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Short Communication

Use of a porous graphitic carbon column to separate *cis* and *trans* isomers in a novel anti-asthma compound

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Introduction

The separation and quantification of *cis* and *trans* isomers is an important objective for the analyst. National drug authorities such as the United States Pharmacopeia (USP) and British Pharmacopoeia (BP) require the control of *cis* and *trans* isomer contents in drug substances where this type of isomerism can exist, e.g. clomiphene citrate, Doxepin Hydrochloride and Dothiepin Hydrochloride [1, 2].

Various chromatographic techniques have been employed to resolve these types of isomer mixtures. Gas chromatography (GC) is specified in the test for *Z* isomer content in the BP monographs for Doxepin Hydrochloride and Dothiepin Hydrochloride [1], the methyl esters of oleic acid (*Z*) and elaidic acid (*E*) were partially resolved by Oesterhalt *et al.* using GC [3] and capillary GC has been partially successful in effecting separation of linoleic acid isomers as reported by Kobayashi [4].

High-performance liquid chromatography (HPLC) has also found extensive use in solving this type of problem, both in normal and reversed-phase modes. *Z* and *E* isomers of clomiphene citrate can be resolved on a Porasil silica column [1, 2]. Steuerle managed to separate six *cis–trans* isomers of vitamin A esters on alumina [5]. Examples of reversed-phase methods include the resolution of *Z* and *E* isomers of 5-dodecen-1-ol acetate, and *Z* and *E* 7-tetradecen-1-ol acetate on μBondapak C18 using methanol–water mixtures as eluents [6].

The discovery that silver ions can complex with olefinic double bonds has been exploited in attempts to resolve *cis–trans* isomers [7]. Applications of 'argentation chromatography' include the separation of stilbene derivatives by normal phase (silica impregnated with silver nitrate) and the resolution of olefinic mixtures by reversed-phase (C-18 bonded phase with silver ions in an aqueous eluent) [8, 9]. Knox *et al.* described the preparation and properties of a stationary phase consisting of porous graphitic carbon (PGC), which was more retentive and exhibited novel selectivity compared to ODS-silica [10]. This paper describes the use of PGC to separate four *cis–trans* isomers of a potential anti-asthma agent (LY170680) and compares the selectivity of PGC towards these isomers against that of an ODS-bonded silica. PGC is found to separate the isomers more quickly and yields much better resolution than does ODS-silica.

Experimental

Reagents

Acetonitrile, dichloromethane and methanol (all HPLC grade) were obtained from FSA Laboratory Supplies (Loughbrough, UK); disodium hydrogen ortho-phosphate (anhydrous) and trifluoroacetic acid (HPLC grade) were obtained from BDH (Poole, Dorset, UK). Distilled water was used.

Apparatus

The HPLC system comprised a Milton Roy Constametric 3000 pump, a Waters Wisp 710B auto-sampler programmed to deliver 10 μl, a Pye–Unicam PU4020 UV detector set at

238 nm and a Kipp and Zonen BD40 chart recorder. Chromatograms were also stored using a Hewlett–Packard HP1000 data system. The HPLC columns used were: two 25 cm × 0.46 cm i.d. Vydac C18 (5 μm) columns (part no. 218 TP 54) and a 10 cm × 0.46 cm i.d. Hypercarb S (7 μm) column (Shandon Scientific Ltd, Cheshire, UK). pH adjustments to one of the mobile phases were monitored by a Beta 52 meter fitted with an EIL glass electrode.

Mobile phases

System 1. The following mobile phase was used to elute material from two coupled Vydac columns: 1 ml trifluoroacetic acid was dissolved in 600 ml water, and the pH was adjusted to 2.4 with 5% w/v di-sodium hydrogen ortho-phosphate (anhydrous) and then 400 ml acetonitrile was added. A flow rate of 1 ml min^{-1} was used.

System 2. A mobile phase consisting of 680 ml methanol, 320 ml dichloromethane and 6.8 ml trifluoroacetic acid was used in conjunction with the Hypercarb column. A flow rate of 1 ml min^{-1} was used.

