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Review

Column-switching techniques in the biomedical analysis of stereoisomeric drugs: why, how and when

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Abstract

The application of stereoselective chromatographic techniques to bioanalytical problems has become a routine procedure. However, this approach is not always straightforward; particularly when the separation involves chromatographic chiral stationary phases. Matrix interferences and more importantly, overlapping metabolite peaks often make direct analysis impractical. One strategy to overcome these problems is to combine two or more columns with different selectivities to produce a multi-dimensional chromatographic system. This review addresses the use of coupled column chromatography in HPLC systems including different coupling methods and the application of the resulting arrangements to bioanalytical analyses.

Keywords: Enantiomer separation; Verapamil; Leucovorin; Tamoxifen; Hydroxychloroquine; 3′-Azido-3′-deoxythymidine; Zidovudine

Contents

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1. Introduction

1.1. Why

Clinical therapy is rapidly moving towards the use of highly specific and potent therapeutic agents. As the therapeutic indices increase and the dosage levels decrease, the bioanalytical problems multiply. The analyst is asked to find smaller amounts of drugs and their metabolites in an increasing variety of matrices ranging from plasma and urine to cellular and tumor tissues. Often, a single chromatographic approach will not suffice.

Bioanalytical chemists have a variety of tools to apply to these problems. In chromatographic assay development, it is possible to use specific extraction techniques (e.g., solid-phase and antibody mediated extractions) before chromatography and specific detectors (e.g., enzyme reactors and mass spectrometers) after. During the chromatographic process itself, it is often feasible to use specific columns to achieve the desired results; for example, an enantioselective separation achieved using a chiral stationary phase (CSP). However, despite extensive developments in column technology, chromatography on a single column may not be able to solve the problem.

An illustration of the type of problems one may encounter is the inability of many CSPs to separate parent drug from metabolites. Both the stereoisomers of the parent and metabolites may be enantiomerically resolved, but the separate enantiomers of the parent and metabolite(s) often overlap. This problem is depicted in Fig. 1 using the enantiomeric separation of verapamil (VER) and norverapamil (NOR) separately and together on an AGP-CSP [1].

One way to overcome this type of problem is to move from single-column chromatography to coupled-column (or multi-dimensional) chromatography. In this approach, columns with different selectivities are used in sequence. A separation which cannot be achieved on one stationary phase is accomplished on the other and vice versa.

1.2. How

Multi-dimensional chromatography is not a new technique. It has been extensively employed in TLC [2], where two-dimensional TLC or multiple plate development with different solvent systems have been used in the analyses of complex mixtures. In HPLC and GC, multidimensionality has been produced by coupling two or more columns with different selectivities. These columns are used sequentially by direct connection, in-line or through a switching valve, or by mechanical transfer of the eluent from one independent system to another.

The theoretical and mechanical aspects of multi-

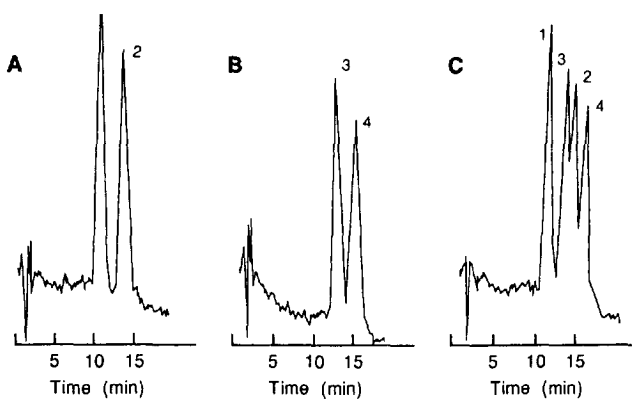


Fig. 1. Representative chromatograms for the separation of racemic verapamil (*R,S*-VER) and racemic norverapamil (*R,S*-NOR) on the AGP-CSP. (A) *R,S*-VER; (B) *R,S*-NOR; (C) *R,S*-VER and *R,S*-NOR. Where 1=*R*-VER; 2=*S*-VER; 3=*R*-NOR; 4=*S*-NOR. Reprinted from Ref. [1].

dimensional HPLC have been extensively reviewed by Roth [3] and Edholm and Ögren [4] and three of many possible coupled HPLC systems are presented in Figs. 2–4 [5–7]. Other issues such as system peak effects in coupled-column systems have also been addressed [8] and, therefore, this presentation will concentrate on the bioanalytical applications of multi-dimensional HPLC, with particular emphasis on enantioselective separations. The intent of this article is to discuss the various reasons for and the different modes of coupled-column chromatography by providing selected examples.

1.3. When

1.3.1. Coupled achiral–chiral chromatographic systems to resolve parent–metabolite overlap

On-line sequentially coupled achiral–chiral columns. An example of a bioanalytical assay which

