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Human α_1-glycoprotein acid as chiral selector in the enantioseparation of midodrine and deglymidodrine racemates by HPLC[1]

M.G. Quaglia [a,*], A. Farina [b], E. Bossù [a], V. Cotichini [b]

[a] *Dipartimento Studi Farmaceutici, Università 'La Sapienza', P. le A. Moro 5, 00185 Rome, Italy*
[b] *Istituto Superiore di Sanità, V.le Regina Elena 269, 00161 Rome, Italy*

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Abstract

Human α_1-acid glycoprotein (α1-AGP) has been used as a chiral stationary phase (CSP) for the enantioseparation of midodrine and deglymidodrine racemates in the same HPLC run. The imobilized AGP resulted as the best chiral selector for the enantioresolution of two compounds. Due to the modification of α1-AGP characters as a result of changing the composition of the mobile phase, an attempt study of the watery mobile phase (ionic strength and pH of the buffer, nature and concentration of the organic modifier) allowed for an increase in the enantioselectivity of the chromatographic system and an optimization of the resolution base-line of both enantiomeric pairs. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

The development of a chiral drug needs an enantioseparation method to define its biological activity correctly. The racemate of a chiral drug must be considered as a mixture of two pharmacological distinguished entities. Each enantiomer can react specifically with the same biological system giving a different pharmacological answer. The racemic mixture generally shows pharmacological activity and side effects which are quantitatively different from those of the optically pure enantiomers. Therefore, the safer use of a chiral drug is assured by the knowledge of pharmacological differences between racemate and pure enantiomers and their behaviour in body fluids. From this point of view, midodrine is an interesting chiral drug.

Midodrine,2 - amino - *N* - [2 - (2,5 - dimethoxy - phenyl)-2-hydroxyethyl] aceta mide, is a cardiovascular drug [1,2] having a chiral carbon in C-2 of the hydroxyethyl portion of molecule.

* Corresponding author.

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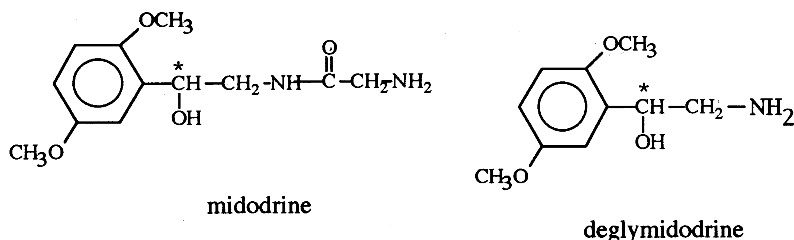

Fig. 1. Molecular structure of midodrine and deglymidodrine.

This drug, used in therapy as a racemic mixture, is described as a prodrug. Actually, midodrine after oral or intravenous administration, undergoes an enzymatic hydrolysis in the systemic circulation, releasing its pharmacologically more active metabolite, deglymidodrine (Fig. 1), which loses the molecule of glycine present in midodrine, but still keeps the chiral carbon [3]. In literature, there are no specific papers about the enantioseparation of midodrine and its active metabolite racemic mixtures, even if the midodrine enantiomers have been separated, together with 56 other chiral drugs, to test the suitability of sulfated cyclodextrins as chiral selector in HPLC and Capillary zone electrophoresis [4,5].

The purpose of this paper is to study a selective method allowing the contemporary enantioseparation of midodrine and deglymidodrine racemates. Actually, a careful investigation about the relationship between chirality and pharmacological activity of midodrine also needs a method resolving deglymidodrine racemate in the same analysis. Therefore, the data related to the best chiral stationary phase (CSP) and conditions for the enantioseparation in the same HPLC run of rac-midodrine and rac-deglymidodrine are reported.

2. Experimental

2.1. Chemicals

All solvents and chemicals were of HPLC grade (Merck, Darmstadt, Germany). The rac-midodrine was kindly supplied by Guidotti Laboratories (Pisa, Italy).

