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Journal of Pharmaceutical and Biomedical Analysis
16 (1998) 1205–1212

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Chiral bioanalysis of warfarin using microbore LC with peak compression[1]

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Received 9 September 1997

1. Introduction

It has been demonstrated in achiral systems, that the application of solid phase extraction (SPE) and microbore LC with peak compression (or on-column sample focusing) is an attractive approach to the analysis of drugs in biological fluids, offering the prospect of access to low limits of detection [1] and ease of automation [2]. The essential element of this approach is in the selection of SPE and microbore LC phases, such that the solvent used to elute analyte from the SPE cartridge is a weak solvent with respect to the stationary phase in the microbore LC column. In this way, it is possible to directly inject large volumes of SPE eluent onto the microbore column with concomitant peak compression so that there is no risk from band broadening to adversely affect the advantage of microbore LC of increased mass sensitivity.

Bearing in mind the need, for the solvent used to elute the analyte from the SPE cartridge, to be a weak solvent with respect to the stationary phase in the microbore LC column, the highly retentive porous graphitic carbon (PGC) material, Hypercarb® was used to demonstrate for the first time that the SPE/peak compression/microbore LC approach could be extended to chiral LC systems [3]. The promise of this illustrative example using warfarin was such that the aim of this subsequent study was to optimise the conditions for this enantioseparation, to the point that it could form the basis of an assay for the determination of warfarin (Fig. 1) enantiomers in biological fluids. Problems still exist in the determination of the enantiomers of warfarin in biological fluids [4] despite the emergence of a number of published methods [5–9]. Because of this and the continuing need to monitor the pharmacokinetics and metabolism of the enantiomers of this racemic drug, especially when co-administered with other drugs, there is a continuing interest in improved methodology for the determination of warfarin enantiomers in biological fluids.

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[1] Presented at the Eighth International Symposium on Pharmaceutical and Biomedical Analysis (PBA '97), Orlando, FL, USA, 4–8 May, 1997.

PII S0731-7085(97)00267-7

2. Experimental

2.1. Materials and reagents

Racemic sodium warfarin was supplied by Sigma, Poole, UK. Acetonitrile (MeCN) HPLC glade, acetic acid (AcOH), triethylamine (TEA) and perchloric acid (all Analar grade) were supplied by BDH, Poole, UK.

Phenyl Bond Elut® cartridges were supplied by Phenomenex, UK. β-Dimethylated cyclodextrin (DM-β-CD) was a gift from Glaxo Wellcome, Ware, UK. *S*-warfarin was a gift from Covance, Harrogate, UK.

2.2. Instrumentation

The HPLC system consisted of a Shimadzu LC-1OAD pump and SPD-6AV W/vis detector. Chromatograms were recorded using the Dionex A1-450 chromatography software system. The column, a Hypercarb® (5 μm; 150 × 1.0 mm i.d.) was custom-packed by Hypersil, Runcorn, UK. A Rheodyne 7125 valve injector fitted with a Tefzel rotor seal (Anachem, Luton, UK) was used with a specially prepared low internal diameter (0.25 mm id.) stainless steel external loop.

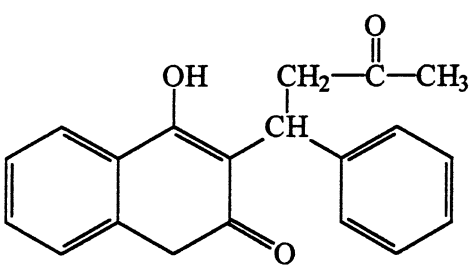

Fig. 1. Structure of warfarin. A coumarin anticoagulant, warfarin is administered in the racemic form. Its enantiomers differ in degrees of stereoselectivity in transport, anticoagulant potency, metabolism, elimination rate and interaction with other drugs [10]. The (*R*)-enantiomer of warfarin is metabolised mainly by reduction to (*RS*)-3′-hydroxywarfarin and is metabolised faster than the (*S*)-enantiomer. However, the (*S*)-enantiomer is three times more active than the (*R*)-enantiomer and binds more strongly with plasma proteins [11]. Typically, a patient will have a steady state plasma concentration of 1.2–2.6 μg ml^{-1} after receiving a daily dose of 5–11 mg orally [12].