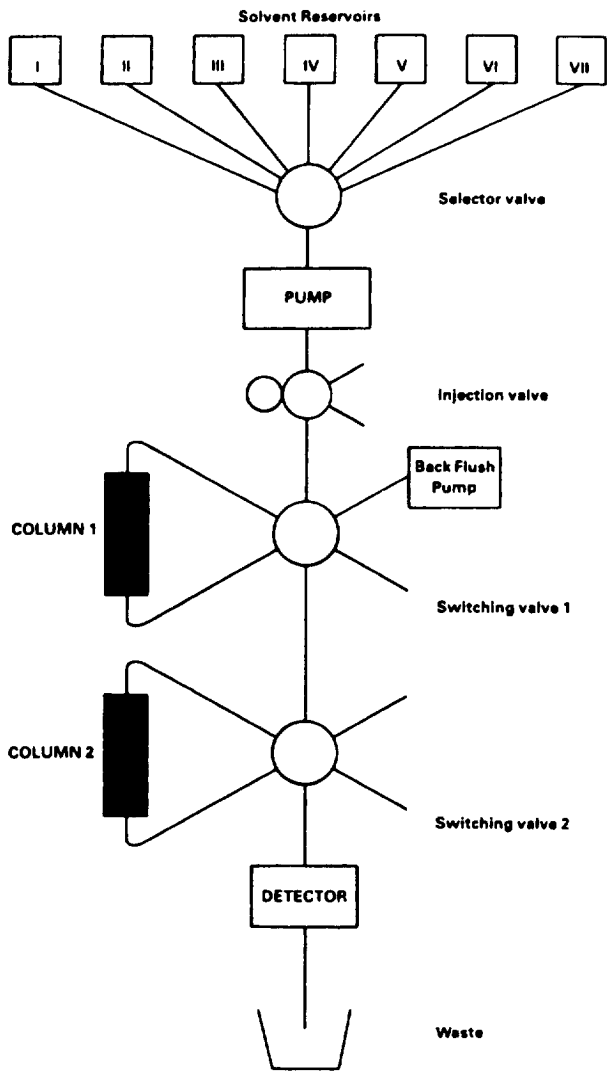


Fig. 2. Diagram of the column switching system with a step gradient elution solvent delivery system. Reprinted from Ref. [5].

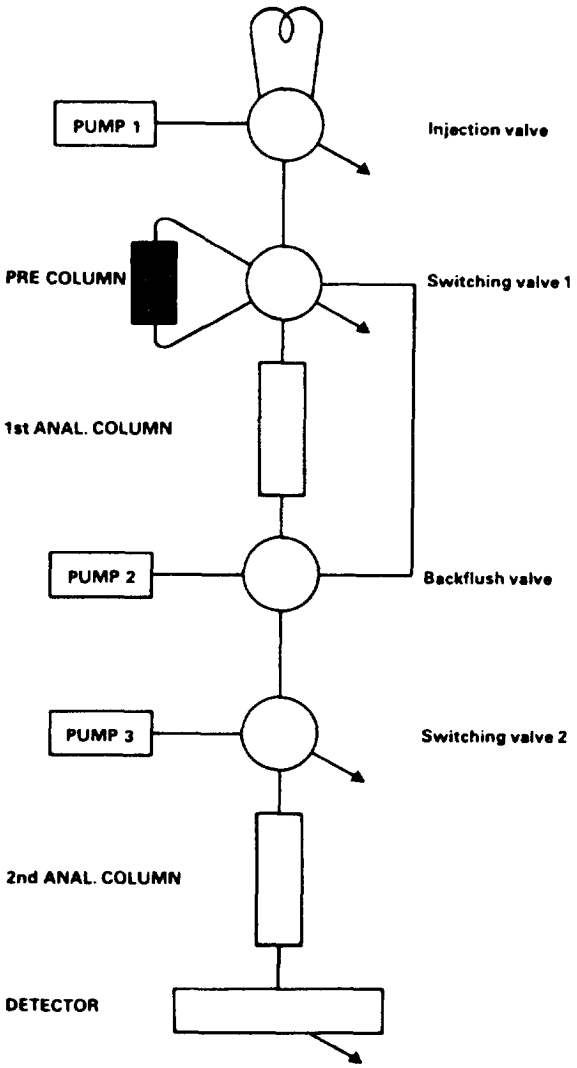


Fig. 3. Schematic diagram of automated precolumn concentration with a coupled column system. Reprinted from Ref. [6].

requires a coupled column system is the determination of *R,S*-VER and *R,S*-NOR plasma concentrations. VER is a calcium channel blocking drug and is widely used in the therapy of hypertension, supraventricular arrhythmias and angina pectoris [9]. VER is a chiral compound which is administered as a racemic mixture of *R*-VER and *S*-VER. The VER enantiomers have different pharmacodynamic and pharmacokinetic properties; for example, *S*-VER is 10 to 20 times more potent than *R*-VER [10] and *S*-VER undergoes extensive first-pass metabolism, resulting in the predominance of *R*-VER in plasma [11]. NOR is a major metabolite of VER and is reported to possess 20% of the coronary vasodilator potency of VER [12]. Nor is also chiral, and its pharmacokinetic disposition is enantioselective [13].

A number of HPLC assays have been reported for the direct determination of the serum concentrations of VER and NOR enantiomers. The initial approach utilized an AGP-CSP [1] and as presented above, a significant overlap was observed when the VER and NOR were chromatographed together, Fig. 1. The problem was overcome by using an achiral–chiral HPLC system where VER and NOR were initially separated from each other and from plasma components on an achiral reversed-phase column (Hisep column). The eluent fractions containing VER and NOR were then selectively transferred via an in-line switching valve to the AGP-CSP [1]. A second HPLC achiral–chiral system has been reported for the determination of VER enantiomers in which an ODS column was coupled to a CSP based upon ovomucoid (OVM-CSP) [14].

Other examples of this approach are the enantioselective methods developed for plasma concentrations of terbutaline [15] and warfarin [16]. In the first instance, terbutaline was separated from other plasma components and metabolites on an achiral phenyl column. The eluent containing terbutaline was collected in a loop and subsequently transferred to a β-cyclodextrin CSP for enantioselective resolution and quantitation of (+)- and (−)-terbutaline. In the second example, the achiral precolumn contained the Pinkerton internal-surface reversed-phase stationary phase and the CSP was based upon immobilized bovine serum albumin (BSA-CSP).

The first three examples of this technique utilized reversed-phase chromatographic conditions. Normal-phase chromatography can also be utilized as is demonstrated by the assay developed for mefloquine plasma levels [17]. In this assay, the precolumn contained an achiral cyanopropyl stationary phase and the analytical column contained a (*S*)-naphthylurea CSP. The mobile phases for the pre- and analytical columns were composed of varying proportions of hexane–2-propanol–methanol.

