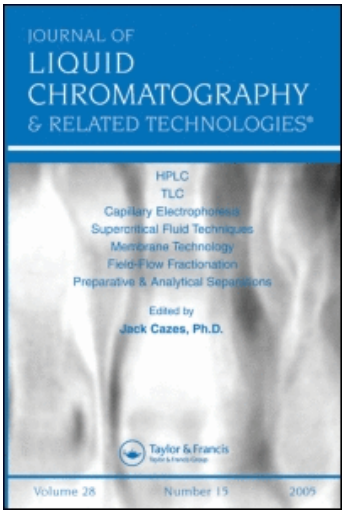

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A Multi-Component Solvent System for the Analysis of a Candidate Antimalarial by Normal Phase HPLC

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A MULTI-COMPONENT SOLVENT SYSTEM FOR THE ANALYSIS OF A CANDIDATE ANTIMALARIAL BY NORMAL PHASE HPLC

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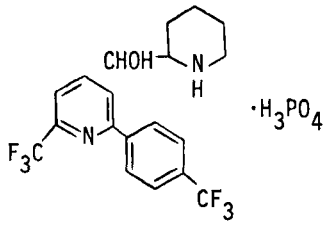
ABSTRACT

The new candidate antimalarial WR 180,409·H_3PO_4 (DL-threo-α-(2-piperidyl)-2-trifluoromethyl-6-(4-trifluoromethylphenyl)-4-pyridinemethanol phosphate) and its internal standard, WR 184,806·H_3PO_4 (DL-2,8-bis(trifluoromethyl)-4-[1-hydroxy-3-(N-t-butylamino)propyl]-quinoline phosphate) are both hydrophobic, highly lipid soluble compounds. Organic extracts of biological fluids of these compounds can be separated and analyzed by normal phase systems. These organic extracts can be chromatographed by means of a Waters μ Bondapak CN column utilizing an organic solvent system composed of two major components (ethylene dichloride and hexane), one minor component (acetonitrile) and two trace modifiers (formic acid and water). Alterations in the proportions of the major components induce predictable shifts in the retention of the antimalarial and its internal standard, while alterations in the proportions of the minor component with respect to these major components induce predictable changes in column selectivity. If the trace modifiers are stabilized in proportionate amounts, proper concentrations of the major components will isolate the antimalarial and internal standard fraction from significant interference while simultaneous alteration of the amount of minor component will effect separation of the antimalarial from the internal standards.

INTRODUCTION

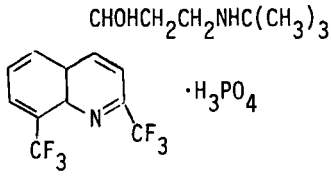
The candidate antimalarial compound, WR 180,409·H_3PO_4 (DL-threo-α-(2-piperidyl)-2-trifluoromethyl-6-(4-trifluoromethyl-

phenyl)-4-pyridinemethanol phosphate) and its internal standard, WR 184,806·H_3PO_4 (DL-2,8-bis(trifluoromethyl)-4-[1-hydroxy-3-(N-t-butylamino)propyl]-quinoline phosphate) when extracted from biological fluids, are insoluble in reverse phase high pressure liquid chromatography (HPLC) solvent systems.



$\cdot H_3PO_4$ (II)

WR 180,409·H_3PO_4



$\cdot H_3PO_4$ (I)

WR 184,806·H_3PO_4

Therefore, they must be chromatographed using normal phase solvent systems. Blood extracts of these lipid soluble, hydrophobic antimalarials possess considerable interference patterns when chromatographed using "conventional" binary normal phase solvent systems. The blood protein interference fraction can sometimes be overcome by changing solvent strengths of a binary mixture or by selecting an alternate solvent. Changing solvent strength with respect to these antimalarials induces undesirable peak selectivity.

The manipulation of a mobile phase to control separation and retention of selected peaks represents the optimum capacity of a HPLC solvent system. A mobile phase system which may be

used to control selectivity and retention of a candidate antimalarial and its internal standard is presented in this paper.

