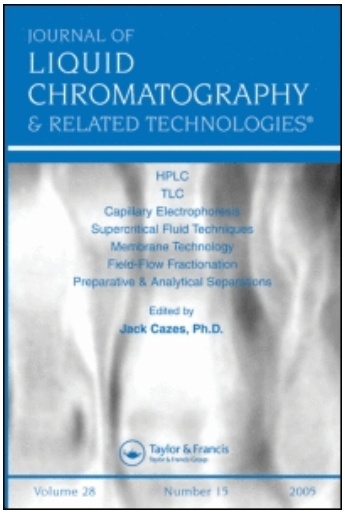

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATIONS OF SULFONAMIDES BY IONIC SUPPRESSION

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ABSTRACT

A high pressure liquid chromatography procedure is reported for extraction and quantitation of 8 sulfonamides in stock solutions and in vitro plasma samples. This assay consists of a single, one-step extraction of sulfonamides from plasma and is sensitive to 10.0 ng/ml at 254 nm without additional concentration of the sample. Four sulfonamides (sulfamerazine, sulfamethazine, sulfapyridine and sulfathiazole) were separated from the plasma matrix by either mobile phase regardless of pH. The sulfonamides with the highest pKa, sulfanilamide (10.5) and sulfaguanidine (11.3), were only separable from plasma in a 50% water/50% methanol mobile phase at pH 7.45. The sulfonamide with the lowest pKa, sulfisoxazole (4.9), and its metabolite, acetylsulfisoxazole (N^4), were separated from plasma by either mobile phase, 50/50 or 60/40 water/methanol, when acetate buffer reduced the pH to 4.00. Standard concentration curves of peak height were the most sensitive at 254 nm when a 60% water/40% methanol mobile phase at pH 4.00 was used. Sulfanilamide and sulfaguanidine were the most responsive to ultraviolet quantitation at 254 nm regardless of ionic suppression or polarity of the mobile phase.

INTRODUCTION

High pressure liquid chromatography (HPLC) has been used to qualitate and quantitate standard solutions of sulfonamides by ion exchange (1,2), or ion pairing (3) and reversed phase (4). In the clinical monitoring of therapeutic levels in biological fluids and the analysis of such compounds in toxicity evaluation, the analytical procedures involving standard solutions, exclusively, are not satisfactory nor reliable.

EXPERIMENTAL

Apparatus

An ALC/GPC 204 liquid chromatograph, equipped with a Model 440 ultraviolet absorbance detector (254 nm) and a U6K septumless injector (Waters Associates, Milford, MA) was used throughout this study. A dual pen recorder (Houston Instruments, Austin, TX) was used to quantitate concentration as a function of peak height (mm). A μBondapak C_{18} reverse phase column was used with an in-line guard column packed with Corasil/C_{18} (Waters Associates, Milford, MA).

Mobile Phase

The mobile phase consisted of double distilled water and methanol (Burdick-Jackson Laboratories, Muskegon, MI) as a 50/50 or 60/40 water/methanol mixture.

The pH of the mobile phase was adjusted with glacial acetic acid (Fisher Scientific, Orlando, FL) to 4.00 or at an isocratic pH of 7.45 as confirmed by a pH electrode (Orion Research, Inc., Cambridge, MA).

All analyses were conducted at ambient temperature (25°C) with a 0.8 ml/min flow rate.

Preparation of Sulfonamides

Ten-milligram samples of sulfanilamide, sulfaguanidine, sulfamerazine, sulfamethazine, sulfapyridine, and sulfathiazole (Fort Dodge Laboratories, Fort Dodge, IA) and sulfisoxazole and acetyl (N^4) sulfisoxazole (Hoffman-LaRoche, Nutley, NJ) were dissolved in methanol (Burdick-Jackson, Muskegon, MI) and double distilled water (1:9) to give concentrations of 100, 50, 25, and 10 μg/ml and 100, 50, and 10 ng/ml. Twenty-μl of these standard solutions were then injected onto the column.

