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# Influence of uncharged mobile phase additives on retention and enantioselectivity of chiral drugs using an $\alpha_1$-acid glycoprotein column

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## ABSTRACT

The influence of uncharged mobile phase additives on retention and enantioselectivity on a chiral $\alpha_1$ acid glycoprotein (AGP) column was investigated. It was observed that it is possible to induce chiral selectivity for several drugs by adding to the mobile phase uncharged modifiers with different hydrophobicities and different hydrogen bonding properties. Modifiers with different hydrogen bonding properties affect the enantioselectivity in different ways. The solute enantiomers seem to compete with the modifier molecules for binding to the chiral stationary phase. The adsorption of 1-propanol and acetonitrile on the AGP column was measured. A monolayer was obtained at mobile phase concentrations of 1.3 *M* (10%) and 2.8 *M* (15%) for 1-propanol and acetonitrile, respectively. These concentrations are in the ranges usually used for chromatographic studies. The effect of 2-propanol on the protein conformation was studied using circular dichroism spectroscopy. It was not possible to detect any change in the conformation of AGP, even in the presence of 40% 2-propanol.

## INTRODUCTION

It is common that the biological activity of racemic drugs resides predominantly in one of the enantiomers. Differences between enantiomers are not limited to pharmacological and toxicological effects, but may also occur in absorption, distribution, metabolism and excretion [1–3]. As a consequence, interest in chiral separations by chromatographic methods has grown considerably in recent years. The AGP column is a chiral column based on immobilization of the human plasma protein $\alpha_1$-acid glycoprotein (AGP) [4,5]. The protein consists of a single peptide chain containing 181 amino acids and five carbohydrate units [6]. There are numerous binding groups on the protein which can be involved in the binding of solutes. The column has been used for the separation of enantiomers of many kinds of chiral drugs such as amines, acids and non-protolytic compounds, which has been reviewed recently [7]. The column has also been used for the separation and determination of the enantiomers of disopyramide, atenolol, chloroquine and metoprolol in biological materials [8–13].

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Capacity factors and separation factors obtained on the AGP column can easily be regulated by adjusting the mobile phase composition. Dramatic effects on the separation factors have been observed after the addition of charged modifiers such as N,N-dimethyloctyl amine [14] and octanoic acid [15]. This paper describes the influence of uncharged modifiers, with different hydrophobicities and different hydrogen-bonding properties, on the retention and the enantioselectivity for hydantoins, barbituric acid derivatives, N,N-diethylaminosuccinimides and 1'-alkyl 2',6'-pipecoloxylidides. In order to obtain a deeper understanding of the uncharged organic modifier-induced changes on the enantioselectivity, the adsorption isotherm of two of the studied modifiers, acetonitrile and 1-propanol, were determined.

Circular dichroism (CD) studies of AGP were also performed in order to elucidate the effect of the modifiers on the protein conformation.

## EXPERIMENTAL

### *Apparatus*

Liquid chromatography was performed with a Waters M 6000 A pump, a Waters U6K injector and a Shimadzu SPD-2A variable-wavelength UV detector. UV detection was carried out at 215 nm. For the gas chromatographic (GC) determinations, a Hewlett-Packard HP 5890 chromatograph with a flame ionization detector was used. The pH was measured with an Orion Research Model 701 digital pH meter equipped with a Ross 8104SC pH electrode. CD spectra were registered using a JASCO (Tokyo, Japan) J600 spectropolarimeter.

### *Chemicals*

Racemic mephenytoin, secobarbital, proxibarbal, thiopental, methylphenobarbital and (−)-methylphenobarbital were kindly supplied by Dr. Jacek Bojarski (Nicolaus Copernicus Academy of Medicine, Krakow, Poland). The 1-alkyl 2',6'-pipecoloxylidides and N-aminoalkylsuccinimides model compounds were gifts from Dr. R. Sandberg (Astra Alab, Södertälje, Sweden). Other test compounds were obtained from their manufacturers. Analytical-reagent grade 1- and 2-propanol were obtained from E. Merck (Darmstadt, F.R.G.). Acetonitrile and methanol (UV grade) were obtained from FSA Laboratory Supplies (Loughborough, U.K.). Racemic 2-butanol, (*S*)-2-butanol and propionitrile were purchased from Fluka (Buchs, Switzerland), 1-butanol (Aristar) from BDH (Poole, U.K.) and ethanol (95.5%) from Kemethyl (Stockholm, Sweden).

### *Liquid chromatographic conditions*

Two different AGP columns were used, one prepared in our laboratory [16] and a commercially available CHIRAL-AGP column (ChromTech, Norsborg, Sweden). The mobile phases were prepared by adding appropriate concentrations of uncharged modifiers in a sodium hydrogenphosphate buffer. The phosphate concentration was 0.01 *M*. The mobile phases were degassed in an ultrasonic bath before being used. The hold-up volume of the column ($V_m$) was determined by injection of water or a mixture deviating slightly in composition from the mobile phase. The flow-rate was 0.9 ml/min and the chromatographic experiments were performed at room temperature.