MATERIALS

The solvents hexane, ethylene dichloride, acetonitrile, ethyl acetate, and methanol were UV spectrograde (Burdick and Jackson Laboratories, Inc.). McGaw Laboratory sterile water for injection was used as a modifier, as well as reagent grade 88% formic acid (Fisher Scientific Co.). The drug, WR 180,409·H_3PO_4, and its internal standard, WR 184,806·H_3PO_4, were assayed at 98% purity (1,2). The drug solutions were made as the salts and all concentrations were expressed as the free base. Chromatographic separation was accomplished using a μ Bondapak CN column (Waters Associates, Inc.) jacketed with a temperature control block (Waters Associates, Inc.). A temperature control water bath was utilized to maintain all mobile phase systems at a constant temperature of 25.0 ± 0.5°C. A Waters Associates Model 6000A solvent delivery system, U6K injector and Model 440 UV absorbance monitor with 280 nm filter completed the high pressure liquid chromatography system. An Omniscribe (Houston Instruments) single channel recorder was used.

All injections were made with either a 25 μl or a 100 μl Precision Sampling Corporation syringe. Filtration of solvent mixtures was conducted using two methods: 1. qualivative filter paper (Schleicher and Schuell, Inc.) and 2. solvent clarification kit (Waters Associates) utilizing a 0.5 μm pore size organic solvent filtration disk.

METHODS

The major and minor components of the mobile phase systems were prepared in the ratios indicated in Table 1. After the solutions were prepared, a water:formic acid solution (50:50 v/v) was added to the mixtures sufficient to saturate each mobile phase system at 25.0°C. The solutions were filtered using qualitative filter paper followed by the solvent clarification kit and placed in a temperature control water bath maintained

TABLE 1

% of Acetonitrile in Various Ethylene Dichloride/Hexane Ratios in the Presence of Trace Modifiers, Water and Formic Acid

EC/H 9/1 + A%*	EC/H 4/1 + A%*	EC/H 7/3 + A%*	EC/H 3/2 + A%*	EC/H 1/1 + A%*
0%	0%	0%	0%	**
0.99%	0.99%	0.99%	0.99%	0.99%
3.8%	3.8%	3.8%	3.8%	3.8%
7.4%	7.4%	7.4%	7.4%	7.4%

*% volume of acetonitrile added calculated as % of final total volume

**not chromatographed

at 25.0 $\pm$ 0.5°C. Sufficient column volumes of mobile phase were utilized to achieve retention reproducibility on repeated injections. Standard solutions of WR 180,409·H_3PO_4 and WR 184,806·H_3PO_4 were prepared in methanol. The flow rate of the solvent delivery system was maintained at 2 ml/min and the chart speed was set at 1.25 cm/min. The volume of injection for the standard solutions was 5 µl.

In order to achieve statistical evaluation, four repetitions of all procedures were conducted. The calculations for capacity (K') or retention as well as selectivity (α) were determined using the standard equations (3). The peak area was obtained from the product of the maximum peak height (centimeters) and the width at peak half-height (centimeters) (4). Peak areas were plotted against actual concentrations in the standard curve determinations.

The blood extract was prepared by spiking a 5 ml blood sample with 50 µl of an equimolar concentration of the antimalarial drug and its internal standard. The blood sample was then mixed. Five ml of 7.4 pH phosphate buffer were added followed by 10 ml

of ethyl acetate. The sample was then mixed for 30 min whereupon it was centrifuged for 10 min. The ethyl acetate layer was removed and evaporated to dryness using a water bath evaporator. Additional ethyl acetate was added to the blood layer and the procedure was repeated two additional times. The sample was reconstituted in 500 µl of the corresponding mobile phase; 50 µl of the sample was injected and chromatographed.

RESULTS

The manipulation of selectivity of the column and retention of candidate antimalarials using isocratic conditions is readily observed in Figure 1. Alterations in the ratio of major components, ethylene dichloride and hexane, induce significant changes in retention (K') of the antimalarial and its internal standard as shown in Parts A and B of Figure 1. The addition of various concentrations of the minor component, acetonitrile, significantly alters the selectivity of the column as is illustrated in Parts C, D, E and F of Figure 1.