One ml *in vitro* plasma samples were incubated with 50 μg/ml of sulfonamide in a 37°C water bath for 1 hour. Twenty μl of this plasma sample was then either injected directly into the system or the 1.0 ml sample was deproteinated with methanol (1:1) (Burdick-Jackson, Muskegon, MI), centrifuged (2000 x g) for 10

min and 20 µl injected. Or, the 1.0 ml sample was filtered through Millex filters (SLHA 02505, Millipore Corp., Bedford, MA) and 20 µl of the element was injected into the liquid chromatographic system. Twenty µl of methanol (Burdick-Jackson Laboratories, Muskegon, MI) were injected into the system between each sample to prevent cross-contamination and to elute any compounds remaining in the column.

RESULTS AND DISCUSSION

Evaluation of Standard Curves

All the standard sulfonamide mixtures were adequately separated with either the 50/50 or 60/40 water/methanol mobile phases at either pH 4.00 or 7.45 (Tables 1 and 2).

In the 50/50 mobile phase (Table 1), the retention times were increased for sulfamerazine, sulfapyridine, sulfisoxazole and acetylsulfisoxazole (N^4) and decreased for sulfamethazine when the pH was reduced from 7.45 to 4.00. The retention times of sulfanilamide, sulfaguanidine and sulfathiazole were not affected by the pH change.

TABLE 1

Retention Time* of Sulfonamides in a Water/Methanol (50/50) Mobile Phase with Acetate Buffer (pH 4.00) and without Buffer (pH 7.45)

	pH 4.00	pH 7.45
Sulfanilamide	3.80	3.80
Sulfaguanidine	3.70	3.70
Sulfamerazine	4.90	4.50
Sulfamethazine	5.00	5.45
Sulfapyridine	4.75	4.45
Sulfisoxazole	5.20	3.20
Acetylsulfisoxazole (N^4)	7.00	2.90
Sulfathiazole	4.00	4.00

*Retention time in minutes from the injection mark.

In the 60/40 mobile phase (Table 2), the retention time of sulfanilamide, acetylsulfisoxazole (N^4) and sulfathiazole were increased as the pH was reduced from 7.45 to 4.00. The retention time of the other sulfonamides, except sulfisoxazole were reduced as pH was reduced.

TABLE 2

Retention Time* (minutes) of Sulfonamides in a Water/Methanol (60/40) mobile Phase with Acetate Buffer (pH 4.00) and without Buffer (pH 7.45)

	pH 4.00	pH 7.45
Sulfanilamide	3.75	3.05
Sulfaguanidine	3.60	3.90
Sulfamerazine	4.10	5.40
Sulfamethazine	4.25	6.10
Sulfapyridine	4.00	5.00
Sulfisoxazole	4.20	4.20
Acetylsulfisoxazole (N^4)	5.00	4.40
Sulfathiazole	3.75	3.30

*Retention time in minutes from the injection mark.

Construction of standard concentration curves (Figures 1 through 4) by plotting peak height at 10, 50 and 100 ng/ml and 10, 25, 50 and 100 µg/ml of sulfonamides in methanol/water (1:9) demonstrated that a difference in sensitivity occurred as a function of ion suppression and polarity of the mobile phase.

In Figure 1, the sensitivity of sulfaguanidine (b) at 254 nm is greater than sulfanilamide (a) at pH 4.00 in a 50/50 mobile phase. If the mobile phase remains at pH 7.45 (Figure 2), this sensitivity is reversed, with the sulfanilamide peak height being greater than sulfaguanidine. There is also a change in the slope of the other 6 sulfonamides as the pH changes in the 50/50 mobile phase (Figures 1 and 2).

