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Note

Direct enantiomeric separation of anticholinergic drugs derived from (±)-cyclohexyl(3-thienyl)glycolic acid on a novel α_1-acid glycoprotein-bonded chiral stationary phase (Chiral-AGP)

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In the pharmaceutical and agrochemical areas there is growing interest in studies of the relationship between molecular stereochemistry and pharmaceutical activity, potency and plasma disposition[1]. Differences in the pharmacokinetic and pharmacodynamic behaviours of drug enantiomers have been found for various families of biologically active molecules, including the important class of anticholinergic agents[2] such as atropine[3]. A recent study[4] of compared antimuscarinic effects of (*R*)- and (*S*)-oxyphencyclimine hydrochloride indicated that the (*R*)-(+)- enantiomer inhibited the binding to cholinergic receptor 39 times more potently than the (*S*)-(−)-enantiomer, and this is a general trend observed[5] for heterocyclic amino alcohol esters derived from (−)-glycolic acids compared with those derived from (+)-glycolic acids [*e.g.*, from (*R*)-(−)- or (*S*)-(+)-cyclohexylphenylglycolic acid[6] (CHPGA)]. The glycolic esters of dialkylamino alcohols considered in this paper (Table I, racemates **1**, **4**, **7** and **10**) also proved to exhibit anticholinergic activity; the influence of molecular stereochemistry is now under investigation.

Accordingly, pharmacologists and clinicians now need reliable and accurate analytical methods to carry out enantiomeric assays in biological samples in order to evaluate the magnitude and consequences of stereochemistry in biological processes. The direct resolution of enantiomers by high-performance liquid chromatography (HPLC) on chiral stationary phases (CSPs)[7,8] is such a method. Among various commercially available CSPs, α_1-acid glycoprotein (α_1-AGP) immobilized on silica gel[9] (Enantiopac; LKB, Stockholm, Sweden) appeared particularly suitable for the resolution of cationic drugs, including anticholinergic agents such as atropine[10,11], tropicamide[12], cyclopentolate[10,11], homatropine[10], methylhomatropine[10,11], oxyphencyclimine[10,13] and mepenzolate bromide[10–13]. These amino alcohol esters (except for mepenzolate bromide) are characterized by the presence of a tertiary amino group and an asymmetric carbon located at either the α- (homatropine, methylhomatropine and oxyphencyclimine) or β-position (atropine, cyclopentolate and

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tropicamide) with respect to the hydroxy group. Mepenzolate bromide differs completely, as the chiral carbon belongs to the cyclic amino moiety. The compounds considered here, derived from (±)-cyclohexyl(3-thienyl)glycolic acid (CHTGA), possess structures closely related to homatropine, methylhomatropine and oxyphencyclimine and were therefore expected to be resolved on the α_1-AGP CSP.

This paper reports the direct LC enantiomeric resolution of this new series of anticholinergic drugs on the commercially available protein CSP, Chiral-AGP (ChromTech, Stockholm, Sweden) developed by Hermansson and co-workers[14,15] and for which plasma α_1-AGP is immobilized according to a novel technique on spherical porous silica particles ($d_p = 5$ μm).

EXPERIMENTAL

Apparatus

Analytical chromatography was performed with a modular liquid chromatograph (Gilson, Villiers-le-Bel, France) equipped with a Model 802C manometric module, a Gilson 811 (1.5-ml) dynamic mixer and a Model 116 variable-wavelength UV detector. The column and solvent were thermostated with a Haake Model D8-V circulator bath (−5 to 150°C) (Roucaire, Vélizy-Villacoublay, France) and a water cooling-jacket. All tubing connections were heat-insulated. UV detection was carried out at 230 nm.

Chiral stationary phases

The 100 × 4.6 mm I.D. Chiral-AGP column was purchased from ChromTech.

Syntheses of anticholinergic compounds 1–12

Caution. These compounds display potent anticholinergic activity and it is advisable to handle them carefully. The glassware was washed thoroughly and residues were disposed of by steeping in 6 M hydrochloric acid.

The preparation of compounds **1–12** (Table I), either as enantiomers or racemates, was carried out by refluxing in a molar ratio of 2-(3-thienyl)-2-cyclohexyl-2-hydroxyacetic acid[16] with the corresponding β- or γ-dialkylaminoalkyl halogenated derivative [purchased as hydrochlorides from Aldrich (Milwaukee, WI, U.S.A.)] in 2-propanol as follows.

