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Assessment of chiral stationary phases for suitability for combined enantiomeric impurity/related substances assays

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ABSTRACT

Chiral stationary phases (CSP) for LC had a major impact on pharmaceutical R&D when they first became commercially available in the 1980s. Even although the use of CSP in pharmaceutical R&D is now very much a mature area, there is still scope for using CSP more effectively to bring about efficiencies. One such instance is the possibility of combining the chiral LC test for the level of a trace enantiomeric impurity in a chiral drug substance and the LC test for related substances into one test. It was envisaged that this could be achieved by carrying out reversed-phase LC on an ODS silica/CSP coupled column system. In evaluating Chiralpak QD-AX, Cyclobond I 2000 DNP and Whelk-O1 CSP using a polar organic – aqueous mobile phase it was found that the Whelk-O1 CSP had good achiral selectivity, the required match of retentivity with the ODS silica material, ACE 5 C18 and also exhibited an encouraging degree of enantioselectivity in the reversed-phase mode. Following consideration of the selectivity of the ACE 5 C18 and Whelk-O1 phases it became apparent that it might be possible to achieve the desired goal of achieving both the enantiomeric impurity and related substances separations in one system by using the Whelk-O1 CSP on its own. This was subsequently demonstrated to be the case using S-naproxen, laevokalim and S-flurbiprofen as illustrative examples.

1. Introduction

The separation of enantiomers by LC has been a major success story since it became routinely possible in the late 1980s [1]. This is so much so the case that in the field of chiral separations of pharmaceuticals there is little left to do by way of fulfilling genuine unmet needs. However, when a single enantiomer drug is tested analytically, the trace enantiomeric impurity is generally determined by a chiral LC test which is separate from the related substances test. Clearly it would be more convenient if the enantiomeric impurity and other related substances could be determined simultaneously using one set of LC conditions. This would also give a check on the specificity of the enantiomeric impurity test. Using N-acetyl-L-tryptophan as a model drug it had been demonstrated that this can be achieved by exploiting a specially tailored combination of achiral and chiral stationary phases (CSP) (R.W.H. Perera, unpublished work on Spherisorb ODS1/Chirobiotic T coupled systems; also R.W.H. Perera and W.J. Lough, poster presentation, Chirality 2004, New York). However when extending this approach to a real drug example, it was found that when using reversed-phase conditions with the CSP it was necessary to use low percentages of the polar organic solvent in order to achieve the optimum chiral separation (Undergraduate projects, B.Sc. Chemical and Pharmaceutical Science, University of Sunderland). As a consequence, this placed a limitation on the achiral phase that could be used in the combination system with the same mobile phase. At this point it was felt that, despite reversed-phase chiral LC method development by screening CSP having already been carried out [e.g. 2,3], further retentivity, enantioselectivity and achiral selectivity (i.e. selectivity between different related substances which are not stereoisomers) characterisation of CSP under reversed-phase conditions would be useful in informing attempts at conducting enantiomeric impurity and related substances determinations with one set of conditions. Achiral selectivity was an issue in that a CSP may exhibit orthogonal achiral selectivity to an achiral phase simply because it has little or no achiral selectivity while the achiral phase does have achiral selectivity. In some cases this may be an advantage but it will be a disadvantage in a combined chiral/achiral system if a contribution from the CSP is needed to bring about resolution of all the related substances from one another. Accordingly it was sought to study those CSP, which might be expected to give retention and enantioselectivity with high amounts of polar organic solvent in the mobile phase, under reversed-phase LC conditions in order to ascertain which of them give large enough enantioresolution and suitable retention to be used in a combination column with an achiral C-18 silica to be able to determine the trace enantiomer and all the other related substances in one test. The use of coupled chiral/achiral columns, often used for drug

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bioanalysis from the early 1990s onwards [4,5], was also used by Phinney et al., [6] in super-critical fluid chromatography (SFC) work for the separation of different drugs (benzodiazepine and β-blocker sets of compounds) from one another as well for the individual drug enantiomer separations. Lindner and co-workers used a reversed phase column to enhance the "chemical selectivity" of a chiral anion-exchanger used for the separation of the enantiomers of a four-compound test mixture [7]. However the application in mind in this instance was more challenging, demanding the separation of the drug and enantiomer in a sample containing several closely related substances without heart-cutting the main peak. This has been achieved for voriconazole [8] using normal phase LC and for propionyl L-carnitine, coupling with an ion-exchange column [9]. However, related substances assay are almost invariably carried out by reversed-phase LC. Therefore here the principle was that it would help to know more about the retention and enantioselectivity of CSP when using the types of reversed-phase mobile phase with a high proportion of the polar organic solvent component typically used for related substances determinations. A match of retentivity with, say, a C18 silica would be needed to allow the phase or column combination to work effectively.

2. Materials and methods

2.1. Instrumentation

The HPLC systems used for the chiral screening in reversed phase each employed a Shimadzu (Milton Keynes, UK) LC-6A pump and SPD-6AV detector. In each case, a manual Rheodyne (Kotati, CA, USA) 7125 loop injection valve, fitted with a 20 μl loop, was used for loading samples. Data was collected using a Dionex PC-based data system with *AI* 450 interface and Chromatographic Automation Software Release 3.33 (Leeds, UK) data system. The sonicator used to degas mobile phases was from GS Group-ULTRAWAVE Ltd, Cardiff, CF2 1YY.

