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The application of achiral/chiral coupled-column high-performance liquid chromatography to biomedical analysis*

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Abstract: The use of achiral/chiral coupled-column HPLC in the direct analysis of the enantiomers of chiral drugs in biological samples is reviewed. The method has been applied to a study of the pharmacokinetics of (+)- and (−)-terbutaline, the resolution of the stereoisomers of leucovorin and the determination of warfarin enantiomers in serum.

Keywords: *Achiral/chiral coupled column HPLC; enantiomers; chiral drugs; terbutaline; leucovorin; warfarin.*

Introduction

The growing awareness of the pharmacological differences between the enantiomers of a chiral drug has created a demand for new bioanalytical methods. These methods must be capable of determining the enantiomeric composition of the drug during the time course of its *in vivo* action. The development of an assay for the quantification of any substance in a biological matrix is often a challenging analytical problem. This problem is further complicated when the substance is chiral and the ultimate goal is the determination of its enantiomeric composition. One approach which has proved useful in the development of such assays is achiral/chiral coupled-column HPLC.

The use of chromatographic systems derived from the coupling of two HPLC columns with different selectivities has been the subject of two recent reviews [1, 2]. The general approach involves the initial fractionation of the analyte on the first column (pre-column) followed by the switching of selected fractions to the second column (analytical column) to obtain the final chromatographic separation. The advantages of coupled-column HPLC systems include the fact that they can be used to analyse serum and urine samples with relatively little pre-column workup and that they can be readily automated.

In an achiral/chiral coupled-column system, the pre-column is an achiral reversed-phase support which is used to isolate the chiral compound from the other components of the matrix. The target solute is then transferred through a switching valve to the HPLC chiral stationary phase (HPLC-CSP) where the enantiomorphs are separated and quantified. One of a number of possible configurations for this system is presented in Fig. 1.

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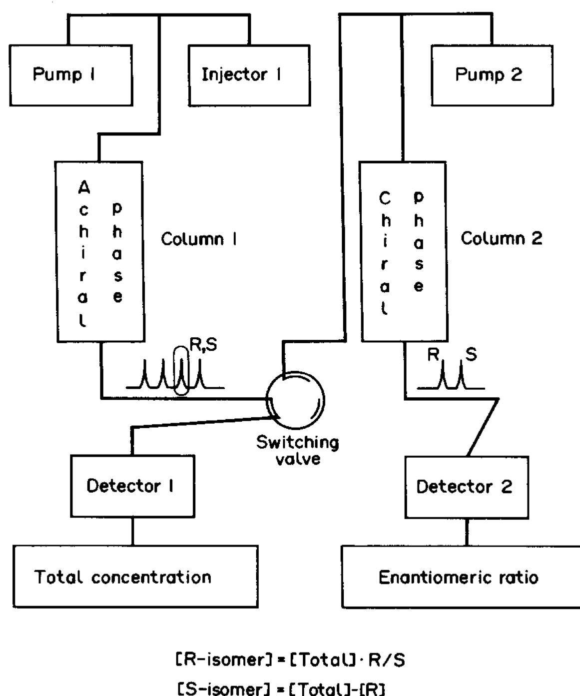

Figure 1
A diagram of a coupled-column system. Reprinted with permission from Ref. 5.

Since the majority of the matrices analysed by coupled-column HPLC are biological, these systems usually operate using reversed-phase chromatographic conditions. Thus, the development of an achiral/chiral coupled-column system requires the use of a CSP which utilises an aqueous mobile phase. A number of phases are available including those based upon β-cyclodextrin (CD-CSP), bovine serum albumin (BSA-CSP) and α_1-acid glycoprotein (AGP-CSP). To date, coupled column systems which utilise the CD-CSP [3] and the BSA-CSP [4, 5] have been reported, as discussed below.

Pharmacokinetics of (+)- and (−)-Terbutaline

Terbutaline is a β_2-receptor agonist widely used in the treatment of asthma. The drug is administered as a racemic mixture in which (−)-terbutaline is the pharmacologically active enantiomorph. Although the pharmacokinetics of the two enantiomers have been studied after the administration of each isomer separately, the pharmacokinetics of the racemate have not been determined. This can now be accomplished using a coupled-column method developed by Edholm *et al.* [3].

