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Practical strategy for the analytical separation of enantiomers by high-performance liquid chromatography

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ABSTRACT

When a pharmacologically active compound containing a chiral centre is identified as a potential development cadidate, it is considered essential to define the pharmacological properties of the pure enantiomers. High-performance liquid chromatography has a critical role in a pharmaceutical research department by providing sensitive measurements of optical purity.

To react effectively to the demands of a large chemistry team, a strategy has been developed. Key racemates are selected for method development during the early stages of each project team's effort. Separation methods for these compounds are identified from a consideration of such factors as physicochemical data, pharmacological potency etc. The methods are evaluated and optimised separations are established to measure optical purity.

Separation methods for analogous molecules synthesised later in a project are predictable from this strategic assessment, and the need for significant extra method development is avoided.

INTRODUCTION

Chiral separations by high-performance liquid chromatography (HPLC) are difficult because unlike achiral compounds, enantiomers have the same physical properties except for their rotation of plane polarised light. It is often held that the direct separation of optical isomer requires a minimum of three simultaneous interactions with a chiral selector, at least one of which is stereochemically controlled [1]. Other workers have reported that a two-point interaction between two chiral structures is sufficient for mutual chiral recognition, provided each of these structures simultaneously contacts a third non-chiral species, *e.g.* a solid surface [2].

A strategy is described which rationalises the complexities of analytical chiral method development using several representative molecules from a series of racemic thromboxane antagonists as examples.

Seperation methods were developed to measure thc optical purity of the enantiomers of each compound to levels defined by pharmacological data.

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EXPERIMENTAL

Materials

Compounds 1–3 (see Table I) were synthesised at Alderley Park. Hexane, propan-2-ol, methanol and acetonitrile were HPLC grade (Fisons, Loughborough, UK). Ethanol was re-rectified absolute and water was doubly distilled from glass. Sodium dihydrogenphosphate and disodium hydrogenphosphate were AnalaR Grade (BDH, Poole, UK). Bovine serum albumin was Fraction V (Sigma) and β-cyclodextrin was purchased from Aldrich (U.K.).

Spherisorb S50DS, S5CN, LiChrosorb 10-μm Diol and covalent Pirkle (*R*)-3,5-dinitrobenzoylphenylglycine columns (all 25 cm × 4.6 mm I.D.) were purchased from Hichrom (Reading, U.K.). A 25 cm × 4.6 mm I.D. Daicel OD column and a 10 cm × 4 mm I.D. Chiral AGP column were supplied by J. T. Baker (Hayes, U.K.) and a 25 cm × 4.6 mm I.D. Cyclobond I column was purchased from Technicol (Stockport, U.K.).

STRATEGY

The strategy is based on a sequential assessment of solute structure and physical data, analytical considerations, chiral selectors, eluent and selector combinations and finally method suitability.

Structure and physical data

A full knowledge of solute structure and reactivity, octanol–water partition coefficient (log *P*) data, pK_a, solubility, UV and stability is required.

Analytical considerations

The physical data are used to develop an achiral HPLC system which is used to measure the solute purity, and to check the stability of the solutes in the selected chiral systems. Potential chiral eluent additives must be optically pure for sensitive measurements to be achieved and the UV spectra of the solute and selected additives are compared to determine the absorbance of the compounds against the background. Other detectors may be considered *e.g.* fluorescence.

Chiral selectors

The interactive sites on the solute and the size(s) of the molecule and its functional groups are assessed. Chiral stationary phases or chiral eluent additives which favour this assessment are chosen, *e.g.* a hydrophobic, ionic selector interacts with an ionic hydrophobic solute. Indirect methods which modify the interactive properties of a solute by achiral derivatisation are also identified, which will allow previously unfavoured selectors to be used. Detectability can be enhanced simultaneously if a strong UV chromophore is incorporated.

