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# Enantiomeric separation of *R,S*-naproxen by conventional and nano-liquid chromatography with methyl-β-cyclodextrin as a mobile phase additive

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**Abstract**

Chiral separations of *R,S*-naproxen mixtures were obtained on an achiral column (ODS) with methyl-β-cyclodextrin as a mobile phase additive using conventional and nano-LC. The optimised mobile phase composition was 20 mmol $l^{-1}$ methyl-β-cyclodextrin, 20% (v/v) acetonitrile, and 50 mmol $l^{-1}$ sodium acetate buffer at pH 3 using hydrochloric acid for pH adjustment. In addition to UV detection at 232 nm, amperometric detection was also investigated. Without using any internal standard, the reproducibility of amperometric detection (+1.05 V vs. Ag/AgCl) over a long analysis cycle in LC was greatly improved by choosing the peak area ratio between *R*- and *S*-naproxen as the analytical readout (the relative standard deviation was 2.11%) and enantiomeric purity could be assessed directly. This method was successfully employed for enantiomeric purity assessment in commercial naproxen tablets. Finally, successful transfer from conventional LC to nano-LC was realised, resulting in over 1000-fold reduction in reagent consumption. 

*Keywords:* Enantiomer separation; Naproxen; Non-steroidal anti-inflammatory drugs

## 1. Introduction

Naproxen, 6-methoxy-α-methyl-2-naphthaleneacetic acid, belongs to an important group of medicines called non-steroidal anti-inflammatory agents and is widely used in the treatment of rheumatic and other inflammatory diseases and for the relief of mild to moderate pain [1]. Naproxen has a chiral centre and the pharmacological activity resides mainly in the *S*-naproxen. While other non-steroidal anti-inflammatory drugs are marketed as racemates, such as ketoprofen and flurbiprofen, naproxen is sold only as *S*-naproxen [2]. Therefore, enantiomeric separation and purity assessment is vital in assuring good quality in the pharmaceutical production of naproxen and in other naproxen-related scientific research work as well.

A variety of chromatographic methods have been proposed for the chiral separation of naproxen enantiomers, mainly by capillary electrophoresis (CE) and LC. In CE, chiral separation of naproxen has been demonstrated with various cyclodextrins [2–5], macrocyclic antibiotics [6,7] and oligosaccharides [8] as background electrolyte additives and

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by capillary electrochromatography (CEC) with a column containing α-chymotrypsin modified silica [9]. While one study was mainly devoted to the quantitative analysis of naproxen enantiomers [3], others were more general and involved fundamental studies, such as peak resolution model establishment [5], acidity and inclusion constant determinations [2], pore size influence studies [9] and comparison of chiral selectors [7].

In LC, chiral separation was obtained on either chiral or achiral stationary phases after derivatisation with different reagents to form ester or amide products [10–13]. In some cases, diastereoisomeric derivatives were produced [12,13]. In addition to the use of complex derivatising procedures, these indirect methods may induce inaccuracy into the determination of enantiomeric ratios owing to the chiral impurities in the derivatising agent and/or the racemising during the derivatisation procedure, particularly when a large difference in the concentration of enantiomers exists [13]. Direct resolution of underivatised naproxen has therefore some advantage. It can be obtained either with chiral stationary phases or with chiral mobile phase additives. Many chiral stationary phases have been employed for this purpose, including chiral stationary phase of Pirkle and co-workers [14,15], proteins and enzymes [16–18], cyclodextrin [10], cellulose types [19,20], terguride-based [21] and molecularly imprinted polymer-based [22]. Although naproxen enantiomers could be separated to different extents with these chiral stationary phases, most of these papers employed naproxen and/or other non-steroidal anti-inflammatory drugs only for column performance tests, and mechanism and comparative studies. Few were devoted mainly to the quantitative determination of enantiomeric purity of naproxen.

Since chiral stationary phases are usually quite expensive and difficult to synthesise, the use of chiral mobile phase additives can be a cheap, convenient, yet effective alternative. However, very few studies have been made on the LC separation of naproxen enantiomers on achiral columns with chiral mobile phase additives. In this paper, chiral separation of naproxen enantiomers on achiral columns with methyl-β-cyclodextrin (methyl-β-CD) as a mobile phase additive was investigated. In addition to UV detection, amperometric detection was investigated for the first time as a detection method for naproxen separation. A novel method to improve the reproducibility in amperometric detection without using either external or internal standards was proposed. The method was applied for the enantiomeric purity assessment of commercial naproxen tablets. In order to reduce the consumption of reagents, particularly the expensive chiral mobile phase additive, the established LC method was transferred to nano-LC.