2.2. Materials

The ACE 5 C18 (15 cm × 4.6 mm i.d.) column used was a gift from Hichrom Ltd., Theale, Berkshire, UK. The columns containing chiral stationary phases that were used, Whelk-O1 (5-μm) (250 mm × 4.6 mm i.d.), Cyclobond I 2000 DNP (250 mm × 4.6 mm i.d.) and Chiralpak QD-AX (250 mm × 4.6 mm i.d.) were gifts from Regis Technologies, Inc., Morton Grove, IL, USA, Sigma–Aldrich Chemie, GmbH, Taufkirchen, Germany and Chiral Technologies Europe, Illkirch, France, respectively. The particle size for all the stationary phases used was 5 μm. The manufacturers do not disclose the pore size of Chiralpak QD-AX. The pore size for the other stationary phases was 100 Å.

Mobile phases were prepared using HPLC – grade methanol, ammonium formate and formic acid (Sigma–Aldrich, Poole, Dorset, UK). Water was distilled and doubly de-ionised using an ELGA Option 3 Water purifier (ELGA, High Wycombe, Bucks, UK). All the drug substances used in chiral screening were from Sigma–Aldrich (Poole, Dorset, UK), Tocris (Bristol, UK) or from a collection of pharmaceutical drug substances available within Sunderland Pharmacy School. Flurbiprofen individual enantiomers and related substances were a gift from Aesica Pharmaceuticals Ltd., UK.

2.3. Methods

Reversed-phase mobile phases were prepared by adding ammonium formate to methanol–water mixtures so that it was present at a 0.02 M concentration. For every 1 l of mobile phase 2 ml of formic acid was added (or *pro rata*) for different volumes. UV detection was used monitoring at λ_{max} or wavelengths used for chromatograms in commercial literature applications except for flurbiprofen, cromakalim, mianserin and ketamine which were monitored at 254 nm. A flow rate of 1.0 ml min^{-1} was used throughout except for the chromatogram of naproxen, its enantiomer and related compounds which was run at 1.5 ml min^{-1}, and the sample injection volume was 20 μl.

A sample solution of S-naproxen, its enantiomer and related compounds at 1.0 mg ml^{-1} in mobile phase with the enantiomer and related compounds present at ~10% w/w was prepared by adding S-naproxen to a solution of racemic naproxen and the related compounds.

Laevokalim was subjected to purposeful degradation by heating a 0.5 mg ml^{-1} solution in mobile phase to 60 °C for 7 h. (No significant levels of degradants had been observed after leaving a solution in mobile phase at ambient temperature in natural light for one week.) The original solution had contained ~0.1% w/w of the enantiomer. The resultant solution was spiked with cromakalim to give a level of 0.3% w/w of the enantiomer.

The sample solution of flurbiprofen at 0.2 mg ml^{-1} in mobile phase with its enantiomer and related substances present at 1% w/w was prepared by adding 0.3 mg of S-flurbiprofen to 300 μl of a mixture of R-flurbiprofen and the related substances present at 0.01 mg ml^{-1} in mobile phase and then diluting this solution ×5 with mobile phase.

2.4. Theory/calculation

Calculations of k and α were carried out from retention times from the data system and R_s from hard copy chromatograms using standard chromatographic equations (e.g. [10]).

3. Results and discussion

The first stage of the study was to select CSP that would be suitable for reversed-phase operation in terms of likely stability and appropriate hydrophobicity of the immobilised moieties. It was already known from previous 'in-house' work (Christopher Edgar, Wimal Perera and Ha Nguyen, B.Sc. Chemical and Pharmaceutical Science, University of Sunderland, final year projects) and the literature (e.g. [11], in screening kit 30% acetonitrile expected to elute all analytes) that for macrocyclic antibiotic CSP and Cyclobond I 2000 in reversed-phase mode and for Chiral-AGP [12] low proportions (usually less than 15–20%) of the polar organic component of the mobile phase are usually needed to obtain significant retention. Attempts to couple such CSP to achiral phases of low retentivity such as cyano- or C8-phases had proved to be unsuccessful because there was not a wide enough range of %organic in the mobile phase with which to easily manipulate retention and selectivity. However it was felt that the Whelk-O1 (Fig. 1a) and Cyclobond I 2000 DNP CSP (Fig. 1b) might be sufficiently hydrophobic in their nature to give similar retentivity to the alkyl-bonded silica stationary phases that are typically used in related substances assays. In a similar vein, it had been found from other previous in-house work involving ion-exchange stationary phases that they could be used with methanol in the mobile phase in the range from 90% to 50% without there being any unacceptably marked increase in k values. In the work of Law and Appleby [13], an isocratic mobile phase of methanol–water–TFA (800:200:2.3, v/v) containing ammonium formate (0.02 M) with an apparent pH of 2.45 was used for the elution of a wide range of organic bases with varying polarities. Similarly anion exchangers were used with mobile phases with high organic content for the separation of organic acids [14]. Accordingly it was decided to also study the commercially available chiral ion-exchange CSP, Chiralpak QD-AX (Fig. 1c).