Eluent–selector combinations

Chiral stationary phases (CSPs). The eluent polarity is chosen to favour the predicted solute–selector interactions without compromising solute solubility, *e.g.* electrostatic and hydrophobic interactions between a selector and an ionic solute are

most effective when the compound is dissolved and eluted in a polar ionising eluent–water. Alternatively separations relying on weak interactions, *e.g.* H-bonds will be favoured in dry, low-polarity eluents.

Chiral eluent additives. The eluent is chosen to enhance the solute–selector interactions, but the solubility of both the solute and the additive must be maintained. Solutes of varying log *P* will dissolve in a range of eluent polarities by choosing the selector carefully, *e.g.* hydrophobic solutes will dissolve in aqueous eluents via complexation with polar selectors *e.g.* cyclodextrins. Applications where polar aminoalcohols are dissolved in apolar eluents after ion pairing with hydrophobic chiral counter ions have been reported [3].

Method suitability

A chromatographic optical purity method is used to measure the amount of a minor enantiomer in resolved material to a level which is usually defined by the pharmacological efficacies of each enantiomer. When methods are identified, they are evaluated against these criteria. If new information implies new target measurements, alternative methods identified during the strategic assessment can be evaluated quickly.

EXAMPLE

Structure and physical data

Three compound were selected for method development because they reflected the range of the log *P* values in a series of racemic thromboxane antagonists (Table I). UV spectra were recorded for each compound.

Analytical considerations

The analytical purity of each solute was measured as >99% using eluents of methanol–water modified with trifluoroacetic acid on a Spherisorb S50DS column. The solutes were allowed to stand in the selected eluents (see below) for 1–2 days and rechromatographed against freshly prepared standards without evidence of decomposition. The optical purity of the two selected chiral additives β-cyclodextrin and bovine serum albumin (BSA) was >99.9%, but the UV absorbance of the protein precluded its use as a selector for compound 3 (Fig. 1).

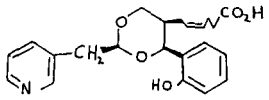
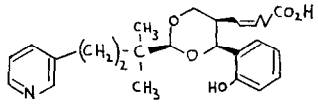
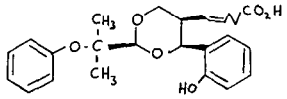
Chiral selectors

α_1 - Acid glycoprotein (AGP) and bovine serum albumin (BSA) were identified as selectors for charged molecules containing hydrophobic moieties. AGP has been used for a range of drugs [4] and several high affinity sites on BSA for carboxylic acids have been reported [5]. Certain solute groups (*e.g.* aryl) are correctly sized to fit into the cavity of a third selector, β-cyclodextrin. Enantioselectivity depends on the degree of inclusion and H-bond interactions between secondary hydroxyls at the cavity mouth and excluded solute groups [6].

Non-aqueous chiral ion-pair systems were unfavourable. Ion-pair formation between the solutes and a chiral counter ion (quinine) would occur, but the lack of an H-bond acceptor β to the acid would preclude a secondary interaction with the C9-OH in quinine.

TABLE I

STRUCTURES AND PHYSICAL DATA

Compound		log P	pK_a acid	S^a (mg/ml)	S^b
1		3.5	4.9	1.0-2.0	1.0
2		5.4	4.9	1.0-2.0	0.5-1.0
3		6.0	4.9	2.0-5.0	0.1-0.5

[a] Solubility in propan-2-ol.
[b] Solubility in sodium phosphate solution pH 7.0.

It was recognised that some unfavoured selectors could be utilised by enhancing the H-bond and charge transfer characteristics of the solutes and a chemical derivatisation to form the α-naphthylamides [7] was defined. The method was not evaluated initially but selective interactions with (*R*)-3,5-dinitrobenzoylphenylglycine [8] (Pirkle) and cellulose tris (3,5-dimethylphenylcarbamate) [9] (Daicel OD) were predicted.