## 2. Experimental

### 2.1. Chemical

The mobile phase was 20% (v/v) HPLC-grade acetonitrile (Labscan, Dublin, Ireland), 20 mmol $l^{-1}$ methyl-β-CD (Sigma–Aldrich, Dorset, UK), and 50 mmol $l^{-1}$ sodium acetate at pH 3. Hydrochloric acid was chosen to adjust the pH in order to provide enough chloride ions in the mobile phase to meet the requirement (10 mmol $l^{-1}$) of the internal UniJet Ag/AgCl reference electrode (Bioanalytical Systems, USA) in amperometric detection. Mobile phase was degassed continuously with helium while in operation.

*R*-, *S*-Naproxen (Clonmel Healthcare, Ireland) and naproxen racemate standard solutions of 1.0 or 2.0 mg $ml^{-1}$ were prepared by dissolving appropriate amounts of *R*-, *S*-naproxen and naproxen racemate in 25 ml or 50 ml calibrated flasks with the mobile phase. Standard solutions of lower concentrations were prepared by dilution with the mobile phase.

### 2.2. Apparatus

An HP 1050 series pump and a manual injector with 20 μl sample loop (Hewlett–Packard, USA) were employed. A 20 cm×4.6 mm I.D. ODS column (Capital HPLC, West Lothian, UK) was used. The pump was operated in an isocratic mode at a flow-rate of 2 ml $min^{-1}$.

For amperometric detection, an LC-4B amperometric detector (Biotech Instruments, UK) equipped with a UniJet radial-flow thin layer cell (Bioanalytical Systems, USA) was used. The working electrode was a glassy carbon electrode of 6 mm

diameter and the reference electrode was Ag/AgCl. The output of the detector was connected to an HP 3392A integrator (Hewlett–Packard) to record and integrate the chromatograms. While investigating the influence of applied potential, the experiments were performed in an flow injection mode with a flow-rate of 1 ml $min^{-1}$. As it is not necessary to have the column connected, the column was replaced by a plain stainless steel tube (80 cm×0.5 mm I.D.) in order to avoid irreproducibility, which would be significant over a long period of use.

For UV detection, an HP 1050 series multiple wavelength detector (Hewlett–Packard) was employed and the chromatograms were recorded and integrated by a computer with HP ChemStation. The wavelength of detection was set at 232 nm.

In nano-LC experiments, a microtech LC pump (Anachem, Bedfordshire, UK), a 20 nl manual valco injection valve (Presearch, Hitchin, UK), and an SPD6A detector (Shimadzu, Japan) fitted with a 3 nl flow cell (from Presearch) were used. An LC-8A preparative liquid chromatograph was used for packing the capillaries.

### 2.3. Preparation of packed capillaries

The packing slurry was prepared by adding 5 μm ODS ($C_{18}$, Hypersil) to methanol (Labscan, Dublin, Ireland) and ultrasonicated for 10 min. The slurry was transferred with a pasteur pipette to a slurry reservoir consisting of a steel HPLC column. The suspension was pumped into a 22 cm×75 μm I.D. capillary (Composite Metals, Worcester, UK) using methanol at 300 bar for 1 h while sonicating. The packing material was retained with a frit prepared using a silica/sodium silicate mixture sintered over a hot flame. The packed column was equilibrated with mobile phase for 30 min before interfacing with the nano-LC system.

### 2.4. Sample analysis

After 20 commercial naproxen tablets (each containing 250 mg of naproxen) were weighed and crushed using a mortar and pestle, a portion of the powder, equivalent to the average weight of the 20 tablets, was dissolved in 25 ml of acetonitrile in a conical flask. After stirring for 10 min, the solution was transferred to 50 ml calibrated flasks and made to the mark with acetonitrile. The sample solutions were then gravity filtered or filtered with a 0.45 μm syringe filter. A volume of 1 ml (for UV detection) or 2 ml (for amperometric detection) of the filtered solution was transferred to a 10 ml volumetric flask and diluted to the mark with the mobile phase.

