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ANALYTICAL AND PREPARATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF THE ENANTIOMERS OF IFOSFAMIDE, CYCLOPHOSPHAMIDE AND TROFOSFAMIDE AND THEIR DETERMINATION IN PLASMA

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SUMMARY

A high-performance liquid chromatography chiral stationary phase (HPLC-CSP) based upon cellulose-tris(3,5-dimethylphenylcarbamate), the OD-CSP, was used to stereochemically resolve three racemic anticancer drugs: ifosfamide (IFF), cyclophosphamide (CTX) and trofosfamide (TFF). The observed enantioselectivities (α) were 1.45 (IFF), 1.21 (CTX) and 1.10 (TFF). The OD-CSP was also used on a preparative scale to isolate the enantiomers of IFF with an optical purity greater than 97%. In addition, an analytical assay was developed for the determination of the enantiomeric composition of IFF and CTX in plasma using achiral–chiral coupled column chromatography. A racemic form of another CSP, the D,L-naphthylalanine-CSP, was used as the achiral pre-column. The sample clean-up prior to HPLC analysis was realized by liquid–liquid extraction with chloroform. The correlation coefficient was 0.987 for both standard curves (20–130 μg/ml) and the controls were within 10% of the real value. The analysis time for each serum sample was less than 30 min.

INTRODUCTION

Ifosfamide (IFF), cyclophosphamide (CTX) and trofosfamide (TFF) are three oxazaphosphorine nitrogen mustard analogues used as anticancer drugs (Fig. 1). All three molecules contain an asymmetrically substituted phosphorus atom and exist in two enantiomeric forms: (+)-(*R*)- and (−)-(*S*)-IFF, (+)-(*R*)- and (−)-(*S*)-CTX, and (−)-(*R*) and (+)-(*S*)-TFF.

These drugs are administered as racemic mixtures and it is possible that there are pharmacodynamic and pharmacokinetic differences within each set

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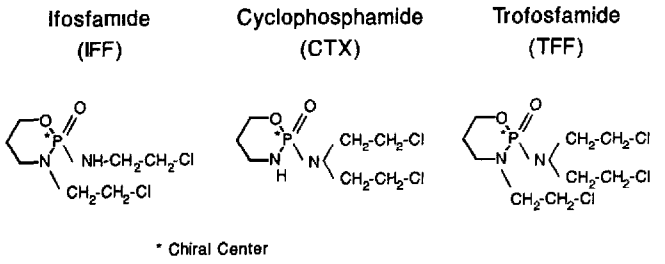

Fig. 1. Chemical structures of the compounds used in this study.

of enantiomers. For example, initial data suggest that in some pediatric patients (*S*)-IFF and (*R*)-IFF differ in their pharmacokinetic disposition [1].

The study of the stereochemical aspects of chiral drugs demands quantities of each isomer for in vitro and in vivo metabolic, pharmacokinetic, toxicity and efficacy studies. The required isomers of CTX, IFF and TFF can be prepared using reported chiral synthetic methods [2,3]. However, since these compounds are currently commercially produced as racemic mixtures, it is more feasible from an economic and time point of view to prepare small amounts of the enantiomerically pure drugs by chromatographic methods.

Blaschke and Maibaum [4] have reported a direct stereochemical resolution of the enantiomers of IFF on a high-performance liquid chromatography (HPLC) column composed of a polyacrylamide chiral stationary phase. The mobile phase was composed of toluene and dioxane and the elution volume was 500 ml. Under these chromatographic conditions, CTX was slightly resolved and TFF was not resolved at all.

In addition to the preparation of the pure enantiomers, the pharmacological study of a chiral drug also requires analytical methods capable of the quantitation in biological fluids of each isomer after the administration of the racemic mixture. Enantioselective assays for CTX and IFF have been reported [5–7].

A specific assay for the determination of CTX enantiomers in plasma has been recently reported [5,6]. The method utilizes the conversion of the CTX enantiomers into diastereomers using chiral derivatizing agents. The diastereomeric derivatives are then resolved on an achiral HPLC system. The assay is able to measure plasma concentrations between 1 and 50 μg/ml. However, the process is quite lengthy requiring two derivatizations and two extractions prior to HPLC analysis. In addition, the resolutions of IFF and TFF were not reported.

