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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC RESOLUTION OF ENANTIOMERS OF 1-PHENYL-2-AMINOPROPANES (AMPHETAMINES) WITH FOUR CHIRAL REAGENTS

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SUMMARY

High-performance liquid chromatography (HPLC) was employed for resolution of enantiomers of chiral ring-substituted 1-phenyl-2-aminopropanes (amphetamines) and 1-phenylethylamine following derivatization with four chiral reagents: (*R*)-(+)-1-phenylethyl isocyanate (PEIC), (—)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (MTPA · Cl), 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC), and 2,3,4-tri-O-acetyl-α-D-arabinopyranosyl isothiocyanate (AITC). Reactions were accomplished under mild conditions (25—70°C) and were complete for all substrates within 60 min. Derivatization with the sugar isothiocyanates (GITC and AITC) and the acyl chloride (MTPA · Cl) was carried out in methylene chloride or acetonitrile in the presence of a base catalyst while derivatization with the isocyanate (PEIC) was performed in methylene chloride. The diastereomeric derivatives were separated by reversed-phase HPLC (C_{18}) with a methanol—water mobile phase. In general, HPLC resolution of the diastereomeric reaction products of GITC or AITC, and MTPA · Cl with the amine substrates was more complete (usually greater than 98% baseline separation) than HPLC resolution of the diastereomeric reaction products of PEIC.

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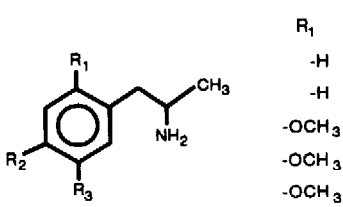
INTRODUCTION

Enantiomers of many drugs possessing chiral centers have different pharmacological activities [1]. Included among these are a variety of amphetamines [2]. For example, (*S*)-(+)-1-phenyl-2-aminopropane (amphetamine) has greater activity than the (*R*)-(—)-enantiomer as a locomotor stimulant [3], hyperthermic agent [4], and ability to elicit stereotypic behavior [3]. The (*R*)-(—)-isomer of the psychotomimetic agent 1-(2,5-dimethoxy-4-methyl)phenyl-2-aminopropane (DOM) has approximately twice the hallucinogenic activity in humans when compared to the racemic mixture, and the (*S*)-(+)-enantiomer is devoid of activity [5]. Long-term neurotoxicity of the serotonergic neurotoxin 1-(4-chloro)-phenyl-2-aminopropane (*p*-chloroamphetamine) is greater for the (*S*)-(+)-enantiomer than for the (*R*)-(—)-enantiomer [6]. Enantiomers of drugs may also differ in their metabolism [7], and this may play a role in their differing pharmacological activities. For example, stereoselective in vitro metabolism of amphetamine [8], DOM [9], and *p*-chloroamphetamine [10] has been described in the literature.

Traditional methods for quantification of enantiomers, e.g. chemical resolution or rotation of polarized light, are not adequate for the determination of trace amounts of enantiomers in biological fluids. Increased interest in stereochemical aspects of pharmacological activity and drug disposition has led to the development of new sensitive and specific methods for the detection of enantiomers in biological fluids, including detection and quantification of one enantiomer in the presence of the other. Most common has been the use of chiral reagents which provide diastereomeric derivatives which can be resolved by chromatographic procedures. Several chiral reagents have been employed for gas chromatographic (GC) resolution of enantiomeric amines including N-pentafluorobenzoyl-S-prolyl-1-imidazolide [11], (—)-α-methyl-α-methoxy-pentafluorophenylacetic acid [12], N-trifluoroacetyl-(D)-prolylchloride [13], and (—)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (MTPA · Cl) [14]. Chiral reagents have also been employed for high-performance liquid chromatographic (HPLC) resolution of amine enantiomers. Chiral reagents used in these procedures have included 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC), 2,3,4,-tri-O-acetyl-α-D-arabinopyranosyl isothiocyanate (AITC) [15, 16], succinimidyl-*l*-α-methoxy-α-methyl-napththaleneacetate [17], and O-methylmandelyl chloride [18]. Our interest in stereochemical aspects of



R_1	R_2	R_3
-H	-H	-H
-H	-Cl	-H
$-OCH_3$	$-CH_3$	$-OCH_3$
$-OCH_3$	$-OCH_3$	$-CH_3$
$-OCH_3$	$-SCH_3$	$-OCH_3$

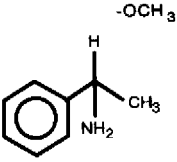


Fig. 1. Structures of the racemic amine substrates.

metabolism and disposition of amphetamines led us to investigate the use of four chiral reagents, (*R*)-(+)-1-phenylethyl isocyanate (PEIC), MTPA · Cl, GITC and AITC for the reversed-phase HPLC resolution of enantiomers of 1-phenylethylamine and the amphetamines shown in Fig. 1.