*Determination of adsorbed 1-propanol and acetonitrile*

The AGP column was equilibrated with mobile phases of phosphate buffers (pH 7.2) containing 0.665–5.32 *M* 1-propanol or 0.951–7.61 *M* acetonitrile. The adsorbed modifier was eluted from the column with 50 ml of 20% ethanol (95.5%) in water. Fractions of 10 ml were collected and analysed. The assays of 1-propanol and acetonitrile were performed by GC using a glass column (4 m × 2 mm I.D.) containing 20% Carbowax 1500 on Chromosorb (80–100 mesh) at 120°C with helium as the carrier gas (20 ml/min). A calibration graph was constructed from peak areas of known concentration of 1-propanol or acetonitrile. The amount of modifiers adsorbed on the AGP column was calculated from the concentration in the eluate after compensation for the content of the solvent in the void volume. The precision of the determination of the modifiers was <2% at all concentrations, expressed as relative standard deviation.

## RESULTS AND DISCUSSION

The addition of uncharged modifiers to the mobile phase is known to decrease the retention and to affect the enantioselectivity on an AGP column [5,7]. In order to obtain a deeper understanding of the mechanism behind these observations, the adsorption of two modifiers to the column, with different hydrophobicities and different hydrogen bonding properties, was studied.

*Adsorption of 1-propanol and acetonitrile on the AGP column*

The amount of 1-propanol and acetonitrile adsorbed on the AGP column was measured by elution of the column with 20% ethanol in water. The concentration of 1-propanol and acetonitrile was then determined by GC as described under Experimental. The concentration range 0.13–5.3 *M* 1-propanol (corresponding to 1–40%, v/v) is much wider than that used in the chromatographic studies (0.13–0.77 *M* or 1–6%, v/v).

Table I summarizes the results for the adsorption of 1-propanol and acetonitrile. The amount of adsorbed modifier increases with increasing concentration of the studied modifier in the mobile phase. At a concentration of 1.3 *M* (10%, v/v) 1-propanol and 2.8 *M* (15%, v/v) acetonitrile the increase levels off, but the amount of adsorbed modifier continues to increase at higher modifier concentrations. This indicates that the modifiers produce multilayers. If the surface area (*S*) per gram of the solid phase is known, the number of layers (*n*) can be calculated by the equation [17]

$$n = \frac{mAN_A}{S \cdot 10^{20}} \quad (1)$$

where *A* is the area of one solvent molecule, the area of an acetonitrile molecule being *ca.* 21 Å$^2$ [18], $N_A$ is Avogadro's number and *m* is the amount of adsorbed modifier in moles per gram of solid phase. According to the adsorption studies a monolayer ($1.3 \cdot 10^{-3}$ mol/g solid phase) of acetonitrile is obtained at a mobile phase concentration of 2.8 *M*. If eqn. 1 is used to calculate the solid phase area that is occupied by this amount of acetonitrile, a value of 167 m$^2$/g is obtained, which is in good agreement with the value given for the underivatized silica (100 m$^2$/g). AGP has a molecular weight of

TABLE I

ADSORPTION OF ACETONITRILE AND 1-PROPANOL ON AN AGP COLUMN

Column, AGP (100 × 4.0 mm I.D.); mobile phase, phosphate buffer (pH 7.2) (0.01 *M* phosphate) containing acetonitrile or 1-propanol.

| Modifier | Modifier concentration in mobile phase | | Modifier adsorbed (mmol/g solid phase) |
|---|---|---|---|
| | *M* | % (v/v) | |
| Acetonitrile | 0.96 | 5 | 0.23 |
| | 1.9 | 10 | 0.58 |
| | 2.8 | 15 | 1.3 |
| | 3.8 | 20 | 1.4 |
| | 5.7 | 30 | 1.7 |
| | 7.6 | 40 | 2.4 |
| 1-Propanol | 0.13 | 1 | 0.24 |
| | 0.67 | 5 | 0.34 |
| | 1.3 | 10 | 0.96 |
| | 2.0 | 15 | 0.99 |
| | 2.7 | 20 | 1.2 |
| | 4.0 | 30 | 2.3 |
| | 5.3 | 40 | 3.5 |

40 000 [6] and the tertiary structure makes the protein very porous and accessible to small molecules, which obviously increases the surface area available for solvent molecules. For 1-propanol a mobile phase concentration of 1.3 *M* is sufficient to produce a monolayer if the area of the 1-propanol molecule is assumed to be equal to that of 2-propanol, which has been determined to be *ca.* 28 Å$^2$ [18].

Several different classes of chiral compounds have been resolved on the AGP column. The concentrations of uncharged modifiers used in these chromatographic studies are usually lower than those giving a monolayer. Normally 1-propanol concentrations below 2 *M* are used on this column as higher concentrations give too low retentions of most compounds. However, it is interesting that chiral recognition can still be achieved at modifier concentrations that give multilayers. Separation factors of 1.12 and 1.25 have been observed for trimipramine and alprenolol, respectively, in the presence of 7.61 *M* (40%, v/v) acetonitrile.

