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ENANTIOMERIC COMPOSITION ANALYSIS OF AMPHETAMINE AND METHAMPHETAMINE BY CHIRAL PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

N-(Trifluoroacetyl)-*l*-prolyl- (N-TFA-*l*-prolyl-) *d*- and *l*-amphetamine diastereoisomers were separated by high-performance liquid chromatography and confirmed by an interfaced mass spectrometer system, using the commercially available N-3,5-(dinitrobenzoyl)phenylglycine chiral column. A separation factor of 1.52 and resolution of 3.8 were observed. N-TFA-*l*-prolyl-*d*- and -*l*-methamphetamine diastereoisomers were only partially resolved.

The chiral stationary phase–solute interactions were studied by varying the mobile phase (2-propanol in hexane). Results indicate the separation mechanism proceeds via dipolar and hydrogen-bond interactions between the chiral stationary phase and the solute. A modified "dipole-stacking" model takes into account these interactions and explains the difference in separability observed for N-TFA-*l*-prolyl-*d*- and -*l*-amphetamine and N-TFA-*l*-prolyl-*d*- and -*l*-methamphetamine.

INTRODUCTION

Possible differences in governmental regulations and pharmacological effects[1] of drug enantiomers necessitate the development of analytical methodologies for the differentiation of these compounds. 1-Phenyl-2-aminopropane (amphetamine) and 1-phenyl-(N-methyl)-2-aminopropane (methamphetamine) are examples of such enantiomeric drugs of pharmaceutical and forensic interest[2]. Specifically, *d*-methamphetamine is a drug of common abuse[3], while *l*-methamphetamine is found in nasal spray[1]. Microscopic observations of diastereoisomeric microcrystals, formed through the reaction of enantiomers with suitable chiral agents, are still commonly used in

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forensic science laboratories for the analysis of these compounds[4]. Analytical methods more suitable for quantitative determinations, such as circular dichroism[5], nuclear magnetic resonance (NMR)[6], radioimmunoassay (RIA)[7] and gas chromatography–mass spectrometry (GC–MS)[8] have also been applied to the analysis of these compounds. More recently, high-performance liquid chromatography (HPLC) has been used, both as a direct[9,10] and an indirect[11,12] method for determining the enantiomeric purity of these compounds.

The indirect approach involves the formation of diastereoisomers through the use of a suitable chiral derivatizing reagent and the subsequent separation of these diastereoisomers on a normal- or reversed-phase HPLC column. The direct approach requires no prior diastereoisomer formation, it relies on the formation of transient diastereoisomeric complexes between the drug enantiomers and the chiral stationary phase or between the drug enantiomers and a chiral mobile phase[13]. The direct approach has become increasingly popular since the development of widely applicable, commercially available HPLC chiral columns in 1980 (Regis, Morton Grove, IL, U.S.A.; J. T. Baker, Phillipsburg, NJ, U.S.A.). We thought a combination of these two approaches, *i.e.*, the separation of diastereoisomers on a chiral column, might improve stereorecognition by making available two chiral interaction sites on the solute molecule to interact with the chiral stationary phase. The degree to which both chiral centers interact would determine the diastereoisomeric separability.

In this present study, we wish to report the resolution of amphetamine and methamphetamine enantiomers, through the use of two chiral phase columns and the derivatization of a chiral agent, N-(trifluoroacetyl)-*l*-prolyl chloride (*l*-TPC), which has been studied to some detail in our previous GC studies[14]. The resolution achieved through this approach is better than those reported in the literature. Furthermore, a mass spectrometer is interfaced to a liquid chromatograph for the identification of the resolved peaks.

MATERIALS AND METHODS

Reagents

d- and *l*-amphetamines were obtained from Aldrich (Milwaukee, WI, U.S.A.). *d,l*-Methamphetamine–HCl was purchased from Sigma (St. Louis, MO, U.S.A.), and the free base was prepared by dissolving the hydrochloride salt in water and extracting with chloroform under basic conditions. Hexane and 2-propanol were HPLC grade and were purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.). The solvents were degassed by filtering them through a 0.45-μm Nylon 66 filter membrane (Alltech Assoc., Deerfield, IL, U.S.A.). The chiral derivatizing reagent, 0.1 *M* *l*-TPC in chloroform, was obtained from Regis. The reagent contained 6% *d*-TPC (see ref. 14 for resolving complications derived from the presence of this impurity). All chemicals were kept dry and were used without further purification.