The direct separation of the isomers of IFF by enantioselective gas chromatography (GC) using a chiral-phase capillary has been reported [1,7]. The method can be used to quantitate serum levels of IFF as low as 0.5 μg/ml and has been used in some initial pharmacokinetic studies of the enantiomers of IFF [1]. However, this approach can not be used for CTX or TFF.

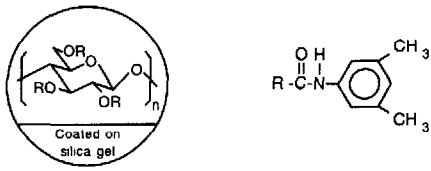

Fig. 2. Structure of the OD-CSP

We have studied the application of an HPLC chiral stationary phase (CSP) based upon cellulose-tris(3,5-dimethylphenylcarbamate), the OD-CSP (Fig. 2) to the stereochemical resolution of CTX, IFF and TFF. The enantiomers of each of the three drugs were resolved on the OD-CSP using a mobile phase composed of hexane and isopropanol or hexane and 2-methyl-1-propanol. An elution volume of less than 20 ml and solvents which allow for UV detection at 210 nm make this approach suitable for analytical determinations as well as for the preparative isolation of the isomers.

For the analytical determinations, an achiral–chiral coupled column approach was used. In this system, the OD-CSP was coupled to an achiral precolumn based on D,L-naphthylalanine. The achiral phase was used to separate either (*R,S*)-IFF (*R,S*)-CTX or (*R,S*)-TFF from the serum components and to quantitate the total drug concentration. The eluent containing the enantiomers was then selectively transferred to the OD-CSP, where the (*R*)- and (*S*)-isomers were stereochemically resolved and the enantiomeric composition determined. This system is accurate, rapid and can be automated for use in large-scale clinical studies.

EXPERIMENTAL

Analytical application: apparatus

The system used for the achiral–chiral coupled column chromatography has been described elsewhere [8]. In general, the process involves two chromatographic systems connected through a Rheodyne Model 7010 switching valve (Rainin, Woburn, MA, U.S.A.) equipped with a 1-ml sample loop. When the eluent fraction from the achiral column containing the analyte was detected, the switching valve was rotated and the eluent flow diverted to the sample loop on the chiral column. After 60 s, the switching valve was rotated again and the eluent containing the analyte was injected onto the chiral column.

Achiral chromatography. The achiral chromatography was performed with a modular liquid chromatograph composed of a Beckman 110 B solvent delivery module pump, a Beckman 166 Gold variable-wavelength UV detector set at 210 nm (Beckman, Houston, TX, U.S.A.), a Shimadzu C-R6A integrator (Shimadzu, Columbia, MD, U.S.A.) and a Rheodyne 7125 injection valve. The injection loop was replaced by a silica guard column (30 mm×2.1 mm I.D.,

Rainin). The column used was a covalent D,L-naphthylalanine column (25 cm × 4.6 mm I.D.; Regis, Morton Grove, IL U.S.A.).

Enantioselective chromatography. The enantioselective chromatography was performed with a similar modular liquid chromatograph. The HPLC chiral stationary phase was cellulose-tris(3,5-dimethylphenylcarbamate) coated on macroporous silica, the Chiralcel OD column (25 cm × 4.6 mm I.D.; J.T. Baker, Phillipsburg, NJ, U.S.A.).

Preparative application: apparatus

The modular liquid chromatograph used for the preparative isolation of the isomers was the same as the one used for the analytical application. The stationary phase was cellulose-tris(3,5-dimethylphenylcarbamate) coated on macroporous silica, the Chiralcel OD column, packed in a semi-preparative column (25 cm × 10 mm I.D.; J.T. Baker). The system was equipped with a Varian 8000 autosampler (Varian, Walnut Creek, CA, U.S.A.) with 200-μl loop and the eluent fractions were collected using a Foxy programmable fraction collector (Isco, Lincoln, NE, U.S.A.).

Chemicals

Racemic IFF was a gift from Bristol-Myers (Evansville, IN, U.S.A.). Racemic TFF was purchased from Asta-Werke (Bielefeld, F.R.G.) and racemic CTX was the commercial lyophilized form (Cytoxan, Mead-Johnson, Syracuse, NY, U.S.A.).