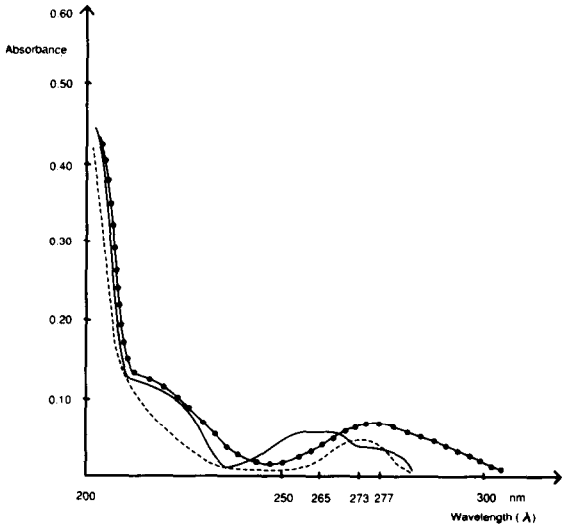


Fig. 1. Comparison of UV spectra of BSA (●—●), compound 2 (———) and compound 3 (– – –).

Eluent–selector combinations

Chiral AGP, Cyclobond I, Pirkle and Daicel OD columns were identified as chiral stationary phases. Bovine serum albumin and β-cyclodextrin were identified as chiral eluent additives.

Chiral stationary phases. A buffered aqueous eluent (pH 7.0) was used with Chiral AGP to ionise the compounds and the protein (pI = 2.7) and to promote an electrostatic effect. Because the carboxylates were water soluble, the amounts of organic modifier (propan-2-ol) could be reduced and the hydrophobic solute–protein interactions were favoured.

Using Cyclobond I, the compounds were dissolved as anions in a buffered aqueous eluent (pH 7.0). The need for an organic solvent (acetonitrile) to force elution was reduced and the hydrophobic cavity–solute interactions were enhanced.

Dry, low-polarity mixtures of alcohols with hexane were identified for separation of the α-naphthylamides using Pirkle and Daicel OD columns. Weak interactions (H-bond, charge transfer) are favoured in apolar media, while solute solubility is maintained through a higher log P.

Chiral eluent additives. Ionising, solvating aqueous eluents at pH 7.0 were selected for use with bovine serum albumin and β-cyclodextrin. The flexibility of the additive technique was exploited by using a range of column polarities (LiChrosorb Diol → Spherisorb S50DS) to identify eluents containing very high amounts of water, in order to dissolve the chiral additives at the high concentrations which favour complexation. When necessary, propan-2-ol and acetonitrile were used as organic modifiers for the protein and cyclodextrin systems, respectively.

TABLE II

CHROMATOGRAPHIC SEPARATION DATA

α = Separation factor; R_s = resolution. A = 50 mM NaH_2PO_4–50 mM Na_2HPO_4 (1/1) pH 7.0. B = 10 mM NaH_2PO_4–50 mM Na_2HPO_4 (1/1) pH 7.0.

Method	Compound 1		Compound 2		Compound 3	
	α	R_s	α	R_s	α	R_s
Chiral AGP[a]	1.10	0.7	1.10	0.7	1.40	0.8
Cyclobond I[b]	1.30	0.9	1.08	0.6	1.06	0.2
Bovine serum albumin[c]	1.10	0.9	1.20	0.9	Not evaluated	
β-Cyclodextrin[d]	1.40	1.7	1.00	0.0	1.00	0.0
Pirkle[e,g]	Not evaluated		Not evaluated		1.10	0.8
Daicel OD[f,g]	Not evaluated		Not evaluated		1.40	1.8

[a] B–propan-2-ol (85:15).
[b] A–acetonitrile (90:10).
[c] A–propan-2-ol (90:10) + 3 mg/ml bovine serum albumin/Spherisorb S5CN.
[d] A–acetonitrile (90:10) + 15 mg/ml β-cyclodextrin/Spherisorb S5CN.
[e] Hexane–propan-2-ol (80:20).
[f] Hexane–ethanol (85:15).
[g] Compound 3 chromatographed as the α-naphthylamide derivative.

Method suitability

Optical purity measurements to levels of 1–2% were targeted to support some early pharmacological work on the solute enantiomers. The direct methods established for the compounds met these criteria (Table II).