## 3. Results and discussion

### 3.1. Optimisation of separation

The optimisation of the chiral separation was carried out using UV detection. A number of parameters were investigated to determine their exact effect on the resultant chromatography. As shown in Fig. 1, pH had a profound effect on the degree of separation. Since naproxen has a carboxylic acid functionality ($pK_a$=4.26) [2], the molecule dissociates in aqueous solution releasing a proton. Ionisation suppression by pH control results in longer retention, as expected, and improves the likelihood of chiral discrimination.

However, pH is merely one of the parameters that affects the chromatography. Buffer concentration has also been shown to have an effect. An increase in buffer concentration from 10 to 50 mmol $l^{-1}$ gave a slight increase in resolution but no increase after 50 mmol $l^{-1}$. This can be explained by the molecule showing a greater preference for the hydrophobic cavity of the cyclodextrin in the presence of a more ionic mobile phase. As expected for a reversed-phase LC method, an increase in the percentage of organic modifier resulted in an increase in eluting power and consequently a decrease in retention. An increase in cyclodextrin concentration yielded a corresponding increase in chiral selectivity ($\alpha$). However, no further increase was observed after 20 mmol $l^{-1}$. Two different cyclodextrins were initially employed, methyl-β-CD and hydroxypropyl-β-CD, the latter showing less selectivity and resolution. Varying the flow-rate from 0.5 to 2.0 ml $min^{-1}$ did not affect the chiral selectivity.

Thus, the optimised mobile phase composition was 20 mmol $l^{-1}$ methyl-β-CD, 20% (v/v) acetonitrile, and 50 mmol $l^{-1}$ sodium acetate buffer at pH 3.

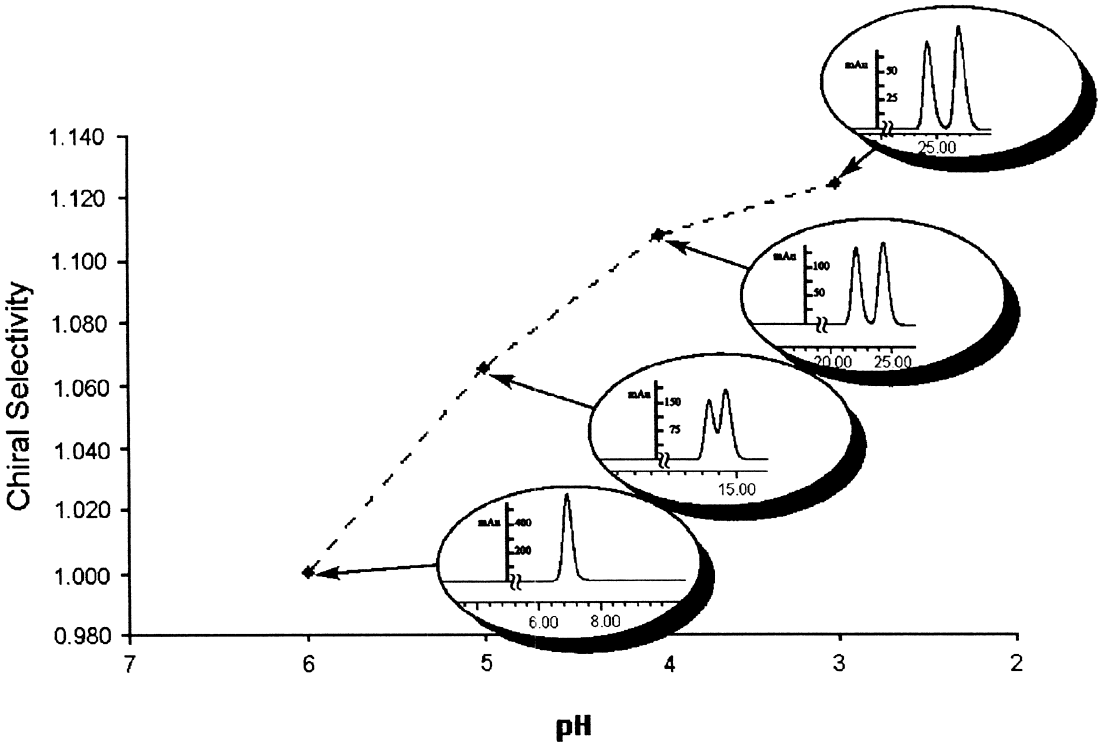


Fig. 1. The influence of pH on the chiral separation.