If the mobile phase is 60% water/40% methanol, sensitivity of the assays for sulfanilamide (a) and sulfaguanidine (b) are

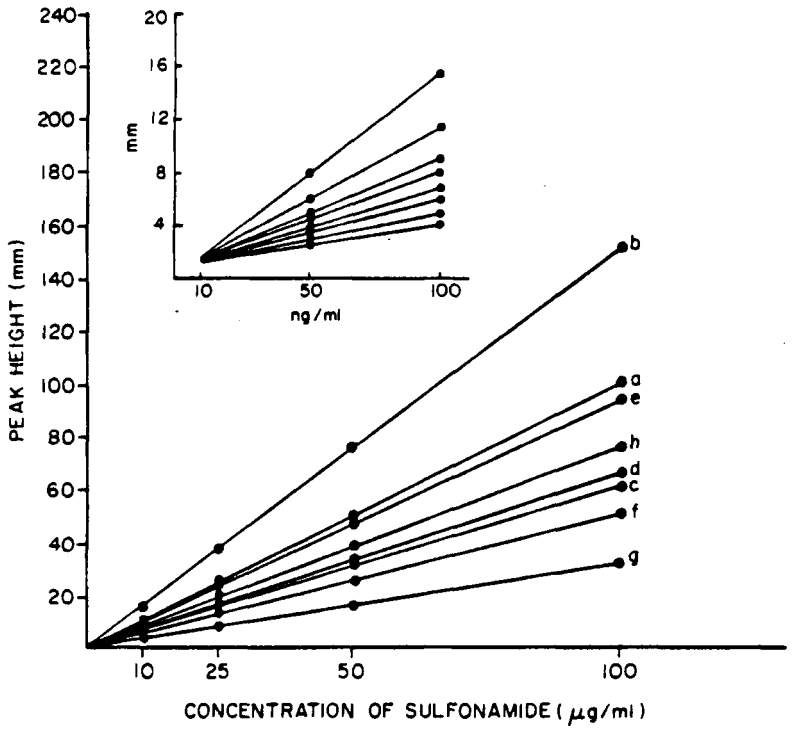

FIGURE 1

Standard concentration curves of sulfanilamide (a), sulfaguanidine (b), sulfamerazine (c), sulfamethazine (d), sulfapyridine (e), sulfisoxazole (f), N^4 acetylsulfisoxazole (g), and sulfathiazole (h) in a water/methanol (50/50) mobile phase with acetate buffer to pH 4.00.

the most sensitive regardless of pH (Figures 3 and 4) at 254 nm. Detection of the remaining 6 sulfonamides vary in sensitivity at 254 nm in the 60/40 mobile phase with the pH change. The most sensitive mobile phase was 60/40 at pH 4.00 as shown by the greater slopes of each sulfonamide (Figure 3). Additional dilution of standard solutions of sulfonamides and increased sensitivity settings to 0.01 A.U.F.S. (absorbance units full scale) on the ultraviolet detector recorded peak heights equivalent to 10.0 ng/ml of sulfonamide from a single 20 µl injection without concentration or reconstitution of the extract.