The selection of compound 3 for development coupled with new pharmacological data indicating a 1:1000 efficacy ratio for the enantiomers demanded more stringent chiral methodology. The resolution of compound 3 was insufficient for measurements below 1–2% (Fig. 2) and a separation of the α-naphthylamide derivative on Pirkle and Daicel columns was evaluated (Table II). The optimum separation was identified (Fig. 3) and used to measure <0.2% of the (−)-enantiomer in the (+)-enantiomer (Fig. 4).

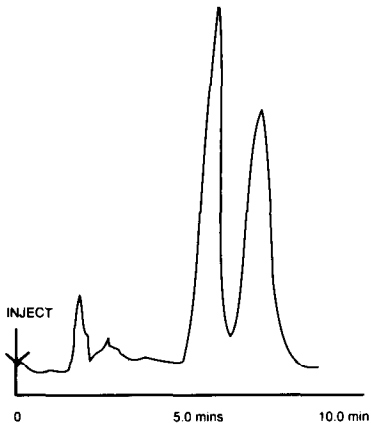


Fig. 2. Preliminary method for compound 3. Column: 10.0 cm × 4.0 mm I.D. Chiral AGP. Eluent: [10m*M* Na_2HPO_4–10 m*M* NaH_2PO_4 (1/1) pH 7.0]–propan-2-ol (85:15). Flow-rate: 0.9 ml/min. Detection: 273 nm.

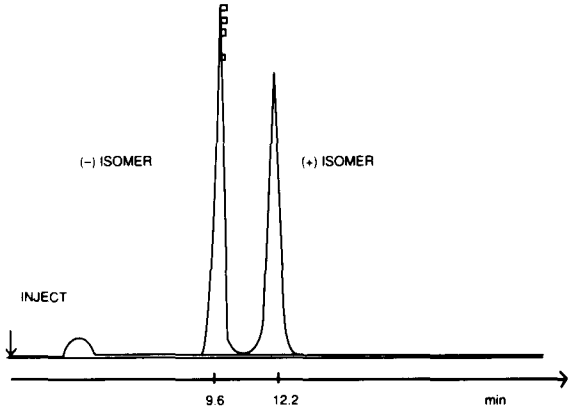


Fig. 3. Final method for compound 3 (α-naphtylamide) Column: 25.0 cm × 4.6 mm I.D. Daicel OD. Eluent: Hexane–ethanol (85:15). Flow-rate: 1 ml/min. Detection: 222 nm.

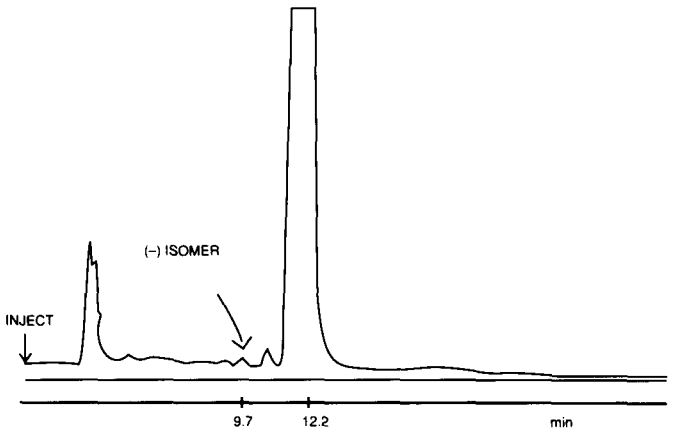


Fig. 4. Optical purity measurement. Column: 25.0 cm × 4.6 mm I.D. Daicel OD. Eluent: Hexane–ethanol (85:15). Flow-rate: 1 ml/min. Detection: 222 nm.

CONCLUSIONS

A strategy to develop chiral HPLC methods has been described. Separations to measure the optical purity of single enantiomers were established which met changing measurement criteria which were defined by pharmacological data. The selection of an enantiomer as a development candidate was not delayed by additional method development.

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