2.3. High-performance liquid chromatography

Subsequent to the method developed to optimise MeCN content, [DM-β-CD] and flow rate, the mobile phase used was MeCN-H_2O-AcOH-TEA (850:150:3:2.5, v/v) containing 20 mM DM-β-CD. The optimised flow rate was 0.048 ml min^{-1} and the optimum wavelength of detection was 305 nm. Standard warfarin solutions were made up in MeCN-H_2O-AcOH-TEA (200:800:3:2.5, v/v), during the course of method development, except when assessing what the appropriate injection volume should be.

2.4. Sample preparation of biological samples

Standard plasma samples spiked with warfarin were prepared using a solution of warfarin in physiological buffer. Protein precipitation was carried out on each plasma sample with 1 ml of a 10% v/v solution of perchloric acid with the samples placed on ice. After centrifugation (at 3000 rpm for 15 min), the supernatant was transferred to a primed Phenyl Bond Elut® cartridge. Priming involved application of 5 ml of MeCN followed by 5 ml of a 0.1% v/v solution of AcOH. After application of the supernatant, the cartridge was washed with 10 ml of MeCN-AcOH (0.1%, v/v) (20:80, v/v) then the sample was eluted with 2 ml of MeCN-AcOH (0.1%, v/v) (50:50, v/v). Aliquots (500 μl) of this were then injected on to the chromatograph. Patient samples were treated in the same way.

3. Results and discussion

3.1. Initial separation conditions

The porous graphitic carbon material, Hypercarb®, has a number of attractive properties [13]. Its high retentivity under reversed-phase LC conditions and its common use as an achiral support for chiral LC, using chiral mobile phase additives made it the ideal choice as the stationary phase in the attempt to extend the use of the SPE/peak compression/microbore LC combination from achiral to chiral systems. The chiral selector used