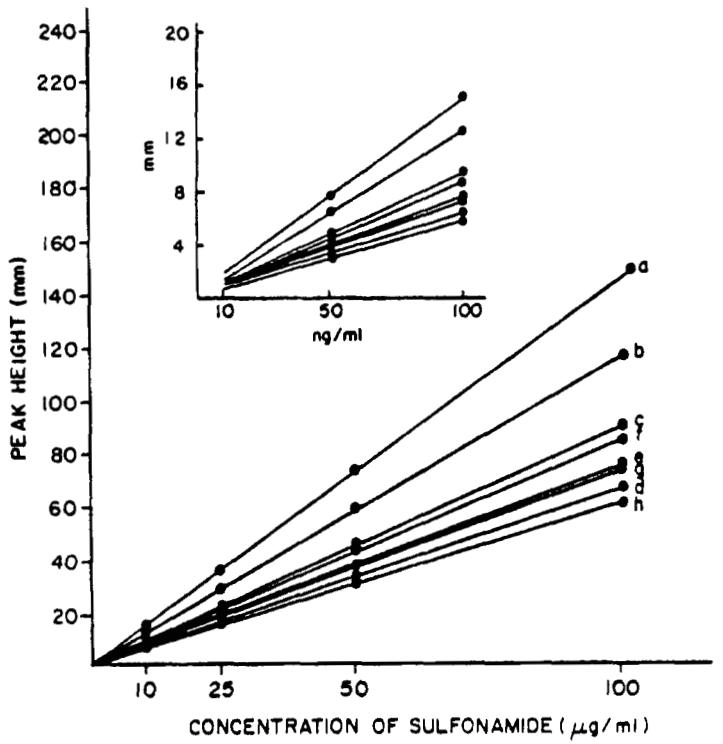

FIGURE 2

Standard concentration curves of sulfanilamide (a), sulfaguanidine (b), sulfamerazine (c), sulfamethazine (d), sulfapyridine (e), sulfisoxazole (f), N^4 acetylsulfisoxazole (g), and sulfathiazole (h) in a water/methanol (50/50) mobile phase with an isocratic pH 7.45.

The slope of the sulfisoxazole (f) curve was the same for the 60/40 mobile phase regardless of pH (Figures 3 and 4). By comparing Figures 1 and 2, one observes that the slope of the sulfamethazine (d) curve was the same for the 50/50 mobile phase at either pH 4.00 or pH 7.45. Sulfaguanidine (b, Figure 1 and 3) and sulfapyridine (e, Figure 2 and 4) curves had the same slope at pH 4.00 and pH 7.45, respectively, regardless of the polarity of the mobile phase. The standard error of peak height on repetitive injection of the same concentration was ± .017.

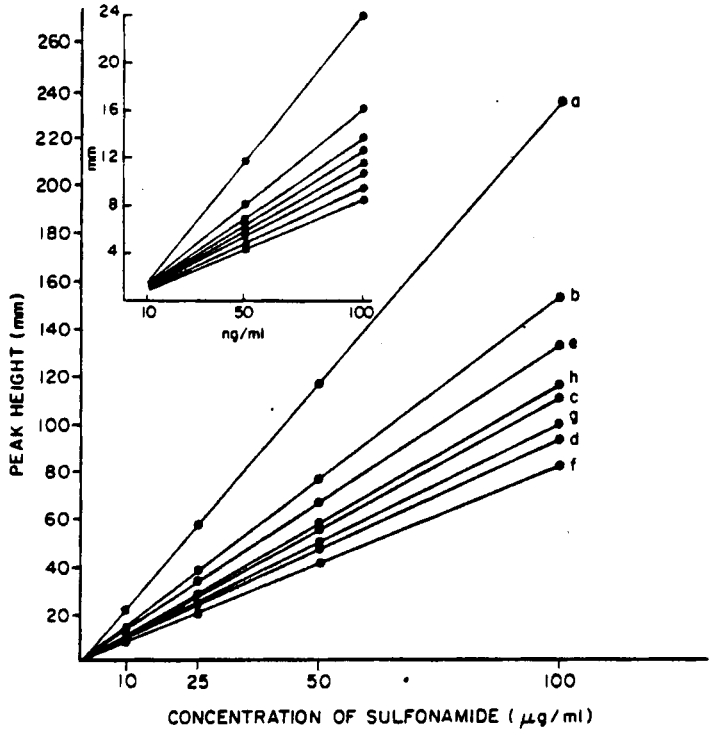

FIGURE 3

Standard concentration curves of sulfanilamide (a), sulfaguanidine (b), sulfamerazine (c), sulfamethazine (d), sulfapyridine (e), sulfisoxazole (f), N^4 acetylsulfisoxazole (g), and sulfathiazole (h) in a water/methanol (60/40) mobile phase with acetate buffer at pH 4.00.

The similarity in slopes is a result of equilibrium shifts. This is a result of the sulfonamide interacting at the liquid-liquid interface with the mobile and stationary phase in order to elute the same quantity of compound.

