP and D-5-HTP (CD detector only).

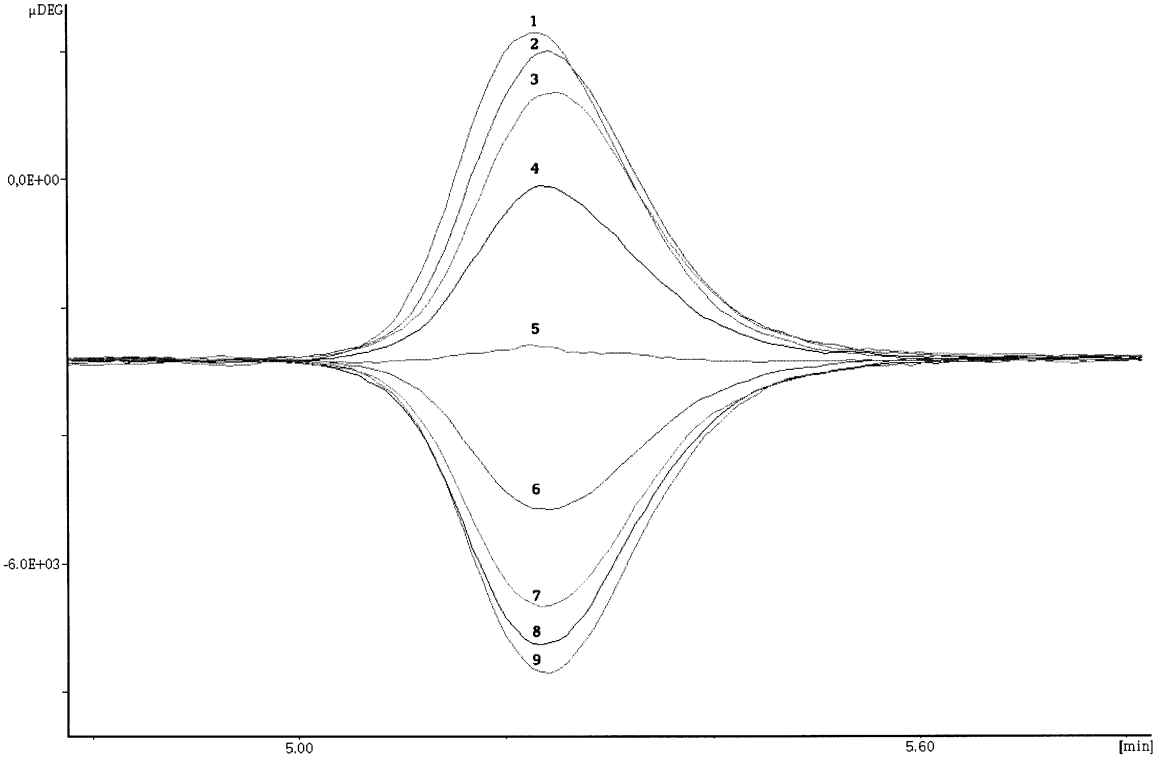

Fig. 2. CD chromatograms of mixtures of L-5-HTP (73.3 µg/ml) and D-5-HTP (71.5 µg/ml. 1: 100% L+0% D; 2: 96% L+4% D; 3: 90% L+10% D; 4: 75% L+25% D; 5: 50% L+50% D; 6: 25% L+75% D; 7: 10% L+90% D; 8: 4% L+96% D; 9: 0% L+100% D.

2.4.4. Huperzine A

The HPLC-CD system described above was used, including the separate UV/Vis detector. Chromatographic conditions were: a Alltima C_{18} column (250×4.0 mm, 5 μm); mobile phase methanol–ammonium acetate (pH 6.0; 80 mM) (27:73, v/v); after 23 min the column was washed for 5.5 min using methanol–ammonium acetate (pH 6.0; 80 mM) (75:25, v/v); flow rate of 1.25 ml/min; column temperature at 25 °C; injection volume of 20 μl; UV and CD detection at 308 nm.

3. Results and discussion

3.1. 5-Hydroxytryptophan

CD and UV spectra of the standard solutions of D- and L-5-HTP were obtained (see Fig. 1), showing the difference between the two enantiomers when using CD. As the CD maximum was determined at 234 nm, further experiments were carried out using UV and CD detection at this particular wavelength.