The solvents were HPLC grade from Burdick and Jackson (Muskegon, MI, U.S.A.). The 2-methyl-propanol was also HPLC grade and was obtained from Aldrich (Milwaukee, WI, U.S.A.). The chloroform used was ACS grade from Fisher Scientific (Fair Lawn, NJ, U.S.A.).

Analytical chromatography

Achiral chromatography. The separation of the three drugs from the plasma components was accomplished on the achiral precolumn using mobile phases composed of hexane–2-propanol, 85:15 (v/v) for IFF and TFF and 90:10 (v/v) for CTX. A flow-rate of 1 ml/min and ambient temperature were used throughout the study.

A silica guard column was used in place of an injection loop and the solutes were loaded onto this column dissolved in 2-propanol. The injector was switched to the inject position and the solutes were washed onto the achiral pre-column. The injector was switched back to the load position 3 min after injection. The guard column was washed with 3 ml of methanol and then reequilibrated with 2 ml of the mobile phase.

Enantioselective chromatography. The mobile phases used for the enantioselective chromatography of IFF and TFF were composed of hexane–isopropanol, 85:15 (v/v) for IFF and 90:10 (v/v) for TFF. The mobile phase used

for the stereochemical resolution of CTX was composed of hexane–2-methyl-1-propanol–acetonitrile (94:5:1, v/v). A flow-rate of 1.1 ml/min and ambient temperature were used throughout the study.

Preparative chromatography

The preparative isolation of IFF required hexane–isopropanol (90:10, v/v) as the mobile phase, at a flow-rate of 4 ml/min. Since a large volume of solvent was consumed, the waste solvent was fractionally distilled and the fraction boiling between 65 and 69 °C (hexane) collected and reused.

IFF [40 μg/ml in hexane–isopropanol (80:20, v/v)] was injected onto the CSP and the eluent fractions containing the individual enantiomers were collected. The fractions from successive injections were pooled and the solvent was evaporated under reduced pressure using a rotary evaporator (Rotovap, Brinkman Instruments, Westbury, NY, U.S.A.). The residue was reconstituted in distilled water, filtered through a 0.45-μm syringe adaptable filter (Millipore, Bedford, MA, U.S.A.) and lyophilized (freeze-dryer, Virtis, Gardiner, NY, U.S.A.).

The purity of the isolated isomers was checked by enantioselective GC using a chiral phase GC column (Chirasil-Val III, Alltech Assoc., Deerfield, IL, U.S.A.) and a Hewlett Packard Model 5890 gas chromatograph equipped with a Model 5790 mass-selective detector (Hewlett Packard, Avondale, PA, U.S.A.). The experimental details have been reported elsewhere [1].

Peak assignment

The chromatographic fractions corresponding to (*R*)- and (*S*)-IFF were identified by comparison with pure enantiomers provided by Prof. G. Blaschke (Westfalishe Wilhelms-Universität Münster, Münster, F.R.G.) and by the polarimetric measurement of the collected eluent (Autopol III, Rudolph Research, Flanders, NJ, U.S.A.). The chromatographic fractions corresponding to the enantiomers of CTX and TFF were identified using an on-line HPLC polarimetric detector developed by Dr. E.S. Yeung (Iowa State University, Ames, IA, U.S.A.).

Sample preparation

The internal standard (35 μl of 1 mg/ml CTX in water for IFF samples, 55 μl of 1 mg/ml IFF in water for CTX samples) was added to a 500-μl serum sample. This was followed by the addition of 4 ml of chloroform and the mixture was vigourously shaken, sonicated and centrifuged at 800 *g* for 3 min. The aqueous phase was discarded, and the organic solvent was evaporated to dryness in a Speed-Vac concentrator (Savant Instruments, Farmingdale, NY, U.S.A.). The residue was reconstituted in 70 μl of isopropanol and injected onto the chromatographic system for analysis. The average recovery was 95%.

Standard curves

A standard curve was constructed from plasma samples containing an IFF or CTX total concentration ranging from 20 to 130 μg/ml. Two determinations were obtained for each concentration. High and low controls (respectively 110 and 30 μg/ml for IFF and 110 and 40 μg/ml for CTX) were run in duplicate in two-day intervals.

The IFF isomers ratio standard curve was determined using known mixtures of the isomers obtained as described in the preparative section of the paper. The total IFF concentration was 110 μg/ml.