*Influence of uncharged modifiers on retention and chiral selectivity*

The retention and the enantioselectivity can be regulated by the addition of an uncharged modifier to the mobile phase. The effect on $k'$ and $\alpha$ depends on the concentration and the properties of the modifier. 2-Propanol, with both hydrogen-donating and -accepting properties, is the most studied modifier on the AGP column (5,7,15,19). In this study the effects of 1-propanol, 2-propanol and acetonitrile on the retention and the enantioselectivity were investigated. Hydantoins and barbituric acid derivatives were used as model compounds and the structures are shown in Fig. 1.

*Retention.* AGP is a glycoprotein with 181 amino acids in a single peptide chain. Many different binding groups are present in the protein, *e.g.*, hydrophobic groups in the tryptophan, phenylalanine and tyrosine residues and cationic and anionic groups in the lysine and aspartic acid residues, respectively. The protein also contains many

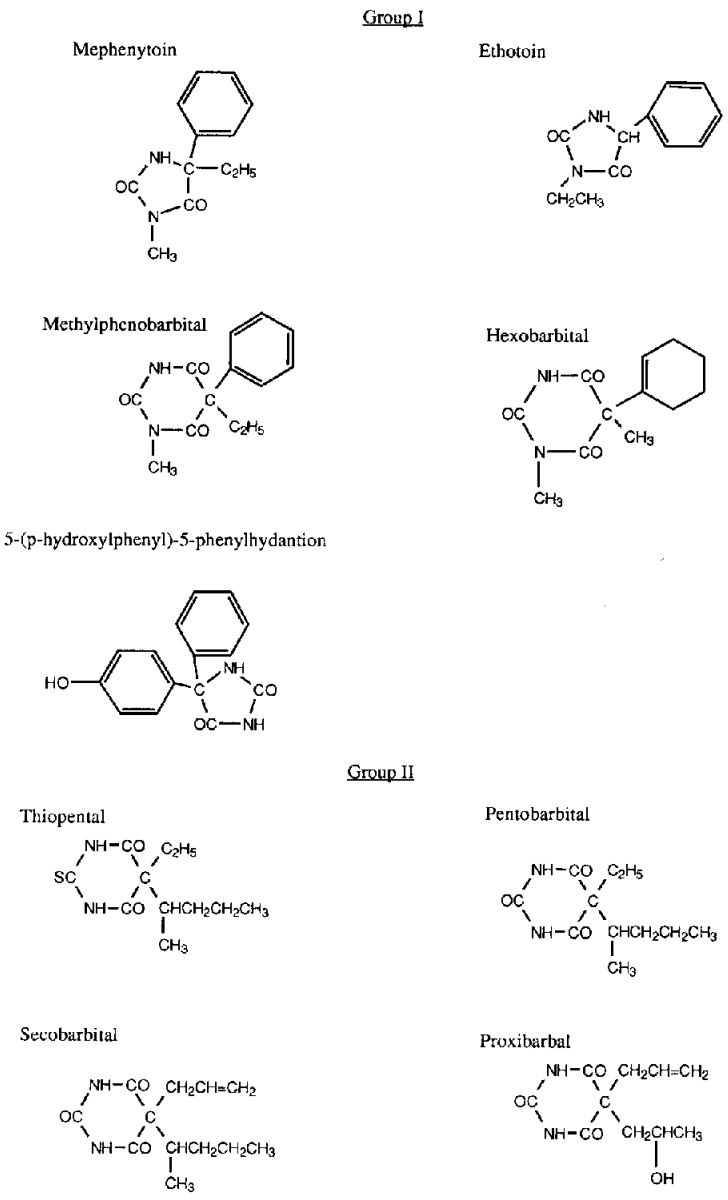



Fig. 1. Structures of the compounds studied.

hydrogen-bonding groups with different properties. All these kinds of binding abilities makes this protein useful for the separation of many compounds with different properties.

Tables II–IV demonstrate that 1- and 2-propanol decrease the capacity factors strongly for all the solutes studied. This is due to competition between the modifier and

TABLE II

INFLUENCE OF CONCENTRATION OF 2-PROPANOL ON THE RETENTION AND ENANTIOSELECTIVITY FOR HYDANTOIN AND BARBITURIC ACID DERIVATIVES

Column, AGP (100 × 4.0 mm I.D.); mobile phase, phosphate buffer (pH 7.2) (0.01 *M* phosphate) containing different concentrations of 2-propanol; flow-rate, 0.9 ml/min.

