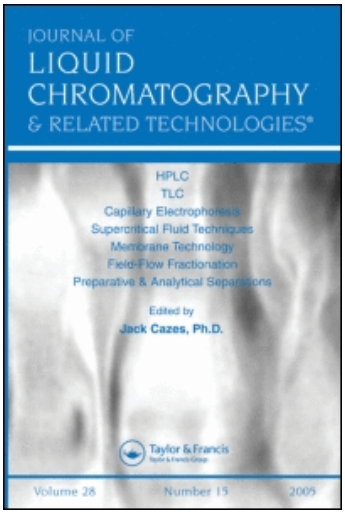

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HPLC SEPARATION OF TWO NOVEL DIKETOPIPERAZINE ISOMERS ON POLYVINYL ALCOHOL FUNCTIONALIZED SILICA GEL

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ABSTRACT

An HPLC method is described for the analytical separation of two novel diketopiperazine isomers isolated from a fungal culture, *Tolypocladium* sp. (SCF-0729). Separation was achieved on a high-performance polyvinyl alcohol silica gel column with a 1-chlorobutane: methanol gradient system. Selectivity, retention and resolution obtained with this support were all highly reproducible. Direct scale up of analytical methodology allowed for the preparative separation of this isomeric pair.

INTRODUCTION

Adsorption chromatography employing native silica gel often provides significant capabilities in the resolution of isomeric mixtures.[1-4] Modifications to the silica (or alumina) backbone such as silver ion impregnation (argentation) can further enhance the separation of isomeric

pairs.[5-6] This capability results from differential pi-pi interactions of olefinic containing compounds with the immobilized metal ion. Despite this capability, significant difficulties arise when labile mixtures, sensitive to moisture and acid, need to be resolved. Recent developments in silica gel surface modifications have expanded the capabilities for isomeric separations. In the case of polyvinyl alcohol (PVA) functionalization, spherical silica particles are coated with mono-molecular vinyl alcohol followed by polymerization of the vinyl alcohol. This process accesses both external and internal surfaces of the support resulting in a phase devoid of acidic silanols.[7] This alcoholic support surface has proven to be a highly selective and stable normal phase matrix for the otherwise difficult separation of mixtures.[8]

In the course of searching for bioactive substances from fungal fermentations, we have isolated Sch 54794 and Sch 54796 from the fermentation of *Tolypocladium* sp (SCF-0729).[9] These diketopiperazines were part of an isomeric mixture that decomposed on silica gel. We wish to describe the baseline separation of this isomeric mixture on polyvinyl alcohol coated silica gel with 1-chlorobutane:methanol as the mobile phase.

EXPERIMENTAL

Materials and Methods

HPLC grade 1-chlorobutane and methanol were obtained from Fluka Chemic AG (Buchs, Switzerland) and Fisher Scientific (Pittsburgh, PA, USA), respectively.

Chromatographic Apparatus

Analytical separations were performed on an integrated Hewlett Packard (Palo Alto, CA) HP 1090 Series II Liquid Chromatographic System comprising a PV-5 ternary solvent delivery module, a 250 µl auto injector, and a photodiode array detector equipped with a standard 8 µl flow cell of 6 mm path length. A YMC Inc. (Wilmington, North Carolina) PVA-Sil 120 Å, 5 µm (4.6 mm x 15 cm) column was employed which was preceeded by a guard column (4.0 mm x 2.3 cm) containing the same stationary phase. A linear gradient of 2-10% methanol in 1-chlorobutane over 20 minutes was utilized to optimize separation and resolution. Samples were dissolved prior to injection in 5% methanol/1-chlorobutane to obtain 1 mg/ml solutions. Analytical flow rate was 1 ml/min and U.V. detection was either at 220 or 275 nm.

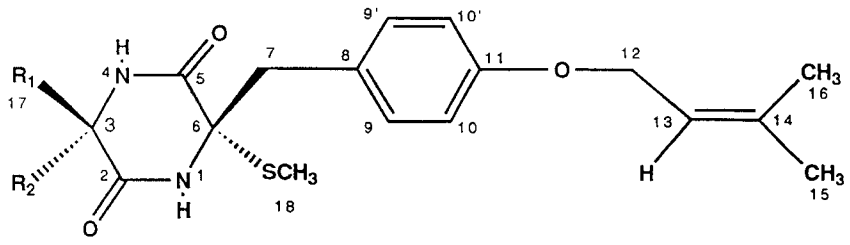
Semi-preparative separations were performed on a system comprised of the following components: a Waters 600 multi-solvent delivery module (Waters Chromatography Division, Milford, MA) equipped with a Rheodyne Model 7125 manual injector (Rainin Instrument Co., Woburn, MA) that contained a 2 ml sample loop. Sample components were detected with a Waters Model 481 variable wavelength UV detector set at 275 nm. Signals were plotted on a Waters Model 740 data module and monitored at an attenuation of 1024. Chromatographic support was a YMC, Inc., PVA-Sil 120 Å, 5 µm column (20 x 250 mm) that was preceded by a 30 x 10 mm guard column. Mobile phase conditions were identical to that described under analytical except flow rate was 8 ml/min. The semi-preparative column received from YMC Inc. was shipped in 0.5% sodium azide. Because PVA-Sil media is primarily used

as a size exclusion support for biomolecular separations, a test mixture of thyroglobulin, albumin, β-lactoglobulin and cytochrome C was separated with 0.2 M NaCl/0.1 M sodium phosphate buffer (pH 7). Before normal phase chromatography could be performed, the column was thoroughly washed with the five bed volumes (300 ml) of water, followed by (300 ml) of methanol before equilibration with mobile phase.

