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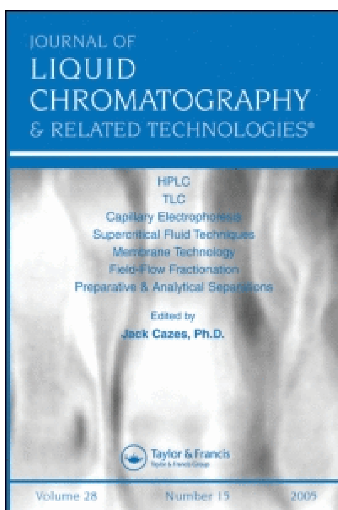

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EXTRACTIONS AND PURIFICATIONS

APPLICATION OF HIGH SPEED CCC FOR THE PURIFICATION OF LISINOPRIL-DIKETOPIPERAZINE DIASTEREOMERS

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ABSTRACT

High-speed Countercurrent Chromatography (HSCCC) has been applied to the separation of three of the possible diastereomers of Lisinopril Diketopiperazine. The diastereomers occur in the synthesis of the ACE inhibitor, Lisinopril, as a result of over heating during a distillation process. Their levels are a measure of process control and typically should be held below 0.2% w/w w.r.t. lisinopril. Pure quantities of the diastereomers are required to serve as standards in determining levels in the final bulk drug. Difficulties often arise during the current procedure for the purifi-

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cation of the pure standards. The potential of HSCCC was examined as an alternative technique for their separation.

INTRODUCTION

Lisinopril is an inhibitor of angiotensin-converting enzyme (ACE) and is adminstered to patients with heart failure.[1] As a result of over heating during a distillation process during its production, Lisinopril undergoes an intermolecular cyclysation reaction producing a cyclic dipeptide Lisinopril diketopiperazine (Lisinopril-DKP). Three diastereomers (Fig. 1) are produced in this reaction and due to stereochemical effects, varying proportions of the diastereomers are formed; SSR > RSR > SSS. The levels of these compounds are a measure of process control and typically should be held below 0.2% w/w w.r.t Lisinopril.

Pure quantities of the diastereomers are required to serve as standards in determining levels in the final bulk drug. During the current procedure for the purification of the pure standards, a normal phase silica preparative High Performance Liquid Chromatography (HPLC) column is utilized. Difficulties often arise when isolating the SSS isomer that is thermodynamically unflavored and tends to epimerise to the SSR isomer. This occurs as a result of interaction with acidic silanols as the stereoisomers pass through the column.

Countercurrent chromatography (CCC) is a term describing modern liquid-liquid chromatography that utilizes a column without a solid support and requires two non-miscible phases.[2] This support-free system provides numerable advantages over other chromatographic techniques, including the elimination of problems such as adsorptive loss of samples, deactivation of samples, and sample contamination. In most variants of CCC, there is no countercurrent flow at all. One liquid phase remains stationary while the second phase is passed through the

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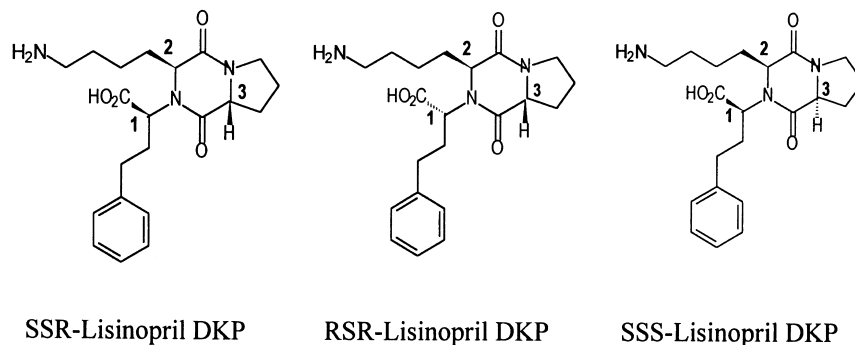

Figure 1. Structures of diastereomers of Lisinopril Diketopiperazine (DKP).

stationary solvent phase. Separation relies on the partitioning of the solute between the two phases.