2.2. Equipment

The chromatographic enantioseparation of midodrine and deglymidodrine racemates has been carried out using a Hewlett-Packard Series 1050 chromatograph equipped with a HP 1050 linear photodiode array detector and an autosampler HP 1050 (Hewlett-Packard, Palo Alto, CA). The chromatograph was controlled and the data evaluated by a ChemStation and a computer HP Vectra 90. The photodiode array detector conditions were:

λ value 225 and 270 nm
Acquisition rate of spectra, 1.280 ms
Bandwidth for each channel, 4
Sensitivity range, 50
Reference wavelength, 450 nm and reference bandwidth 50.

2.3. Chromatographic conditions

The chromatographic resolution of two racemates was carried out using a Chiral AGP (100 × 4 mm I.D., 5 μm); provided by Daicel Chemical Industries. A solution containing potassium dihydrogen phosphate/disodium hydrogen phosphate 10 mM at pH 6 has been used as mobile phase at a flow rate of 0.8 ml min^{-1}. The conditions of the other columns tested were:

Chiralcel OD-H (25 cm length and 0.40 cm I.D, 5 μm): mobile phase hexane:ethanol (85:15) containing 0.2% of diethylamine at a flow rate of 0.8 ml min^{-1}. Chiralcel OD-R (25 cm length and 0.46 cm I.D.): mobile phase 200 mM $NaClO_4$, pH 7 with different percentage of CH_3CN. Flow rate 0.5 ml min^{-1}.

2.4. Standards and working standards preparation

The commercial rac-midodrine, with an attested purity of 98.2%, has been considered as standard. The standard of rac-deglymidodrine has been obtained by hydrolysis of rac-midodrine. A solution of rac-midodrine standard (1 mmole) in 1 N NaOH (20 ml) was refluxed for 3 h under stirring. After cooling, the solution was extracted with ethyl acetate (6 × 10 ml). The organic layer was washed with water, dried (Na_2SO_4) and evaporated until dry. The residue was crystallized from ethyl acetate; yield 95%, m.p. = 95–96°C as base. The chromatographic control, made with the α1-AGP column, showed that the hydrolysis compound was a racemic mixture with a different retention time with respect to rac-midodrine. The analysis of rac-midodrine and rac-deglymidodrine, made by chiral and reversed phase chromatography confirmed the purity of compounds. IR and NMR spectra confirmed the identity of deglymidodrine.

Two different calibration curves for the quantitative analysis of rac-midodrine or rac-deglymidodrine standards have been made. The peak area values of the first eluted enantiomer (E1) and the second one (E2), obtained by injecting different concentrations of rac-midodrine or rac-deglymidodrine, have been plotted against the concentration of the two racemates (Fig. 2).

3. Results and discussion

A complete investigation regarding the relationship between stereochemistry and the activity of midodrine requires an identical investigation on its main metabolite, deglymidodrine. Due to deglymidodrine racemate not being commercially available, it was obtained by the hydrolysis of rac-midodrine standard, as reported in the experimental section.

The enantioresolution of midodrine racemate has been successfully obtained using a cellulose tris(3,5-dimethylphenylcarbamate) adsorbed on macroporous silica gel as CSP (Chiralcel OD-H). This CSP however is not able to resolve base-line deglymidodrine racemate as well as rac-midodrine (Fig. 3). The Chiralcel OD-R is in a better position to resolve both racemates separately, but when they are in the same solution the separation of two enantiomer pairs is not possible. Actually, though an attempt study of chromatographic parameters has been made, the selectivity of the method is limited: only a partial resolution of both racemates (Fig. 4) when 13% of acetonitrile to the mobile phase (200 mM $NaClO_4$, pH 7) has been obtained.