Derivatization procedure

The TPC derivatization procedure recommended by the supplier was used to form the diastereoisomeric N-(trifluoroacetyl)-*l*-prolyl- (N-TFA-*l*-prolyl-) *d,l*-amphetamine and N-TFA-*l*-prolyl-*d,l*-methamphetamine (Fig. 1). A typical derivatization procedure is as follows: 15 μl of *d,l*-amphetamine was added to 0.50 ml

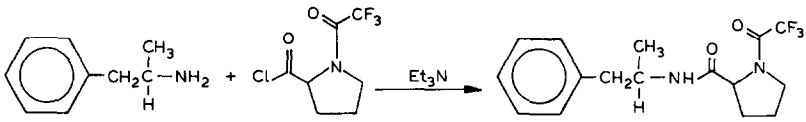


Fig. 1. Derivatization of amphetamine with N-(trifluoroacetyl)-*l*-prolyl chloride. Et_3N = Triethylamine.

chloroform in a sealable vial, followed by the addition of 1.0 ml of the *l*-TPC reagent. The mixture was allowed to stand for 5 min before the addition of 20 µl of triethylamine to take up excess unreacted *l*-TPC. After 15 min of intermittent shaking the solution was washed with 1.0 ml of 6 *M* hydrochloric acid to remove the ammonium salt. The mixture was finally washed with 1.0 ml of distilled water and then dried over anhydrous magnesium sultate. The solution was filtered before being submitted to HPLC analysis.

HPLC and HPLC–MS analysis

Samples were analyzed on a modular isocratic HPLC system consisting of a Laboratory Data Control (Riviera Beach, FL, U.S.A.) Constametric III pump, a variable-wavelength UV monitor set at 254 nm, and a chromatographic control module. The sample injector was a Rheodyne (Berkeley, CA, U.S.A.) injector with a 20-µl sampling loop.

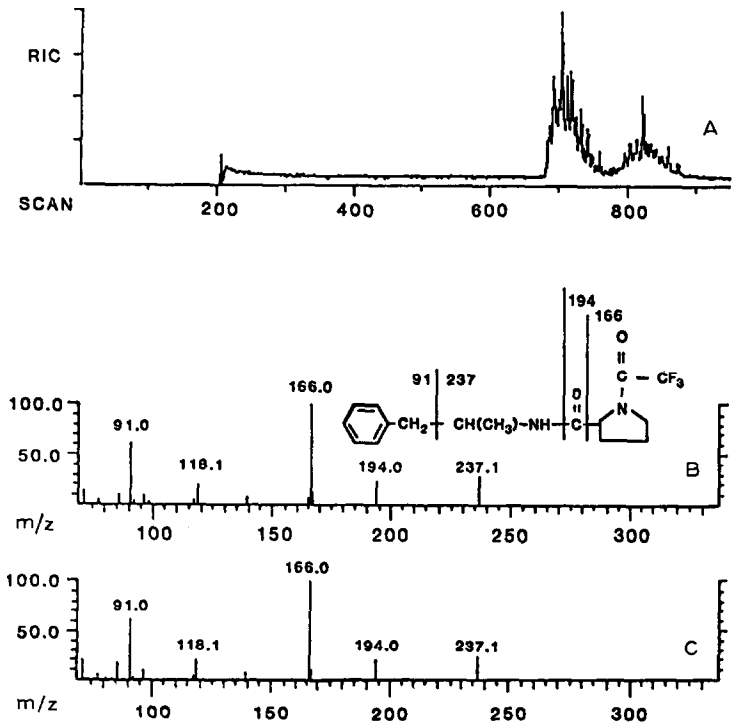


Fig. 2. Reconstructed HPLC–MS total ion chromatogram (A) and the mass spectra of N-TFA-*l*-prolyl-*l*-amphetamine (B) and N-TFA-*l*-prolyl-*d*-amphetamine (C).

The chiral columns used in the study were a Regis Pirkle Covalent Phenylglycine column (25 cm × 4.5 cm I.D.) and a Regis Pirkle Ionic Phenylglycine column (25 cm × 4.5 cm I.D.).

A Waters Assoc. (Milford, MA, U.S.A.) 6000A solvent delivery system with injector was interfaced to a Finnigan 4000 MS/INCOS system through a Finnigan moving belt LC–MS interface. The system was used to confirm the identification of subject compounds. The mass spectrometer was operated on electron impact mode at 70 eV, with scan range of *m/z* 71–450 at 2 s per cycle. The filament was not turned on until the passage of the solvent front. A typical LC–MS ion chromatogram along with the *l*-TPC-derivatized amphetamine mass spectra are shown in Fig. 2.