The acetonitrile-induced alterations in selectivity of the column with respect to varying ratios of the major components, ethylene dichloride and hexane, are illustrated in Figure 2. With alterations in the ethylene dichloride/hexane ratio the selectivity appears to have a non-linear relationship with respect to various acetonitrile concentrations.

Alterations in the ratio of major components lead to shifts in polarity related to the equation $P = .01 (\%a\ Pa + \%b\ Pb + \%c\ Pc)$, where %a, %b and %c are the percent of ethylene dichloride, hexane and acetonitrile respectively in the mixture, while Pa, Pb and Pc are polarity (solvent strength) of ethylene dichloride, hexane and acetonitrile respectively (5,6). A linear relationship can be derived from this equation as illustrated in Figure 3.

The ability to manipulate selectivity and retention is useful for the analysis of blood extracts. Protein fractions extracted from blood along with the candidate antimalarials cause considerable interference which may be overcome by altering

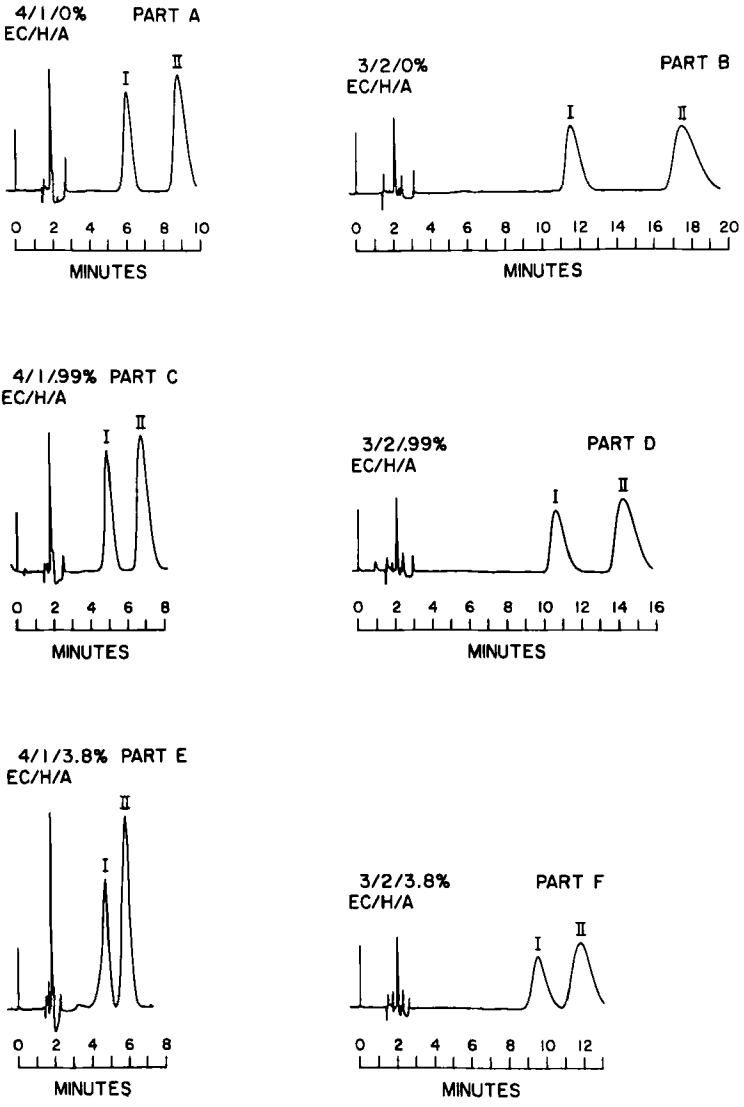


FIGURE 1

Part A and B. The effects of solvent strength on column retention times for the candidate antimalarial WR 180,409·H_3PO_4, and its internal standard, WR 184,806·H_3PO_4. EC = Ethylene Dichloride; H = Hexane; A = Acetonitrile; Peak I = WR 184,806·H_3PO_4, Peak II = WR 180,409·H_3PO_4.

solvent strength. When the antimalarial peaks are separated from the interference, alteration in the minor component, acetonitrile, causes an increase in selectivity, thereby making the resultant chromatogram more amenable to quantification as is demonstrated in Figure 4.