| Compound | Concentration of 2-propanol (*M*) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | | 0.13 | | 0.32 | | 0.52 | | 0.75 | |
| | $k'_1$ | α | $k'_1$ | α | $k'_1$ | α | $k'_1$ | α | $k'_1$ | α |
| Methylphenobarbital | 49.9[a] | 1.0[a] | 4.28 | 1.12 | 1.84 | 1.22 | 1.10 | 1.27 | 0.708 | 1.24 |
| Hexobarbital | 7.76 | 1.95 | 1.83 | 1.67 | 0.89 | 1.50 | 0.56 | 1.42 | 0.43 | 1.33 |
| Mephenytoin | 17.6[a] | 1.0[a] | 1.73 | 1.23 | 0.906 | 1.26 | 0.625 | 1.24 | 0.453 | 1.23 |
| Ethotoin | 5.60 | 6.06 | 0.83 | 3.34 | 0.47 | 2.16 | 0.36 | 1.64 | 0.31 | 1.40 |
| 5-(*p*-Hydroxyphenyl)-5-phenylhydantoin | 6.55 | 1.13 | 3.35 | 1.12 | 2.34 | 1.16 | 1.82 | 1.14 | 1.57 | 1.11 |
| Pentobarbital | 12.9 | 1.90 | 3.01 | 1.92 | 1.53 | 1.74 | 1.00 | 1.61 | 0.75 | 1.50 |
| Secobarbital | 19.1 | 1.39 | 4.96 | 1.23 | 2.44 | 1.12 | 1.56 | 1.07 | 1.08 | 1.0 |
| Thiopental | 39.4 | 1.96 | 11.7 | 1.82 | 5.81 | 1.64 | 3.18 | 1.63 | 2.16 | 1.47 |
| Proxibarbal | 1.32 | 1.0 | 0.309 | 1.0 | 0.185 | 1.0 | 2.30 | 1.0 | 0.076 | 1.0 |

[a] Data obtained on the CHIRAL-AGP column.

the solutes for the binding groups of the protein. More drastic effects were obtained with 1- and 2-propanol, compared with acetonitrile, which is the result of the strong adsorption of these modifiers as discussed above.

TABLE III

INFLUENCE OF CONCENTRATION OF 1-PROPANOL ON THE RETENTION AND ENANTIOSELECTIVITY FOR HYDANTOIN AND BARBITURIC ACID DERIVATIVES

Conditions as in Table II, with 1-propanol in place of 2-propanol.

| Compound | Concentration of 1-propanol (*M*) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | | 0.13 | | 0.27 | | 0.40 | | 0.77 | |
| | $k'_1$ | α | $k'_1$ | α | $k'_1$ | α | $k'_1$ | α | $k'_1$ | α |
| Methylphenobarbital | 49.9[a] | 1.0[a] | 3.14 | 1.14 | 1.50 | 1.17 | 1.05 | 1.16 | 0.642 | 1.0 |
| Hexobarbital | 7.76 | 1.95 | 1.46 | 1.49 | 0.708 | 1.37 | 0.363 | 1.32 | 0.350 | 1.0 |
| Mephenytoin | 17.6[a] | 1.0[a] | 1.38 | 1.32 | 0.750 | 1.35 | 0.589 | 1.31 | 0.389 | 1.22 |
| Ethotoin | 5.60 | 6.06 | 0.688 | 2.39 | 0.470 | 1.43 | 0.343 | 1.0 | 0.267 | 1.0 |
| 5-(*p*-Hydroxyphenyl)-5-phenylhydantoin | 6.55 | 1.13 | 3.17 | 1.13 | 2.08 | 1.13 | 1.57 | 1.09 | 1.24 | 1.0 |
| Pentobarbital | 12.9 | 1.90 | 2.28 | 1.71 | 1.11 | 1.47 | 0.684 | 1.34 | 0.489 | 1.18 |
| Secobarbital | 19.1 | 1.39 | 3.85 | 1.20 | 2.00 | 1.13 | 1.44 | 1.08 | 0.782 | 1.0 |
| Thiopental | 39.4 | 1.96 | 9.32 | 1.65 | 4.98 | 1.45 | 3.58 | 1.32 | 1.84 | 1.13 |
| Proxibarbal | 1.32 | 1.0 | 0.234 | 1.0 | 0.108 | 1.0 | 0.124 | 1.0 | 0.077 | 1.0 |

[a] Data obtained on the CHIRAL-AGP column.

TABLE IV

INFLUENCE OF CONCENTRATION OF ACETONITRILE ON THE RETENTION AND ENANTIOSELECTIVITY FOR HYDANTOIN AND BARBITURIC ACID DERIVATIVES

Conditions as in Table II, with acetonitrile in place of 2-propanol.