Preparative reverse phase chromatography was performed with instrumentation as described in the semi-preparative section. A YMC Inc. C-18 column 120 Å, 15 um/irregular media (30 x 500 mm) was preceded by a (30 x 50 mm) guard column. The *cis/trans* diketopiperazine mixture was enriched, but co-eluted under a 20 minute linear gradient of 80-90% methanol in water at 20 ml/min.

RESULTS AND DISCUSSION

As shown in Figure 1, Sch 54794 (**1**) and Sch 54796 (**2**) are *cis/trans* diketopiperazine isomers that were obtained from the fermentation of *Tolypocladium* sp. (SCF-0729). Purification of the isomeric pair involved ethyl acetate extraction of the fermentation broth (8L). This yielded 3 g of an oily residue that was dissolved in methanol: methylene chloride (1:1). After removal of insoluble material, the bioactive soluble portion was concentrated *in vacuo* and then precipitated with hexane. The precipitate (1.8 gm) was further purified on a C-18 preparative column. This yielded 500 mg of the *cis/trans* isomeric mixture which could not be resolved utilizing silica, neutral or basic alumina, nor by HP-20P or LH-20, chromatographic approaches.



Sch 54794 (1) : $R_1 = H$ $R_2 = SCH_3$

Sch 54796 (2) : $R_1 = SCH_3$ $R_2 = H$

FIGURE 1: Structure of diketopiperazines

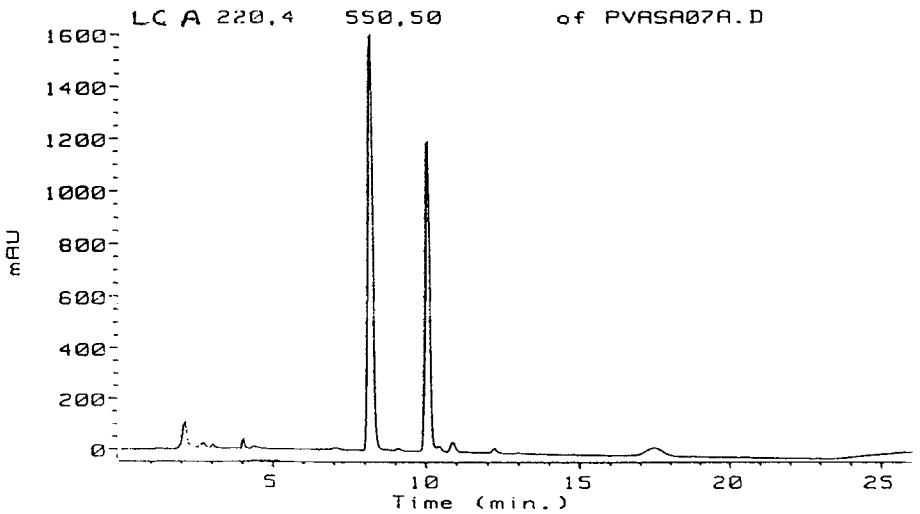


FIGURE 2: Analytical separation of *cis/trans* mixture (50 µg) (see Experimental section for details)

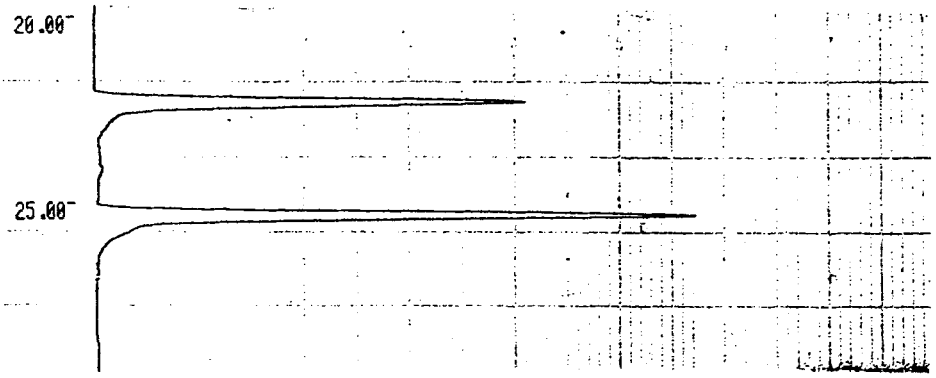


FIGURE 3: Semi-preparative separation of diketopiperazine *cis/trans* mixture (see Experimental section)