CD chromatograms from the series of mixtures of D- and L-5-HTP in different ratios are plotted in one figure (Fig. 2). Decrease of the CD signal and shifting of the polarity is observed due to the decrease of concentration of one of the enantiomers and increase of concentration of the other one. The UV signal is only slightly influenced, because it depends on the total concentration of 5-HTP, which in this particular case varies from 71.5 to 73.3 μg/ml.

A curve of the CD/UV peak area ratio ($\Delta A/A$) versus the enantiomeric purity (e.p.) is linear ($\Delta A/A = 6.4 \times 10^{-5}$ e.p. $+ 4.2 \times 10^{-5}$; $R^2 = 0.9998$) as is shown in Fig. 3. E.p. is defined here as {[L]-[D]}/{[L]+[D]} × 100%, therefore, e.p. = 100% corresponds to 100% L-5-HTP and e.p. = −100% to 100% D-5-HTP. As $\Delta A/A$ is independent of the concentration the enantiomeric purity of a sample can be determined based on this curve, presumed that the UV signal in not influenced by other compounds. HPLC-UV-CD analysis of 78.6 μg/ml

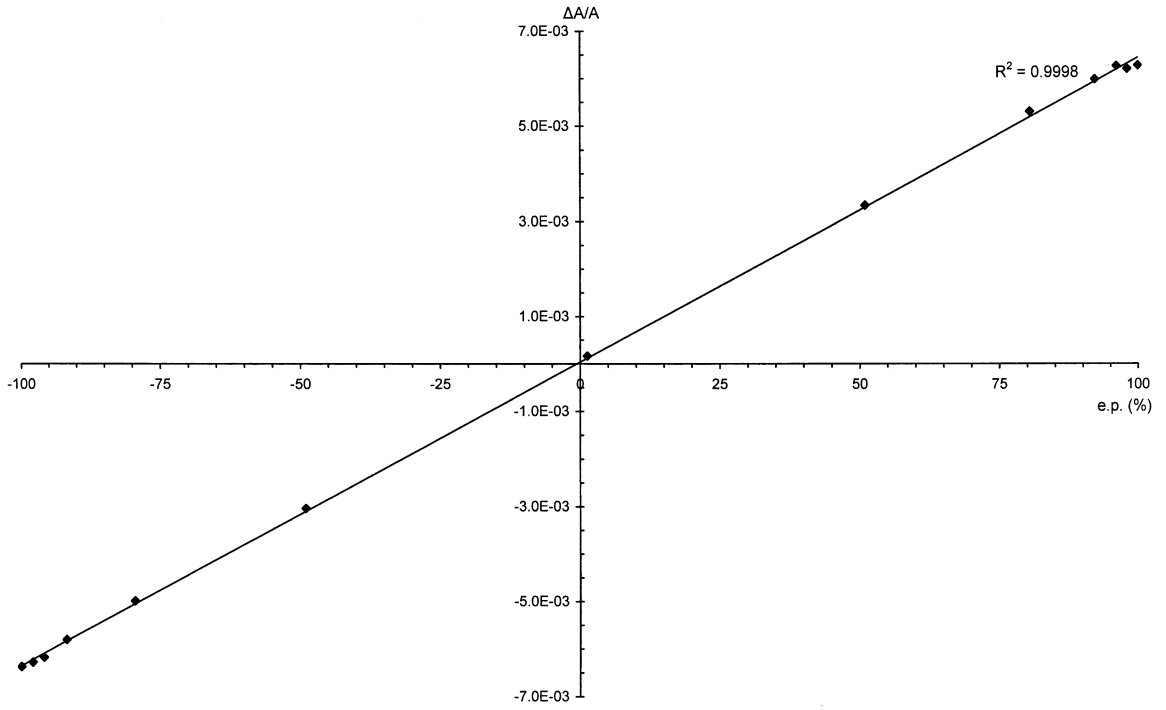

Fig. 3. Curve of $\Delta A/A$ vs. enantiomeric purity (e.p.); $x = 100$ corresponds to 100% L-5-HTP, $x = -100$ to 100% D-5-HTP.

of D,L-5-HTP resulted in a UV peak area of 943519 μAU and a CD peak area of 150 μDEG. Based on Fig. 3 the e.p. of D,L-5-HTP is 2%, therefore, it contains 51% L- and 49% D-5-HTP.