In-line sequentially coupled achiral–chiral columns.

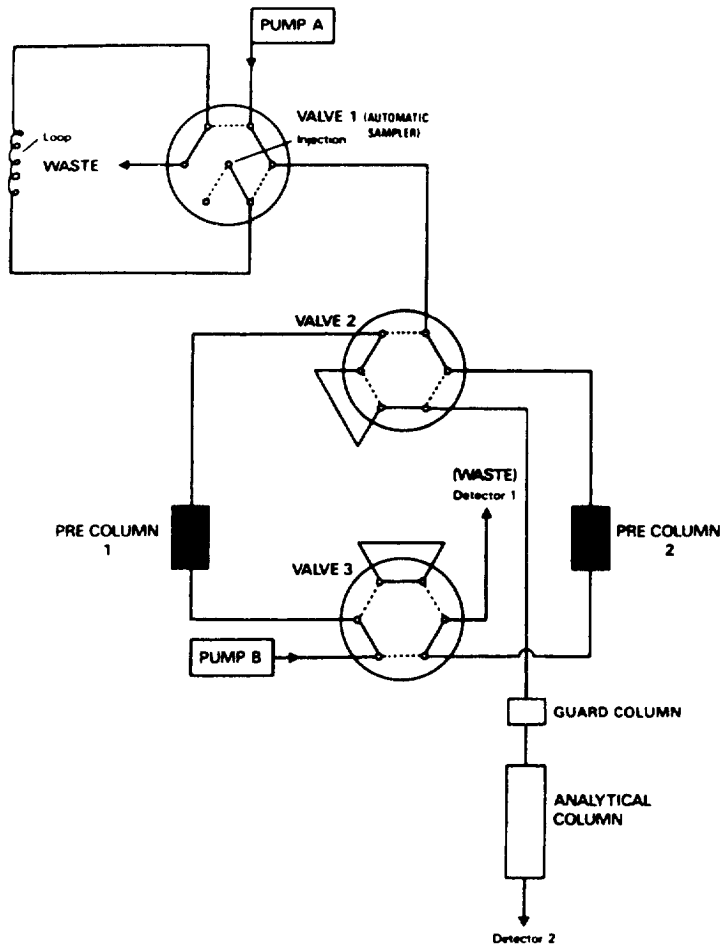


Fig. 4. Alternating precolumn switching technique for sample enrichment using an autosampler (valve 1) and two six-port valves that connect two short precolumns with an analytical column and an additional guard column. Reprinted from Ref. [7].

A different approach to the analysis of VER and NOR enantiomers in plasma which does not require mechanical column switching was developed using a CSP composed of amylose 3,5-dimethylphenylcarbamate coated on silica (AD-CSP) [18]. The AD-CSP enantioselectively resolved *R*- and *S*-VER (α= 1.27) and *R*- and *S*-NOR (α=1.16) without overlap, Fig. 5 and Table 1. However, the determination of the elution profiles of additional key VER metabolites revealed an overlap between *R*-VER and the D617 metabolite and *S*-NOR and the PR25 metabolite, Table 1. This overlap was eliminated by the addition of a diol silica column (LiChrocart DIOL, 50×4.0 mm I.D.) as an in-line precolumn. The effect of the achiral precolumn was to alter the retention times of all of the components with the greatest effect on the interfering metabolites, Table 1.

The use of an achiral precolumn is often the easiest and most reliable method to overcome parent drug/metabolite overlap. Another example of the utility of this technique is the validated assay for the determination of metyrapone and the enantiomers of its chiral metabolite metyrapol in human plasma and urine [19]. In this case, an overlap between metyrapone and (−)-metyrapol was overcome using a silica gel precolumn.

This approach can also be used with reversed-phase chromatographic conditions. Indeed, Enquist

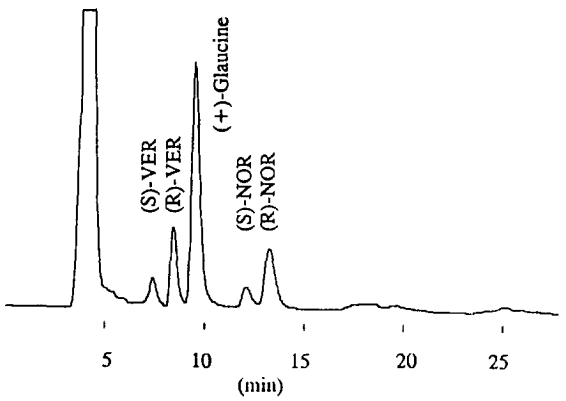


Fig. 5. Representative chromatogram for the separation of *R,S*-VER and *R,S*-NOR in plasma on a coupled achiral (Diol)–chiral (AD-CSP) HPLC system. The plasma was obtained from a healthy volunteer in whom steady-state plasma levels of VER had been established after oral dosing of a sustained release formulation of 240 mg *R,S*-VER every 12 h. Reprinted from Ref. [18].

and Hermansson reported the first use of in-line coupled achiral–chiral chromatography in the analyses of disopyramide [20] and atenolol [21] enantiomers in biological matrices. Both assays employed an AGP-CSP coupled to an achiral precolumn containing either a C_8 (disopyramide) or diol (atenolol) stationary phase.

Off-line sequential achiral–chiral columns. In some instances, it is impossible to find a suitable on-line or in-line coupled achiral–chiral system. Another alternative is to separate the target compounds from each other and matrix interferences on one chromatographic system, collect the eluents containing these compounds, concentrate these fractions and inject the resulting solution onto a second system for enantioselective separation. In this approach, quantitation of the target compounds must be achieved on the first system to avoid errors stemming from the collection, concentration and transfer of the analytes. It may be noted that there can be a reduction in sensitivity on the second column due to loss of analyte from incomplete collection or mechanical loss during concentration and transfer of the collected sample.