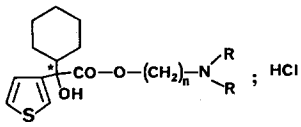
In a round-bottomed flask were placed 4.8 g (20 mmol) of 2-(3-thienyl)-2-cyclohexyl-2-hydroxyacetic acid, 20 mmol of the corresponding halogenated derivative (freshly prepared)[17] and 60 ml of 2-propanol. The mixture was refluxed with magnetic stirring under a nitrogen atmosphere for 2 h (**1–6**) or 8 h (**7–12**). The solid which appeared after cooling to room temperature was collected by filtration under suction and washed with 10 ml of cold 2-propanol. Crystallization from ethanol afforded pure **1–12** as hydrochlorides. Physical data are listed in Table I and ^{1}H NMR data in Table II.

Solvents

Aqueous buffer solutions were prepared either directly[18] from mixtures of 0.2 *M* solutions of $NaH_2PO_4 \cdot 2H_2O$ and $Na_2HPO_4 \cdot 2H_2O$ (extra-pure grade, Merck, Darmstadt, F.R.G.), or from a commercially available sodium phosphate buffer (25

TABLE I

PHYSICAL DATA REFERRING TO THE SYNTHESIS OF COMPOUNDS 1–12



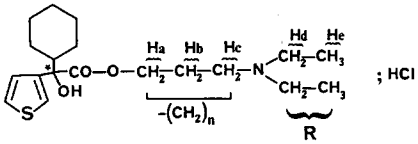
1-12

No.	*n*	*R*	*Configuration*	$[\alpha]^{25}_{365}$ (°) *(water, c = 1)*	*Melting point (°C)*	*Yield (%)*
1	2	CH_3	Racemate		186–188	43
2			*R*	−16.8	208–210	36
3			*S*	+16.1	208–210	35
4	2	C_2H_5	Racemate		205–207	96
5			*R*	−17.6	212–214	92
6			*S*	+18.0	212–214	93
7	3	CH_3	Racemate		168–169	65
8			*R*	−17.7	194–196	55
9			*S*	+18.4	194–196	36
10	3	C_2H_5	Racemate		169–170	75
11			*R*	−10.7	152–154	62
12			*S*	+10.5	151–153	67

TABLE II

200 MHz ^{1}H NMR SPECTRA OF RACEMIC COMPOUNDS **1**, **4**, **7** AND **10**

Chemical shifts in ppm from internal standard [2,2,3,3-$^2H_4$]3-(trimethylsilyl)propionic acid (sodium salt) (TSP-d_4) in ^2H_2O (c = 20 mg ml^{-1}) at 295 K. Racemates and enantiomers showed identical spectra.



Compound	*3H (aromatic)*	*11H (cyclohexyl)*		*2Ha*	*2Hb*	*2Hc*	*4Hd*	*6He*
		$-(CH_2)_5-$	*$-CH-$*					
(±)-**1**: $n=2$, R = CH_3	7.28 (m) 7.50 (m)	0.9–1.9 (m)	2.39 (m)	4.52 (t)	–	3,52 (t)	–	2.87 (s)
(±)-**4**: $n=2$, R = C_2H_5	7.26 (m) 7.48 (m)	0.9–1.9 (m)	2.35 (m)	4.52 (t)	–	3.52 (t)	3.18 (q)	1.27 (t)
(±)-**7**: $n=3$, R = CH_3	7.27 (m) 7.48 (m)	0.9–1.85 (m)	2.35 (m)	4.28 (m)	2.09 (m)	3.01 (d.d)	–	2.82 (s)
(±)-**10**: $n=3$, R = C_2H_5	7.24 (m) 7.49 (m)	0.9–1.9 (m)	2.36 (m)	4.30 (m)	2.07 (m)	3.01 (d.d)	3.17 (q)	1.24 (t)

m*M*, pH 6.88) (Merck) (in both instances diluted to afford 8 m*M* buffers). Deionized water was doubly distilled on a Buchi-Fontavapor 285 apparatus (Roucaire). The pH of the aqueous buffer eluent was controlled with a Model Minisis 8000 pH/millivoltmeter (Tacussel, Villeurbanne, France) and Tacussel glass TB/HS and Tacussel C8 calomel reference electrodes. Aqueous solvents were filtered through 0.65-μm DAWP Millipore membrane filters (Touzart et Matignon, Vitry-sur-Seine, France) and then degassed with helium.

2-Propanol was of LiChrosolv grade purchased from Merck. Solutes were dissolved, in an ultrasonic bath, in the 8 m*M* sodium phosphate buffer (pH 7.0). The concentrations of the solutes were around $4 \cdot 10^{-2}$ mg ml^{-1}, corresponding to an amount injected of about 2 nmol (20 μl).

RESULTS AND DISCUSSION

Enantiomeric resolution of (±)-CHTGA by crystallization of diastereomeric salts with either (−)- or (+)-ephedrine and assignment of absolute configuration by circular dichroism measurements have been reported previously[16]. The control of optical purity was carried out by HPLC using a β-cyclodextrin-bonded CSP[19] (Cyclobond-I; Astec, Whippany, NJ, U.S.A.); this CSP is able to resolve a series of aromatic carboxylic acids[19], including (±)-CHPGA[20,21] (precursor of the anticholinergic ester oxyphenonium bromide), but not the ester derivatives.