3.2. Interferon

Both INF-α-2a and INF-α-2b resulted in a negative CD signal. The CD signal was linear over the range of 5 to 100 μg/ml (INF-α-2a: $Y = -3261.5X + 15\,281$, $R^2 = 0.9957$; INF-α-2b: $Y = -12\,476X + 49\,842$, $R^2 = 0.9985$; different ranges on the detector were used). The LOD was estimated to be 5 μg/ml for INF-α-2a and 2.5 μg/ml for INF-α-2b. As an example the UV and CD chromatogram of sample **2** containing approximately 35 μg/ml INF-α-2a is presented in Fig. 4.

3.3. Amphetamine

In Fig. 5 the CD chromatograms of the mixtures of (d,l)-amphetamine sulphate/yohimbine·HCl, D-(+)-dexamphetamine sulphate/yohimbine·HCl, and of sample **4** are presented. Yohimbine and D-(+)-dexamphetamine are detected, however, the racemate (d,l)-amphetamine is not. Therefore, in sample **4** the presence of D-(+)-dexamphetamine together with yohimbine is demonstrated.

3.4. Huperzine A

The UV spectrum of (−)-huperzine A was recorded showing a maximum at 230 nm and a local maximum at 308 nm. The CD signal at 308 nm was negative. As interference of a coeluting compound in sample **5** was observed in the UV chromatogram qualitative and quantitative analy-

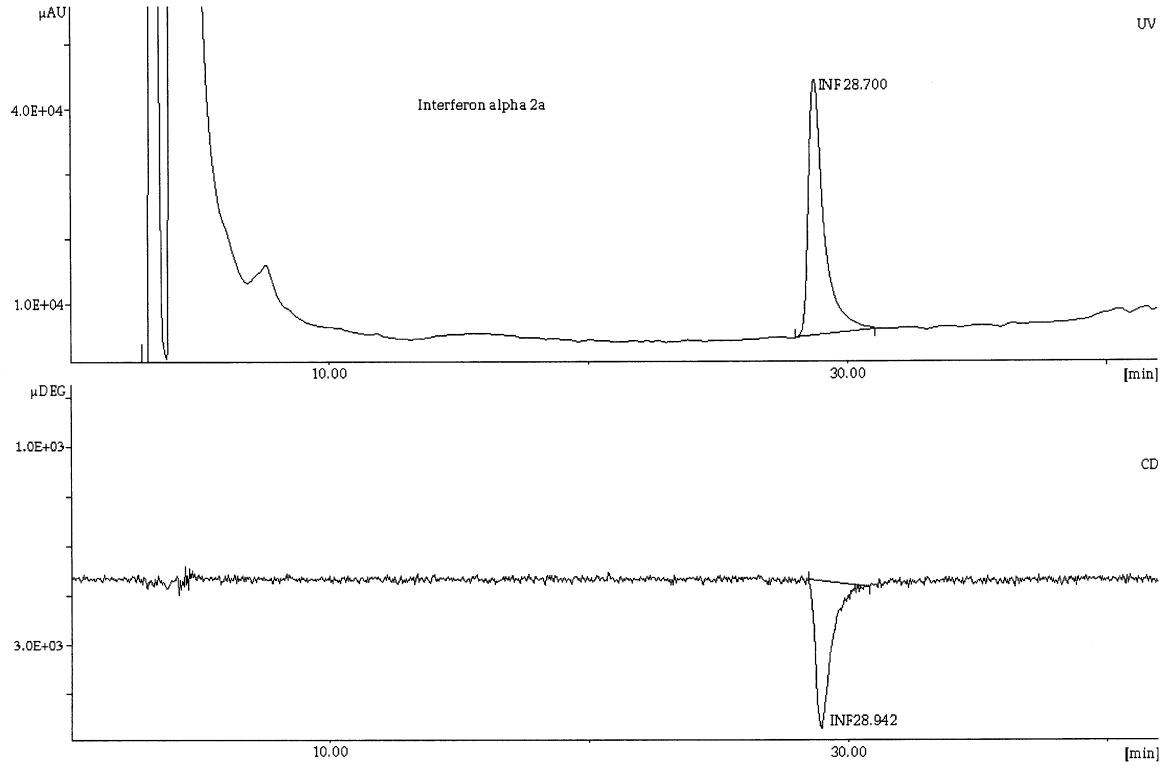

Fig. 4. UV and CD chromatogram of sample **2** containing approximately 35 μg/ml INF-α-2a (separate CD and UV detectors).