MATERIALS AND METHODS

Reagents

(*RS*)-(+)- and (*S*)-(+)-amphetamine sulfate were obtained from Smith, Kline and French Labs. (Philadelphia, PA, U.S.A.). (*RS*)-(±)-*p*-chloroamphetamine hydrochloride was obtained from Regis Chemical (Morton Grove, IL, U.S.A.). (*RS*)-(±)- and (*S*)-(−)-1-phenylethylamine were obtained from Aldrich (Milwaukee, WI, U.S.A.). (*RS*)-(±)- and (*R*)-(−)-1-(2,5-dimethoxy-4-methyl)-phenyl-2-aminopropane hydrochloride, (*RS*)-(±)-1-(2,5-dimethoxy-4-thiomethyl)phenyl-2-aminopropane hydrochloride and (*RS*)-(±)-1-(2,4-dimethoxy-5-methyl)phenyl-2-aminopropane hydrochloride were generous gifts of Dr. Neal Castagnoli, University of California (San Francisco, CA, U.S.A.). (*R*)-(+)-1-Phenylethyl isocyanate was purchased from Fluka Chemical (Hauppauge, NY, U.S.A.). (−)-α-Methoxy-α-(trifluoromethyl)phenylacetic acid was purchased from Aldrich. 2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate and 2,3,4,-tri-O-acetyl-α-D-arabinopyranosyl isothiocyanate (AITC) were obtained from Polysciences (Warington, PA, U.S.A.). N-acetyl-L-leucine was purchased from ICN Pharmaceuticals (Plainview, NY, U.S.A.) and N-acetyl-D-leucine was obtained from Sigma (St. Louis, MO, U.S.A.). All solvents were chromatographic or reagent grade.

(*R*)-(−)-*p*-Chloroamphetamine and (*S*)-(+)-*p*-chloroamphetamine were resolved as diastereomeric salts of N-acetyl-D-leucine and N-acetyl-L-leucine as previously described [10]. The acid chloride of (−)-α-methoxy-α-(trifluoromethyl)phenyl acetic acid (MTPA · Cl) was prepared by reaction with freshly distilled thionyl chloride as previously described [14]. The resulting oil was stored in the cold in methylene chloride (0.5 mmol/ml).

Derivatization procedures

PEIC. To 12-ml conical centrifuge tubes was added 0.1 mg of the amphetamine or 1-phenylethylamine dissolved in 100 μl methylene chloride. To each tube was added approximately 10% molar excess PEIC (100 μl, 6.8 μmol/ml solution in methylene chloride), and the reaction allowed to proceed at room temperature for 60 min. Solvents were then removed under a gentle stream of nitrogen. To each tube was added 1 ml of 0.1 *M* sodium hydroxide followed by vigorous shaking or vortexing for 15 min. Following addition of 1 ml of 20% sodium hydroxide and 3.0 ml methylene chloride, tubes were shaken on a mechanical shaker (15 min), and the aqueous layer separated by centrifugation (1000 *g*) for 15 min. The organic layer was removed and washed with 2 ml of 0.1 *M* hydrochloric acid. Aliquots (100 μl) of the methylene chloride extracts were diluted to 1 ml with methanol, and 20-μl aliquots of the diluted solution analyzed by HPLC. Diluted aliquots of the reaction mixtures were also directly injected for HPLC analysis.

MTPA · Cl. To 12-ml conical tubes was added 0.1 mg of the amphetamine or

1-phenylethylamine dissolved in methylene chloride (200 μl). To each tube was added 100 μl MTPA · Cl (100 μl, 0.5 mmol/ml solution in methylene chloride) and pyridine (50 μl). The reaction was allowed to proceed at 70° C for 30 min. Tubes were cooled in an ice water bath, 1 *M* hydrochloric acid added (1 ml), and tubes were shaken on a mechanical shaker for 5 min. Following centrifugation (1000 *g*, 15 min) the aqueous phase was discarded and 15% sodium carbonate (0.5 ml) added with additional methylene chloride (200 μl). The tubes were again shaken for 5 min and the organic layer removed following centrifugation (1000 *g*, 15 min). Aliquots (40 μl) of the methylene chloride extracts were diluted to 1 ml with methanol, and aliquots (20 μl) of the diluted solution analyzed by HPLC. The presence of pyridine precluded direct HPLC analysis of the MTPA · Cl reaction mixtures.

