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APPLICATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC CHIRAL STATIONARY PHASES TO PHARMACEUTICAL ANALYSIS

DIRECT ENANTIOMERIC RESOLUTION OF AMIDE DERIVATIVES OF 1-PHENYL-2-AMINOPROPANE

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SUMMARY

A series of amide derivatives of the enantiomers of 1-phenyl-2-aminopropane (amphetamine) were resolved by high-performance liquid chromatography on a chiral stationary phase (CSP), (*R*)-N-(3,5-dinitrobenzoyl)phenylglycine, which is commercially available in ionically and covalently bonded columns. The separation factor ranged from 1.01 to 1.09 and was consistently larger when the compounds were chromatographed on the covalent column. Chromatographic parameters correlate in detail with a solute–CSP interaction model requiring up to four binding sites and a fifth repulsive interaction which is steric in origin and which provides the key chiral discriminant.

INTRODUCTION

The resolution of enantiomers has been approached in a variety of ways. Two of the most widely researched and applicable avenues of investigation are indirect and direct chromatographic resolutions. The indirect approach involves the synthesis of diastereoisomers and their separation on achiral gas–liquid chromatographic (GLC) or high-performance liquid chromatographic (HPLC) supports. The direct approach resolves the molecules as enantiomers, utilizing chromatography on chiral stationary phases (CSPs). Both of these approaches have been reviewed[1,2].

Amphetamine (1-phenyl-2-aminopropane) is a pharmacologically active asymmetric molecule whose (*S*) and (*R*) enantiomeric forms, dextro- and levoamphetamine, respectively, possess different biological activities[3]. Accordingly, the resolution and quantification of amphetamine have received a great deal of attention.

A number of indirect methods have been reported for the synthesis and GLC separation of diastereoisomeric amides of amphetamine[4–10]. Direct resolution of the enantiomers has been somewhat more elusive. Weinstein *et al.*[11], Lochmüller and Souter[12], and Lochmüller and Hinshaw[13] chromatographed amphetamine on a variety of GLC CSPs without success. Lochmüller and Hinshaw recently reported[14] the

direct GLC resolution of amphetamine on two CSPs: carbonylbis[(*S*)-valine isopropyl ester] and peptide siloxane phases.

However, the direct resolution of amphetamine by HPLC has not been reported although a number of HPLC CSPs have been developed[15–23]. Optically active amines and amides have been resolved by Baczuk *et al.*[16] on a CSP synthesized by linking L-arginine to a polydextran medium; by Sousa *et al.*[19] using chiral crown ethers bonded to silica; and by Pirkle *et al.* on a fluoroalcohol-bonded CSP[22] and on an ionically bonded CSP, (*R*)-N-(3,5-dinitrobenzoyl)phenylglycine[23].

The (*R*)-N-(3,5-dinitrobenzoyl)phenylglycine CSP developed by Pirkle is now commerically available as a prepacked column in either the ionically or covalently bonded form. The availability of these columns and their reported broad applicability[23] raise the possibility of rapid, accurate and reproducible regulatory assays for enantiomeric composition of drug substances by direct enantiomeric analysis.

Direct enantiomeric resolution offers advantages such as generally easier sample preparation, decreased analysis time, and simultaneous chemical as well as optical purity analysis[1]. If derivatization is necessary, the direct method is also more advantageous than the indirect method. The reaction of an enantiomeric mixture with an achiral reagent produces a product ratio which is the same as the initial enantiomeric proportion because the enantiomers react at the same rate with a non-optically active reagent. This is not the case with the indirect method, which involves the formation of diastereoisomers[2]. Two enantiomers may have quite different rate constants when reacted with a chiral reagent, resulting in unequal amounts of the two diastereoisomer products. Furthermore, in the case of the trace analysis of one enantiomer in the presence of the other, there is the possibility that a trace isomeric contamination of the chiral derivatizing agent may give a false positive result. This hazard is involved in the indirect method but not in the direct method.

As a part of our laboratory's ongoing program in the development of regulatory assays for the stereochemical purity of commercial pharmaceuticals, we have investigated the applicability of the covalent and ionic CSP columns to the direct resolution of amphetamine.

We here report the synthesis and direct resolution of a series of amide derivatives of amphetamine using both the ionically and covalently bonded forms of the (*R*)-N-(3,5-dinitrobenzoyl)phenylglycine CSP developed by Pirkle *et al.*[23].

EXPERIMENTAL

Apparatus

The chromatography was performed with a Spectra-Physics (Santa Clara, CA, U.S.A.) Model 8000 liquid chromatograph equipped with an SP 8000 data system, a Spectra-Physics Model 8310 UV–visible detector set at 254 nm, and a temperature-controlled column compartment.