| Compound | Concentration of acetonitrile (*M*) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | | 0.19 | | 0.38 | | 0.57 | | 0.76 | |
| | $k'_1$ | $\alpha$ | $k'_1$ | $\alpha$ | $k'_1$ | $\alpha$ | $k'_1$ | $\alpha$ | $k'_1$ | $\alpha$ |
| Methylphenobarbital | 49.9[a] | 1.0[a] | 10.0 | 1.0 | 6.32 | 1.0 | 4.50 | 1.0 | 3.49 | 1.0 |
| Hexobarbital | 7.76 | 1.95 | 3.71 | 1.67 | 2.62 | 1.57 | 2.00 | 1.48 | 1.55 | 1.41 |
| Mephenytoin | 17.6[a] | 1.0[a] | 3.77 | 1.17 | 2.42 | 1.19 | 1.78 | 1.22 | 1.42 | 1.20 |
| Ethotoin | 5.60 | 6.06 | 1.39 | 3.93 | 0.981 | 3.13 | 0.793 | 2.63 | 0.162 | 2.43 |
| 5-(*p*-Hydroxyphenyl)-5-phenylhydantoin | 6.55 | 1.13 | 5.14 | 1.18 | 4.32 | 1.20 | 3.81 | 1.21 | 3.12 | 1.21 |
| Pentobarbital | 12.9 | 1.90 | 5.88 | 1.78 | 4.01 | 1.70 | 2.98 | 1.62 | 2.28 | 1.54 |
| Secobarbital | 19.1 | 1.39 | 10.92 | 1.30 | 7.10 | 1.26 | 5.08 | 1.23 | 3.90 | 1.20 |
| Thiopental | 39.4 | 1.96 | 27.4 | 1.90 | 19.3 | 1.83 | 13.5 | 1.76 | 9.38 | 1.74 |
| Proxibarbal | 1.32 | 1.0 | 0.649 | 1.0 | 0.386 | 1.0 | 0.284 | 1.0 | 0.221 | 1.0 |

[a] Data obtained on the CHIRAL-AGP column.

It is interesting to compare the capacity factors obtained for the enantiomers of pentobarbital and thiopental, as their structures are very similar: thiopental has a thiocarbonyl group whereas pentobarbital has a carbonyl group located in the same position in the ring structure. The thiocarbonyl group seems to play an important role in the adsorption of this solute, as this group gives the enantiomers of thiopental 3–5 times higher capacity factors than those of pentobarbital. Differences in electronegativity might influence the binding.

*Chiral selectivity*. Dramatic effects on the enantioselectivity have been observed with cationic and anionic mobile phase additives [14,15]. For example, the tertiary amine N,N-dimethyloctylamine (DMOA) can improve the enantioselectivity for certain cationic solutes. This was observed for propiomazine and promethazine using AGP as a chiral complexing agent in the mobile phase [20], and with immobilized protein (CHIRAL-AGP) [21]. DMOA has also been reported to improve strongly the enantioselectivity of 2-aryl propionic acids [14].

Uncharged organic modifiers can also be used in order to affect both the enantioselectivity and the retention. Usually both the retention and the enantioselectivity decrease with increasing concentration of an uncharged modifier in the mobile phase [5,19]. However, for the local anaesthetics mepivacaine and bupivacaine, it has been reported that an increase in the 2-propanol concentration from 1 to 8% did not significantly affect the separation factors, despite the fact that the retention was drastically reduced [19].

Recently, it has also been demonstrated that it is possible to induce and increase the chiral selectivity by adding uncharged modifiers to the mobile phase [7]. The effects on the enantioselectivity of three different modifiers with different hydrogen-bonding properties and hydrophobicities are presented in Tables II–IV. The test solutes can be divided into two groups, those with the chiral carbon in the ring system, group I, and

those with the chiral carbon in the attached side-chain, group II (see Fig. 1). The enantiomers of mephenytoin, methylphenobarbital and 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (group I) gave separation factors between 1.0 and 1.13 using a mobile phase without modifier, whereas the separation factors obtained for ethotoin and hexobarbital were 6.06 and 1.95, respectively. Ethotoin and hexobarbital do not have as large substituents on the chiral carbon as the other solutes in group I which seems to be favourable for the chiral recognition in the absence of a modifier.

It is interesting that 1- and 2-propanol (with both hydrogen-donating and -accepting properties) improve the chiral selectivity for both methylphenobarbital and mephenytoin. However, acetonitrile, with only hydrogen-accepting properties, induces a selective increase in the separation factor, $\alpha$, for only mephenytoin, as demonstrated in Figs. 2 and 3. Improvement of the enantioselectivity for 5-(*p*-hydroxyphenyl)-5-phenylhydantoin was obtained with 2-propanol and acetonitrile but not with 1-propanol. The separation factors for these solutes increased initially with increasing concentration of the modifiers in the mobile phase. The separation factors reached a maximum at about 0.3–0.5 *M* of the modifiers; at concentrations above 0.5 *M* the enantioselectivity decreased slightly. For ethotoin and hexobarbital, closely related to mephenytoin and methylphenobarbital, respectively, the enantioselectivity decreased with increasing concentration of all three modifiers tested.

For the solutes in group II (pentobarbital, thiopental and secobarbital), separation factors $\geq 1.39$ were obtained in phosphate buffer (pH 7.2). The enantioselectivity for these solutes decreased with increasing concentration of organic modifier in the mobile phase. The least hydrophobic modifier, acetonitrile, decreased the enantioselectivity less than 1- and 2-propanol. The resolution of the enantiomers of secobarbital and mephenytoin is demonstrated in Fig. 4a and b.