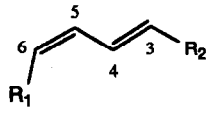
Test solutions

Solutions containing 1 mg ml^{-1} LY170680 in methanol were injected into both HPLC systems to assay isomer levels. A specially synthesized sample containing approximately equal amounts of each isomer was used to develop the chromatographic separation.

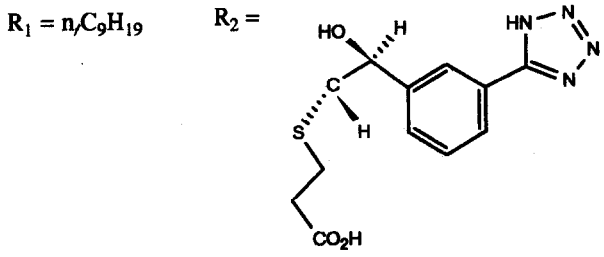
Results and Discussion

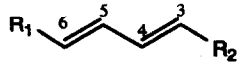
Method development

Figure 1 shows the structure of LY170680 and three other potential isomeric arrangements of the conjugated diene system. The systematic name for LY170680 is: 5-(3-[2(*R*)-

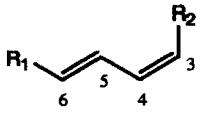


1. Trans - cis isomer (3 E, 5 Z)

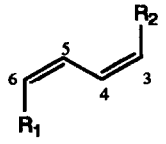




2. Trans - trans isomer (3 E, 5 E)



3. Cis - trans isomer (3 Z, 5 E)



4. Cis - cis isomer (3 Z, 5 Z)

Figure 1
Structure of LY170680 and isomers.

(carboxyethylthio)-1(*S*)-hydroxypentadeca-3(*E*), 5(*Z*)-dienyl]-phenyl)-1*H*-tetrazole. The required molecular species is the *trans–cis* isomer (Fig. 1—1); however during its synthesis, some of the other three isomeric species may be formed. These other isomers (*cis–trans*, *cis–cis*, *trans–trans*) (Fig. 1—2–4) are known to be less active than the *trans–cis* isomer, thus methods to monitor these by-products were required. The structure of LY170680 and knowledge of its chemical stability were taken into consideration when deciding upon a suitable strategy for method development.

The presence of the carboxylic acid group precluded the use of GC as a method unless the substance was derivatized. This would lengthen the method and may even alter the isomeric content. An HPLC separation of the chiral isomers of LY170680 has already been reported from these laboratories [11]. Derivatization was investigated, but the presence of the tetrazole group led to several products being formed when methylation with diazomethane was attempted.

HPLC was considered to be the best approach because LY170680 was capable of being chromatographed without derivatization. Normal-phase methods are usually more successful for separating geometric isomers, because the retention mechanism is based on adsorption, so procedures using silica columns were examined.

Exhaustive experimentation was carried out to find an eluent which would resolve the synthetic isomer mixture; eluents consisting of hexane, chloroform, methyl *t*-butyl ether, various alcohols and acetic acid in many combinations were tried. Partial separation of isomers 1, 2, 3 and 4 was obtained in 2 h, but a full separation could not be achieved. Different types of silica were tried and the columns were also cooled in ice baths, but adequate resolution could not be obtained. Alternative systems based on adsorption chromatography were explored: an alumina column was found to exhibit very strong retention towards the isomers which could not be eluted and a nitrile-bonded phase yielded broader peaks than silica. Argentation chromatography was found to be unsuitable because poorly separated, broad peaks were observed.

Owing to the inability of normal-phase methods to separate LY170680 isomers, several reversed-phase methods were tried, of which the best used two coupled 25-cm Vydac C18 columns in combination with the mobile phase described in system 1 [D. Osborne, personal communication]. Figure 2 shows a chromatogram obtained from the injection of a 1 mg ml^{-1} solution of LY170680 (batch 5888). The *trans–trans* isomer was easily separated from the main compound (*trans–cis*); in fact its separation has never been a problem as it can be resolved on 15 cm ODS phases in less than 20 min with methanol–water–acetic acid eluents.