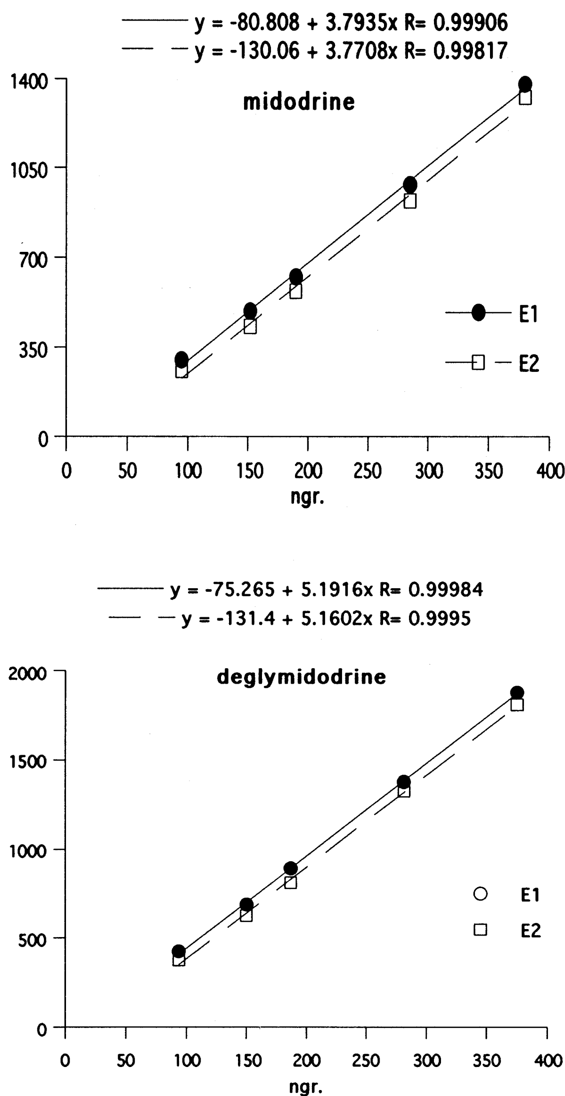

Fig. 2. Calibration curves obtained by plotting the area of first (E1) and second (E2) eluted enantiomers of midodrine and deglymidodrine vs. the concentration of racemates injected.

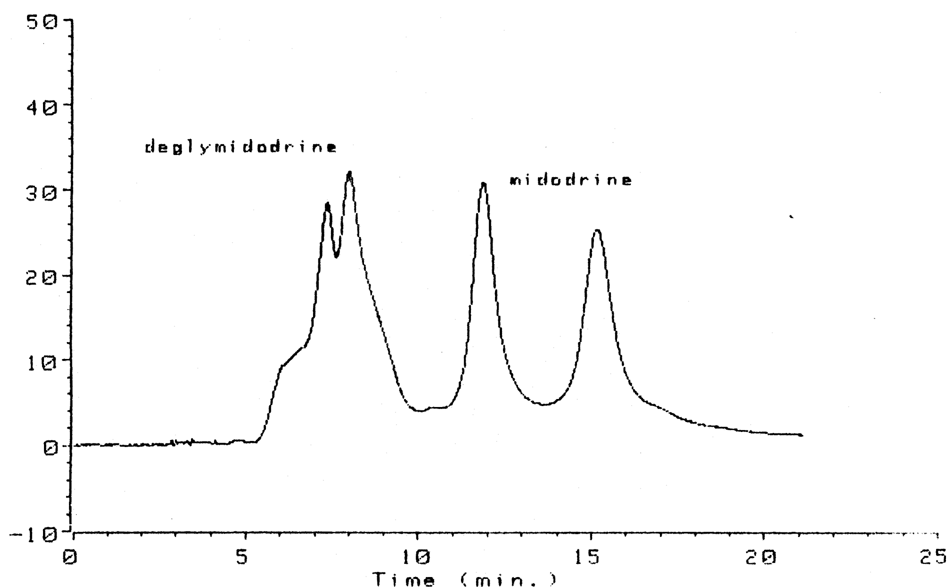

Fig. 3. Enantioseparation of midodrine (a) or deglymidodrine (b) racemates obtained with a Chiralcel OD-H. Chromatographic conditions described in Section 2.