For proxibarbal no enantioselectivity could be observed using mobile phases containing 1-propanol, 2-propanol or acetonitrile and the enantiomers of proxibarbal did not separate in pure phosphate buffer.

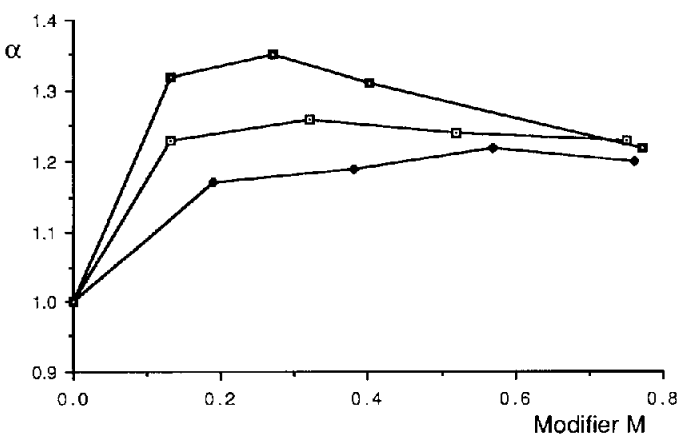



Fig. 2. Influence of mobile phase additives on the separation factor of mephenytoin. Column. AGP (100 × 4 mm I.D.); mobile phase, 0.01 *M* phosphate buffer (pH 7.2) containing different amounts of uncharged modifier; flow-rate, 0.9 ml/min. ■, 1-Propanol; □, 2-propanol; ◆, acetonitrile.

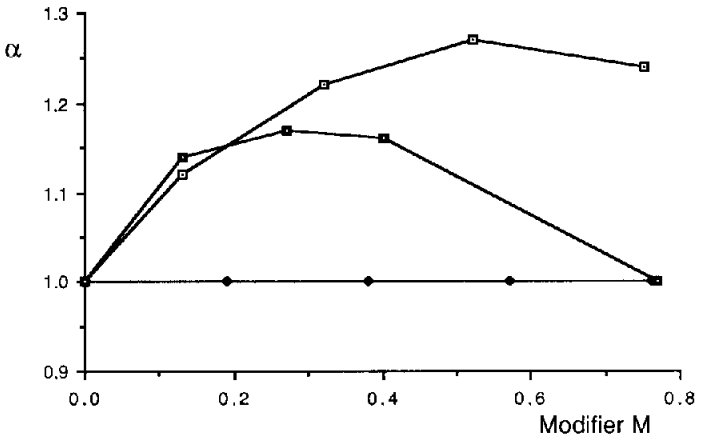



Fig. 3. Influence of mobile phase additives on the separation factor of methylphenobarbital. Conditions and symbols as in Fig. 2.

There are two reasonable explanations for the effects of uncharged modifiers on the enantioselectivity. One is that the modifier competes with the solute enantiomers for binding to groups with different hydrogen-bonding properties in the binding site(s). Therefore, modifiers with different hydrogen-bonding properties affect the enantioselectivity in a different way. The other is that the uncharged modifiers cause reversible changes in the protein conformation. The effect of uncharged organic modifiers of the protein conformation was studied using CD. CD spectra of native AGP (25 $\mu M$) were recorded in phosphate buffers (pH 7.0) with and without 2-propanol. The CD spectra were identical and are presented in Fig. 5. With this technique it was not possible to detect any change in the conformation of AGP, even in the presence of as high a concentration as 40% of 2-propanol. However, it is possible to affect the conformation of AGP by adding charged modifiers to a solution of AGP. Fig. 5 demonstrates a CD spectrum of AGP dissolved in phosphate buffer (pH 7.0) containing 0.015 $M$ sodium dodecyl sulphate. The negative peak with a maximum at

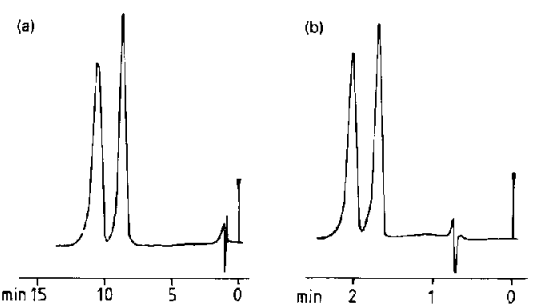



Fig. 4. (a) Resolution of the enantiomers of secobarbital. Column, AGP (100 × 4 mm I.D.); mobile phase, phosphate buffer (pH 7.2) containing 0.36 $M$ acetonitrile. (b) Resolution of the enantiomers of mephenytoin. Column, AGP column (100 × 4 mm I.D.); mobile phase, phosphate buffer (pH 7.2) containing 0.13 $M$ 1-propanol.