Clinical samples

The clinical samples analyzed in this study were obtained from pediatric patients treated for osteosarcoma. IFF (1.6 g/m^2) was administered as a single 15-min infusion. Blood samples were collected at various times, allowed to clot, and the plasma was harvested. The plasma was then frozen at −20°C until analyzed.

RESULTS AND DISCUSSION

Stereochemical separation of CTX, IFF and TFF

The enantiomeric oxazaphosphorines used in this study were resolved on an HPLC-CSP based upon cellulose-tris(3,5-dimethylphenylcarbamate), the OD-CSP (Fig. 2). Representative chromatograms are presented in Fig. 3, and Table I summarizes the relative retention times and observed stereoselectivities. The separations took less than 20 min, and baseline separations were achieved for IFF and CTX.

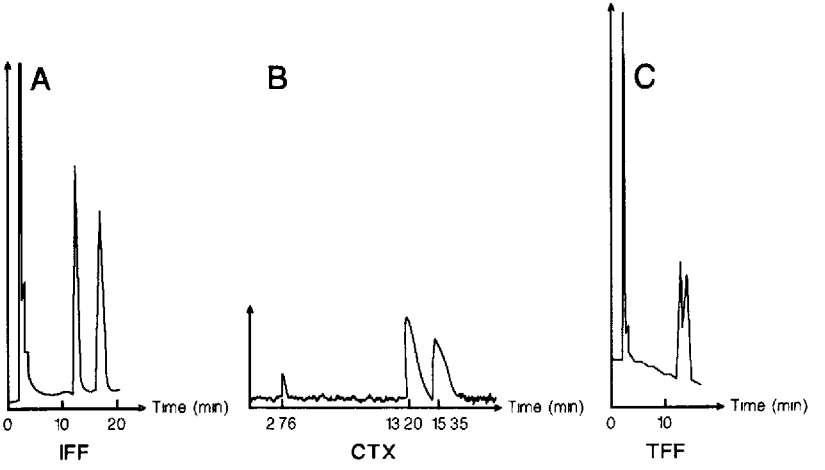

Fig. 3. Stereochemical separation of the enantiomers of (A) IFF, (B) CTX and (C) TFF on the OD-CSP. For chromatographic conditions see text.

TABLE I

CHROMATOGRAPHIC RESULTS ON THE OD-CSP

Compound	$k'(R)^a$	$k'(S)^b$	α^c
IFF	3.20	4.62	1.43
CTX	3.78	4.56	1.21
TFF	3.03	3.34	1.10

[a]Capacity factor for the (*R*)-enantiomer.
[b]Capacity factor for the (*S*)-enantiomer.
[c]Stereochemical selectivity, i.e. $k'(S)/k'(R)$.

The mobile phases were composed of hexane modified with an alcohol. The modifier used in the separations of IFF and TFF was 2-propanol. However, with this alcohol in the mobile phase, an adequate stereochemical resolution could not be achieved for CTX. A different modifier, 2-methyl-1-propanol, was used instead. This alcohol was chosen based on previous work on a related derivatized cellulose CSP which suggested that the use of sterically bulky alcohols can increase stereoselectivity [9]. A small amount of acetonitrile was added to the mobile phase used with CTX to reduce retention and increase efficiency.

The retention order on the OD column was found to be the same for the three drugs: the (*R*)-isomer was eluted first. It is of interest to note that the spatial arrangement around the phosphorus atom is the same for (*R*)-IFF and (*R*)-TFF, but it is opposite for (*R*)-CTX.

Determination of IFF and CTX in plasma

The direct analysis of extracted plasma samples containing IFF and CTX on the OD-CSP were attempted, but interfering plasma components made quantitation impossible. Therefore, a coupled column chromatographic system was investigated. In this system, the IFF and CTX were separated from the interferences, quantitated on an achiral precolumn and then switched to the OD-CSP for the determination of the enantiomeric composition.

The previously reported achiral–chiral coupled column assays have been based on chromatographic systems which use aqueous mobile phases [8,10,11]. However, since CTX, IFF and TFF were stereochemically resolved on the OD-CSP using a non-aqueous mobile phase, the achiral portion of the system had also to use normal-phase conditions.