Investigations carried out on the Chiral-AGP column (Table III) showed that a baseline resolution could be achieved, depending on the mobile phase composition and solute structure (Fig. 1). Several observations can be made from Table III: (a) whatever the *n* value, N-diethylamino substitution leads to both higher retention (for both enantiomers) and selectivity than N-dimethyl substitution; (b) whatever the N-dialkylamino substitution, the retention and selectivity decrease with lengthening of the alkyl "joining block" (as termed in ref. 2) from $n=2$ to $n=3$; (c) the general trend with increasing pH is a concomitant higher retention and selectivity gain, however, for (±)-**1** and (±)-**10** the selectivity is altered to a limited extent compared with the retention.

Enhancement of retention and selectivity with increasing steric bulkiness at the basic nitrogen atom seems to be a general trend with the α_1-AGP CSP[9], already emphasized by Schill *et al.*[10] for a series of compounds related to metoprolol and by Hermansson and co-workers[15,22] for a series of N-dialkyl-substituted succinimides. The latter compounds have some structural similarities with the glycolates in this paper (Fig. 2), but for N-dialkylsuccinimides an increase in *n* from 2 to 3 resulted in a decrease in selectivity, unlike the aforementioned results for thienylglycolates. Such discrepancies can be attributed to other specific structural features of these two series of compounds (cyclic structure for succinimides, etc.) and to the replacement of an ester group for an amide group at the asymmetric centre (Fig. 2).

The increase in retention observed for both enantiomers on changing R can be ascribed mainly to stronger hydrophobic interactions with the protein. For the solute (±)-**10** the selectivity variations on decreasing the 2-propanol content in the mobile phase, are slight; this means that enantiomers are affected to a similar extent by addition of 2-propanol (k' represents the ratio of the sum of the solute–CSP interactions to the sum of solute–mobile phase interactions); accordingly, 2-propanol, which

TABLE III

INFLUENCE OF THE MOBILE PHASE pH ON THE RESOLUTION OF RACEMIC ANTICHOLINERGIC AGENTS **1**, **4**, **7** AND **10**

Column, Chiral-AGP; mobile phase, 8 m*M* sodium phosphate buffer with 2-propanol added;flow-rate, 0.9 ml min^{-1}; t_0 = 1 min; temperature, 20°C; UV detection at 230 nm.

Solute	*2-Propanol (%, v/v)*	*Elution order*	*pH 6.61*			*pH 6.80*			*pH 7.00*			*pH 7.20*			*pH 7.43*		
			k_2'[a]	α[b]	R_s[c]	k_2'	α	R_s	k_2'	α	R_s	k_2'	α	R_s	k_2'	α	R_s
(±)-**1**: n=2, R=CH_3	10	(*R,S*)	10.6	1.48	2.7	13.4	1.58	3.0	13.3	1.54	2.6	21.1	1.57	2.7	26.7	1.55	1.5
(±)-**4**: n=2, R=C_2H_5	10	(*R,S*)	17.6	2.00	3.7	22.9	2.11	4.0	24.2	2.27	4.5	31.2	2.24	3.6	49.5	2.33	2.0
(±)-**7**: n=3, R=CH_3	6	(*S,R*)	13.6	>1.0*	–	17.8	1.18	0.6	19.5	1.17	0.9	31.8	1.16	0.8	46.0	1.29	1.2
(±)-**10**: n=3, R=C_2H_5	6	(*R,S*)	17.7	1.21	1.2	24.2	1.23	1.2	23.2	1.25	1.2	38.1	1.24	1.2	49.1	1.24	1.0
	10	(*R,S*)	7.8	1.18	1.0	9.6	1.19	1.1	9.1	1.23	1.1	14.4	1.21	1.1	24.8	1.20	0.8

[a] Capacity factor of the second eluted enantiomer was calculated from dead retention time t_0 (t_0 = 1 min) as follows: $k' = (t_r - t_0)/t_0$.

[b] Selectivity $\alpha = k_2'/k_1'$.

[c] R_s (resolution factor) = 2 (distance of the two enantiomer peak positions / sum of the band widths of the two peaks at their bases) : $R_s = 2(t_{r2} - t_{r1}) / (w_1 + w_2)$.

The asterix indicates the beginning of separation.