RESULTS AND DISCUSSION

Resolution of diastereoisomers and enantiomers

Chromatographic data for the separation of *l*-TPC derivatized *d,l*-amphetamine and *d,l*-methamphetamine are given in Table I. Although N-TFA-*l*-prolyl-*d,l*-amphetamine diastereoisomers were well separated by both the covalent and the ionic columns (as shown in Fig. 3 and Table I), the latter column is slightly more efficient. As shown in Table I, maximum resolution (3.8) and separation factor (1.52) were achieved with the mobile phase composed of 2-propanol–hexane (1:99). These results are superior when compared to those reported in the literature using conventional approaches. Specifically, the best reported separation factors involving the use of a

TABLE I

CHROMATOGRAPHIC DATA FOR N-TFA-*l*-PROPYL-AMPHETAMINE AND N-TFA-*l*-PROLYL-METHAMPHETAMINE

2-Propanol (in hexane) (%)	*Capacity factor*	*Separation factor*	*Resolution*	*Column*
N-TFA-l-prolyl-d,l-amphetamine				
0.5	20.8	1.78	–	Ionic
1.0	9.90	1.52	3.8	Ionic
3.0	4.59	1.25	0.80	Ionic
6.0	2.70	–	–	Ionic
0.5	26.2	1.60	–	Covalent
1.0	12.2	1.40	2.7	Covalent
3.0	5.02	1.18	–	Covalent
6.0	2.76	–	–	Covalent
N-TFA-l-prolyl-d,l-methamphetamine				
0.5	19.1	1.10	–	Ionic
1.0	12.1	1.08	–	Ionic
3.0	8.43	–	–	Ionic
6.0	2.95	–	–	Ionic
0.5	19.5	1.09	–	Covalent
1.0	12.0	1.08	–	Covalent
3.0	5.64	1.07	–	Covalent
6.0	2.76	–	–	Covalent

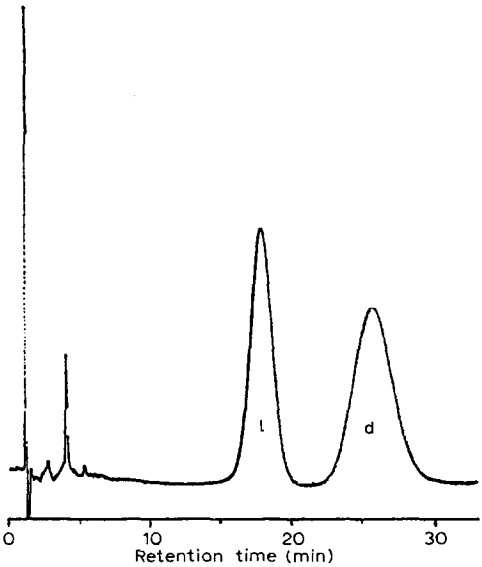
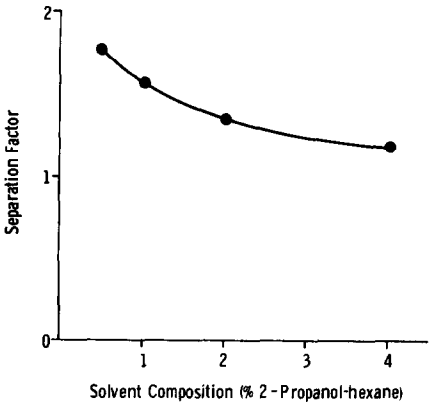


Fig. 3. Resolution of diastereoisomers of N-TFA-*l*-prolyl-amphetamine. Elution order: N-TFA-*l*-prolyl-*l*-amphetamine, N-TFA-*l*-prolyl-*d*-amphetamine.

non-chiral column on chiral derivatization products[11] and a chiral column on non-chiral derivatization products[9] are 1.47 and 1.05, respectively.

Information provided by Fig. 2B and C indicates the mass spectra of these diastereoisomeric pair are, as expected, practically identical. Therefore, the fragmentation pattern as depicted in Fig. 2B could not help differentiate the elution order of these two compounds. However, it was concluded that N-TFA-*l*-prolyl-*l*-amphetamine (*SR*) was eluted from the column prior to N-TFA-*l*-prolyl-*d*-amphetamine (*SS*) as determined by observing peak area ratio of a sample containing three parts of *l*-amphetamine and one part of *d*-amphetamine. The methamphetamine counterparts were not as well separated: the best separation factor, 1.10, was achieved with 2-propanol–hexane (1:99); no difference in column efficiency could be observed in this analysis. Since only racemic methanphetamine was used in this study, the elution order of the derivatized *d*-methanphetamine and *l*-methamphetaine was not empirically determined. However, our previous GC study[14] indicated that methamphetamine and amphetamine enantiomers follow the same elution order.