Figure 2 illustrates the excellent separation of this mixture on PVA-Sil. Retention times for the pure *cis* isomer (**1**) and pure *trans* isomer (**2**) were 8.2 and 10.1 minutes respectively; void volume (Vo) = 2.2 mL, efficiency N=35,315 plates calculated for the *trans* isomer. Multiple chromatographic analyses were performed on this mixture and yielded highly consistent results. (standard retention time error < 0.10 min for 10 replicate runs)

The ratio of *cis/trans* components for the isomeric mixture was subsequently shown to vary when the culture was refermented. A nearly complete reversal of abundance (1:2) was encountered when a subsequent fermentation was evaluated.

Figure 3 represents the purification of each isomer by direct scale-up under semi-preparative conditions. With 40 mg of the mixture from C_{18}, 10 mg of pure *cis* isomer t_{R1} = 22.3 min. and 20 mg of pure *trans* isomer t_{R2} = 25.4 min. were obtained.

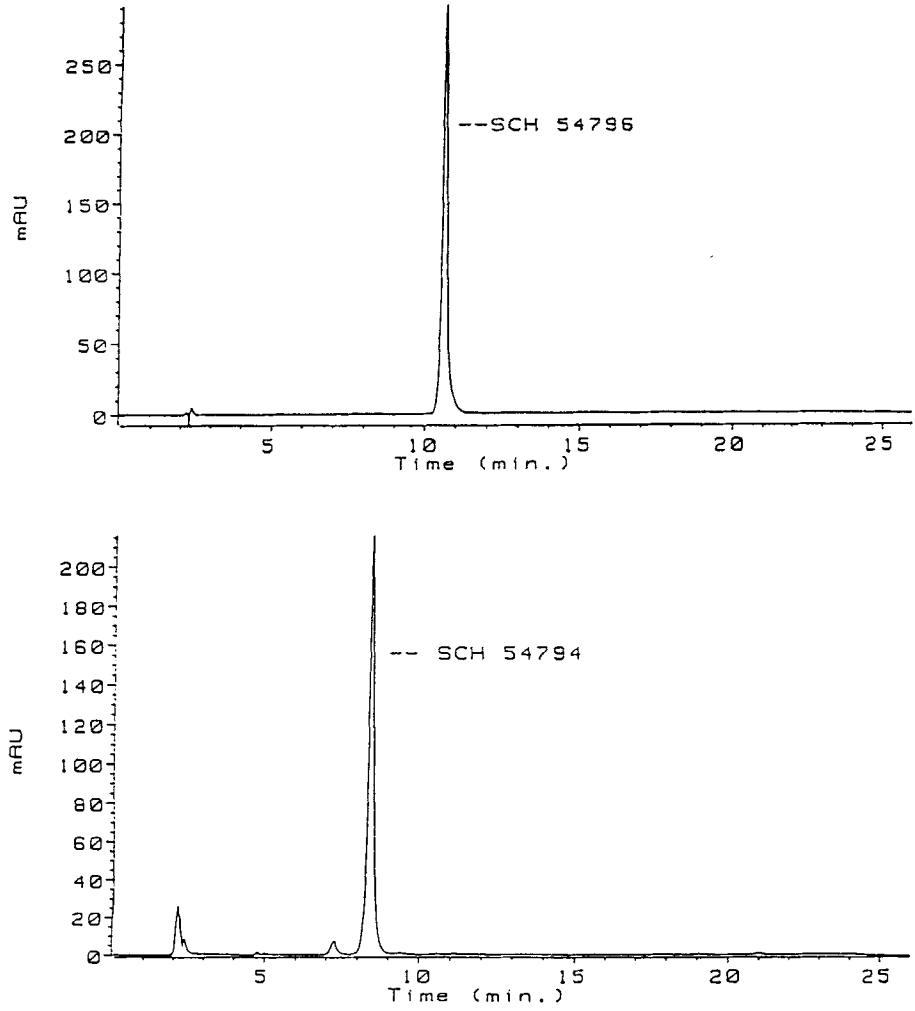


FIGURE 4: Purified *cis* and *trans* diketopiperazines (5μg each)

Analytical re-evaluation of fractions generated (see Figure 4) confirmed the complete separation of *cis* and *trans* diketopiperazine isomers.

CONCLUSION

Polyvinyl alcohol functionalized silica gel has been demonstrated to provide unique capabilities for the isomeric separation of diketopiperazines from *Tolypocladium* sp. (SCF-0729). This rigid and stable stationary phase is well suited for the separation of this acid labile mixture. The uniformity of the polyvinyl alcohol surface combined with its lack of non-specific adsorption yielded high sample recoveries. Selectivity, retention and resolution obtained with this phase were all highly reproducible. Direct scale up of analytical methodology allowed for the baseline preparative separation of this difficult isomeric mixture. Future applications of this chemically modified silica support are expected.

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