Earlier studies on the chiral resolution of IFF and CTX had demonstrated that these compounds were retained but not stereochemically resolved on an HPLC-CSP based upon D-naphthylalanine. Subsequent studies revealed that this column could be used to separate CTX, IFF and TFF from plasma constituents using mobile phases composed of hexane–2-propanol. This column was chosen as the pre-column. To insure that an undetected chiral separation

did not occur on the pre-column, a racemic form of this stationary phase, D,L-naphthylalanine, was used.

With a mobile phase of hexane–2-propanol (85:15, v/v), the capacity factors for CTX, TFF and IFF on the achiral pre-column were 4.56, 6.32 and 6.68, respectively. This permits the use of CTX as the internal standard for IFF and TFF determinations and IFF as the internal standard for CTX analysis. A blank serum extract and a spiked sample (70 μg/ml each) are shown in Fig. 4A and B, respectively. Since TFF seems to be of less clinical importance, only IFF and CTX were studied further, but the results indicate that the same approach can be used for TFF.

The standard curves for total IFF and CTX plasma concentrations, i.e. (*R*)- plus (*S*)-IFF and (*R*)- plus (*S*)-CTX determined on the achiral pre-column were linear over the range investigated. The equation describing the IFF curve was $y=77.0x+15.9$ with a correlation coefficient of 0.989 and the equation describing the CTX curve was $y=79.8x+9.18$ with a correlation coefficient of 0.989. The calculated coefficients of variation for the IFF and TFF were 5.4 and 10.9%, respectively, for the high controls and 0.4 and 1.8%, respectively, for the low controls. Although the lower limit of the standard curve was 20 μg/ml, a concentration of less than 10 μg/ml of each compound could be detected.

In some instances, a late eluting peak (approximately 50 min) was observed on the achiral pre-column. In order to eliminate this peak, a silica guard column was put in place of the injection loop and the injection valve switched back to the load position 3 min after the injection. The strongly retained com-

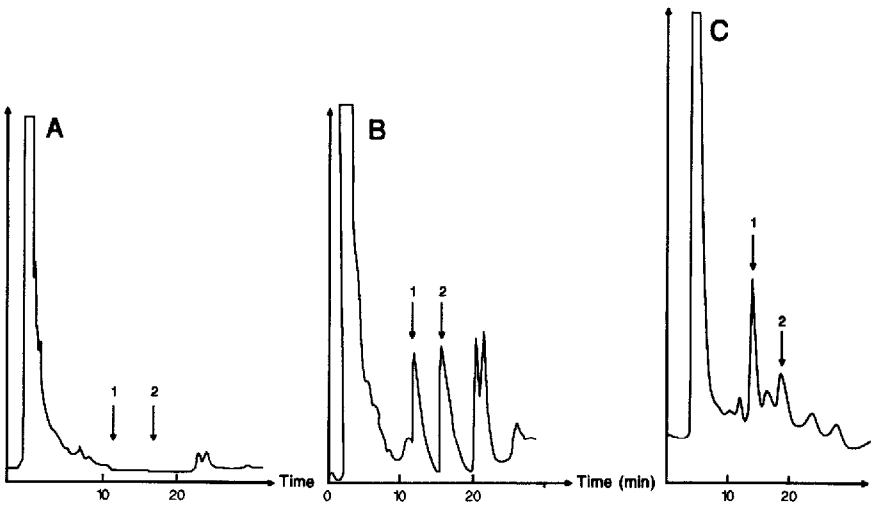

Fig. 4. Representative chromatograms on the achiral pre-column for (A) an extracted blank plasma sample, (B) a plasma sample spiked with CTX (70 μg/ml) and IFF (70 μg/ml) and (C) a plasma sample from the clinical study. Peaks: 1 = CTX; 2 = IFF. For chromatographic conditions see text.

pounds were washed from the guard column with the methanol. This reduced the total analysis time to below 30 min.

The results from the chromatography on the OD-CSP after the transfer of eluent from a serum blank and from a spiked serum sample (100 μg/ml) are presented in Fig. 5A and B, respectively. The ratio of the (*S*)-IFF to (*R*)-IFF peak heights from the analysis of a serum sample spiked with racemic IFF was unity. The plot of this peak-height ratio versus the ratio of (*S*)-IFF/(*R*)-IFF serum concentrations ranging from 1:3 to 3:1 was linear. This allowed for the direct calculation of the enantiomeric composition from the determination of the peak-height ratio.