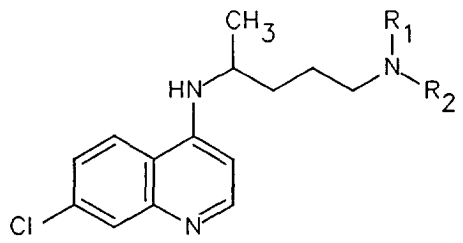
Table 1
Retention times of verapamil and its major metabolites on an AD-CSP without (A) and with (B) a diol pre-column coupled in series

Compound	Retention time (min)	
	A	B
(*R*)-Verapamil	7.7	8.4
(*S*)-Verapamil	6.7	7.4
(*R*)-Norverapamil	11.7	13.4
(*S*)-Norverapamil	10.5	12.2
D617	7.4	17.0
PR22	15.5	18.6
	18.2	21.2
PR25	10.7	32.8
(+)-Glaucine	8.4	9.5

See Ref. [18] for experimental details.

One of the first systems reported utilizing this approach was an assay for the plasma and urine concentrations of the enantiomers of disopyramide (DP) and its monodesisopropyl metabolite (MDP) [20]. The initial assay for DP using a C_8 precolumn (see Section 1.3.1.2 above) could not be used to measure MDP levels because the metabolite overlapped with endogenous substances. Thus, the initial chromatography was changed and a silica gel stationary phase was used with a polar organic mobile phase. The eluents containing DP and MDP were collected and since the polar organic mobile phase was incompatible with the AGP-CSP, the fractions were evaporated to dryness. The residues were dissolved in the aqueous mobile phase used with the AGP-CSP and injected onto the CSP.

Another example is the sequential achiral–chiral system developed for the determination of the enantiomers of hydroxychloroquine, *R*-HCQ and *S*-HCQ, and the enantiomers of its three main metabolites, desethylhydroxychloroquine, desethylchloroquine and bisdesethylchloroquine, Fig. 6 [22]. All of the compounds could be enantioselectively separated on the AGP-CSP but the stereoisomers overlapped and only 7 peaks could be obtained, Table 2. The problem was solved by initially separating the target compounds from matrix interferences and each other on a cyano-bonded stationary phase, collecting the eluents containing the solutes, concentrating the fractions and injecting the resulting solutions onto the AGP-CSP. Fig. 7 and its insert present the results from an analysis of a plasma sample taken from a rabbit after the subcutaneous administration of 13 mg/kg HCQ. The sample was initially chromatographed on the cyano-bonded



	R_1	R_2
Hydroxychloroquine	CH_2CH_3	CH_2CH_2OH
Desethylhydroxychloroquine	H	CH_2CH_2OH
Desethylchloroquine	CH_2CH_3	H
Bisdesethylchloroquine	H	H
Chloroquine (internal std)	CH_2CH_3	CH_2CH_3

Fig. 6. The structures of hydroxychloroquine (HCQ), its metabolites and chloroquine.

stationary phase, Fig. 7, and the assay of the eluent from the achiral phase containing HCQ was accomplished on the AGP-CSP, on Fig. 7(insert).

1.3.2. Coupled chiral–achiral chromatographic systems to increase assay sensitivity

Leucovorin (LV), Fig. 8A, is a reduced folate which is used in cancer chemotherapy. LV is administered as a diastereomeric mixture in which the diastereomers differ in chirality at carbon 6 of the tetrahydropteridine ring while the configuration of the chiral center on the *L*-glutamic acid moiety is fixed in both forms of the molecule. Initial studies have indicated that the (6*S*)-isomer is the active form [23] and that, after administration, it is rapidly converted to an active metabolite, 5-methyltetrahydrofolate (5-METHF), Fig. 8B [24]. It is also known that the plasma half-lives of (6*S*)-LV and 5-METHF are significantly shorter than that of (6*R*)-LV [24], but the effect of the (6*R*)-isomer on the metabolism and disposition of (6*S*)-LV is unknown.

Although LV and 5-METHF are diastereomeric molecules, they cannot be resolved by achiral chromatography. This is due to the distance between the asymmetric centers. Their structures also do not readily lend themselves to further derivatization with a homochiral derivatizing agent. The initial analytical problem was solved using a coupled achiral–chiral chromatographic system where the precolumn contained a phenyl stationary phase and the CSP was a BSA-CSP [25]. The achiral chromatography separated the peaks and only the LV and 5METHF are switched onto the BSA-CSP for stereoisomeric analysis.

The achiral–chiral coupled system was useful when large clinical doses of LV were administered, but the method could not be validated for submicrogram quantities of LV and 5-METHF. The major problem was the low efficiency of the BSA-

Table 2
Chromatographic parameters of hydroxychloroquine and its metabolites on the achiral and chiral stationary phases

Compound	Ultremex Cyano Column[a]	Chiral-AGO Column[b]		
	k'[c]	k'[d]	α_{RS}[e]	R_{RS}[f]
Bisdesethylchloroquine	1.61	12.11	1.25	1.29
Desethylhydroxy-chloroquine	2.56	10.19	1.32	1.39
Desethylchloroquine	3.93	11.44	1.39	1.97
Hydroxychloroquine	5.33	8.67	1.39	2.08
Chloroquine (I.S.)	9.34	NA	NA	NA

See Ref. [22] for details.

[a] Chromatographic conditions: mobile phase, N,N-DMOA phosphate (0.02 *M*)–ammonium acetate (0.06 *M*) (40:60, v/v, pH adjusted to 4.5); flow-rate, 0.6 ml/min; temperature, ambient.

[b] Chromatographic conditions: mobile phase, phosphate buffer (0.03 *M*, pH 7.0)–ethanol–acetonitrile (79:20:1, v/v); flow-rate, 0.9 ml/min; temperature, ambient.