The analytical system was constructed using a Nucleosil phenyl HPLC column as the pre-column and a CD-CSP as the analytical column. Serum samples were pre-treated using solid-phase extraction columns and the eluate containing the terbutaline was injected on to the pre-column. The initial separation of racemic terbutaline from the other components in the serum was achieved using a mobile phase composed of 0.01 M ammonium acetate (pH 4.6). The eluate from the pre-column which contained (+)- and

(−)-terbutaline was collected in a loop and subsequently transferred to the analytical column using a six-port switching valve. The stereochemical resolution of (+)- and (−)-terbutaline was achieved using a mobile phase of methanol–0.05 M ammonium acetate (pH 6.0) (10:90, v/v) and an amperometric detector was used to quantify the terbutaline. Under these chromatographic conditions, the method was able to accurately determine nM concentrations of each isomer.

Resolution of the Stereoisomers of Leucovorin

Leucovorin (Lv) is a reduced folate which is used to treat or prevent host toxicity in cancer patients due to the administration of methotrexate; Lv is also used to potentiate the anti-tumour effects of 5-fluorouracil. The drug is administered as an equal mixture of two diastereomers which differ only in the configuration at the 6-carbon of the tetrahydropteridine ring, (6S)-Lv and (6R)-Lv (Fig. 2A). The major chromatographic problem in resolving the two diastereomers arises from the fact that the two chiral centres, in the glutamic acid residue and in the 6-position of the tetrahydropteridine ring, respectively, are so far apart that the internal energies of the two diastereomers are essentially equivalent. Consequently, standard achiral chromatographic methods cannot be used to resolve the two forms of the drug. Thus a chiral selector was found to be necessary for the successful recognition of these two isomers, exploiting the chiral configuration in the tetrahydropteridine ring. In the commercially available drug, the chirality of the glutamic acid residue is strictly controlled.

Initial studies [6] have indicated that (6S)-Lv is the biologically active form of the drug and that it is rapidly converted to the active metabolite 5-methyltetrahydrofolate (5-METHF). In addition, the plasma half-lives of (6S)-Lv and 5-METHF are significantly shorter than that of (6R)-Lv.

The initial methods for the determination of the serum concentrations of (6S)-Lv, (6R)-Lv and 5-METHF were based upon biochemical assays of intact serum samples or of eluate fractions from an HPLC separation [6, 7]. These methods were lengthy and inaccurate. In order to facilitate clinical and pharmacokinetic studies, Wainer and Stiffin [4] have developed a coupled-column chromatographic system capable of determining the stereoisomeric composition of Lv and 5-METHF in serum.

The chromatographic system comprised an achiral pre-column (μBondapak phenyl) and a chiral analytical column (BSA-CSP). Serum samples were prepared by precipitation of serum proteins with methanol, centrifugation and evaporation of the supernatant. The residue was reconstituted in water and injected on to the chromato-

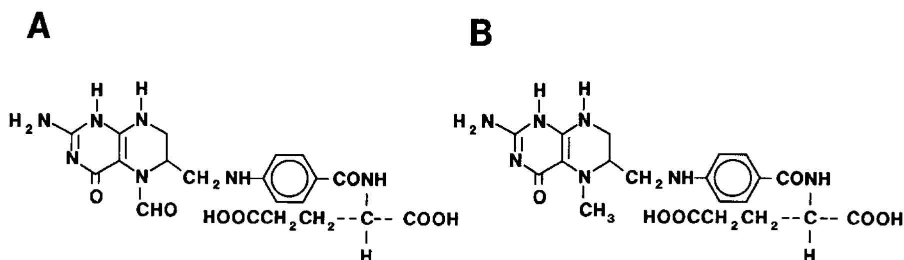

Figure 2
Structures of: (A) leucovorin and (B) 5-methyltetrahydrofolate. Reprinted with permission from Ref. 4.