High-speed countercurrent-chromatography (HSCCC) developed in the early 1980s, relies on centrifugal force for the retention of the stationary phase.[3] HSCCC offers advantages over other liquid-liquid techniques of efficient separation in a relatively short period of time and a wider range of suitable solvent systems.[4]

In this study, the potential of HSCCC was examined as an alternative technique to preparative HPLC for the isolation of the three diastereomers of Lisinopril-DKP.

EXPERIMENTAL

Chemicals

Isobutanol, water, acetonitrile (all HPLC grade), ethanol (AnalR grade), ammonium formate (AnalR grade), ammonia solution, S.G. 0.88 (HPLC grade) were purchased from Fisher Scientific (Loughborough, UK). N-butyronitrile (98%) and isobutanol (99%) were purchased from Acros (Geel, Belgium). Trifluoroacetic acid and formic acid (99%) were purchased from Sigma-Aldrich (Dorset, UK).

AstraZeneca (Macclesfield, UK) kindly supplied the mixture of lisinopril-diketopiperazine diastereomers (ssr, rsr and sss). Helium and nitrogen gases were purchased from BOC Ltd. (Surrey, UK).

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Apparatus/Instrumentation

The separations were performed using a Quattro Labprep Countercurrent Chromatograph ("J" type) (AECS, Bridgend, UK). The instrument consists of 2 bobbins, each wound with two coils of PTFE tubing (1.6mm i.d.). The coils are of different volumes and the one employed in this study had a volume of 80mL. The end of the coil was connected to a Waters' photodiode array detector (Model 990) and a Gilson fraction collector (Model 231). A Rheodyne injector equipped with a 1 mL loop was connected to the inlet of the coil. The pumping system comprised of an HP1100 series quaternary pump (Agilent Technologies, Germany).

Measurement of Partition Coefficients

Samples of Lisinopril-DKP (2mg) were weighed into 8 mL vials, to which 3 mL of each phase of the pre-equilibrated biphasic solvent systems were added.

The vials were capped and shaken vigorously to thoroughly equilibrate the sample with the two phases. Equal volumes (1 mL) of each layer were analyzed by HPLC to determine the partition coefficients of each of the diastereomers.

Preparation of the Biphasic Solvent Systems and Sample Solution

The initial two-phase solvent system used in the separation of the diastereomers consisted of isobutanol / water (1/1 v/v). This system was also used slightly modified by trifluoroacetic addition: isobutanol / TFA / water (1/0.01/1 v/v/v). The solvent systems were thoroughly purged using a flow of helium gas and allowed to equilibrate at room temperature in a separatory funnel. Prior to use, the two phases were separated.

For the initial separation, the sample solution was prepared by dissolving 300 mg of the diastereomeric mixture in 3 mL of the solvent mixture consisting of equal volumes of each phase. For the following separation, selected fractions (determined by HPLC analysis) were combined and blown down to dryness in a stream of nitrogen. The residue was made up in a solvent mixture (1 mL) consisting of equal volumes of each phase of the second solvent system.

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HSCCC Separation Procedure

The first separation was initiated by completely filling the column (80 mL volume) with the stationary (upper) phase. The aqueous mobile phase was then pumped into the column at a flow-rate of 2 mL/min in a tail-to-head direction while the column was rotated at 800 rpm in a reverse (anti-clockwise) direction. The oven temperature was maintained at 30°C throughout the experiments. The stationary phase displaced was collected and measured to determine the stationary phase retention. The sample solution was injected through the Rheodyne sample loop (1 mL) into the column. The effluent was monitored with the photodiode array detector at 220 nm and fractions (2 mL) were collected using the fraction collector. Fractions of interest were analyzed by high-performance liquid chromatography (HPLC).

Analysis of CCC Fractions by High Performance Liquid Chromatography

The system used consisted of a Hewlett-Packard 1050 Series liquid chromatograph equipped with a multi-wavelength detector. An aliquot (100 µL) of each fraction to be analyzed was made up to 1 mL in a solvent consisting of ace-

tontrile-water (80/20 v/v). Analytical conditions were as follows: Column, Zorbax RX-C8 (4.6 mm ID x 250 mm, 5 μm) (Hichrom, Reading, UK); Eluent, 0.01 M Ammonium formate, pH 5 (formic acid): acetonitrile (84/16 v/v); flow rate, 1 mL/min; column temperature, 30°C; detection, 215 nm and injection volume, 10 μL.