GITC and AITC. To 12-ml screw-capped centrifuge tubes was added 0.05 mg amphetamine or 1-phenylethylamine dissolved in 50 μl methylene chloride. To each tube was added approximately 10% molar excess AITC or GITC (50 μl, 7.6 μmol/ml solution in acetonitrile or methylene chloride containing 0.2% triethylamine). The reaction was allowed to proceed at room temperature for 60 min. To each tube was added 1 ml of 1 *M* hydrochloric acid, and tubes were shaken on a mechanical shaker for 5 min. Following centrifugation (1000 *g*, 15 min) the aqueous phase was discarded and 1 *M* sodium hydroxide (1 ml) added. The tubes were again shaken for 5 min and the organic layer removed following centrifugation (1000 *g*, 15 min). Aliquots (10 μl) of the extracts were diluted to 1 ml with methanol and 20—50-μl aliquots analyzed by HPLC. Diluted aliquots of the reaction mixtures were also directly injected for HPLC analysis.

To determine the amount of the underivatized amphetamines or 1-phenylethylamine remaining at the end of reaction with PEIC, MTPA · Cl, GITC, and AITC, aliquotos of the reaction mixtures (1 μl) were analyzed directly by a nitrogen—phosphorus GC method [14] employing a 10% Carbowax—2% KOH column.

Derivatization time course studies

To samples of (*RS*)-(±)-amphetamine (0.5 mg, 0.5 ml methylene chloride) was added 0.5 ml of PEIC, GITC or AITC reagent solutions (see above). The reactions were carried out at room temperature. At the appropriate times, 40-μl aliquots were withdrawn and diluted with 960 μl of methanol. Aliquots (20 μl) of this diluted solution were then analyzed by HPLC.

For time course of derivatization studies with MTPA · Cl, individual samples of (*RS*)-(±)-amphetamine (100 μl of 1 mg/ml methylene chloride) and pyridine (40 μl) were prepared. MTPA · Cl reagent solution (100 μl, see above) was added to each sample and the reaction allowed to proceed at 70° C. After desired reaction times, samples were placed in an ice bath, 1 *M* hydrochloric acid (1 ml) and methylene chloride (200 μl) were added, the samples shaken for 15 sec and the mixtures frozen with a dry ice—acetone bath. After all reactions were completed, the samples were thawed and shaken for 5 min prior to centrifugation (10 min, 1000 *g*). The aqueous layer was discarded, 0.5 *M* sodium hydroxide (1 ml) added, and the mixture shaken for 5 min. Following centrifugation (10 min, 1000 *g*), 20-μl aliquots of the organic layer were analyzed by HPLC.

HPLC analysis

HPLC analysis of the diastereomeric derivatives was accomplished with a reversed-phase octadecyl column (250 × 4.5 mm, 5-μm particle size, IBM Instruments, Danbury, CT, U.S.A.) and methanol—water mobile phase with a flow-rate of 1—2 ml/min. An IBM CL/9533 ternary gradient liquid chromatograph, LC/934 or LC/9541 Data System, printer plotter, and Millipore Waters Assoc. sample processor (WISP) 710B (Milford, MA, U.S.A.) were used for HPLC analyses. An IBM LC/9523 variable UV detector was employed for UV absorbance signal detection. Mobile solvent conditions for analysis of the derivatives were as follows: PEIC, methanol—water (60:40) isocratic mobile phase; MTPA · Cl, methanol—water (60:40) isocratic mobile phase for 20 min followed by a linear gradient to 100% methanol at 40 min; GITC and AITC, methanol—water (55:45) isocratic mobile phase. Eluting materials were detected by UV absorbance at 220 nm or 254 nm.