Alterations in the components to improve separation have no effect on linearity of the standard curve, as shown in Figure 5. In four significantly different mobile phase systems, the standard curves do not deviate from linearity.

DISCUSSION

Alterations in the ratio of major components of the solvent system, ethylene dichloride and hexane, induce a shift in the equilibrium equation; Xm + n Sads $\rightleftarrows$ Xads + n Sm (7). In this equation, Xm and Xads represent candidate antimalarial molecules in the mobile phase and the adsorbed state respectively. Free mobile phase molecules of ethylene dichloride and hexane are represented by Sm while the mobile phase molecules adsorbed on the surface are represented by Sads. The number of adsorbed solvent molecules, n, must be displaced by the adsorption of the candidate antimalarial, X. When the solvent, ethylene dichloride and hexane, interacts strongly with the surface sites, the equilibrium is shifted to the left and the candidate antimalarials

FIGURE 1 (legend continued)

Part C, D, E and F. The effects of solvent strength on column retention times with respect to various acetonitrile concentrations on column selectivity for the candidate antimalarial WR 180,409·H_3PO_4, and its internal standard, WR 184,806·H_3PO_4. Peak I = WR 184,806·H_3PO_4; Peak II = WR 180,409·H_3PO_4.

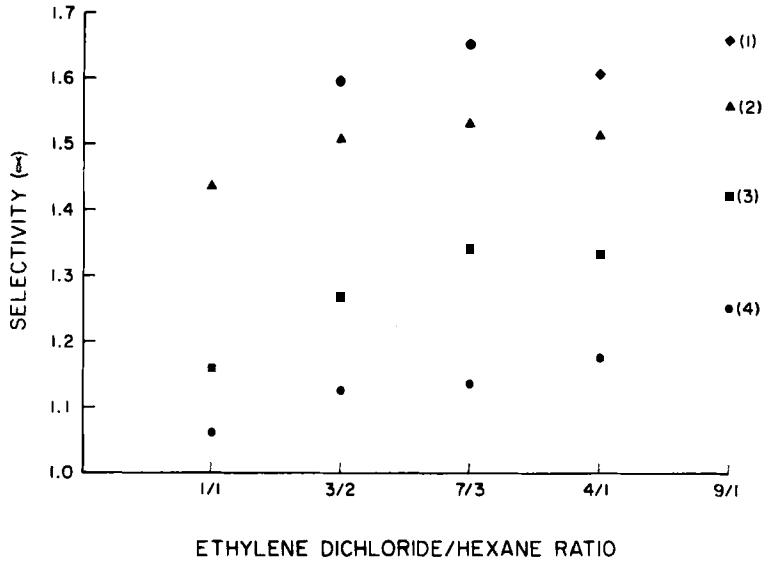


FIGURE 2

Column selectivity with respect to varying acetonitrile concentrations at several ethylene dichloride/hexane ratios. ◆ (1) Acetonitrile = 0%; ▲ (2) Acetonitrile = 0.99%; ■ (3) Acetonitrile = 3.8%; ● (4) Acetonitrile = 7.4%. The 1/1 ratio of ethylene dichloride/hexane with 0% acetonitrile was omitted because of extremely long retention volumes needed to elute the candidate antimalarial and its internal standard.

remain in solution and capacity (K') decreases. Under ideal conditions, this equation is functional for alterations in the major components of the mobile phase (Figure 1: Parts A and B).

The μ Bondapak CN column is designed to include a very broad range of polar to non-polar solutes. In the general concept of normal phase liquid chromatography the stationary phase is polar and the mobile phase is relatively non-polar, therefore a decrease in polarity of the mobile phase will cause an increase in the retention of the compounds on the column. This basic concept is illustrated by reducing the polarity of the mobile phase by increasing the relative concentration of the less polar hexane.