The ionically bonded column was a stainless-steel, Regis-packed, Pirkle Type 1-A column (25 cm × 4.6 mm I.D.) with an α-aminopropyl packing of 5-μm spherical particles modified with the (*R*)-N-3,5-dinitrobenzoyl (3,5-DNB) derivative of D-phenylglycine (Regis, Morton Grove, IL, U.S.A.).

The covalently bonded column was a stainless-steel, Regis-packed Pirkle covalent phenylglycine column (25 cm × 4.6 mm I.D.) with a silica packing of 5-μm

spherical particles which first were derivatized with γ-aminopropyl groups; the terminal amine was then linked to the above chiral phenylglycine via amide linkages (Regis).

Materials

Acetyl, lauryl, benzoyl, *p*-anisoyl, 3,5-DNB and trimethylacetyl chlorides were purchased from Aldrich (Milwaukee, WI, U.S.A.). The 1- and 2-naphthoic acids were purchased from the same supplier. Racemic *p*-methoxyamphetamine was generously supplied by the Mid-Atlantic Regional Laboratory of the U.S. Drug Enforcement Administration. All HPLC organic solvents were purchased from Burdick & Jackson (Muskegon, MI, U.S.A.). The remaining chemicals were reagent grade and used as purchased.

General synthesis procedures

The amphetamine amides were synthesized from the free base and the appropriate acid or acid chloride according to the procedure described by Dale *et al.*[24]. The resulting amides were recrystallized from methanol, methanol–water or water.

Chromatographic conditions

The mobile phase was hexane–isopropanol (97:3). A flow-rate of 2 ml/min and a column temperature of 20°C were maintained throughout the analysis.

Order of enantiomeric elution

The elution order of the (*R*)- and (*S*)-isomers was determined by chromatographing a 3:1 mixture [(*S*):(*R*)] of the two isomers. The mixture was prepared by using known amounts of the pure (*S*)-isomer and the racemic mixture.

RESULTS AND DISCUSSION

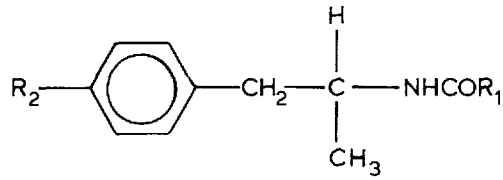
The chromatographic results obtained with the covalently bonded CSP are presented in Table I. The separation (α) and resolution (R_s) factors for the aliphatic amides (compounds 1, 3 and 4) ranged from 1.03 to 1.05 and from 0.30 to 0.53, respectively. For amides with π-basic substituents (6–9), α values ranged from 1.06 to 1.09 and R_s values ranged from 0.82 to 1.34. For the amide containing a π-acid substituent (5), the α value was 1.03 and the R_s value was 0.44. In every case determined*, the (*S*)-isomer eluted before the (*R*)-isomer.

The results of the chromatography of amides 1, 5, 6 and 9 on the ionically bonded CSP are presented in Table II. For amides 1, 6 and 9, the α and R_s values were lower than those obtained on the covalently bonded CSP. These results are contrary to recent reports which state that the covalently bonded CSP is less efficient than the ionically bonded CSP[25]. The 3,5-DNB derivative (5) had the same α and R_s values on both columns. The (*S*)-isomers of amides 1, 6 and 9 eluted before the corresponding (*R*)-isomers. However, there was a reversal in the elution order of

* *p*'-Methoxyamphetamine was available only as the racemate and the order of elution was not determined.

TABLE I

CHROMATOGRAPHIC RESULTS OBTAINED WITH THE COVALENTLY BONDED CSP COLUMN



Compound	R_1	R_2	k_1'*	α	R_s
1	Methyl	H	15.4	1.05	0.53
2	Methyl	Methoxy	32.8	1.06	1.03
3**	Lauryl	H	16.2	1.03	0.30
4***	*tert.*-Butyl	H	2.7	1.03	0.33
5	3,5-DNB	H	28.8	1.03	0.44
6	Phenyl	H	11.4	1.07	0.82
7	*p*-Methoxyphenyl	H	25.1	1.06	1.03
8	1-Naphthyl	H	41.8	1.06	0.89
9	2-Naphthyl	H	24.2	1.09	1.34

* k_1' is the capacity ratio for the initially eluted enantiomer, which is the (*R*)-isomer in all cases except for compound 2, where the elution order is unknown.

** Chromatographed at a flow-rate of 0.5 ml/min.

*** Chromatographed at a flow-rate of 1.0 ml/min.

amide 5, *i.e.* the (*R*)-isomer eluted before the (*S*)-isomer. The significance and interpretation of this unique reversal are under further investigation.

The chromatographic results can be explained using the "three point" chiral recognition model proposed by Dalgliesh[26]. Dalgliesh postulated that chiral recognition requires a minimum of three simultaneous interactions between the CSP and solute. At least one of these interactions must be stereochemically dependent and may be either attractive or repulsive. The relative strengths of the resulting diastereoisomeric complexes determine the resolution and elution order of the two enantiomers.