Separation from in vitro Plasma Samples

Six of the 8 sulfonamides were separable from in vitro human plasma with a 50/50 water/methanol mobile phase at pH 4.00 (Figure 5). Sulfanilamide and sulfaguanidine were not separable from endogenous serum peaks. Sulfapyridine was the most sensitive sulfonamide with this mobile phase. With a pH of 7.45, sulfanila-

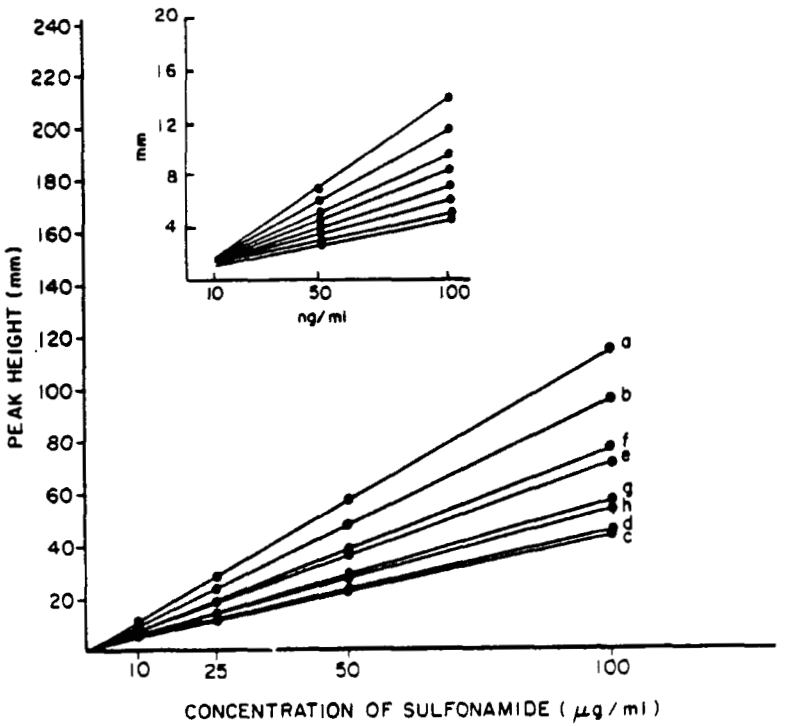

FIGURE 4

Standard concentration curves of sulfanilamide (a), sulfaguanidine (b), sulfamerazine (c), sulfamethazine (d), sulfapyridine (e), sulfisoxazole (f), N^4 acetylsulfisoxazole (g), and sulfathiazole (h) in a water/methanol (60/40) mobile phase with an isocratic pH 7.45.

mide and sulfaguanidine were separable from human plasma with the 50/50 mobile phase (Figure 6). Sulfanilamide was the most sensitive using the 50/50 mobile phase at pH 7.45.

The same 6 sulfonamides were separable in the 60/40 mobile phase at pH 4.00 (Figure 7) as were separable in the 50/50 mobile phase at pH 4.00. These 6 sulfonamides had a pKa less than 7.0 and should have no ionic charge at pH 4.00. The lower pH suppressed the ionic charge, eliminating the ion exchange factor, and permitted separation based on the partition coefficient in each mobile phase. The 60/40 mobile phase at pH 4.00 was the most

sensitive of the 4 mobile phases as shown by the standard curve and was confirmed by the detector sensitivity being reduced for Figure 7.

At pH 7.45, only 4 sulfonamides were separable with the 60/40 mobile phase (Figure 8) and this was the least sensitive of all the mobile phases. Apparently, with the increased pH and greater polarity of the mobile phase, the other 4 sulfonamides were eluted with some portion of the endogenous serum peaks.

There were no differences in peak height using either of the preparative techniques listed. The unbound or free sulfonamide was equally quantitated if the *in vitro* plasma sample was deproteined with methanol, filtered or injected directly into the system.