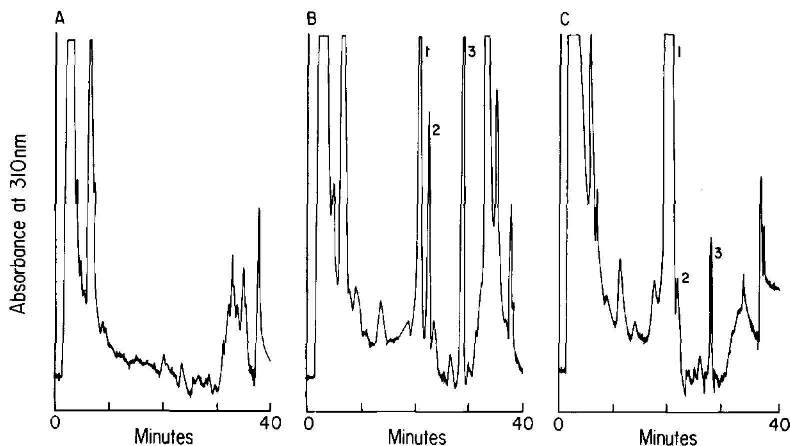

Figure 3
Representative chromatograms on the achiral phenyl support of: (A) extracted blank serum; (B) serum with added leucovorin (12 $\mu g\ ml^{-1}$), 5-methyltetrahydrofolate (1.25 $\mu g\ ml^{-1}$) and internal standard; and (C) serum sample from a patient 15 min after the end of a 4-h infusion of leucovorin. Peaks: 1 = leucovorin; 2 = 5-methyltetrahydrofolate; 3 = internal standard. Reprinted with permission from Ref. 4.

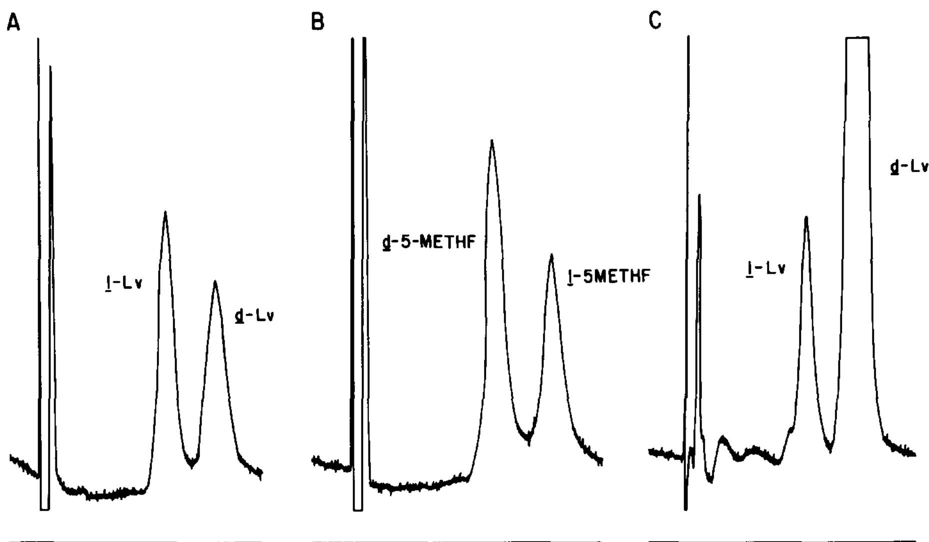

Figure 4
Representative chromatograms on the BSA-CSP after switching from the achiral pre-column of: (A) a leucovorin standard: (B) a 5-methyltetrahydrofolate standard; and (C) leucovorin from a sample taken 30 min after a 4-h infusion. Reprinted with permission from Ref. 4.

graphic system. Lv and 5-METHF were separated from interfering substances on the achiral phenyl column using a gradient elution programme and the total amount of each solute was quantified using UV detection at 310 nm. Representative chromatograms are presented in Fig. 3.

The eluate fractions containing either the Lv or the 5-METHF fraction can be selectively switched to the BSA-CSP for chiral resolution. On this column, the

stereoisomers of Lv are resolved with a stereochemical selectivity (α) of 1.44; for the stereoisomers of 5-METHF, $\alpha = 1.57$ (Fig. 4). The stereoisomeric compositions of the Lv and 5-METHF samples are reflected in the peak areas of each isomer.

This method has been successfully used in the analysis of Lv in clinical samples in man [8] and from pharmacological studies in animals (I.W. Wainer, unpublished results). The coupled-column system has been automated for use in the clinical monitoring of Lv.

Determination of Warfarin Enantiomers in Serum

Warfarin (W) is an enantiomeric molecule which is administered as a racemic mixture. The enantiomorphs of W, (S)-W and (R)-W, differ in their potency, rate of elimination and plasma protein binding. One of the key issues associated with the clinical use of W is the large number of drug interactions which occur between this agent and other commonly administered drugs. To date, over 55 drug interactions involving warfarin have been reported [9] and many of these interactions are stereoselective.