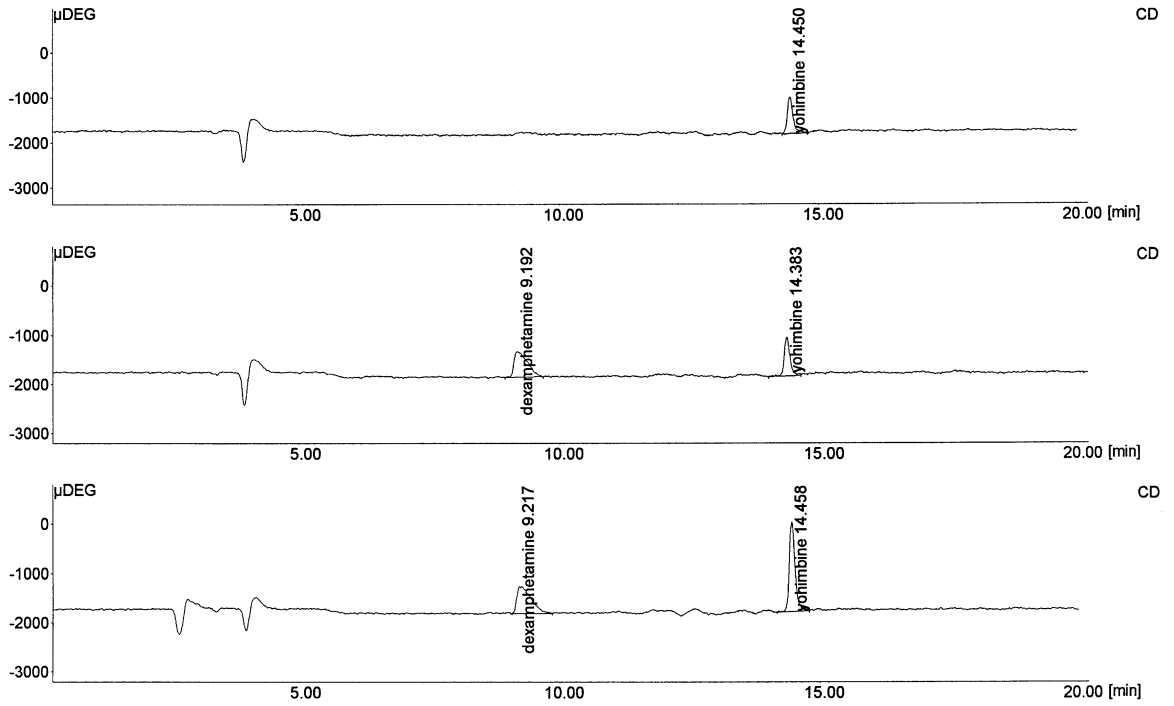

Fig. 5. CD chromatograms; upper trace: mixture of (d,l)-amphetamine sulphate and yohimbine·HCl; middle trace: mixture of D-(+)-dexamphetamine sulphate and yohimbine·HCl; lower trace: sample **4**, a Viagra falsification.

sis was carried out based on the CD chromatogram at 308 nm in which interference was not observed (see Fig. 6). The calibration curve was linear over the range from 7 to 32 µg/ml ($Y = 1\,420\,355X + 535.7$; $R^2 = 0.9997$). The repeatability of the peak area was determined (RSD = 2.4%; $n = 5$). The LOD in standard solution and sample was determined to be 1.17 µg/ml and 3.08 µg/capsule, respectively. Sample **5** contained 22 µg (−)-huperzine A/capsule.