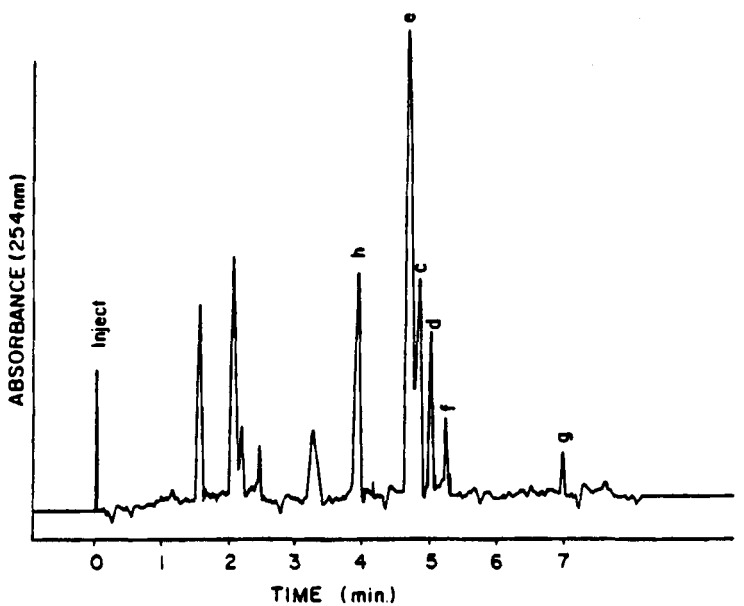

FIGURE 5

Chromatogram of sulfamerazine (c), sulfamethazine (d), sulfapyridine (e), sulfisoxazole (f), N^4 acetylfulsifosazole (g) and sulfathiazole (h) separated from human plasma *in vitro* with a 50/50 water/methanol mobile phase with acetate buffer at pH 4.00. Detector sensitivity 0.5 A.U.F.S.

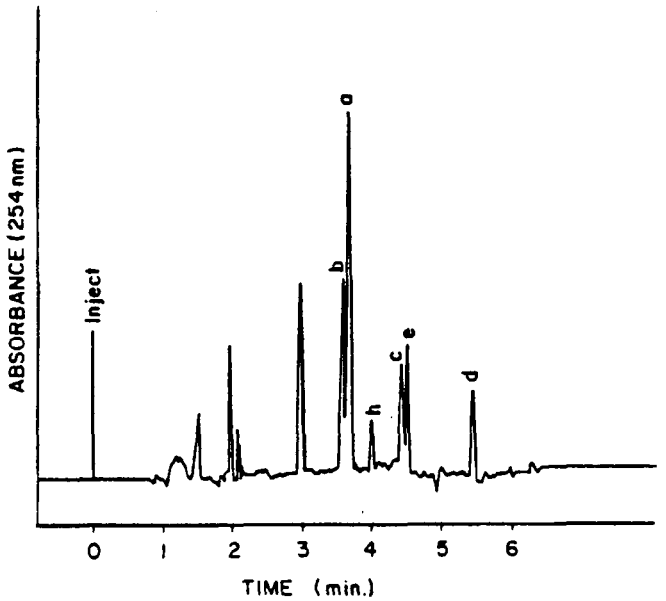

FIGURE 6

Chromatogram of sulfanilamide (a), sulfaguanidine (b), sulfamerazine (c), sulfamethazine (d), sulfapyridine (e) and sulfathiazole (h) separated from human plasma in vitro with a 50/50 water/methanol mobile phase at pH 7.45. Detector sensitivity 0.5 A.U.F.S.

CONCLUSIONS

A specific and sensitive HPLC method for the analysis and separation of 8 sulfonamides from human plasma has been developed and evaluated. Four sulfonamides (sulfamerazine, sulfamethazine, sulfapyridine and sulfathiazole) could be separated from a plasma matrix in a 50/50 or 60/40 methanol/water mobile phase regardless of pH. Sulfanilamide and sulfaguanidine were only separable from plasma in a 50% water/50% methanol mobile phase at pH 7.45 due to their increased pKa of 10.5 and 11.3, respectively. Sulfisoxazole and acetylsulfisoxazole (N^4) were separated with either mobile phase by ionic suppression with acetate to reduce the mobile phase pH to 4.00.