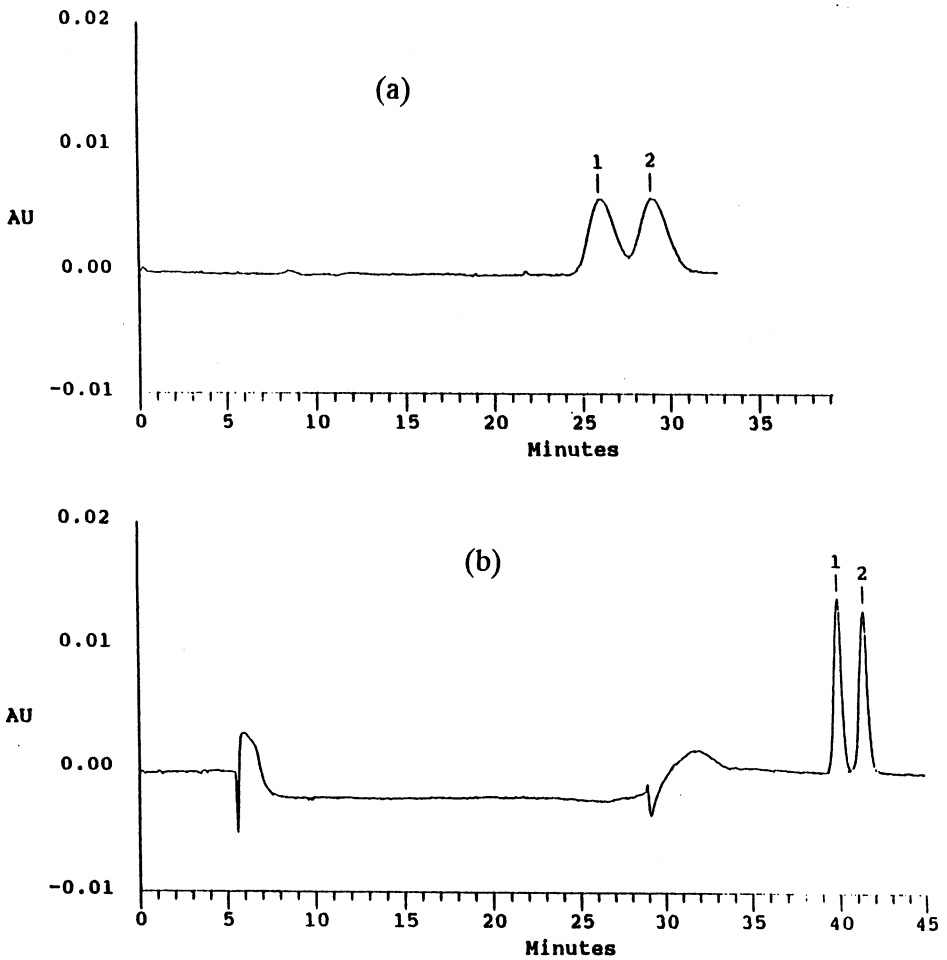

Fig. 2. Peak compression. In both cases, flow 0.024 ml min^{-1}, mobile phase [MeCN-H_2O-AcOH-TEA (800:200:3:2.5, v/v)] containing 10 mM DM-β-CD. Chromatograrn (a) is a 20 μl injection of 200 ng of warfarin in mobile phase; chromatogram (b) is 500 μl of 200 ng of warfarin in MeCN-H_2O-AcOH-TEA (200:800:3:2.5, v/v).

in the mobile phase was dimethylated-β-cyclodextrin (DM-β-CD). The peak compression effect achieved using an injection solvent which is non-eluting is clearly illustrated in the chromatograms in Fig. 2, where 200 ng of racemic warfarin was injected in 20 μl of mobile phase and also in 500 μl of MeCN-H_2O-AcOH-TEA (200:800:3:2.5, v/v). The warfarin enantiomer peaks are wider with injection in 20 μl of mobile phase, this volume being large enough to give volume overload on the 150 × 1.0 mm id. column used. However when using the non-eluting solvent, large volumes may be injected without introducing extra-column band-broadening through volume overload at the head of the column. Therefore the peak compression or on-line sample focusing effect taking place has potential as a convenient concentration step in drug bioanalysis. Also with respect to the convenient use of this effect in drug bioanalysis it is important to note that it is not adversely affected by the absence of a chiral selector in the injection solvent.

However, before this methodology could be used as the basis of a method for the determination of warfarin enantiomers in biological fluids there were some issues that needed to be ad-

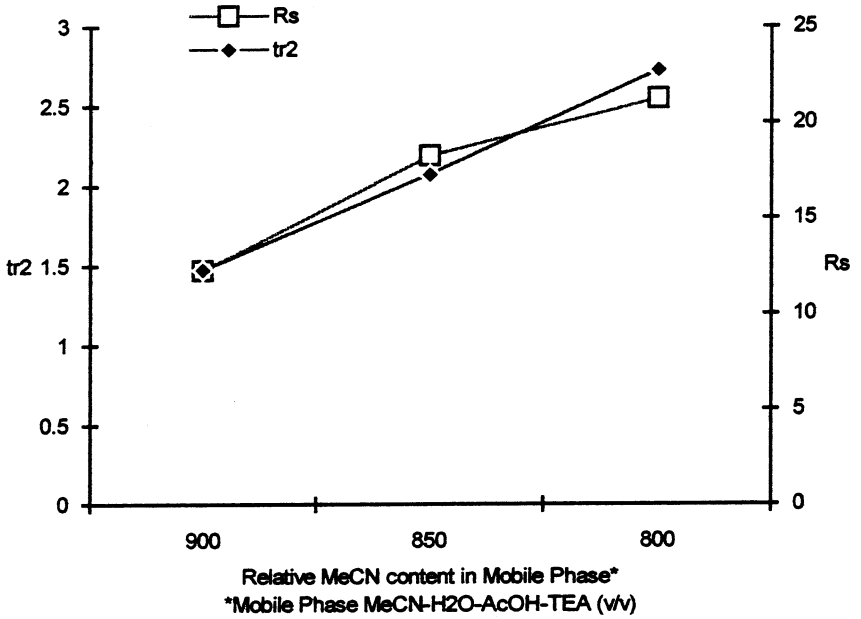

Fig. 3. Effect of organic modifier on resolution and retention.