TABLE II

COMPARISON OF COVALENT AND IONIC CSP COLUMNS

*Compound**	α (R_s)	
	Covalent column	*Ionic column*
1	1.05 (0.53)	1.01 (0.00)
5	1.03 (0.44)	1.03 (0.45)**
6	1.07 (0.82)	1.02 (0.40)
9	1.09 (1.34)	1.06 (1.13)

* See structure shown in Table I.

** The (*S*)-isomer elutes before the (*R*)-isomer in this instance, whereas the (*R*)-isomer elutes before the (*S*)-isomer in the other cases.

For the amphetamine amides (Fig. 1A) there are four possible sites for bonding interactions with the CSP. Three of these sites are common to all of the derivatives: the phenyl ring of the phenethyl moiety (site 1), which can act as a π–π donor–acceptor site; the amide hydrogen (site 2), which can act as a hydrogen bond donor; and the amide carbonyl (site 3), which can act as a hydrogen bond acceptor. The existence of an additional π–π donor–acceptor site (site 4) depends upon the structure of the derivatizing agent.

The corresponding bonding sites on the CSP (Fig. 1B) can be deduced, as Pirkle has suggested, by treating the *N*-3,5-DNB derivative of phenylglycine as being "locked" in a conformation where the amide hydrogen is essentially in the plane of and *trans* to the amide carbonyl oxygen[27]. This conformation provides the CSP with four readily available bonding sites: the 3,5-DNB ring (site 1′), which acts as a π-acidic receptor site; the carboxyl function of the phenylglycine in the ionically bonded CSP, or the corresponding amide carbonyl in the covalent analogue (site 2′), which can act as a hydrogen bond acceptor; the amide hydrogen (site 3′), which can act as a hydrogen bond donor; and the phenyl ring of the phenylglycine molecule (site 4′), which can act as a π–π donor–acceptor site.

The results from the chromatography of the amide derivatives on the covalently bonded CSP (Table I) support the assumption that the bonding interactions take place between sites 1 and 1′, 2 and 2′, 3 and 3′, and, when possible, between sites 4 and 4′.

Sites 1–3 are suggested from separation of the aliphatic amide compounds 1–4, where these sites are the only available bonding loci. Sites 1′–3′ were chosen as the corresponding interacting groups on the CSP— from conformational and bonding considerations starting from the assumption that the phenyl ring of the phenethyl moiety (site 1) would preferentially interact with the π-acidic 3,5-DNB portion (site 1′) of the CSP.

This assumption is supported by the increased separation and resolution resulting from the addition of a methoxy group in the *para*-position of the amphetam-

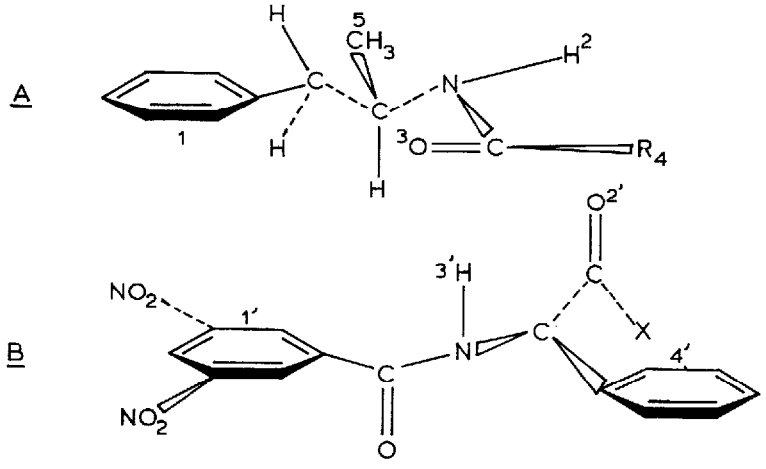


Fig. 1. Bonding interaction between solute and CSP. (A) (*S*)-1-phenyl-2-aminopropane amide; (B) CSP. X = $-NH(CH_2)_3-$, covalently bonded column; X = $-O^-$, ionically bonded column.

ine molecule (2). This substitution enhances the π-basicity of site 1 and strengthens the π–π interaction between sites 1 and 1′.

The importance of the amide hydrogen (site 2) is suggested by the fact that when this hydrogen was replaced by a methyl group, the amide derivatives could not be resolved. Thus, under the chromatographic conditions, the acetyl and benzoyl amides of the N-methyl derivative of amphetamine [(*R*)- and (*S*)-methamphetamine] had capacity ratios (k') of 10.3 and 13.5, respectively, but no separation was observed.