Mobile phase compositions were varied between 0.5 and 4% and 0.5 and 13% 2-propanol (in hexane) for the studies of separation factors and capacity factors. Results are plotted in Figs. 4 and 5. Data plotted in Fig. 4 are averages of triplicates and duplicates. Individual values are shown as follows: 4% 2-proponol: 1.21, 1.21, 1.21; 2% 2-proponol: 1.36, 1.35, 1.34; 1% 2-proponol: 1.56, 1.56; 0.5% 2-proponol: 1.77, 1.76. These data established the reliability of the relative retention data of the diastereoisomeric pair. It is apparent that as the mobile phase polarity increases, the separation and the capacity factor of the diastereoisomers decrease. Conversely, if the mobile phase polarity is too low, peaks become smeared and retention times become inconveniently long. These results are to be expected taking into account the nature of the solute–mobile phase–stationary phase interactions.



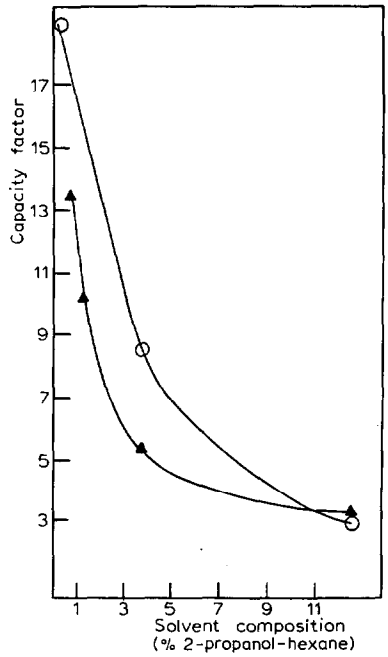


Fig. 4. Separation factor as a function of solvent composition for N-TFA-*l*-prolyl-*d*- and -*l*-amphetamine obtained at 2.0 ml/min on the ionically-bound column.

Fig. 5. Capacity factor as a function of solvent composition for N-TFA-*l*-prolyl-amphetamine (○) and N-TFA-*l*-prolyl-methamphetamine (▲) obtained at 2.0 ml/min. on the ionically-bound column.

Accuracy of quantitative analysis

Quantitative analysis of amphetamine enantiomers by chiral derivatization process is complicated by the presence of *d*-TPC. Since there is a small percentage of *d*-TPC in the *l*-TPC that was used for derivatization, four compounds, N-TFA-*l*-prolyl-*l*-amphetamine (*l-l*), N-TFA-*d*-prolyl-*d*-amphetamine (*d-d*), N-TFA-*l*-prolyl-*d*-amphetamine (*l-d*), and N-TFA-*d*-prolyl-*l*-amphetamine (*d-l*), may result. These four compounds were well resolved in our GC studies[14], but only two peaks (Fig. 3) are observed in the current study. Comparison with results obtained from the GC study indicates that the first peak in Fig. 3 includes *l-l* and *d-d*, while the second peak is composed of *l-d* and *d-l*. The contributions of *d-d* and *d-l*, which were derived from the unintended *d*-TPC impurity, to the respective peaks will affect the accuracy in the quantitative analysis of the enantiomeric composition. This is especially true when the enantiomeric ratio is large and *d*-amphetamine is the minor component in the sample. However, since the composition of *d*-TPC is known, it is possible to calculate and to correct the error derived from this source. True and apparent enantiomeric compositions are calculated in Table II as a function of selected values of percent *d*-TPC and enantiomeric composition ratios. Percent errors are also included in this table. Based on this calculation, analysts may judge the tolerable impurity levels of *d*-TPC and whether it is necessary to apply correction to the apparent results. It should be noted that the *d*-TPC contents in commercial *l*-TPC products are clearly labeled, and are usually in 2% levels.