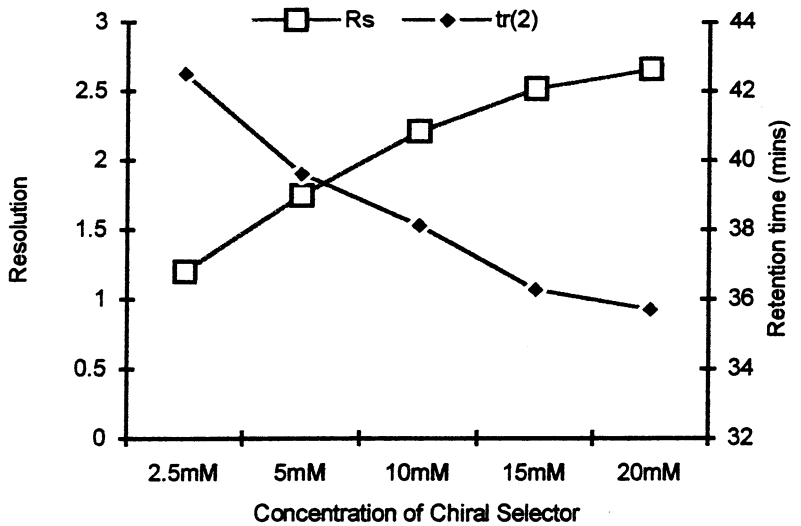

Fig. 4. Effect of chiral selector concentration on retention and resolution.

dressed. First of all, the analysis time needed to be reduced, and secondly the mobile phase composition needed to be adjusted so that peak compression could be obtained with an injection solvent containing 50% acetonitrile since this amount was needed in the eluent from the Phenyl Bond Elut® cartridge in order to elute warfarin.

With respect to analysis time, there was fortunately scope both to increase flow rate and increase the organic content of the mobile phase, to reduce retention times since there was scope for reducing resolution from the initial conditions and still having sufficient resolution to have a reliable baseline separation. A major contribution

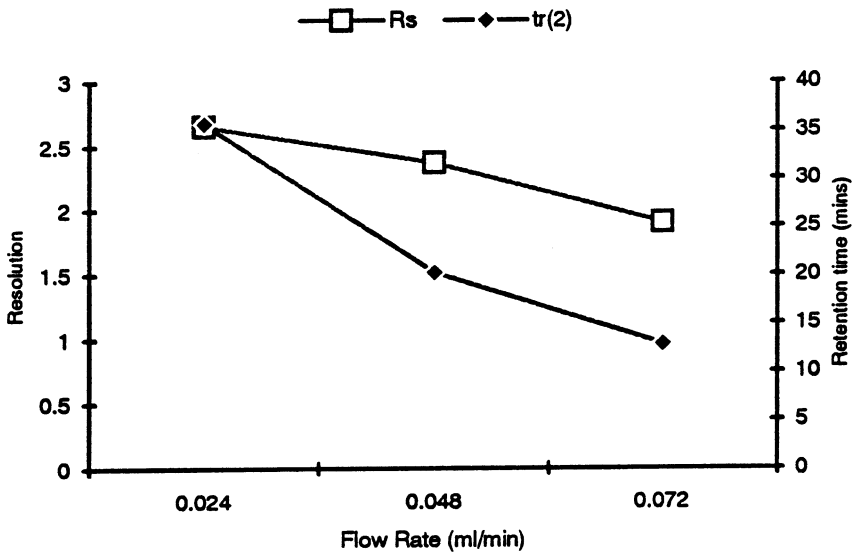

Fig. 5. Effect of varying flow rate on resolution and retention.

to the analysis time was the time taken for the large volume from the loop to be transferred to the column. Since work with standards indicates that the limit of detection (2 ng ml^{-1} for each enantiomer with a 500 μl injection volume) is sufficiently low to enable therapeutic samples to be analysed comfortably without having to use maximum allowable injection volumes, the possibility of striking a balance between detectability and chromatographic run time could be considered.

With respect to the mobile phase composition needing adjustment so that peak compression could be obtained, it would be necessary to obtain resolution of warfarin enantiomers with a mobile phase containing as much acetonitrile as possible. Otherwise an additional step of post-SPE adjustment of solvent composition is required, which would involve a limited amount of dilution with aqueous acetic acid. Again there would be scope for trading off resolution against the option of increasing the acetonitrile content of the mobile phase. Also, it would be practical to investigate the possibility of maintaining resolution at higher mobile phase acetonitrile compositions by using a higher cyclodextrin composition.