RESULTS AND DISCUSSION

Selection of the Two-Phase Solvent Systems

In HSCCC, successful separations require the use of a suitable biphasic solvent system, which provides an ideal range of partition coefficients (K) for the sample under investigation. The choice of possible solvent systems for the components under investigation was achieved by following a generic methodology strategy.[5] The screening method indicated that the compounds possessed a high polarity. K is defined as the ratio; solute concentration in the stationary phase divided by solute concentration in the mobile phase. Efficient separations require K to have a value close to 1 and the separation factor (α) ($\alpha = K_1 / K_2$, where K_1 and K_2 are partition coefficients of two solutes and $K_1 > K_2$) between two components to be greater than 1.5. This minimum α-value is required for baseline separation in semi-preparative CCC equipment, providing a moderate partition efficiency of approximately 800 theoretical plates.[6] For a pure compound, the K value can be determined by measuring the UV absorbencies of each phase after partitioning between the two phases. In this case where only a mixture is available, an HPLC method is applied where the components are separated into three peaks.

After partitioning of the sample between the phases of a solvent system, aliquots were taken from the upper and lower phases and analyzed by HPLC. The ratios of the peak heights of the corresponding peaks in the two chromatograms were used to determine the value of K.

Numerous polar solvent systems were examined to achieve a suitable value for K (Table 1). In all cases, the diastereomers exhibited partitioning solely into the aqueous (lower) phase with no trace in the organic layer. pH control produced no significant improvement in the partitioning of the components.

Increased polarity may be attained by the addition of a volatile acid, e.g., butanol-trifluoroacetic acid-water (1:0.001:1 to 1:0.01:1, v/v), butanol-acetic acid-water (4:1:5, v/v/v).[7] However, due to the nature of the compounds being susceptible to epimerisation under severe acidic conditions, the use of these solvent systems were avoided.

In this study, the use of partition coefficients provided insufficient results for the determination of a suitable solvent system. An optimization procedure

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Table 1. Solvent Systems Examined in the Selection of a Suitable Two-Phase Solvent System

Solvent System	Settling Time (seconds)
Isobutanol-Water (1:1)	15
Isobutanol-Ethanol (1:0.2:1)	25
Isobutanol-Methanol-Water (1:0.3:1)	>120
Isobutanol-10mM Ammonium Formate, pH 3 (1:1)	10
Isobutanol-10mM Ammonium Formate, pH 4 (1:1)	7
Isobutanol-10mM Ammonium Formate, pH 5 (1:1)	10
Isobutanol-10mM Ammonium Formate, pH 6.2 (1:1)	10
Isobutanol-10mM Ammonium Formate, pH 9 (1:1)	15
Butyronitrile-Water (1:1)	6
Butyronitrile-Methanol-Water (1:0.9:1)	40

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(carried out directly using the CCC) produced the successful separations achieved. Many of the polar solvent systems (Table 1) and several others were examined in the optimization procedure adopted. It was established that a number of the solvent systems produced the expected results as indicated by the partition coefficient experiments, i.e. $K \ll 1$, and the solutes eluted close together near the solvent front resulting in no peak resolution. However, a few of the systems exhibited some resolution of the diastereomeric components.

Separation of the Diastereomers of Lisinopril-DKP by HSCCC

The use of one HSCCC separation alone did not prove to be sufficient as a total isolation procedure for the three diastereomers. A 100 mg quantity of the diastereomer mixture was partially separated using the solvent system of isobutanol-water (1:1) (Fig. 2). The retention of the stationary phase was 66%. The total separation time was 32 minutes with a total elution volume of 65 mL. The fractions collected were analysed by HPLC and the absorbance (peak areas) of the components at 215 nm was plotted to produce the elution curve. It was shown, that the rsr diastereomer was sufficiently separated from the ssr and sss distereomers, which were found to coelute. As can be seen, the components eluted very close to the solvent front. Fractions 26-32 containing the pure rsr diastereomer were combined and blown down to dryness in a stream of nitrogen.