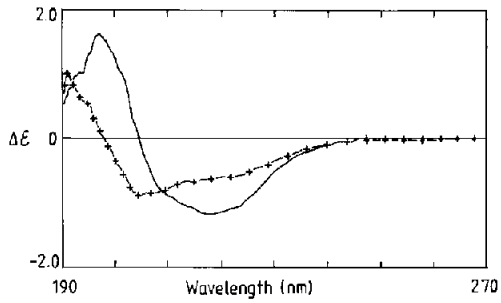



Fig. 5. (———) CD spectrum of native AGP dissolved in phosphate buffer (pH 7.0) or in phosphate buffer (pH 7.0) containing 40% (v/v) 2-propanol (spectra are identical). (+——+) CD spectrum of native AGP in phosphate buffer (pH 7.0) containing 0.015 *M* sodium dodecyl sulphate.

205 nm and a shoulder at about 225 nm clearly demonstrates a change in the secondary structure of AGP, probably a transformation of parts of the protein molecule with a $\beta$-conformation or an unordered structure into an $\alpha$-helical form. This observation is in accordance with that obtained by Jirgensons [22]. Hence it is reasonable to assume that the uncharged organic modifier-induced changes in the enantioselectivity depend on competition between the solute enantiomers and the modifier for the binding groups present in the binding sites of the protein. However, small changes in the protein conformation resulting in the exposure of new binding groups in the binding site(s) cannot be neglected when a small molecule is bound to a protein or an enzyme [23,24].

The effects of other alcohols and nitriles than 1- and 2-propanol and acetonitrile on retention and the chiral selectivity for methylphenobarbital and mephenythoin were also studied and the results are summarized in Table V. In this study the commercially available CHIRAL-AGP column was used. All the alcohols studied, except methanol, induced chiral selectivity for both methylphenobarbital and mephenytoin. The separation factor for methylphenobarbital increased with increasing length of the alkyl chain of the alcohol and with alcohols with a branched alkyl chain. The best separation factor was obtained in presence of 2-butanol. It is interesting that the two tested nitriles induce a selective increase in $\alpha$ only for mephenytoin. The highest separation factor for mephenytoin was obtained by adding propionitrile to the mobile phase. It was also observed that the effect on both $k'$ and $\alpha$ was equivalent with (*R*,*S*)-2-butanol and (*S*)-2-butanol.

The effect of different uncharged modifiers on the enantioselectivity was also studied with two other homologous series, 1-alkyl 2′,6′-pipecoloxylidides and N,N-diethylaminosuccinimides.

Table VI presents capacity factors and separation factors obtained for a series of 1-alkyl 2′,6′-pipecoloxylidides using 1-propanol, 2-propanol and acetonitrile as mobile phase additives. Mobile phases containing 1-propanol gave the best separation conditions for this series of compounds, as high enantioselectivity and low retention were obtained. A capacity factor of 15.8 and no enantioselectivity were obtained for the enantiomers of the ethyl homologue using a mobile phase containing 0.75 *M* (4.0%, v/v) acetonitrile, whereas a $k'_1$ value of 4.63 and a separation factor of 1.30 were obtained using 0.75 *M* (5.8%, v/v) 1-propanol.

TABLE V

INFLUENCE OF UNCHARGED MODIFIERS ON $k'$ AND $\alpha$ FOR METHYLPHENOBARBITAL AND MEPHENYTOIN

Column, CHIRAL-AGP; mobile phase, phosphate buffer (pH 7.2) (0.01 *M* phosphate) containing different modifiers; flow-rate, 0.9 ml/min.

| Modifier | Concentration (*M*) | Methylphenobarbital | | Mephenytoin | |
|---|---|---|---|---|---|
| | | $k'_1$ | $\alpha$ | $k'_1$ | $\alpha$ |
| – | | 49.9 | 1.0 | 17.6 | 1.0 |
| Methanol | 0.25 | 37.3 | 1.0 | 13.8 | 1.0 |
| | 0.44 | 28.6 | 1.0 | 10.9 | 1.0 |
| | 1.48 | 13.0 | 1.0 | 5.26 | 1.0 |
| Ethanol | 0.18 | 20.3 | 1.0 | 7.65 | 1.10 |
| | 0.88 | 4.52 | 1.15 | 2.20 | 1.13 |
| | 1.41 | 2.53 | 1.14 | 1.42 | 1.08 |
| 1-Propanol | 0.13 | 7.42 | 1.17 | 3.24 | 1.24 |
| | 0.27 | 3.63 | 1.18 | 1.90 | 1.23 |
| | 0.66 | 1.76 | 1.0 | 1.19 | 1.12 |
| 1-Butanol | 0.11 | 3.16 | 1.29 | 1.76 | 1.15 |
| | 0.22 | 1.68 | 1.14 | 1.25 | 1.0 |
| | 0.44 | 1.05 | 1.0 | 0.86 | 1.0 |
| 2-Propanol | 0.13 | 10.1 | 1.19 | 4.33 | 1.12 |
| | 0.27 | 5.07 | 1.29 | 2.92 | 1.16 |
| | 0.66 | 1.95 | 1.29 | 1.30 | 1.09 |
| (*R,S*)-2-Butanol | 0.11 | 3.81 | 1.33 | 2.07 | 1.19 |
| | 0.22 | 2.09 | 1.33 | 1.37 | 1.17 |
| | 0.44 | 1.14 | 1.25 | 1.01 | 1.0 |
| (*S*)-2-Butanol | 0.22 | 2.08 | 1.32 | 1.39 | 1.16 |
| Acetonitrile | 0.19 | 20.8 | 1.0 | 8.65 | 1.06 |
| | 0.38 | 15.9 | 1.0 | 5.53 | 1.15 |
| | 0.95 | 6.22 | 1.0 | 2.72 | 1.16 |
| Propionitrile | 0.14 | 8.02 | 1.0 | 3.18 | 1.33 |