[c] Retention factor.

[d] Retention factor of first eluted enantiomer.

[e] Enantioselectivity factor.

[f] Stereochemical resolution.

NA=Not applicable.

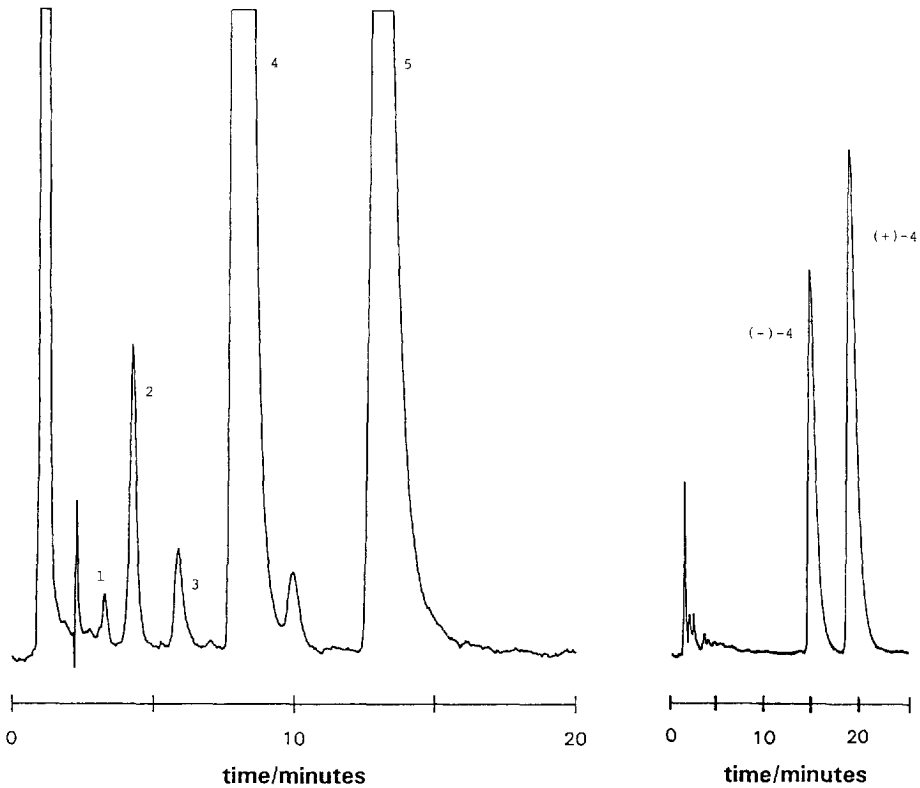


Fig. 7. (Left) Representative chromatogram of hydroxychloroquine (HCQ) and its metabolites in a plasma sample taken from a rabbit after the subcutaneous administration of 13 mg/kg HCQ and analyzed on an achiral cyano-bonded stationary phase. Peaks: 1 = bisdesethylchloroquine; 2 = desethylhydroxychloroquine; 3 = desethylchloroquine; 4 = hydroxychloroquine; 5 = chloroquine (I.S.). (Right) Representative chromatogram of the enantiomers of hydroxychloroquine (HCQ) [(−)-4, (+)-4] in a plasma sample taken from a rabbit after the subcutaneous administration of 13 mg/kg HCQ as determined by initial chromatography on an achiral cyano-bonded stationary phase and subsequent analyses on an AGP-CSP of the eluent from the achiral system containing HCQ. For experimental details see Ref. [22].

CSP. At the low end of the concentration curves, the LV and 5-METHF peaks tended to merge with background noise in the chromatogram.

One method of overcoming the low efficiency of the BSA-CSP is to reverse the order of the columns. In the resulting chiral–achiral coupled system, the

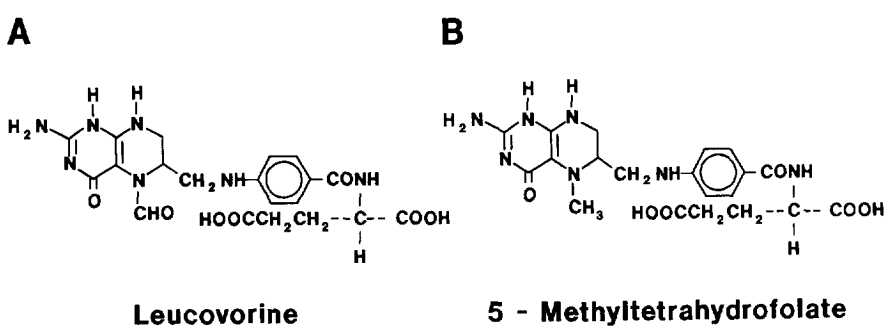


Fig. 8. The structure of (A) leucovorin and (B) 5-methyltetrahydrofolate.

achiral phase(s) would be used to trap and compress the stereoisomers which were separated on the CSP. The overall effect should be an increase in efficiency and selectivity.

Walhagen and Edholm [26] have described such a chiral–achiral coupled system and successfully used it to separate and quantitate the enantiomers of metoprolol, terbutaline, bupivacaine and oxazepam. The system described by Walhagen and Edholm [26] utilized either AGP-CSP or a BSA-CSP as the initial column and a variety of achiral hydrophobic phases for trapping and separating the resolved enantiomers.

The system developed for LV and 5-METHF is presented in Fig. 9 [27]. In this configuration, the BSA-CSP was used to separate (6*R*,*S*)-LV and 5-METHF from interfering plasma components and from each other and to stereochemically resolve the diastereomeric (6*S*)- and (6*R*)-LV. The eluent containing (6*S*)-LV was directed onto one C_{18} column and the eluent containing (6*R*)-LV and 5-METHF was directed onto a second C_{18} column. The eluotropic strength of the mobile phase used on the BSA-CSP was not enough to move LV or 5-METHF on the C_{18} column, and the compounds were compressed at the head of the achiral columns. After the columns were loaded, (6*S*)-LV, (6*R*)-LV and 5-METHF were sequentially eluted from the respective columns. The method was validated for plasma levels of each component ranging from 15 to 500 ng/ml and was able to detect LV concentrations of as low as 5 ng/ml. Representative chromatograms are presented in Fig. 10.