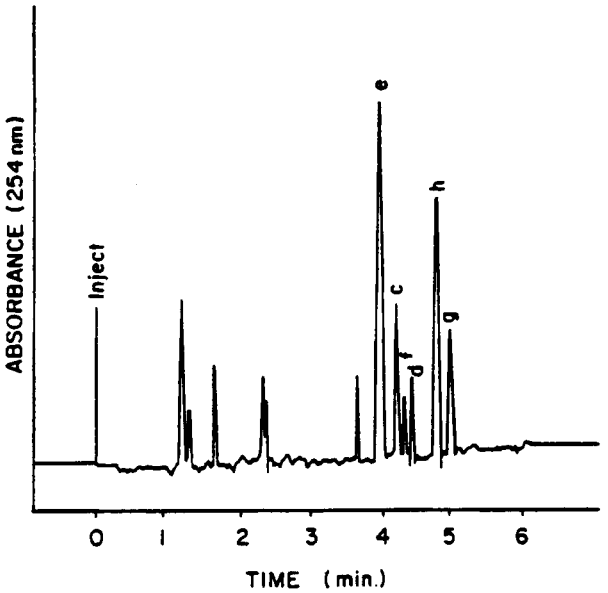

FIGURE 7

Chromatogram of sulfamerazine (c), sulfamethazine (d), sulfapyridine (e), sulfisoxazole (f), N^4 acetylsulfisoxazole (g) and sulfathiazole (h) separated from human plasma in vitro with a 60/40 water/methanol mobile phase with acetate buffer at pH 4.00. Detector sensitivity 1.0 A.U.F.S.

Sensitivity of the assays was 10.0 ng/ml of sulfonamide from 20 µl injections of spiked plasma samples without concentration or reconstitution of the extract. Assays for sulfanilamide and sulfaguanidine were the most sensitive in the water/methanol mobile phase at 254 nm. A 60% water/40% methanol with acetate buffer to pH 4.00 was the most sensitive of the assays.

In order to separate all 8 sulfonamides, one should attempt a separation using the 50/50 or 60/40 mobile phase with a less drastic pH change. By using the 60/40 mobile phase, one would not sacrifice sensitivity for separation.

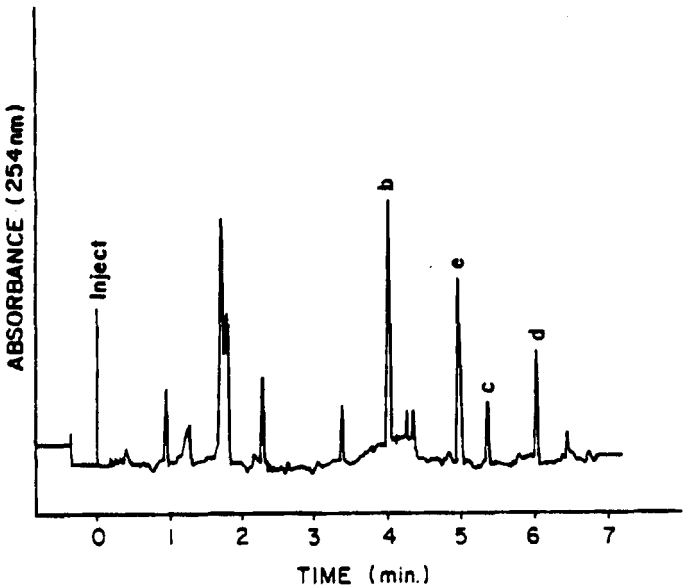

FIGURE 8

Chromatogram of sulfaguanidine (b), sulfamerazine (c), sulfamethazine (d) and sulfapyridine (e) separated from human plasma in vitro with a 60/40 water/methanol mobile phase at pH 7.45. Detector sensitivity 0.5 A.U.F.S.

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