The chiral selectivity is highly affected by the length of the alkyl chain bound to the piperidine nitrogen, as discussed previously [16]. The enantioselectivity for the unsubstituted compound (PPX) was >3 times higher than for the methyl-substituted compound with 2-propanol in the mobile phase. Separation factors of 4.27 and 4.96 were obtained for the PPX enantiomers using mobile phases containing 2-propanol and 1-propanol, respectively. Acetonitrile, with only hydrogen-accepting properties, drastically reduced the enantioselectivity for the enantiomers of PPX (see Table VI), which clearly demonstrates that the hydrogen-bonding properties of the modifier strongly affect the enantioselectivity.

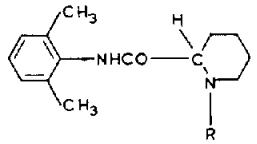
For the series of N,N-diethylaminosuccinimides, it is also more advantageous to use 1-propanol as mobile phase modifier, even though the separation factor is 1.6 times higher when using acetonitrile (see Table VII). This is due to the fact that the retention for the last eluted enantiomer is twelve times lower with 1-propanol as mobile phase additive and the separation factor is >1.4 in all instances.

In conclusion, it is important to note that it is possible to induce and increase chiral selectivity for chiral compounds by adding certain uncharged modifiers to

TABLE VI

INFLUENCE OF UNCHARGED MODIFIERS ON $k'$ AND $\alpha$ FOR 1-ALKYL-2',6'-PIPECOLOXYLIDIDES

Column, AGP (100 × 4 mm I.D.); mobile phase, phosphate buffer (pH 7.2) containing different modifiers; flow-rate, 0.9 ml/min. S = The last-eluted enantiomer.



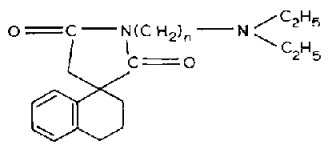
| R | 0.75 *M* 1-propanol | | 0.75 *M* 2-propanol | | 0.75 *M* acetonitrile | |
|---|---|---|---|---|---|---|
| | $k'_s$ | $\alpha$ | $k'_s$ | $\alpha$ | $k'_s$ | $\alpha$ |
| H (PPX) | 12.6 | 4.96 | 11.1 | 4.27 | 16.5 | 1.93 |
| $CH_3$ | 3.99 | 1.51 | 3.95 | 1.40 | 12.0 | 1.17 |
| $C_2H_5$ | 4.63 | 1.30 | 4.21 | 1.23 | 15.8 | 1.0 |
| $C_3H_7$ | 7.83 | 1.67 | 8.56 | 1.40 | 45.6 | 1.37 |
| $C_4H_9$ | 10.6 | 1.47 | 12.7 | 1.24 | 79.6 | 1.26 |
| $C_5H_{11}$ | 13.8 | 1.19 | 18.9 | 1.07 | | – |
| $C_6H_{13}$ | 18.1 | 1.0 | 31.0 | 1.15 | – | – |

the mobile phase. The adsorption of methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol, acetonitrile and propionitrile to the protein is reversible and the columns are very stable and can be used for long periods without being negatively affected by these modifiers. This was tested by running test compounds frequently

TABLE VII

INFLUENCE OF UNCHARGED MODIFIERS ON $k'$ AND $\alpha$ FOR DIETHYLAMINESUCCINIMIDES

Conditions as in Table VI. $k'_2$ = The last-eluted enantiomer.



| n | 0.75 *M* 1-propanol | | 0.75 *M* 2-propanol | | 0.75 *M* acetonitrile | |
|---|---|---|---|---|---|---|
| | $k'_2$ | $\alpha$ | $k'_2$ | $\alpha$ | $k'_2$ | $\alpha$ |
| 2 | 12.1 | 1.48 | 23.2 | 1.60 | 147 | 2.40 |
| 3 | 6.84 | 1.75 | 10.5 | 2.12 | 85.7 | 3.26 |
| 4 | 5.95 | 1.41 | 9.37 | 1.70 | 71.9 | 2.51 |
| 5 | 9.73 | 1.54 | 18.2 | 1.83 | 124 | 2.21 |

during this study for the determination of the capacity factors and the enantioselectivity for the test compounds.

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