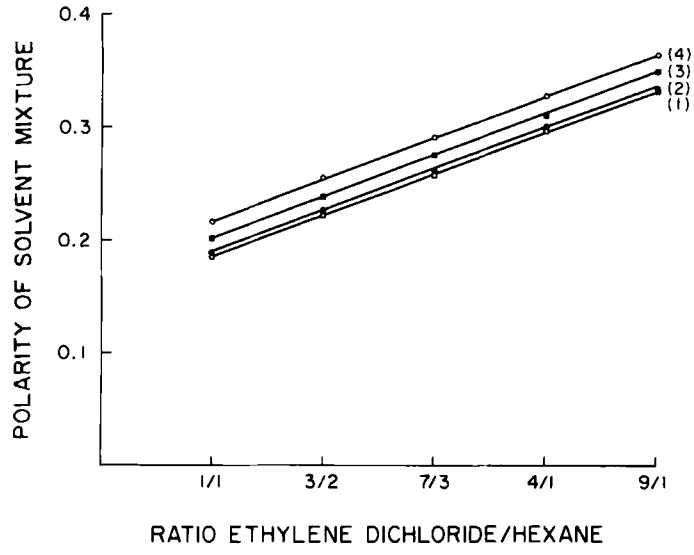


FIGURE 3

The polarity of the solvent mixture with respect to the ethylene dichloride/hexane ratio at varying concentrations of acetonitrile (A). (1) Acetonitrile = 0%; (2) Acetonitrile = 0.99%; (3) Acetontrile = 3.8%; (4) Acetonitrile = 7.4%.

Decreasing the polarity of the mobile phase in this manner induced the anticipated increase in retention of the antimalarial and its internal standard as is illustrated in Figure 1, Parts A and B.

The development of a tertiary mobile phase with the addition of acetonitrile induces a special effect in one of the compounds in addition to the basic equilibrium phenomena described previously.

An apparent alteration in column selectivity is observed with small additions of the minor component, acetonitrile. Although the addition of acetonitrile will induce insignificant alterations in polarity (Figure 3) which may be responsible for minor shifts in the retention of peak I (Figure 1: Parts C, D, E and F), these minor alterations in polarity may not be responsible for the significant alterations in the retention of peak II (Figure 1: Parts C, D, E and F).

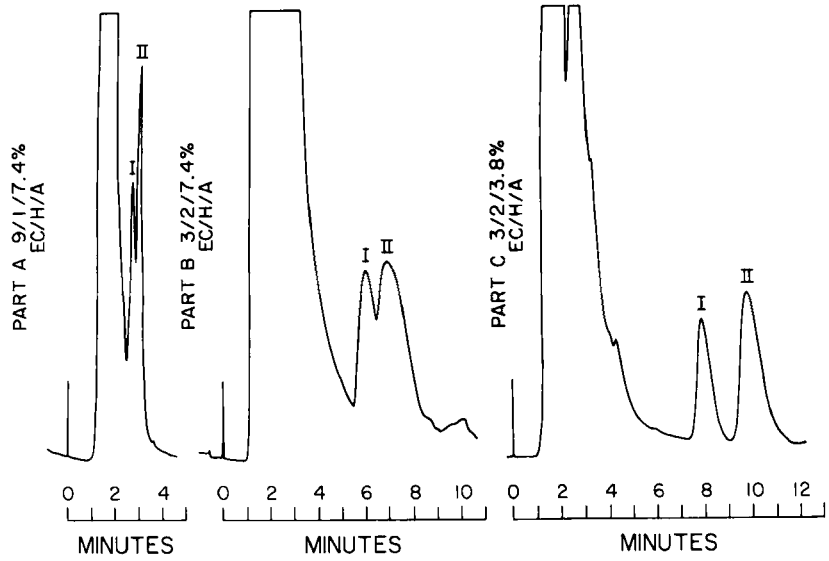


FIGURE 4

A blood extract containing the candidate antimalarial WR 180,409·H_3PO_4, and tis internal standard, WR 184,806·H_3PO_4. Part A and Part B demonstrate the ability to isolate the antimalarial (peak II) and its internal standard (peak I) from significant interference patterns, while simultaneous alterations of minor component (Part C) will effect separation of the candidate antimalarial from the internal standard. A = Acetonitrile; EC = Ethylene Dichloride; H = Hexane; Peak I = WR 184,806·H_3PO_4; Peak II = WR 180,409·H_3PO_4.