The flow-rate and detection wavelength were set at 2.0 ml $min^{-1}$ and 232 nm, respectively.

With the optimised mobile phase composition and flow-rate, baseline separation of *R*- and *S*-naproxen mixture was achieved with both UV detection and amperometric detection. Although the peak height of the longer retained solute is somewhat lower than the first peak, there is no difference in peak area between *R*- and *S*-naproxen peaks when their concentrations are equal. The chiral selectivity and resolution ($R_s$) were found to be 1.13 and 1.51, respectively.

### *3.2. Influence of applied potential*

As shown in Fig. 2, the applied potential has a strong influence on the sensitivity as expected. The higher the potential, the greater the sensitivity. However, the baseline noise in chromatographic analysis and background current increased accordingly. To maintain both high sensitivity and stable baseline, +1.05 V vs. Ag/AgCl was selected for the following amperometric detection. Both *R*- and *S*-naproxen standards were tested separately and no difference in sensitivity was observed.

### *3.3. Reproducibility of amperometric detection*

While operating in a flow injection analysis mode with a sampling frequency of 30 s $h^{-1}$, the precision was very good. A RSD of 0.69% ($n=5$) was

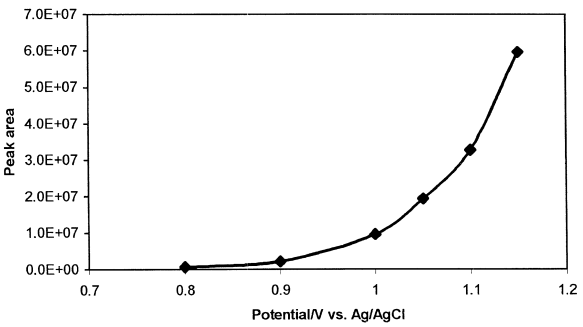


Fig. 2. The influence of applied potential on sensitivity (0.041 mg $ml^{-1}$ of *R*-naproxen).

obtained. However, in the chromatographic separation of naproxen enantiomers, 30 min is required for elution. For six consecutive injections in 3 h, the RSD for peak area was as high as 13.9%. Even though better precision might be obtainable with temperature control and other techniques, such as conditioning the electrode surface periodically, it is not practical to use external standard series to calibrate the instrument. Obviously, internal standard calibration is preferable.

The addition of an internal component may be tedious in operation and introduce unnecessary impurities. In an enantiomeric separation, when the most important and most frequently required information is the enantiomeric purity, i.e. the ratio of the two enantiomers, the following approach is possible. As there is no difference in their electrochemical characteristics, and their retention times are usually very close, the peak area or peak height ratio between the two enantiomers could be the ideal analytical readout for enantiomeric purity assessment. The precision of amperometric detection will be improved greatly without the addition of an internal standard because the conditions of the electrode surface is required to be stable only for very short periods of time, typically a few minutes, rather than a few hours. In this manner, when peak area ratio was chosen as the readout, a RSD of 2.1% was obtained for six consecutive injections with a constant *R*- and *S*-naproxen concentration ratio while varied in their total amounts from 0.04 to 1.7 mg $ml^{-1}$. The precision is acceptable for enantiomeric purity assessment.

### *3.4. Linearity between peak area ratio and concentration*

A series of standard solutions containing 0.35 mg $ml^{-1}$ of *S*-naproxen and different amounts of *R*-naproxen were prepared and separated by the above chromatographic procedure using amperometric detection. The peak area ratios between *R*-naproxen and *S*-naproxen were plotted against the concentration of *R*-naproxen in the standard solutions. The linear relationship was maintained over the range of 0.01 to 1.0 mg $ml^{-1}$ with a regression coefficient ($R$) of 0.9985.