4. Conclusions

The applicability of HPLC-CD to a variety of samples, such as pharmacologically active, low and high molecular weight standards, drug formulations, and food supplements is demonstrated.

Distinction between a racemate and the presence of an excess of one of the enantiomers can simply be made by CD detection. To distinguish between the one and the other enantiomer the availability of one of the enantiomers as a standard is required. Based on the difference between the concentration calculated from the UV peak area and the one calculated from the CD peak area the e.p. can even be estimated. When both enantiomers are available as standards the e.p. of a sample can be calculated from the curve of the CD/UV peak area ratio versus the e.p. generated from analyses of the standards; the total concentration of both enantiomers can be calculated from the UV peak area.

HPLC-CD has a higher selectivity than HPLC-UV, due to the fact that only chiral compounds are detected using CD detection. This also implies that less interferences will occur and therefore HPLC-CD can be a good alternative to HPLC-UV when interference of matrix compounds is observed in the UV chromatogram. However, it should be noted that HPLC-CD is less sensitive than HPLC-UV, because CD detection is based on absorption differences between right and left circularly polarised lights.

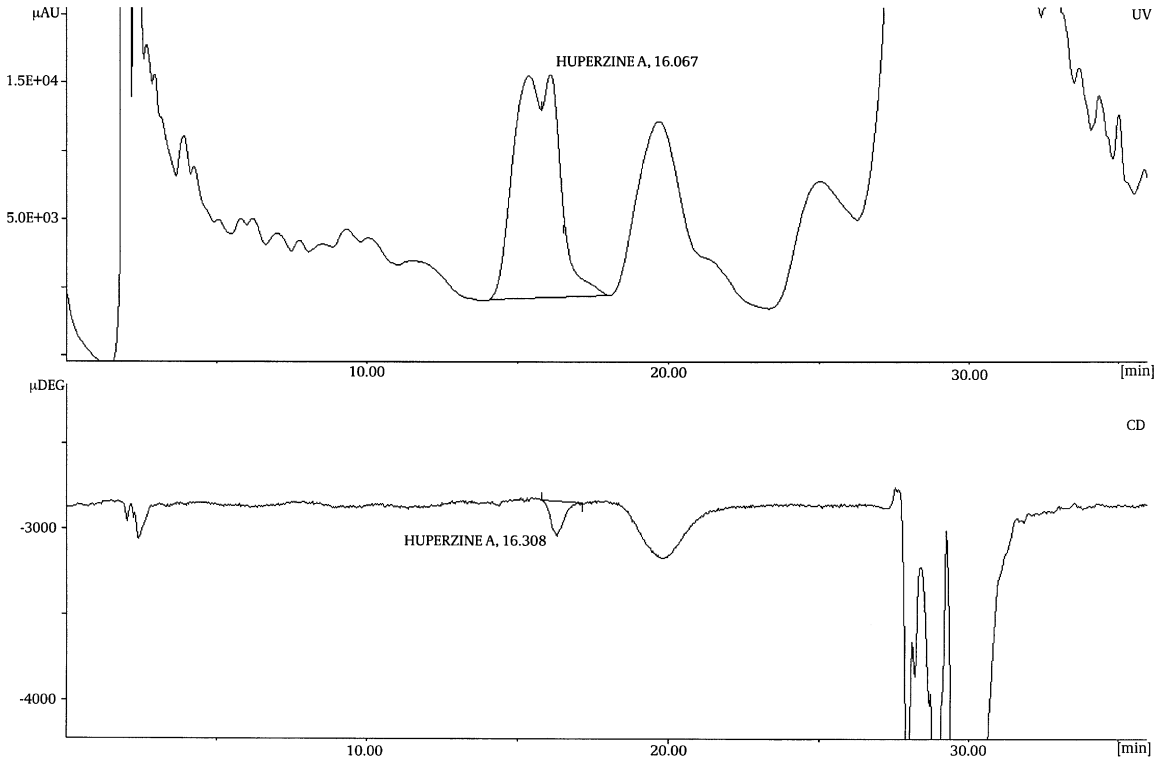

Fig. 6. UV and CD chromatogram of sample **5**, a food supplement, containing (−)-huperzine A (separate CD and UV detectors).

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