1.3.3. Coupled achiral–achiral system for the extraction of labile compounds

Tamoxifen (TAM) is a nonsteroidal antiestrogen used in the treatment of all stages of breast cancer [28,29] as well as experimentally to treat recurrent cerebral astrocytomas [30]. The standard clinical dose is 20 mg per os daily for breast cancer and 200

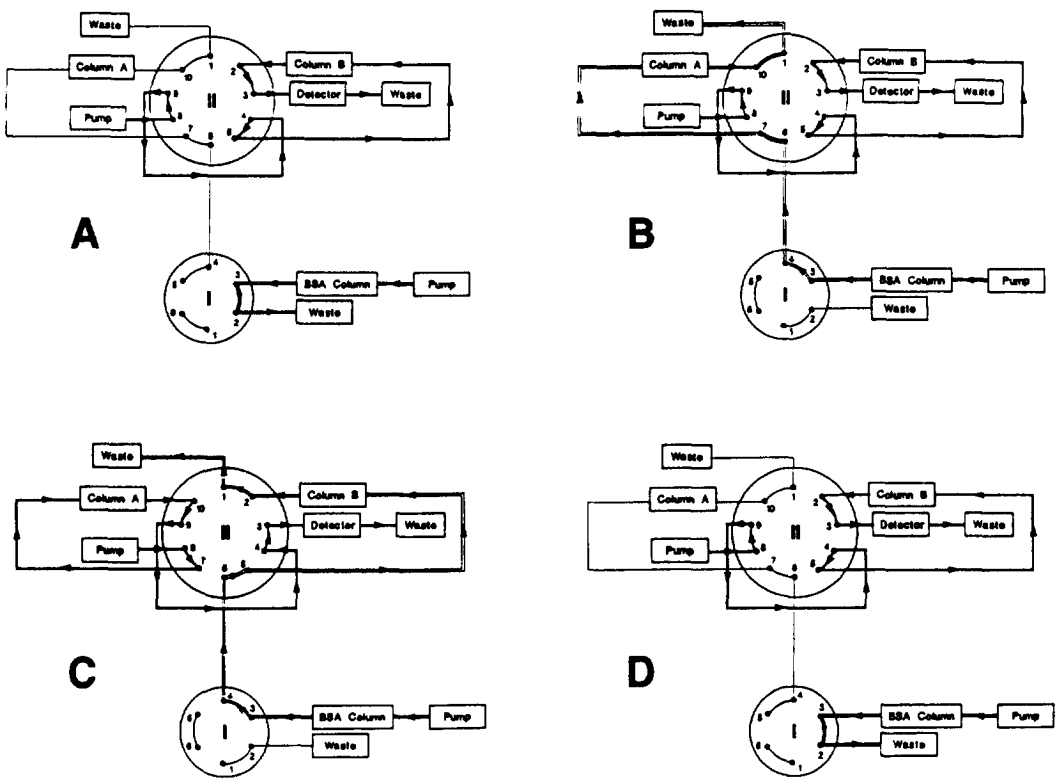


Fig. 9. The chiral (BSA-CSP)–achiral (C_{18}) HPLC system used to determine low levels of (6*S*)-leucovorin, (6*R*)-leucovorin and (6*S*)-5-methyltetrahydrofolate. See Ref. [27] for details.

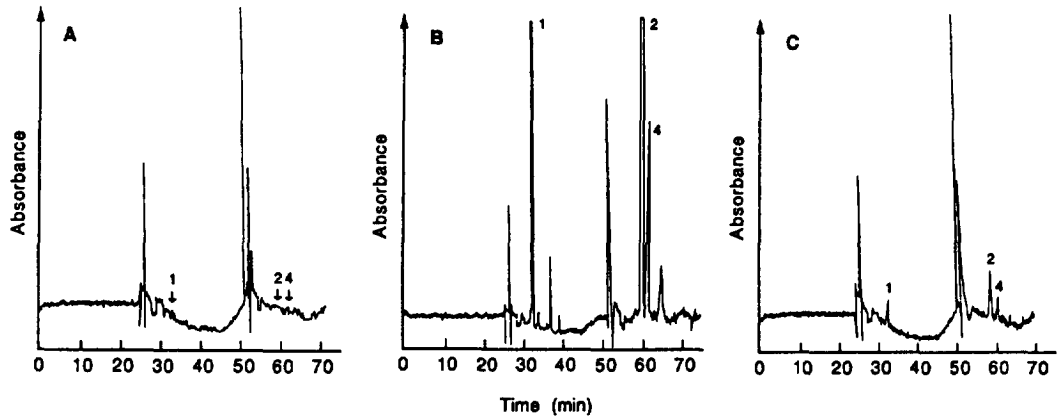


Fig. 10. Analysis of canine plasma samples on the chiral (BSA-CSP)–achiral (C_{18}) coupled system. (A) Blank plasma; (B) plasma sample obtained 1 h after i.v. administration of a 25-mg dose of (6*R*,*S*)-leucovorin. Peaks: 1=(6*S*)-leucovorin; 2=(6*R*)-leucovorin; 3=(6*S*)-5-methyltetrahydrofolate. Reprinted from Ref. [27].

mg twice daily for cerebral astrocytomas. TAM is extensively metabolized in the human. Three of the metabolites found in plasma, 4-hydroxytamoxifen (Met B), 4-hydroxy-N-desmethyltamoxifen (Met BX), and tamoxifen-ol (Met Y), are products of hydroxylation and two others are formed by N-demethylation, N-desmethyltamoxifen (Met X) and N-desdimethyltamoxifen (Met Z) [31–35].