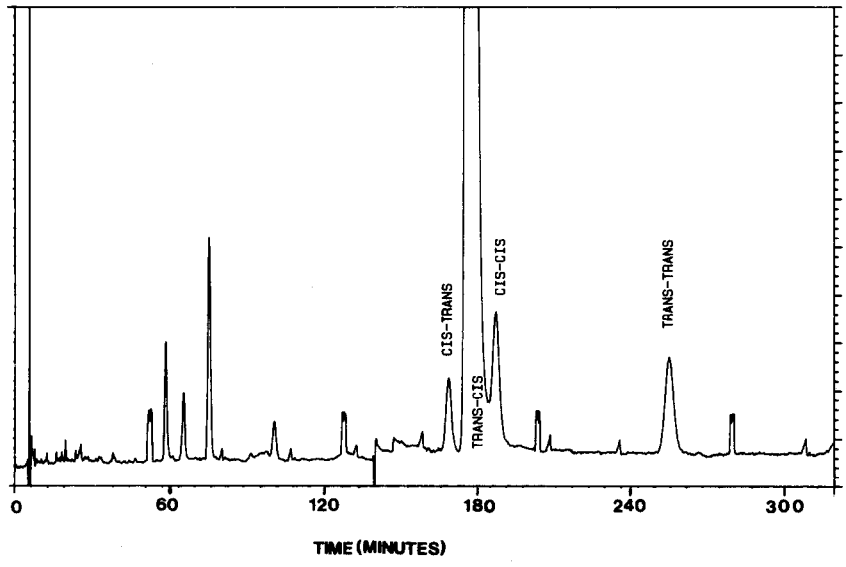


Figure 2
Chromatogram showing separation of geometric isomers from a 1 mg ml^{-1} LY170680 solution using Vydac C18 (system 1).

The separation of the *cis–trans* and *cis–cis* isomers has always been a bigger challenge; the chromatogram shows marginal separation of the *cis–trans* from the main peak with the *cis–cis* eluting on the tail of the main component. Consequently, peak area measurements would be less reproducible and accurate compared to a system that produced better resolution. Another important consideration is that caused by loss of column efficiency: any such loss would compromise the already minimal resolution of these isomers. Final problems with this method were the high column back pressure (approximately 4000 psi) and the very long analysis time (5.5 h).

The use of a porous graphite carbon (PGC) column was subsequently investigated because of its potentially useful selectivity, especially towards isomers. Using the mobile phase described in system 2, all four isomers were well resolved from each other. Peak assignments were made by injecting pure LY170680 (*trans–cis*) and pure *trans–trans*. The identity of each of the two remaining components were assigned by injecting a solution known to contain mostly *cis–trans* and some *cis–cis* [S. Morgan, personal communication]. A 1 mg ml^{-1} solution of LY170680 (batch 5888) was prepared and chromatographed; a typical chromatogram is shown in Fig. 3. It is apparent that this chromatogram is superior to that obtained for the same batch using the Vydac system. The PGC column efficiency is 20,000 plates m^{-1}, which is rather low for high-performance columns, but is adequate for the current analysis. Isomer separation is better on PGC and the entire components are eluted in 40 min (Table 1). The differing elution order of the isomers reflects the novel selectivity of the PGC column. PGC is based on a new form of graphite; hence at the molecular level, the surface consists of delocalized electrons which are capable of interacting with the electrons from injected solutes (pi–pi interaction). The diene system in LY170680 has pi bonding so it is reasonable to surmize that there may exist an electronic interaction between the pi electrons of LY170680 and the delocalized surface electrons. The different spatial arrangement of the pi electrons in each of the isomers would cause this interaction to be slightly modified for each isomer and hence effect a separation.