Among the stationary phases investigated, only a silica-bonded α_1-acid glycoprotein (Chiral-α_1-AGP) allowed a contemporary resolution of midodrine and deglymidodrine racemates in the same chromatographic conditions.

α_1-AGP [6–9] is an acidic protein (isoelectric point 2.5) having negatively charged groups. The peptide chain of α_1-AGP links by asparagine residues five carbohydrate units. Two different binding sites are on α_1-AGP and the main one is most likely to be a hydrophobic pocket, formed by an enrichment of hydrophobic amino acid residues such as tryptophan, phenylalanine, leucine and isoleucine. Protolytic amino acid residues and numerous hydrogen bonding groups are in the binding sites. Midodrine and deglymidodrine are two basic compounds, therefore the enantioseparation of their racemates using α_1-AGP as CSP could be due to the interaction with hydrogen bonding groups and hydrophobic amino acid residues.

The immobilized AGP has the property of modifying its character by changing the buffer's cation type, ionic strength and pH, nature and concentration of organic modifier in the mobile phase. The changes in these parameters have a significant effect on enantioselectivity and retention.

Our experiment has been performed in the pH range 5–7, which means that the protein has a net negative charge. Thus, our compounds, which are cationic, can be retained by an interaction with negatively charged groups in the binding sites [10–12]. This hypothesis is supported by the strong effects on the capacity factor (K) of midodrine and deglymidodrine changing the pH of mobile phase (Table 1). The reported data show that the resolution increases increasing the pH from 5.5 to 7, showing the best resolution value at pH 6. Also, an increase in the retention time has been noted, with a maximum value at pH 7. Considering that the isoelectric point of AGP is at a pH value of 2.5, we can suppose that at pH 7, the enantiomers of midodrine and deglymidodrine are completely ionized and strongly bonded to the anionic groups which are present in the binding sites of AGP. As a consequence, an evident increase in the retention time has been noted.

The selectivity in the enantioseparation of midodrine and deglymidodrine with this CSP has been optimized with an attempt investigation regarding the influence of the buffer type, concen-