A more polar solvent system was required to retain the compounds in the column longer and which would increase the probability of peak resolution to be attained. Modification of the isobutanol-water system was necessary to achieve the increased polarity. The addition of ammonium formate, acidic conditions

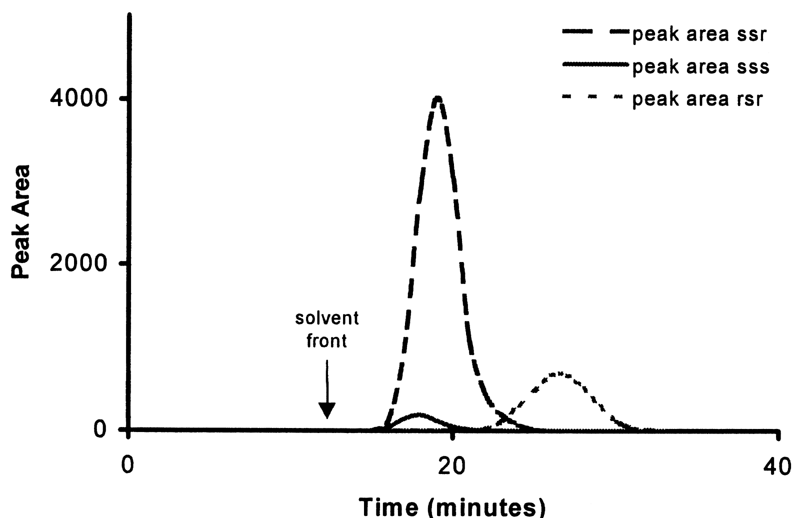

Figure 2. Reconstructed elution curve of the HSCCC separation of Lisinopril-diketopiperazine diastereomers using the solvent system isobutanol – water (1:1). Peak areas were obtained from HPLC fraction analysis. CCC experimental conditions: Column volume (100 mL), flow rate (2 mL/min), rotation (800 rpm), aqueous mobile phase (T-H direction), oven temperature (30°C), stationary phase retention (66%).

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(formic acid / ammonium formate) and basic conditions (ammonia/ ammonium formate) did not exhibit any real improvements in peak resolution. However, at pH 3 there was evidence of the three peaks moving away from the solvent front and, more significantly, movement of the sss diastereomer from under ssr diastereomer.

Although severe acidic conditions should have been avoided, the examination of the highly polar isobutanol-TFA-water (1:0.01:1) system led to the increased retention of the diastereomers in the column. The retention of the stationary phase was 66%, the total separation time less than 90 minutes, with the total elution volume of 180 mL. HPLC analysis of the effluent fractions indicated that partial resolution of the diastereomers had occurred (Fig. 3).

The combination of the two solvent systems was successfully applied to the separation of the various diastereomers. Initially, the sample mixture (100mg) was injected into the isobutanol-water (1/1 v/v) system, fractions 26-32 were combined and blown down to dryness, as above, resulting in the isolation of reasonably pure (95%) rsr diastereomer. 9mg of the diastereomer were recovered, equating to a yield of 47%. Those fractions 15-21 containing ssr and sss diastereomers were combined and again blown down to dryness.