The Vydac ODS phase would be expected to separate substances on the basis of differences in lipophilicity; the diene system isomers of LY170680 do not exhibit sufficient lipophilic

Table 1
Retention time data (min)

Isomer	System 1	System 2
cis–trans	170	25.5
trans–cis	180	16
cis–cis	185	31.5
trans–trans	260	2.2

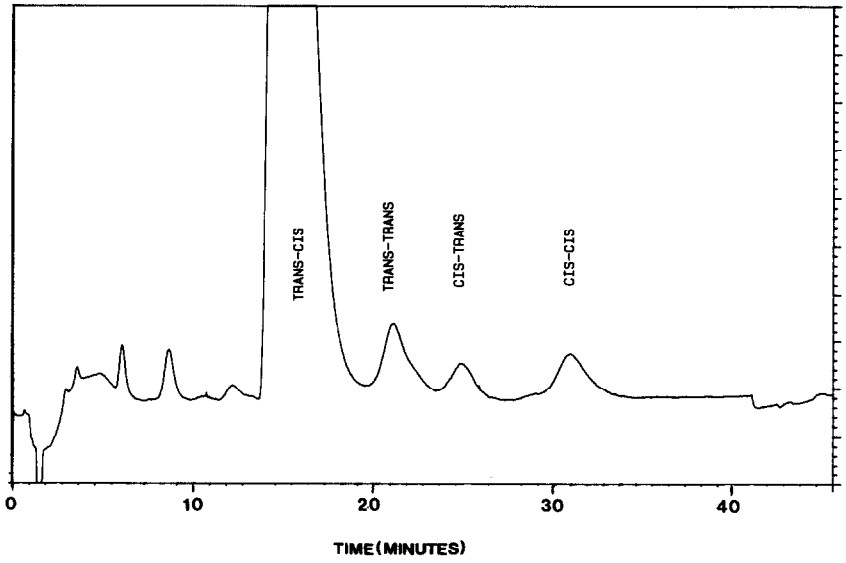


Figure 3
Chromatogram showing separation of geometric isomers from a 1 mg ml^{-1} LY170680 solution using a PGC column (system 2).

differences, so a separation would be very difficult to achieve by this means.

Samples from other experimental batches were also examined using the PGC column. Isomer levels were calculated by the following procedure: a 100-fold dilution of the test solution was injected and the area of the major peak (*trans–cis*) recorded. The test solution 1 mg ml^{-1} was also injected and areas of the minor isomer components were also recorded: ratios were then calculated for each of these to the peak area obtained using the 100-fold dilution to give the percentage w/w. The results are shown in Table 2. For these purposes, the assumption was made that each isomer exhibited identical absorptivity at 238 nm. The levels of different isomers can be seen to vary between batches, but this is not surprising because each batch was produced by a slightly modified synthesis or final purification step.

Linearity and precision

Linearity of response was assessed by recording the peak areas from five solutions of LY170680 over the 0.005–0.04 mg ml^{-1} concentration range. A correlation coefficient of 0.999 was observed; the *y*-axis intercept was 126.8 and the slope was 574438, thus demonstrating linearity.

Precision was determined from replicate analysis of a 1 mg ml^{-1} solution (batch 5888); the RSD values for isomers 2, 3 and 4 were: 5.1, 7.2 and 7.6% respectively ($n = 5$) at the 1% w/w level, which is quite acceptable. The method was found to be robust; a second analyst obtained closely similar values for the isomer content of batch 5888; the RSD values rose only slightly to 6.2, 7.5 and 8.0% ($n = 5$) for isomers 2, 3 and 4, respectively.

Table 2
Isomeric content of some LY170680 batches (% w/w)

Batch	*cis–trans*	*trans–trans*	*cis–cis*
5888	0.64	1.00	1.21
521PP9	none	0.14	none
629PP8	0.55	0.38	none

Conclusion

A rapid and simple HPLC method has been developed for assaying the geometric isomers of a pharmaceutical substance that contains a conjugated diene system as part of its structure.

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