There are several methods described in the literature for the determination of TAM and its metabolites but most do not overcome the difficulties inherent with the recovery and analysis of the target compounds. These problems include the high percentage of protein binding of TAM and its metabolites, the extreme light sensitivity of the target compounds, the low plasma concentrations of the hydroxylated metabolite Met B and the marked differences in extraction and elution properties between the metabolites with and without hydroxy and/or dimethylaminoethoxy sidechains.

A fully automated method has been developed for the determination of TAM and its metabolites based upon an in-line extraction process combined with column switching to a coupled analytical column [36].

The sensitivity of the assay is increased by the photochemical conversion of TAM and its metabolites to their highly fluorescent phenanthrenes. With a UV detector set at 260 nm, the absorbance of the phenanthrene is more intense than native tamoxifen by a factor of 1.5. However, by using fluorescence detection, the sensitivity ratio of the cyclization product vs. tamoxifen increased to a factor greater than 25 [37]. The conversion takes place in-line by irradiating the post-column effluent in a photochemical reactor.

The problem of protein binding is solved by the addition of acetonitrile to the plasma sample (1:1, v/v). An aliquot of the deproteinated sample is injected onto the sample loading portion of the chromatographic system which consists of a semi-permeable surface (SPS) nitrile guard column. SPS packing material is made up of two phases. A covalently bonded polyoxyethylene polymer forms a hydrophillic outer phase while the inner hydrophobic phase consists of reversed-phase material, in this case nitrile, bonded to the silica surface of the pores.

The compounds of interest are retained at the top of the SPS guard column while most of the endogenous substances from the plasma are washed off with de-ionized water to the waste. The mobile phase is then changed to buffer–acetonitrile and the switching valve position is turned to direct the flow through the extraction (SPS) column and the analytical column to a photochemical reactor placed in-line before the fluorescence detector.

Off-line sample preparation, i.e., deproteination, occurs in an amber microfuge tube and is accomplished in minutes, thereby eliminating the problem of photodegradation. Use of an SPS guard column

eliminates the need for off-line sample extraction for sample clean-up. The dual nature of SPS columns (hydrophobic/hydrophillic) results in good recoveries and prevents peak broadening of both the more hydrophobic demethylated metabolites and the more hydrophillic hydroxy metabolites.

The chromatography as described results in extremely clean chromatograms with base line separation of TAM and its metabolites. The method was validated and used in clinical studies involving breast and glioma patients. Fig. 11 shows chromatograms of drug-free plasma, spiked drug-free plasma and

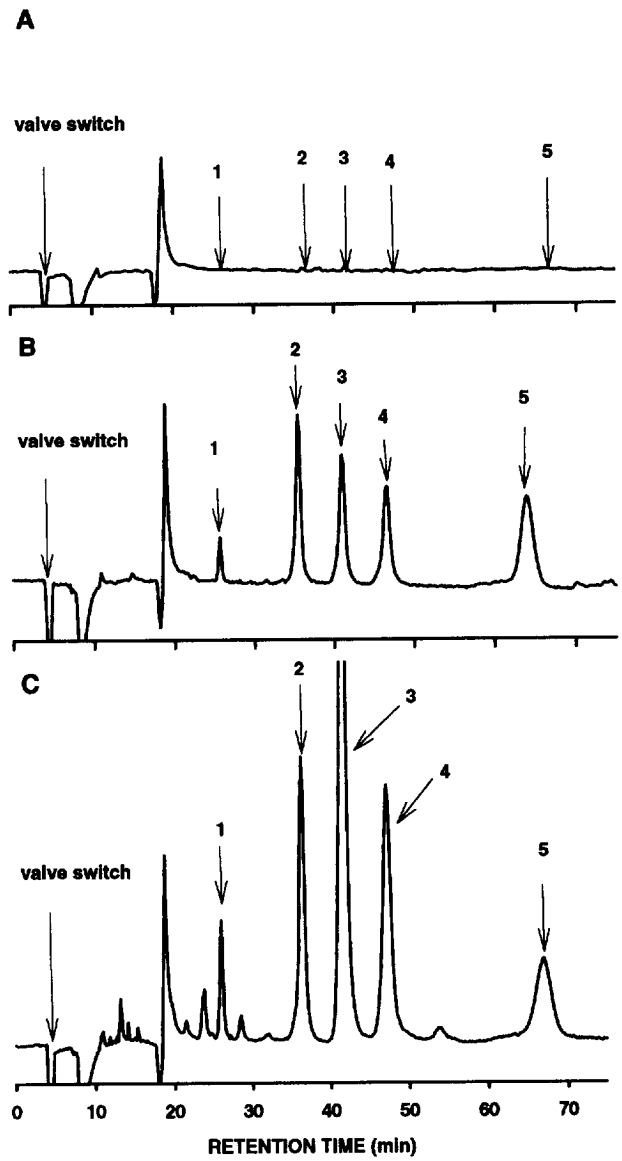


Fig. 11. Representative chromatograms for tamoxifen and its major metabolites in serum obtained on a coupled achiral–achiral HPLC system. (A) Drug-free plasma; (B) spiked plasma; (C) plasma from a patient after 4 weeks on a dose of 200 mg tamoxifen twice daily. Peaks: 1=4-hydroxytamoxifen; 2=N-desdimethyltamoxifen; 3=N-desmethyltamoxifen; 4=tamoxifen; 5=tamoxifen-ol. See Ref. [36] for details.

plasma from a patient receiving 200 mg tamoxifen twice daily for 4 weeks.