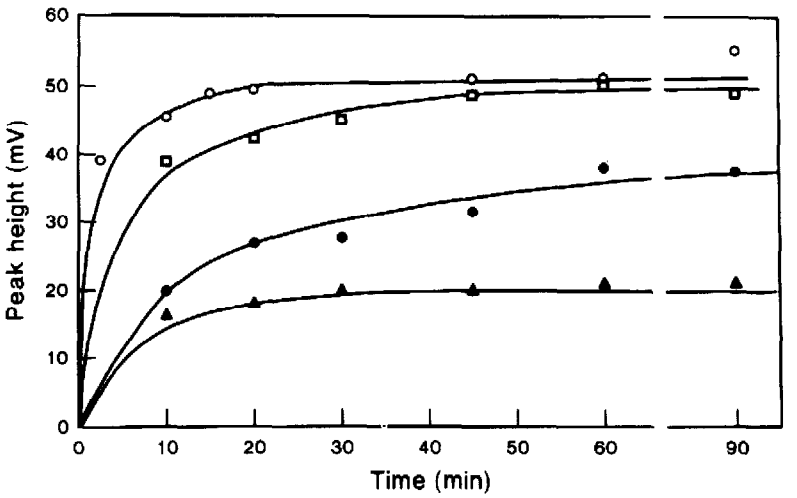
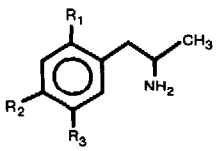


Fig. 2. Time course of derivatization of (*RS*)-(±)-1-phenyl-2-aminopropane (amphetamine) with MTPA · Cl (○); GITC (□); AITC (●); and PEIC (▲).



			R			
R_1	R_2	R_3	PEIC	MTPA•Cl	GITC	AITC
-H	-H	-H	0.36	1.20	1.17	1.14
-H	-Cl	-H	0.39	1.38	1.61	1.55
1-phenylethylamine			0.00	0.84	1.31	1.17
$-OCH_3$	$-CH_3$	$-OCH_3$	0.96	0.89	1.54	1.73
$-OCH_3$	$-OCH_3$	$-CH_3$	0.63	0.98	1.73	1.55
$-OCH_3$	$-SCH_3$	$-OCH_3$	--	1.08	1.19	1.07

Fig. 3. Tabular presentation of the optimum resolution achieved for all substrates with the four chiral reagents by reversed-phase (methanol—water) HPLC analysis as described in Materials and methods. Resolution factor (R) is defined as $2d/(w_1 + w_2)$ where d is the separation between maxima, and w_1 and w_2 are the widths of the peaks at baseline.

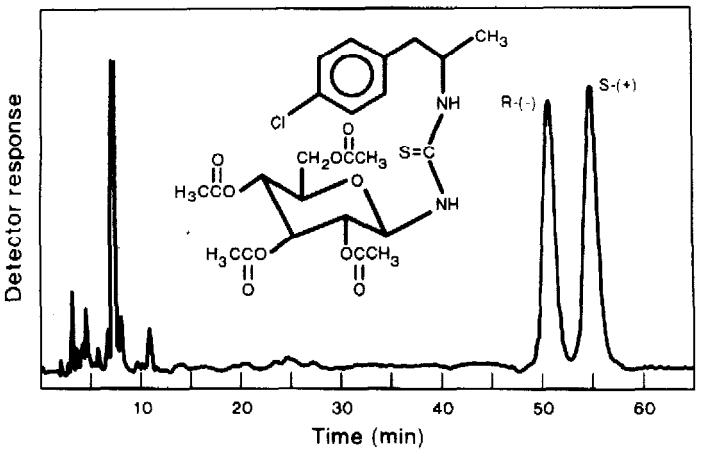


Fig. 4. Reversed-phase HPLC chromatogram of separation of the diastereomeric thiourea derivatives formed from (*RS*)-(±)-*p*-chloroamphetamine with GITC. Mobile phase is methanol—water (55:45) at 1 ml/min.

RESULTS

All amphetamines and 1-phenylethylamine (or the hydrochloride or sulfate salts) formed a pair of diastereomeric products with each of the four chiral reagents under mild reaction conditions. HPLC analysis did not reveal the presence of additional UV-absorbing reaction products following derivatization, and reaction products were stable under reaction and workup conditions. Reaction mixtures could be analyzed directly by HPLC without sample cleanup. Alternatively, hydrolysis and extraction procedures could be employed to remove excess reagent and to isolate underivatizated amines (if any) prior to HPLC analysis. Derivative formation was complete after 60 min, and no amine substrates were detectable at that time by HPLC or GC analysis (data not shown). The time course of derivatization of amphetamine with the four reagents is shown in Fig. 2.