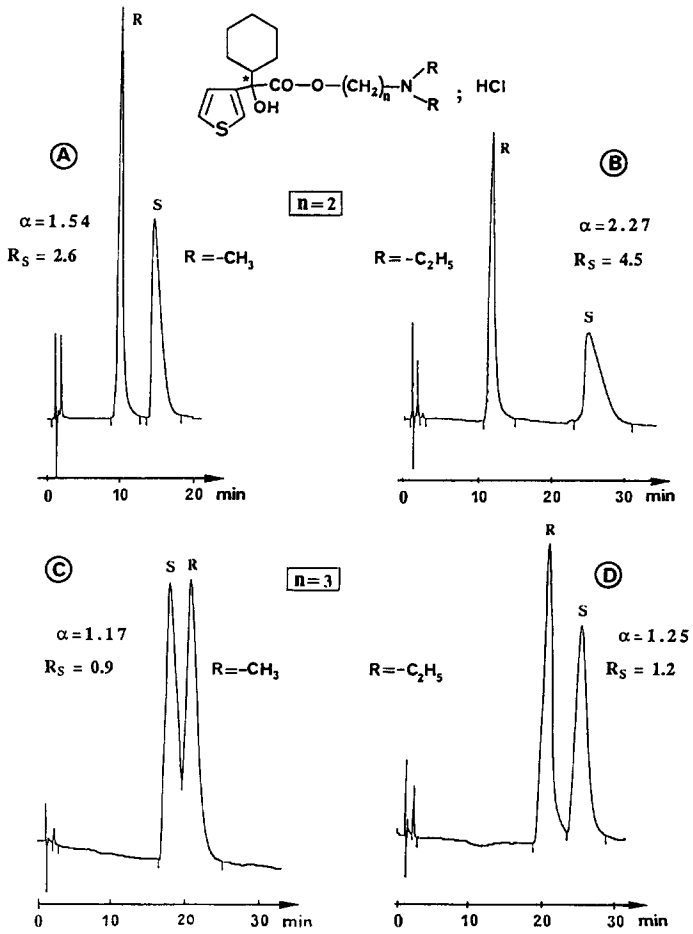


Fig. 1. Influences of the distance between the asymmetric carbon and the nitrogen atom and the N-diaminoalkyl substitution on the resolution of amino alcohols glycolic esters. Column: Chiral-AGP. (A) (±)-**1**; (B) (±)-**4**; (C) (±)-**7**; (D) (±)-**10**. Mobile phase: 8 m*M* sodium phosphate buffer (pH 7.0) + (A) and (B) 10% and (C) and (D) 6% (v/v) 2-propanol. Other conditions as in Table III.

can compete with the solute on the protein binding sites through hydrophobic and/or hydrogen bonding interactions, regulates the retention and to a minor extent the enantiorecognition process for such cationic drugs.

Small variations in the mobile phase pH may be responsible for a significant improvement in selectivity [*e.g.*, for solutes (±)-**4** and (±)-**7**]. The influence of pH on stereoselectivity may result from conformational modifications of the protein involving its ionic binding sites (protein secondary or tertiary structure).

Moreover, regarding the solute structure, the selectivity is markedly affected by the *n* value, *i.e.*, the distance between the cationic ammonium site and the α-hydroxy

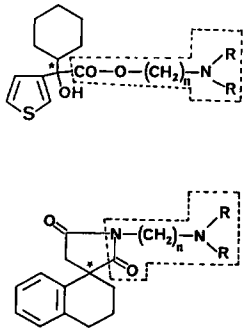


Fig. 2. Structures of (A) N-dialkylamino alcohol glycolic esters (this work) and (B) N-dialkylaminosuccinimides studied by Hermansson *et al.*[22]. Dotted regions mark similar chemical moieties. A possesses an ester function and B an amide function.

group (on the asymmetric carbon), which is probably engaged in hydrogen bonding formation with the protein. This finding suggests that a specific spatial fitting of solute structure with the protein is required for enantiorecognition. Further, different protein binding sites or functional groups of the same binding sites may be involved in the chiral recognition mechanism, depending on the nearest functional environment at the asymmetric centre (*e.g.*, the relative positions of the α-hydroxy group and the basic nitrogen atom), thus leading to differences in the enantiorecognition ability of the CSPs. Accessibility to the protein binding sites is governed by the conformational state: modifications of intramolecular hydrogen bonding and/or electrostatic interactions due to addition of 2-propanol or mainly to pH changes will alter the type and the shape of the binding sites and thus affect its enantiorecognition ability.

Compound (±)-**7** with $n = 3$ and R = methyl was expected to be the worst resolved [assumptions (a) and (b) on p. 409]; nevertheless, the observed inversion of elution order, compared with other solutes, was not predictable (Table III).

CONCLUSION

The use of an aqueous buffered mobile phase with α_1-AGP protein-derived CSP is advantageous for the analysis of biological samples and the good resolutions obtained on the Chiral-AGP column make it suitable for the enantiomeric purity control of thienylglycolates **1–12**. Considering such cationic drugs, ionic binding to the protein and additional hydrogen bond formation and hydrophobic interactions are suggested to account for chiral recognition mechanisms.

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