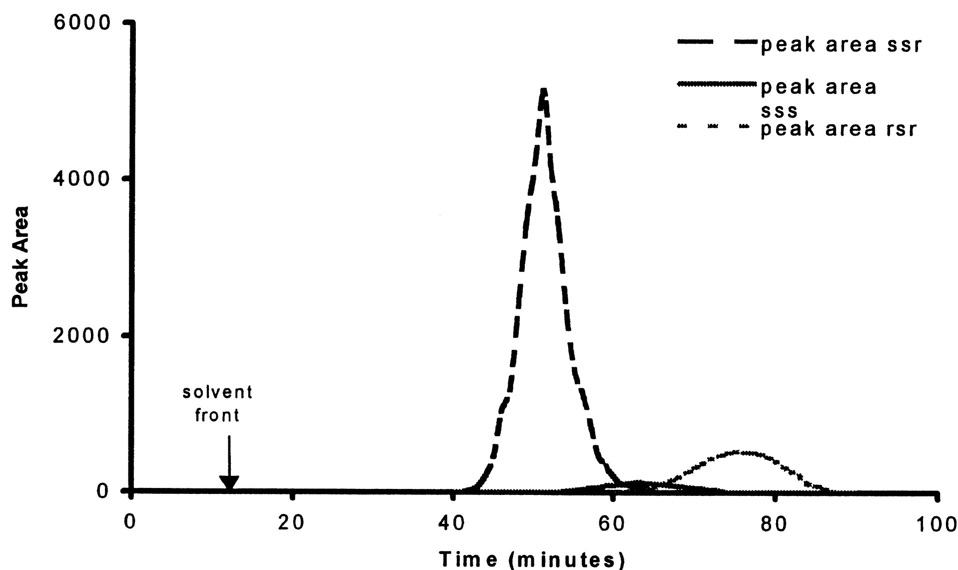

Figure 3. Reconstructed elution curve of the HSCCC separation of Lisinopril-diketopiperazine diastereomers using the solvent system isobutanol - trifluroacetic acid - water (1:0.01:1). Peak areas were obtained from HPLC fraction analysis. CCC experimental conditions: Column volume (100 mL), flow rate (2 mL/min), rotation (800 rpm), aqueous mobile phase (T-H direction), oven temperature (30°C), stationary phase retention (66%).

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The residue, made up in solution (1 mL), was subjected to the second CCC separation using the acidified solvent system isobutanol-TFA-water (1/0.01/1 v/v/v). HPLC analysis was carried out on the fractions that produced the elution curve in Fig. 4. Although some peak overlap occurs, each of the diastereomers can be isolated in high purity (97%). Fractions containing pure ssr lisinopril-DKP were combined into a pre-weighed vial and, similarly, those containing pure sss.

Each sample was blown down to dryness and re-weighed. 37.5 mg of ssr (48% yield) and 1.1 mg of sss (32% yield) was obtained. The percentage recoveries of the pure diasteromers are shown to be significant when considering the peak overlap of the diastereomers during the HSCCC separations. The fractions containing both the diastereomers once blown down and redissolved can be re-injected into this solvent system to undergo a further HSCCC separation.

The overall results of this present study indicates that HSCCC is a useful technique for the separation of diastereoisomeric Lisinopril diketopiperazines using two polar solvent systems in succession. The results presented here, sug-

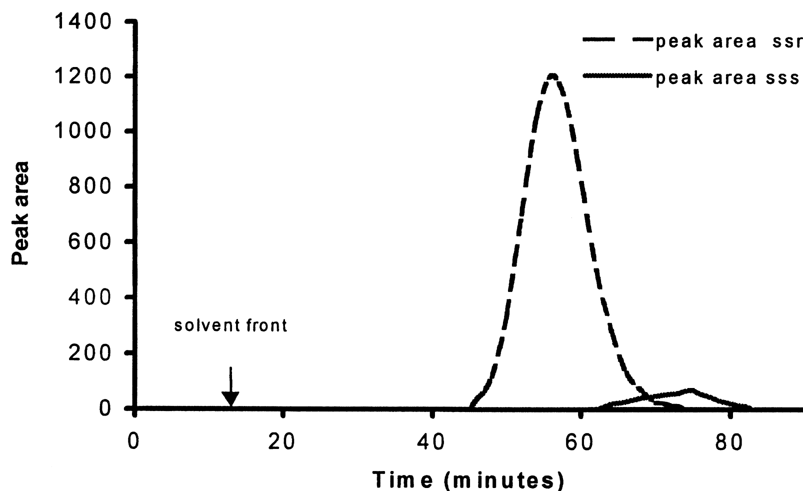

Figure 4. Reconstructed elution curve of the HSCCC separation of ssr and sss Lisinopril-diketopiperazine diastereomers (combined fractions 15 – 21 from isobutanol-water separation) using the solvent system isobutanol – trifluoroacetic acid - water (1/ 0.01 /1 v/v/v). Peak areas were obtained from HPLC fraction analysis. CCC experimental conditions: Column volume (100 mL), flow rate (2 mL/min), rotation (800 rpm), aqueous mobile phase (T-H direction), oven temperature (30°C), stationary phase retention (66%).

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gest the great potential of this technique for separating other closely related compounds.

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