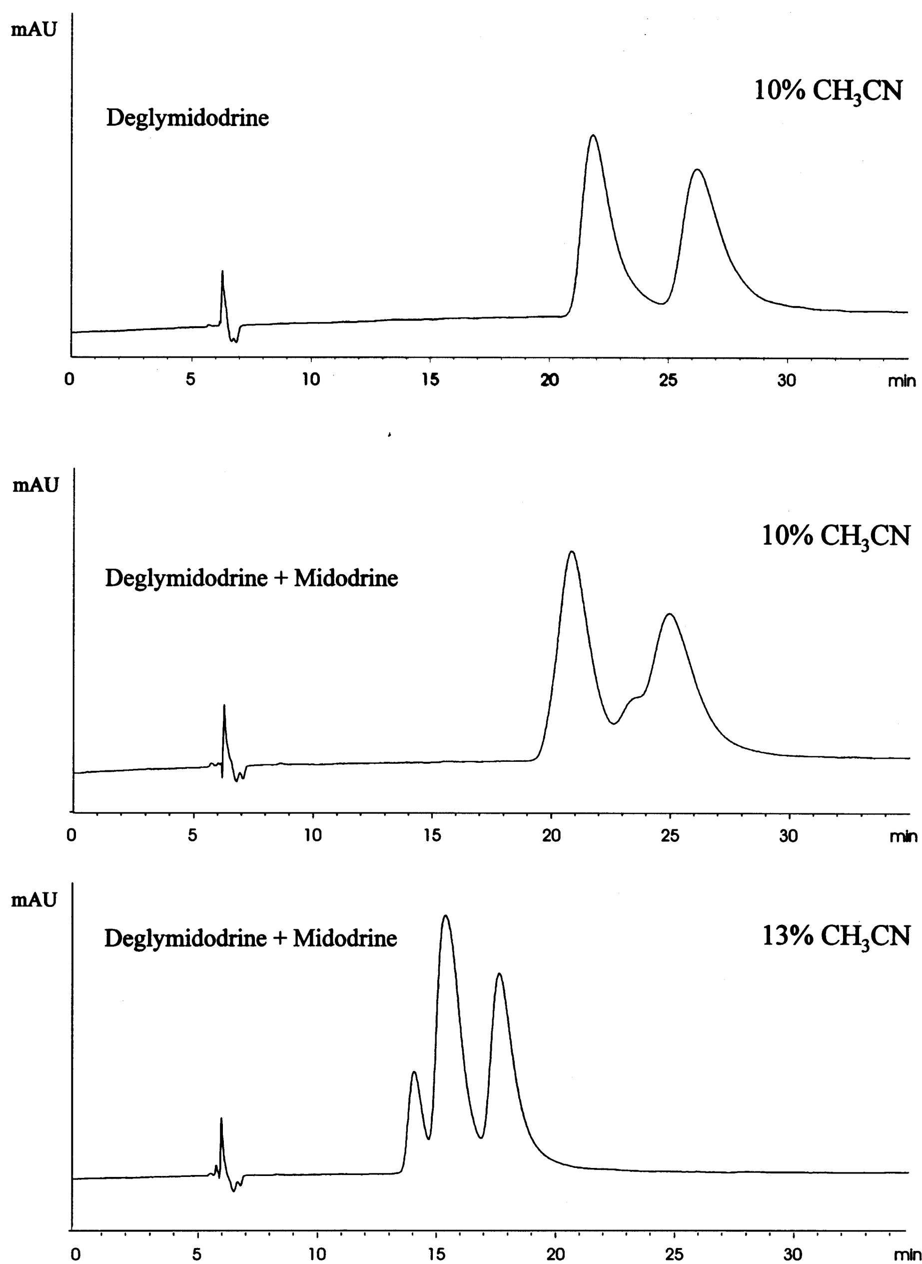

Fig. 4. Enantioseparation of midodrine or deglymidodrine racemates obtained with a Chiralcel OD-R. Chromatographic conditions described in Section 2.

tration and pH value, including the usefulness of the addition of an organic modifier (acetonitrile) in the mobile phase. Table 2 reports the changes in α, K_1 and K_2 values of the two eluted enantiomers (E1 and E2) obtained by changing the percentage of the organic modifier in the mobile phase (10 mM phosphate buffer at pH 6). The best enantioseparation of both racemates has been obtained in ~ 20 min with a phosphate buffer at pH 6 without an organic modifier (Fig. 5).

Table 1

	pH	K_1	K_2	R	α
Midodrine	5.0	4.35	5.96	2.20	1.37
Deglymidodrine	5.0	3.57	4.96	2.39	1.39
Midodrine	5.5	6.06	8.52	2.46	1.41
Deglymidodrine	5.5	5.04	6.96	2.63	1.38
Midodrine	6.0	10.85	15.08	3.62	1.39
Deglymidodrine	6.0	9.31	12.75	3.78	1.37
Midodrine	7.0	23.03	31.87	3.06	1.38
Deglymidodrine	7.0	21.84	28.47	2.65	1.30

A suitable method for the enantioseparation of midodrine and deglymidodrine is necessary not only to collect the separated enantiomers, but mainly to verify that it does not cause any inversion in vitro and in vivo.