Resolution of diastereomeric reaction products by reversed-phase HPLC varied widely according to substrate and reagent. In general, better resolution was achieved following derivatization with AITC, GITC and MTPA · Cl than with PEIC (data summarized in Fig. 3). For example, optimum resolution values (R) for the separation of the diastereomeric PEIC derivatives were less than 1 while values greater than 1 were obtained for the separation of the AITC, GITC and MTPA · Cl diastereomeric reaction products. The R values of 1.0 and 1.5 indicate 98% and 99.7% baseline resolution of diastereomeric pairs, respectively. The HPLC chromatogram of the diastereomeric products from one derivatization reaction (*p*-chloroamphetamine and GITC) is shown in Fig. 4. Actual retention times for the diastereomeric reaction products under optimum HPLC conditions (described in Materials and methods) are reported in Table I. In general, retention times may be reduced by one-half (by varying mobile phase conditions) with 3—20% loss in resolution with MTPA · Cl, GITC and AITC. Enantiomers were available for some substrates allowing determination of the order of antipode elution. When determined, the (*R*)-(—)-antipode was

TABLE I

RETENTION TIMES OF DIASTEREOMERIC REACTION PRODUCTS AT OPTIMUM RESOLUTION

Reagent	Substrate*			Retention time** (min)	
	R_1	R_2	R_3	*R*—	*S*—
PEIC	—H	—H	—H	13.5	14.0
	—H	—Cl	—H	23.6	24.2
	1-phenylethylamine			13.0	13.0
	$-OCH_3$	$-CH_3$	$-OCH_3$	26.3	28.0
	$-OCH_3$	$-OCH_3$	$-CH_3$	26.3***	27.2***
	$-OCH_3$	$-SCH_3$	$-OCH_3$	—	—
MTPA · Cl	—H	—H	—H	9.7	10.4
	—H	—Cl	—H	13.9	14.9
	1-phenylethylamine			22.1	22.8
	$-OCH_3$	$-CH_3$	$-OCH_3$	33.0	33.5
	$-OCH_3$	$-OCH_3$	$-CH_3$	32.5***	38.9***
	$-CH_3$	$-SCH_3$	$-OCH_3$	30.8***	31.4***
GITC	—H	—H	—H	26.4	28.5
	—H	—Cl	—H	49.2	53.1
	1-phenylethylamine			15.3	17.2
	$-OCH_3$	$-CH_3$	$-OCH_3$	46.4	51.0
	$-OCH_3$	$-OCH_3$	$-CH_3$	43.2***	47.0***
	$-OCH_3$	$-SCH_3$	$-OCH_3$	35.6***	38.0***
AITC	—H	—H	—H	18.2	16.8
	—H	—Cl	—H	35.4	32.3
	1-phenylethylamine			11.4	10.4
	$-OCH_3$	$-CH_3$	$-OCH_3$	33.1	30.2
	$-OCH_3$	$-OCH_3$	$-CH_3$	31.2***	28.7***
	$-OCH_3$	$-SCH_3$	$-OCH_3$	24.8***	23.5***

*See Fig. 3.
**Enantiomeric assignment based on retention times of pure enantiomers except where noted.
***Order of elution based on results with substrates for which enantiomers were available.

eluted prior to the (*S*)-(+)-antipode for PEIC, GITC, and MTPA · Cl derivatives. The opposite order of elution was observed for AITC derivatives. The results with AITC and GITC are consistent with those of Nimura et al. [16] for HPLC elution of AITC and GITC catecholamine derivatives.

DISCUSSION

HPLC resolution of enantiomers of amphetamines and related compounds has not been widely reported in the literature. Examples include resolution of DOM following derivatization with succinimidyl-*l*-α-methoxy-α-methyl-1-naphthaleneacetate [17], resolution of amphetamine amides with a chiral column [19], and resolution of catecholamines and amino acids with GITC and AITC [15, 16]. Our interest in stereochemical aspects of metabolism

prompted an investigation of methods which could be used for the resolution of amphetamines and which would be appropriate for biological studies. Based on the results of our studies, resolution of other amphetamines and related amines should be possible with one or more of the four chiral reagents studied. HPLC procedures may be preferred to GC procedures when derivatives are not adequately volatile or stable for GC analysis. The described HPLC methods are adequate for biological analysis as well. We can readily quantitate less than 100 ng derivative on column with detection at 254 nm, and sensitivity may be increased by detection at 220 nm (data not shown). An added advantage of HPLC resolution procedures such as these is the ability to recover analyzed materials. We are employing HPLC separation of amine enantiomers following derivatization with AITC or GITC as a preparative method for isolation of radiolabeled amine enantiomers [20].

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