The column selectivity changes observed in Figure 2 demonstrate the significance of small additions of the minor component, acetonitrile, in the ability to achieve ideal separations of these compounds.

The selectivity alterations observed in Figure 2 could have been caused by other variables. Some authors have demonstrated the great influence of small increases of water and temperature on retention (8-12). The water and formic acid content were stabilized by saturation at constant temperature and therefore assumed to remain constant. The formic acid was used in addition

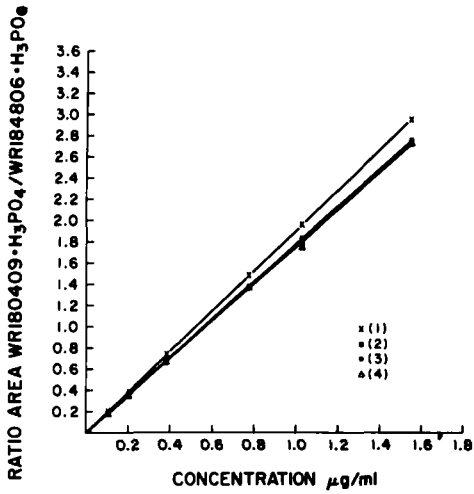


FIGURE 5

Calibration curves of the ratio of the area for the chromatogram of the antimalarial WR 180,409·H_3PO_4 (peak II) with respect to the area of the chromatogram of its internal standard (peak I) at several concentrations (µg/ml) of the antimalarial WR 180,409·H_3PO_4. Four calibration curves (1-4) were established at several combinations of major and minor components.

(1) 4 EC / 1 H / 3.8% A (2) 3 EC / 2 H / 0% A (3) 3 EC / 2 H / 3.8% A (4) 9 EC / 1 H / 0% A

EC = Ethylene Dichloride; A = Acetonitrile; H = Hexane

Standard Deviation at Several Combinations of Major and Minor Components

EC/H/%A 9/1/0%	EC/H/%A 3/2/3.8%	EC/H/%A 3/2/0%	EC/H/%A 4/1/3.8%
2.738 ± .061	2.720 ± .049	2.776 ± .071	2.952 ± .084
1.828 ± .053	1.763 ± .076	1.745 ± .051	1.955 ± .053
1.361 ± .016	1.342 ± .042	1.40 ± .063	1.482 ± .076
.702 ± .015	.678 ± .019	.698 ± .032	.741 ± .019
.350 ± .021	.314 ± .014	.366 ± .023	.365 ± .015
.196 ± .014	.171 ± .014	.186 ± .015	.202 ± .011

to water to deactivate the column and to prevent peak tailing. With these variables controlled, the acetonitrile appears to be the only component which could be causing the alterations demonstrated in Figures 1 and 2.

The flexibility of this mobile phase system is demonstrated in Figure 4. Since protein fractions from blood extracts create considerable chromatographic interference, the manipulation of major components (ethylene dichloride and hexane) will isolate the antimalarial and the internal standard fraction while simultaneous concentration changes of the minor component, acetonitrile, will effect separation of the antimalarial from the internal standard. The ability to selectively control retention of the antimalarial and its internal standard facilitates the evaluation and quantitation of the resulting chromatograms.

Although significant changes in column selectivity are induced by the alteration of mobile phase systems, the standard calibration curves (Figure 5) remain linear over a wide range of mobile phase systems. The maintenance of linearity of the corresponding calibration curve enables the use of simple linear regression plots in the quantitative analysis of the selected candidate antimalarial.

CONCLUSION

The manipulation of selectivity of a solvent system with the addition of a minor component, acetonitrile, has been demonstrated. Acetonitrile appears to be inducing selectivity alterations independent of the concentration of the major components of the mobile phase, ethylene dichloride and hexane.

* * * * * *

Commercial materials and equipment are identified in this report to specify the investigative procedure. Such identification does not imply recommendation or endorsement or that the materials and equipment are necessarily the best available for the purpose. Furthermore, the opinions expressed herein are those of the authors and are not to be construed as those of the Army Medical Department.

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