The chromatograms presented in Figs. 4C and 5C are from the analysis of a patient sample collected 7 h after the administration of a single 15-min infusion of IFF (1.6 g/m^2). The results of three serum assays are presented in Table II. It is of interest to note that for patient 2 there were significant differences in the (*R*)- and (*S*)-IFF concentrations. This is consistent with the

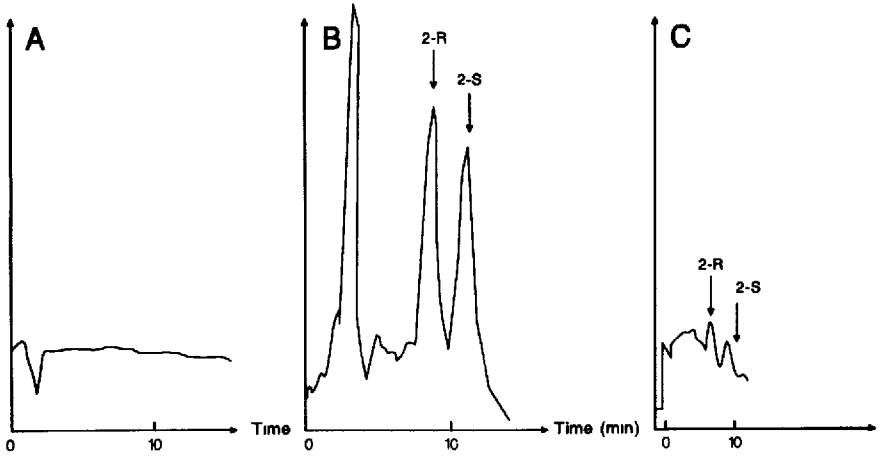

Fig. 5. Representative chromatograms on the OD-CSP for (A) an extracted blank plasma sample, (B) a plasma sample spiked with (*R,S*)-IFF (100 μg/ml) and (C) a plasma sample from the clinical study. Peaks: 2-R = (*R*)-IFF; 2-S = (*S*)-IFF. For chromatographic conditions see text.

TABLE II

SERUM CONCENTRATION OF (*R*)- and (*S*)-IFF

Patient No.	Time (h)	(*R*)-IFF (μg/ml)	(*S*)-IFF (μg/ml)
1	6	18.0	18.0
2	5	19.9	16.7
2	7	20.5	13.1

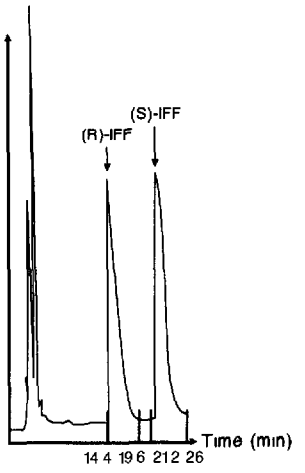

Fig. 6. Representative chromatogram from the preparative chiral separation of (*R*)-IFF and (*S*)-IFF on the OD-CSP after the injection of 8 mg of (*R,S*)-IFF. For chromatographic conditions see text.

initial studies of (*R*)- and (*S*)-IFF pharmacokinetics using enantioselective GC [1].

Preparative isolation of IFF

The chromatographic separation of IFF was adapted for the preparation of gram quantities of the pure enantiomers. The semi-preparative column (25 cm × 10 mm I.D. column packed with the OD-CSP) was loaded with 8 mg of (*R,S*)-IFF (i.e., 4 mg of each isomer), Fig. 6. The separation was achieved in 25 min with an 85% recovery of the individual enantiomers. Since the system was automated, it functioned continuously and 196 mg of each isomer were prepared per day.

The purity of the isolated isomers was checked by enantioselective GC. The lower detectable percentage of one isomer present in the other was 3%. Using this method, no (*R*)-IFF was found in the (*S*)-IFF isomer and vice versa, and, therefore, the purities were 97% or greater.

CONCLUSION

This study demonstrates that the OD-CSP can be used for the analytical and preparative stereochemical resolution of the optical isomers of IFF, CTX and TFF. The results also indicate that HPLC-CSPs which use non-aqueous mobile phases can be coupled to achiral normal-phase columns, and the resulting systems used for the direct analysis of biological samples.

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