3.2. Effect of organic modifier on resolution and retention

The effect of organic modifier on retention time and resolution, with 20 mM DM-β-CD in the mobile phase is shown in Fig. 3. As can be seen from the graph, as the percentage of organic modifier decreases, both retention and resolution increase. The optimum mobile phase chosen on the basis of this plot, was MeCN-H_2O-AcOH-TEA (850:150:3:2.5) a significantly better resolution was achieved with this composition without compromising analysis time.

3.3. Effect of concentration of chiral selector on resolution and retention

As illustrated in Fig. 4, increasing the concentration of chiral selector in the mobile phase, increases resolution whilst retention time of the second eluting peak decreases. At 20 mM, a plateau appears to have been reached, at higher concentrations of chiral selector a dramatic rise in resolution is not seen and likewise a dramatic fall in retention time is absent.

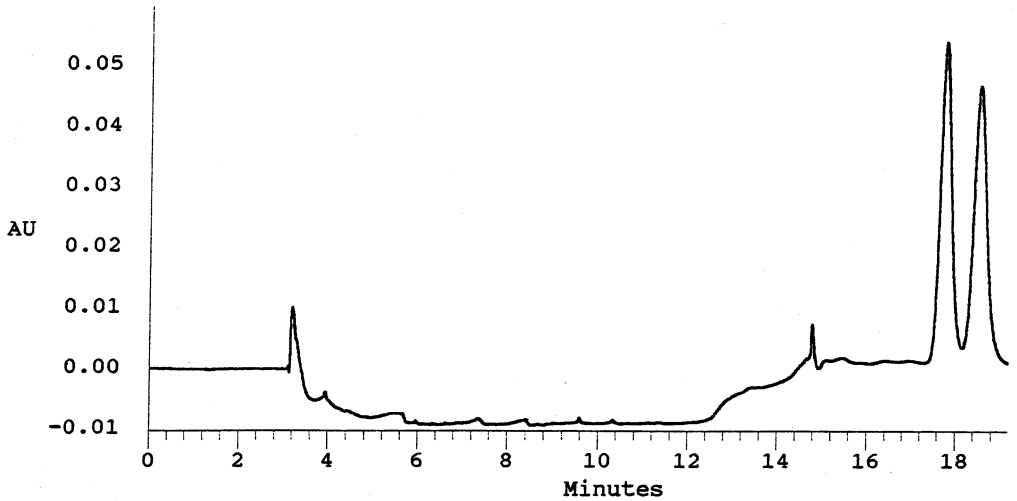

Fig. 6. Plasma spiked with racemic warfarin (3 μg ml^{-1}). Flow 0.048 ml min^{-1}; mobile phase 20 mM DM-β-CD in MeCN-H_2O-AcOH-TEA (850:150:3:2.5, v/v); injection volume 500 μl; injection solvent MeCN-0.1% AcOH (50:50, v/v).

3.4. Effect of flow rate on resolution and retention

Flow rate is not a variable that would be considered worth varying to look for significant changes in resolution. However, since the retention mechanism could possibly involve inclusion complexation and therefore potentially slower mass transfer, the chance of losing resolution by increasing flow rate could not be ignored. The effect of increasing the flow rate giving a decrease both in resolution and retention time of the second eluting peak is shown in Fig. 5. Although some degree of resolution is lost, resolution was still baseline up to a flow rate of 0.072 ml min^{-1}. At this flow rate, therefore, the run time can be more than halved compared with conditions which had been used initially. However, while this flow rate gives rise to no back pressure problems when the mobile phase is in the column, there is a significant increase in back pressure when the injection solvent mixes with the mobile phase at the head of the column during sample loading. Therefore, in considering the conditions that should be used for subsequent drug bioanalysis, it was decided to select a flow rate of 0.048 ml min^{-1} thereby giving scope for pressure changes with viscosity of solvent in the column and opting for more reliability in achieving resolution, reducing the run time below 20 min which was considered to be quite acceptable in the context of probable total analysis time. Similarly it was decided to hold the injection volume at 500 μl, thereby giving priority to having scope for detecting low levels of warfarin enantiomers over reducing the run time.

The optimum chromatographic conditions decided upon therefore was a mobile phase of 20 mM DM-β-CD in MeCN-H_2O-AcOH-TEA (850:150:3:2.5, v/v) at a flow rate of 0.048 ml min^{-1} with an injection volume of 500 μl using the SPE MeCN-AcOH (0.1%, v/v) (50:50, v/v) eluent as the injection solvent.