1.3.4. Coupled achiral–achiral system for extraction of highly polar compounds

Zidovudine (3′-azido-3′ deoxythymidine, AZT) is probably the most widely used drug in the treatment of acquired immunodeficiency syndrome (AIDS). It is rapidly metabolized to its glucuronide (3′-azido-3′-deoxy-5′-β-D-glucopyranuronosylthymidine, G-AZT) with a half-life of approximately one hour. The simultaneous determination of AZT and its glucuronide GAZT in biological matrices is inherently problematic. Both AZT and GAZT are highly

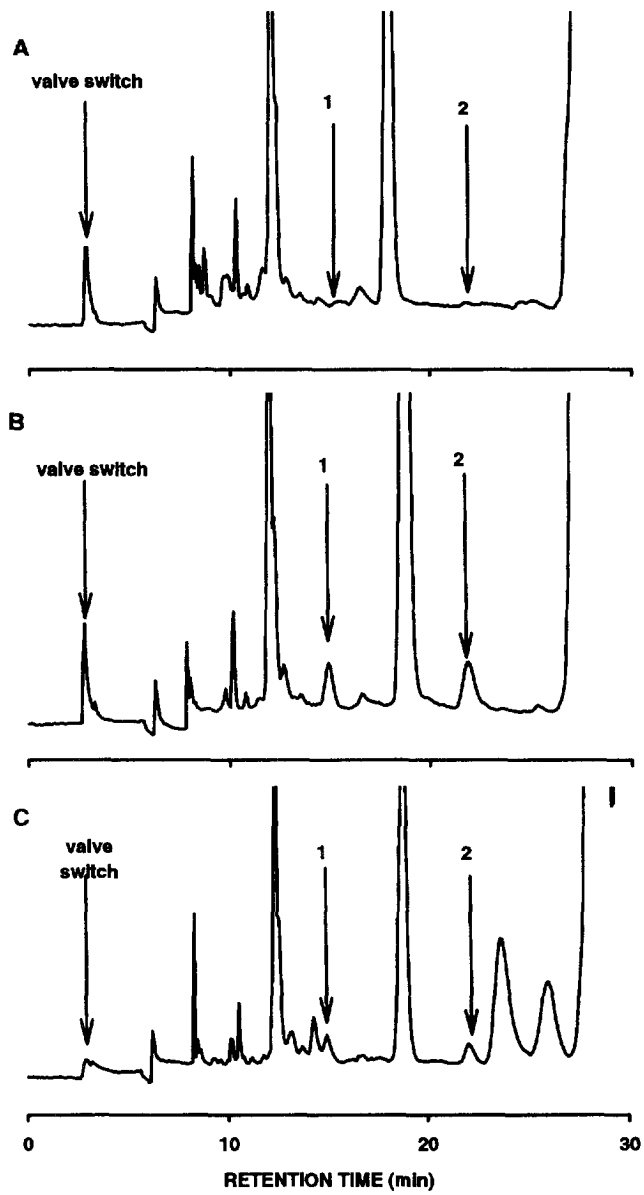


Fig. 12. Representative chromatograms for AZT and its glucuronide metabolite (GAZT) in plasma obtained on a coupled achiral–achiral HPLC system. (A) Drug-free plasma; (B) plasma spiked with GAZT and AZT; (C) plasma from an AIDS patient 4 h after an i.v. dose of liposomal AZT. Peaks: 1=GAZT; 2=AZT.

polar compounds, making their efficient extraction and quantification by standard techniques difficult.

There are several published HPLC methods that attempt to overcome this problem by using complicated, labor-intensive and expensive sample pretreatments [38–42]. Some methods ignore the more difficult to quantify glucuronide [41]. Since the inactive GAZT is formed in the liver with a large first pass effect, comparison of the GAZT to AZT ratio can be used as a measurement of hepatic function and drug deactivation. A method was developed to determine AZT and GAZT in plasma using coupled column chromatography. Plasma samples were heat inactivated to destroy the HIV virus and the proteins were precipitated with trichloroacetic acid (TCA). After dilution with phosphate buffer (pH 3.0) and centrifugation, an aliquot of the supernatant was injected onto the chromatographic system. The system consisted of an Ultrabiosep ODS guard column as an extraction column coupled by a switching valve to an ODS analytical column.

Ultrabiosep is an internal surface reversed-phase (ISRP) material designed for the determination of drugs in serum or plasma by direct injection. However, for this method, precipitation of the proteins with TCA is necessary to eliminate interference peaks not eluted from the ISPR column during the wash step. After injection of the deproteinated sample, GAZT and AZT are retained on the Ultrabiosep column as it is washed briefly with buffer. Separation occurs when the mobile phase is changed to buffer–acetonitrile, the switching valve is rotated to divert the flow leaving the ISRP from waste to the analytical ODS column.

By using an inexpensive guard column for in-line sample clean-up, the difficulty in extraction of highly polar compounds is eliminated. Recoveries were greater than 95% for both AZT and GAZT. In addition, pre-analysis sample handling is minimal and the chromatography is fully automated. Fig. 12 shows sample chromatograms of drug-free plasma, spiked drug-free plasma and plasma from an AIDS patient on AZT.

2. Conclusion

The use of a multi-dimensional chromatographic strategy in bioanalytical analysis through the application of coupled-column techniques is valid and validatable. The approach is relatively direct, reproducible and accurate. However, it can also be time consuming and costly. A preferable tactic would be to employ only a single column and isocratic elution techniques, although with the current column technology, in particular the available chiral stationary phases, single column systems are often not possible. However, column technology is advancing at a rapid pace and this may soon be a moot point.

Other analytical strategies to overcome matrix and metabolite interferences include the use of CE and mass spectrometric detection. The bioanalytical use of the former technique is limited by low sensitivity while the latter requires that parent and metabolites produce distinct ions. An attractive option is the combination of the two techniques and the bioanalytical application of this strategy will soon be tested.

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