TABLE II

d-TPC-DERIVED ERROR IN QUANTITATIVE ENANTIOMERIC COMPOSITION DETERMINATION OF AMPHETAMINE

d-TPC (%)	*True ratio*		*Apparent ratio*		*Error* (%)
	l-amphet. amine	*d-amphet. amine*	*l-amphet. amine*	*d-amphet. amine*	
0	80	20	80	20	0
	60	40	59.6	40.4*	1.7**
2	80	20	78.8	21.2	7.1
	90	10	88.4	11.6	15
	60	40	59	41	4.1
5	80	20	77	23	16
	90	10	86	14	32
	60	40	58	42	7.9
10	80	20	74	26	29
	90	10	82	18	49

* Sample calculation: *l-l*, 60 · 98/100 = 58.8; *d-l*, 40 · 98/100 = 39.2; *l-d*, 60 · 2/100 = 1.2; *d-d*, 40 · 2/100 = 0.8. First peak = *l-l* + *d-d* = 58.8 + 0.8 = 59.6; second peak = *d-l* + *l-d* = 39.2 + 1.2 = 40.4.

** Sample calculation: [(60/40 − 59.6/40.4)/(60/40)] · 100 = 1.7%.

Separation mechanism

Of the stereorecognition mechanisms which have been proposed for solute–stationary phase interactions for phenylglycine columns[15], a modified "dipole-stacking" model may account for the effective resolution observed for the compound studied. These interactions are depicted in Fig. 6. The stationary phase is pictured as being conformationally rigid with the amide hydrogen trans to and coplanar with the amide carbonyl.

Five possible sites can be envisioned on the chiral stationary phases: these are: (1) the 3,5-(dinitrobenzoyl)phenylglycine (DNB) ring, a π-acid; (2) the amide carbonyl, adjacent to the 3,5-DNB ring, which can act as a dipole source or as a hydrogen bond acceptor; (3) the amide hydrogen, a hydrogen bond donor or dipole

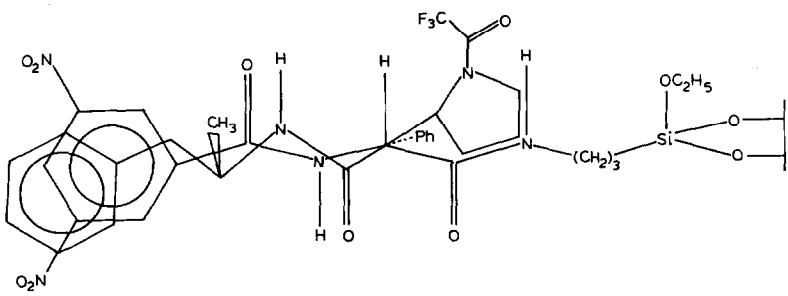


Fig. 6. Modified dipole-stacking model depicting interaction of N-TFA-*l*-prolyl-*d*-amphetamine with 3,5-DNB phenylglycine stationary phase. Ph = Phenyl.

source; (4) the phenylglycine's phenyl group which can interact as a π–π donor–acceptor; and (5) the second carbonyl which can act as a hydrogen bond acceptor or as a dipole source. The observed separation may be considered as results of interaction on all of these five sites; four of these are electronic in nature, the other one being steric. The first three of the electronic interactions are as those described by Pirkle and Welch[15], namely π–π interactions between the 3,5-DNB on the stationary phase and the phenyl ring on the solute, and the electrostatic bonding of the amide dipoles. The fourth interaction may be a dipolar interaction between the second carbonyl on the stationary phase and the highly polarized N–C–F of the prolyl component. Being highly electronegative, the fluorines tend to withdraw electrons from the trifluorinated carbon to facilitate its interaction with the carbonyl oxygen of the stationary phase, with the carbonyl carbon of the stationary phase interacting with the nonbonding electrons on the nitrogen of the solute. The fifth interaction is steric in nature and occurs at the methyl group attached to the chiral carbon on the amphetamine. The steric hindrance exerted by this group restricts the π–π interaction between the 3,5-DNB ring of the stationary phase and the phenyl portion of N-TFA-*l*-prolyl-*l*-amphetamine. On the other hand, the methyl group of N-TFA-*l*-prolyl-*d*-amphetamine is positioned away from the surface of the π–π interaction, and contributes little hindrance to the solute–stationary phase interaction. This model also accounts for the small separation observed for N-TFA-*l*-prolyl-*d,l*-methamphetamine. The lack of hydrogen-bonding potential and the diminishing dipole at the amide nitrogen site minimize the effects of the four electronic interactions.

CONCLUSION

The combined use of a chiral derivatizing reagent and chiral LC column achieved a better resolution of *d* and *l*-amphetamine in comparison with those reported in the literature. Additionally, HPLC–MS provides positive identification of the resolved peaks. A dipole-stacking mechanism accounts for the observed resolution, and stereorecognition is believed to occur through a steric, repulsive interaction between the chiral phases and the solute methyl group.

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