The method suggested by this paper could also be used to follow the quantitative aspect of hydrolysis of midodrine in deglymidodrine in vivo. Therefore, to verify the linear relationship between peak areas and concentrations, a fixed volume of solutions containing a known and increasing amount (concentration range between 95 and 380 ng) of midodrine or deglymidodrine racemates has been injected. By plotting the area of each separated enantiomer against the concentration of each racemate, two curves have been obtained. The very good linearity of the two curves has been tested by coefficient R values of E1 and E2 enantiomers respectively of 0.9991 and 0.9982 for midodrine, and 0.9998 and 0.9995 for deglymidodrine. Furthermore, the percentage ratio between E1 and E2 areas of each pair of enantiomers in the concentrations range mentioned above has also been observed. In the midodrine racemate, the enantiomer E2 is 45.5% (medium average of five analyses) with respect to enantiomer E1, while in the deglymidodrine, it is 46.6%.

The precision and reproducibility of the proposed chiral HPLC procedure was confirmed by eight replicate determinations by injecting 5 μl of solution containing 10 and 5 mcg ml^{-1} of midodrine or deglymidodrine racemates, respectively. The analyses made with the more concentrated solution gave a relative standard deviations (RSD) of 0.8% for midodrine and 0.75% for deglymidodrine. The low detectable amount (LOD) of each enantiomer at a signal to noise ratio of 3:1 was $\sim$8 ng, while the quantitation limit (LOQ) at a signal to noise ratio of 9:1 was $\sim$25 ng.

In conclusion, the α_1-AGP, among the other chiral stationary phases tested, resulted to be the more selective for the enantioseparation of midorine and deglymidodrine racemates in the same HPLC run. Actually, this CSP is the only one which allows one to follow the destiny of racemates or pure enantiomers in the serum samples and then to verify the safe use of midodrine.

Table 2

	% CH_3CN	K_1	K_2	R	α
Midodrine	0	10.85	15.08	3.62	1.39
Deglymidodrine	0	9.31	12.75	3.78	1.37
Midodrine	2	3.61	4.02	1.00	1.11
Deglymidodrine	2	3.51	4.99	3.04	1.43
Midodrine	3	2.90	3.02	0.44	1.04
Deglymidodrine	3	2.87	3.92	3.30	1.36
Midodrine	5	2.29	2.29	—	—
Deglymidodrine	5	2.35	3.07	2.80	1.30

t_R E1, retention time of the first eluted enantiomer.
t_R E2, retention time of the second eluted enantiomer.
K_1 and K_2, capacity factors of the first and second eluted enantiomers.

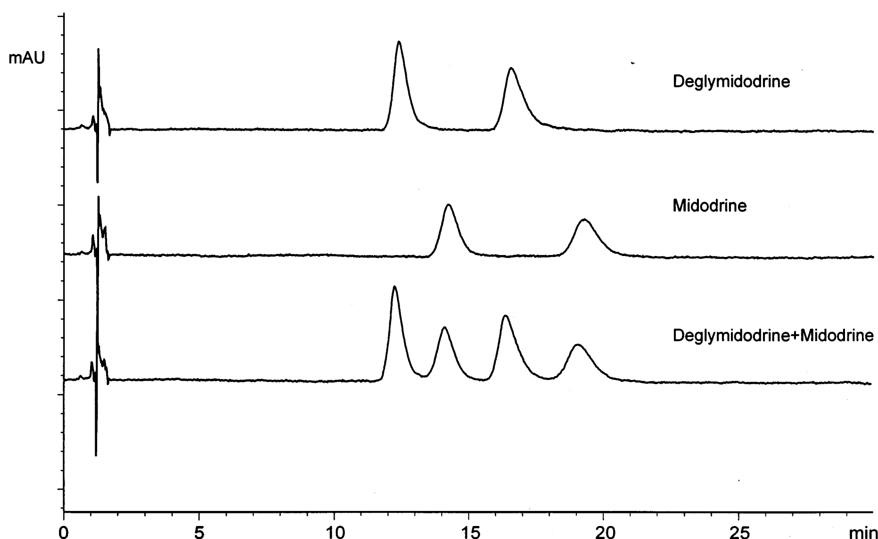

Fig. 5. Enantioseparation of midodrine or deglymidodrine racemates obtained with a Chiralcel AGP. Chromatographic conditions described in Section 2.

Acknowledgements

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