### *3.5. Enantiomeric purity assessment*

Because the active ingredient in naproxen tablets is *S*-naproxen and the tablets are marketed as *S*-naproxen, enantiomeric purity determination is essential for quality control in pharmaceutical production of naproxen tablets. Two kinds of naproxen tablets manufactured by two different pharmaceutical companies were analysed for enantiomeric purity using both amperometric detection and UV detection. Their chromatograms obtained under identical conditions with amperometric detection were shown in Fig. 3. It is evident that the enantiomeric purity of the tablets manufactured in company (b) is much higher than that of the tablets manufactured in company (a). The concentration of *R*-naproxen in the tablets manufactured in company (b) was so low that no peak was recorded for *R*-naproxen by amperometric detection. The enantiomeric excess of *S*-naproxen was thus found to be 100%. When detected by UV, it was 99.8%.

However, the concentration of *R*-naproxen in the tablets manufactured in company (a) was high enough to form two well-defined peaks. The enantiomeric excess of *S*-naproxen was found to be 95.5%, which was very close to 95.7% obtained by UV detection.

### *3.6. Transferring to nano-LC*

Even though the mobile phase in conventional LC

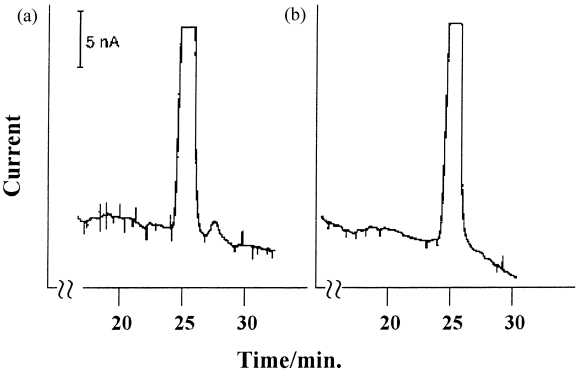


Fig. 3. Partial chromatograms of injected extracts for two commercial naproxen tablets (a and b) using amperometric detection (+1.05 V vs. Ag/AgCl). The retention times for *S*- and *R*-naproxen are 25.3 and 28.4 min, respectively.

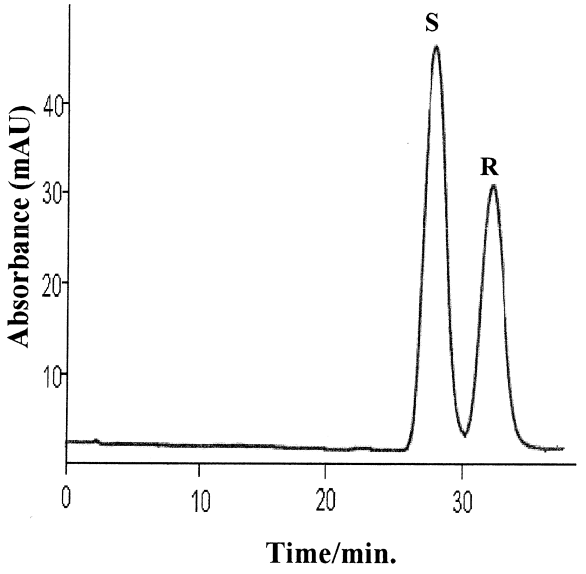


Fig. 4. Chromatogram showing the separation of *S*- and *R*-naproxen by nano-LC (flow-rate is 800 nl $min^{-1}$).

can be recycled, it is much desirable to reduce the consumption of reagents, especially the expensive chiral mobile phase additive if there is a need for routine use of the method. This can be realised by transferring the established method from conventional LC to nano-LC in which the flow-rate is in the nl $min^{-1}$ range. During transferring, both the concentration of β-CD and the percentage of acetonitrile were varied but the optimum conditions were the same as those used for conventional HPLC. Baseline separation of naproxen enantiomers was also realised in nano-LC (Fig. 4, $\alpha=1.13$, $R_s=1.69$). The consumption of mobile phase can be reduced from 60 ml in conventional LC to 50 μl in nano-LC for each sample.

## 4. Conclusions

Chiral separation of *R,S*-naproxen was achieved on an achiral ODS column with methyl-β-CD as a mobile phase additive. Both UV and amperometric detection are possible. In amperometric detection, the reproducibility can be improved by taking the peak area ratio of the two enantiomers as the analytical readout without using any added internal standard. Also, the most important information required in chiral separation, the enantiomeric excess, can be obtained directly from peak area ratio without using either spiked internal or external standards. Finally, the method was successfully transferred into the nano-LC system by which reduced consumption of expensive chiral mobile phase additive has been realised.

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