The effect of the interaction between sites 4 and 4′ is evident in the enhanced separation and resolution of compounds 6–9. In these cases, the amide molecules possess an additional site for π–π interactions. Consideration of the geometry of

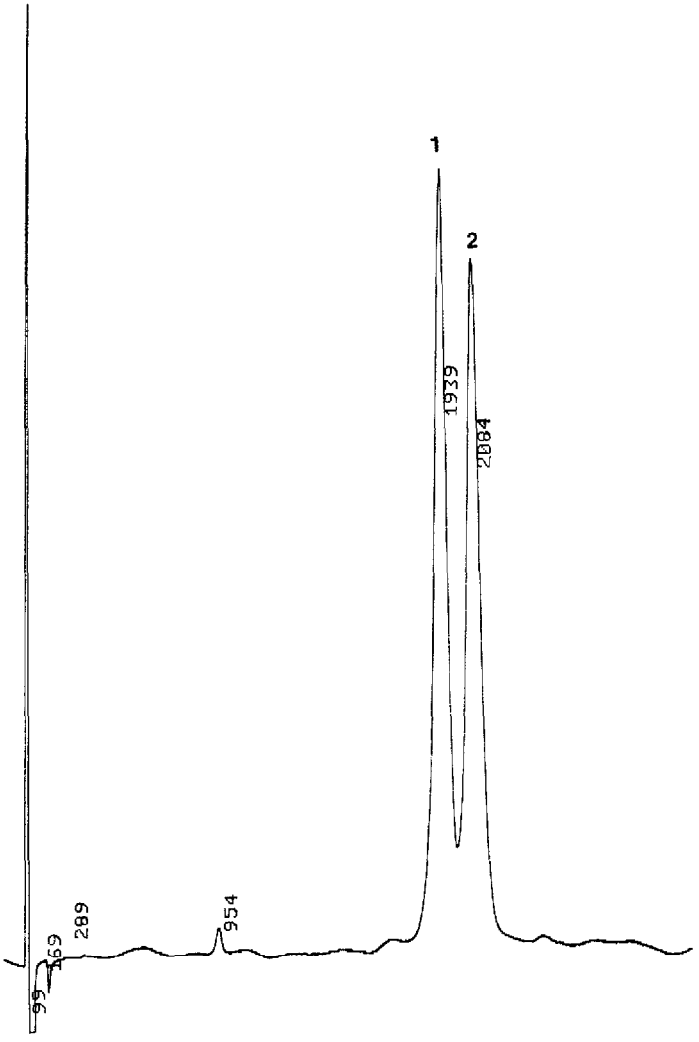


Fig. 2. Resolution of a racemic mixture of the enantiomers of 1-phenyl-2-aminopropane as the 2-naphthoyl derivatives. Peaks: 1 = (*R*)-isomer, 2 = (*S*)-isomer.

the complex demonstrates that the 2-naphthoyl derivative has the maximum overlap between the two sites, and, as expected, has the largest separation and resolution (Fig. 2).

Examination of the solute–CSP complexes through the use of space-filling models reveals that although the four interactions cited above may be responsible for the formation of the complexes, they are not *per se* the source of the chiral recognition. This is not surprising in view of the fact that two of the three major bonding sites for the amphetamine molecule (sites 2 and 3, and in addition site 4) are on the same bond of the asymmetric carbon. When the solute enters into the complex, the CSP cannot effectively differentiate between the two enantiomers since there is no bonding interaction under direct stereochemical control. The same is true, in reverse, for the CSP where sites 1′ and 3′ are on the same bond.

The space-filling models reveal that there may be an additional interaction, a steric interaction involving the α-methyl group of the amphetamine molecule (Fig. 1A, site 5), which can be the stereochemically controlled interaction and the source of the observed resolution and elution order.

The reasons for the differences in separation and resolution capabilities of the two CSPs are not readily discernible, but they most likely lie in the electronic and steric differences between a molecule containing an amide bond at site 2′ and one containing a carboxylate anion involved in an ion pair at the same site.

CONCLUSIONS

The results of this study demonstrate that both the ionic and covalent CSP columns have applicability to the development of regulatory assay. Initial studies utilizing the 2-naphthoyl derivative (9) indicate that the assay should be able to detect trace amounts of the (*R*)-isomer in the presence of the (*S*)-isomer. Further quantitative studies are in process.

This study also demonstrates that such application will not be straightforward. As Pirkle *et al.*[22] have pointed out, the net result of the chromatography is probably a weighted average of competing modes of interaction. In the current study there are not only competing interactions, but also competing conformations which contribute to the observed separations.

It is clear from the results on both columns and from the differences between them that the situation is highly susceptible to secondary influences which add to its complexity. Although this complexity may increase the difficulty of devising and interpreting some separations, it also permits separations such as the one observed in this study to be made and should be welcomed both as a challenge and as an opportunity.

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