The pharmacological and pharmacokinetic differences between (R)- and (S)-W and the existence of stereoselective drug interactions make it necessary to rapidly and accurately determine the serum concentrations of these isomers. A coupled-column achiral/chiral chromatographic system capable of accomplishing this has been developed by Chu and Wainer [5].

In this system, an achiral Pinkerton internal-surface reversed-phase (ISRP) column [10] was used as the pre-column and a BSA-CSP was used as the analytical column. Serum samples were prepared by precipitation of serum proteins with acetonitrile, centrifugation and evaporation of the supernatant. The residue was reconstituted in phosphate buffer (pH 10) and injected on to the chromatographic system. (R,S)-W was separated from interfering substances on the ISRP column and the total amount of the drug was measured using UV detection at 225 nm. Representative chromatograms are presented in Fig. 5.

The eluate fraction containing (R,S)-W can be selectively switched to the BSA-CSP for chiral resolution. On this column, (R)- and (S)-W are resolved with a stereochemical

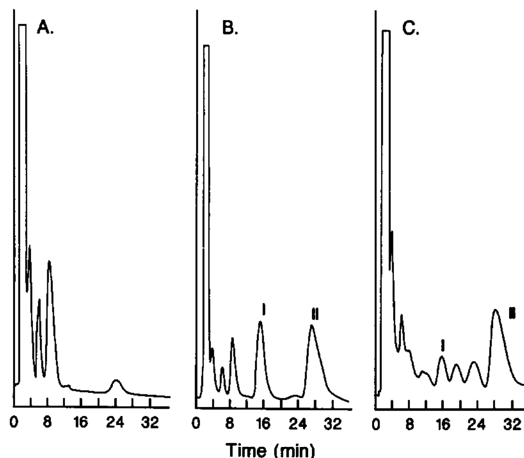

Figure 5
Representative chromatograms on the achiral ISRP column for: (A) a blank serum sample; (B) a serum sample spiked with 10 μg ml^{-1} (R,S)-warfarin; and (C) a serum sample from a clinical study. I = (R,S)-warfarin; II = internal standard. Reprinted with permission from Ref. 5.

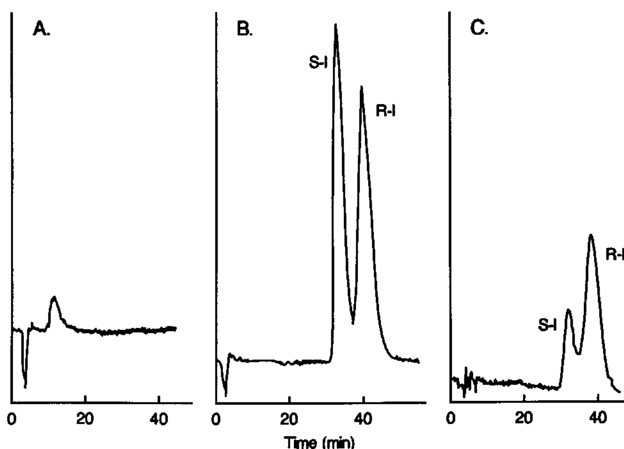

Figure 6
Representative chromatograms on the BSA-CSP after switching from the achiral pre-column of: (A) a blank serum sample; (B) a serum sample spiked with 5 μg ml^{-1} (R,S)-warfarin; and (C) a serum sample from a clinical study. S-I = (S)-warfarin; R-I = R-warfarin. Reprinted with permission from Ref. 5.

selectivity (α) of 1.19 (Fig. 6). The enantiomeric composition of W is reflected in the peak areas of each isomer.

This method has been successfully used in the analysis of samples from the clinical use of W [5] in humans. The coupled-column system has been automated for use in the clinical monitoring of (R,S)-W.

Conclusion

A number of CSPs use aqueous mobile phases and are able to stereochemically resolve enantiomeric compounds without pre-column derivatisation. These phases can be easily coupled to achiral reversed-phase pre-columns and the resulting coupled-column systems used for the direct analysis of biological samples. The assays described in this overview are representative examples of this approach and give an indication of the potential of contemporary current technology.

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