3.5. Order of elution of enantiomers

It was determined using a sample of racemic warfarin spiked with *R*-warfarin, that the *S*-enantiomer eluted first. This was fortuitous since the *S*-enantiomer is metabolised more rapidly than the *R*-enantiomer [7], the problem of integrating a small peak for the *S*-enantiomer on the tail of a large peak of the *R*-enantiomer could have been encountered had the retention order been the other way round.

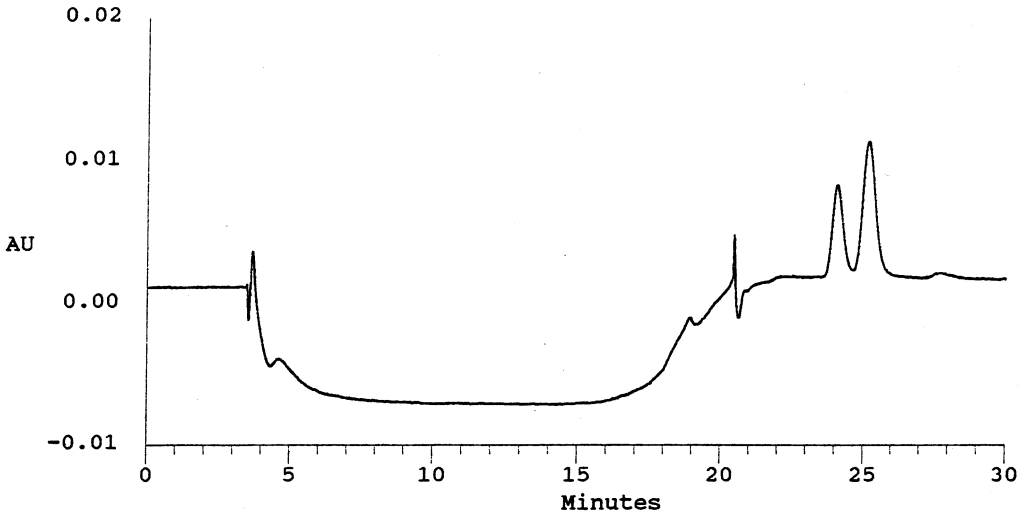

Fig. 7. Plasma sample from patient receiving warfarin therapy. Flow 0.036 ml min^{-1}, mobile phase 20 mM DM-β-CD in MeCN-H_2O-AcOH-TEA (850:150:3:2.5, v/v); injection volume 500 μl; injection solvent MeCN-0.1% AcOH (50:50, v/v).

3.6. Bioanalysis

A standard solution of 3 μg ml^{-1} warfarin in plasma was prepared and extracted using a previously described solid phase extraction procedure. The eluant (500 μl) was injected on to the chromatograph to produce the chromatogram shown in Fig. 6. This is well within the steady state concentration range of warfarin in plasma. Thus, this shows that these conditions could provide the basis for a suitable method for monitoring patients' plasma concentrations of the enantiomers of warfarin.

The chromatogram obtained from a plasma extract of a patient receiving warfarin therapy (extracted in the same way is shown in Fig. 6)(Fig. 7). This shows that no metabolites interfere with the analyte peaks and that no late running metabolite peaks are present. In fact, by using a solid phase extraction step, a selective clean-up of the plasma sample is obtained, removing polar metabolites to give a 'cleaner' chromatogram. Because of the absence of these metabolites and the fact that if present they would elute before the warfarin enantiomer peaks, the analysis time is significantly shorter than the validated method of Naidong and Lee [8].

4. Conclusion

The SPE/peak compression/microbore LC approach to drug bioanalysis has obvious benefits for the determination of warfarin enantiomers in biological fluids, the most striking advantage being the ability to achieve lower limits of detection with UV detection than is achieved in other methods which use fluorescence detection. Given the high retentivity of Hypercarb® and its versatility as an achiral support for use with chiral mobile phase additives, there is little reason why the methodology described here should not find widespread use in the determination of enantiomers in biological fluids, thereby avoiding contamination problems of expensive chiral stationary phases or the necessity to use complex valve switching systems which beset many existing methods.

Acknowledgements

This work was supported by the Analytical Division of the Royal Society of Chemistry through a grant for an SAC studentship for A.S. Prangle, which is gratefully acknowledged.

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