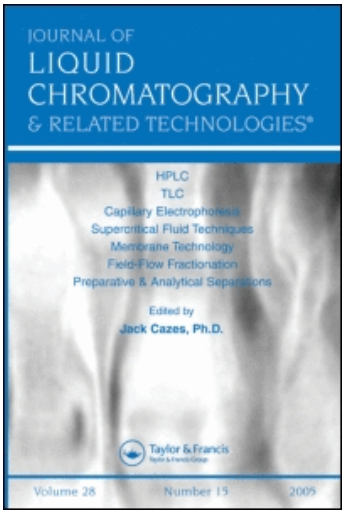

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EVALUATION OF REVERSED-PHASE, RADIALLY-COMPRESSED, FLEXIBLE-WALLED COLUMNS FOR THE SEPARATION OF LOW MOLECULAR WEIGHT, UV-ABSORBING COMPOUNDS IN SERUM

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ABSTRACT

Radially-compressed, flexible-walled columns were compared to rigid-walled columns for the reversed-phase separation of twenty compounds of biological importance. The radially-compressed columns were found to be efficient and reproducible, and enable the analysis time of human serum to be reduced by 50%. The effect of pH, flow-rate, and changing methanol concentration upon the separation of some compounds on the radially-compressed column is also presented.

INTRODUCTION

High performance liquid chromatography (HPLC) has been widely used for the analysis of nucleic acid components in physiological samples. Several different packing materials have been used in rigid-walled columns of various lengths and configurations (1-16). With microparticle, chemically-bonded, reversed-phase packings in narrow-bore, stainless-steel columns ranging from 25 to 30 cm in length, sensitive and efficient separations of nucleosides and bases were achieved (17-22). However, problems have surfaced in the routine analysis of large numbers of samples. Since rapid analyses are required, high flow-rates (>2.0 ml/min) must be used. With high flow-rates, the microparticle packings

produce void volumes within the column causing the reduction of column efficiency and peak doubling. In addition, during rapid routine work, the adsorption of biological materials to the non-bonded silica sites makes column regeneration difficult.

Recently, a new concept in analytical HPLC column technology was introduced (23). The approach is similar to that used in a preparative HPLC system (24) where a compression chamber is used to radially-compress a flexible-walled cartridge packed with micro-particle, spherical material. Theory predicts (25-29) that the radial compression of the cartridge can create an efficient and homogenous packing which is non-voiding and enable rapid separations and short analysis times. In addition, the short, wide-diameter, flexible-walled cartridges should allow high flow-rates to be used without the damaging effects of high back-pressures. Furthermore, due to the versatility of the compression system, it should be possible to use regeneration techniques with greater success. Therefore, we investigated a prototype radial compression system for use in our laboratory where a large number of samples must be rapidly, reproducibly, and sensitively analyzed for the low molecular weight, UV absorbing constituents in serum.

MATERIALS AND METHODS

Apparatus

A Waters Associates (Milford, MA) ALC 204 liquid chromatograph equipped with Model 6000A solvent delivery systems, Model 660 solvent programmer, Model 440 dual-wavelength detector, and Model U6K injector was used throughout the study.

A Model SF 770 Spectroflow Monitor and a Model FS 970 L. C. Fluorometer (Schoeffel Instrument Division, KRATOS Inc., Westwood, NJ) were used for peak identification. Retention times and peak areas were electronically acquired on a HP 3380A integrator (Hewlett-Packard, Avondale, PA).

Columns

The rigid-walled columns used were obtained from Whatman, Inc. (4.6 x 250 mm, Partisil-10 ODS, Clifton, NJ), Waters Associates (3.9 x 300 mm, μBondapak C_{18}), and ES Industries (4.6 x 300 mm, Chromegabond, Marlton, NJ). A prototype Radial Compression Separation System™ from Waters Associates was used. The system consists of a compression chamber which mechanically compresses a flexible-walled cartridge. Three levers reside on the top of the compression chamber. Each lever accuates a piston which forces fluid around a Viton® jacket around the column. As the fluid fills the chamber surrounding the jacket, it compresses the column along the column walls. Two or three pistons may be used to radially-compress these flexible-walled cartridges. The cartridges (8 x 100 mm) were packed with totally-porous, spherical, microparticle reversed-phase material. This packing differs from the packings used for conventional columns in having a spherical particle shape and a lower carbon loading.

Chromatographic Conditions

For the majority of the analyses, a linear gradient of 0.02 M KH_2PO_4, pH 5.7, was used as the initial eluent and 3:2 methanol-water (by volume) solution as the final eluent. The linear gradient was programmed from 0 to 40% of the methanol-water eluent, over a 35 minute period at a flow-rate of 1.5 ml/min. The radial compression chamber was maintained at ambient temperature and three pistons were used to exert the compression pressure. Where different chromatographic conditions were used, they are given in the text or in the legends of the figures.

Chemicals

All standard compounds and enzymes were purchased from Sigma Chemical Company (St. Louis, MO). Solutions of the standard compounds and enzymes were prepared in double distilled, deionized

water buffered with reagent grade potassium dihydrogen phosphate (Mallinckrodt, Inc., St. Louis, MO).

Sample Preparation

A standard solution of twenty low molecular weight, biologically important compounds was prepared at a concentration of approximately 10^{-4} M. Serum samples were processed according to protocol used routinely in our laboratory (22,30) and were stored at -20°C.

Peak Identification

Identification of the peaks was made on the basis of retention times, absorbance ratios, co-chromatography with reference compounds (19), enzymic peak-shifts (19,22), stopped-flow UV scanning (31), and fluorescence (32).

RESULTS

The radially-compressed columns were found to be as efficient as the rigid-walled columns, under the same chromatographic conditions, as shown by the separation of twenty low molecular, UV-absorbing compounds which are of biological importance and may be found in serum (Figures 1 and 2).

The efficiency of the radially-compressed column was calculated from triplicate analyses of an isocratic separation of cytosine and cytidine using a flow-rate of 1.0 ml/min with the 0.02 M KH_2PO_4, pH 5.7, eluent. An average of 47,000 plates/meter was determined. The rigid-walled columns were found to have 40,000 to 50,000 plates/meter.

In the gradient elution mode, peak widths and retention times are similar for both types of columns under identical conditions. However, with the flexible-walled column significantly reduced retention times and peak widths can be achieved by increasing the flow-rate to 3.0 ml/min. No loss of resolution was noted. In addition, the high back-pressures which develop when high flow-

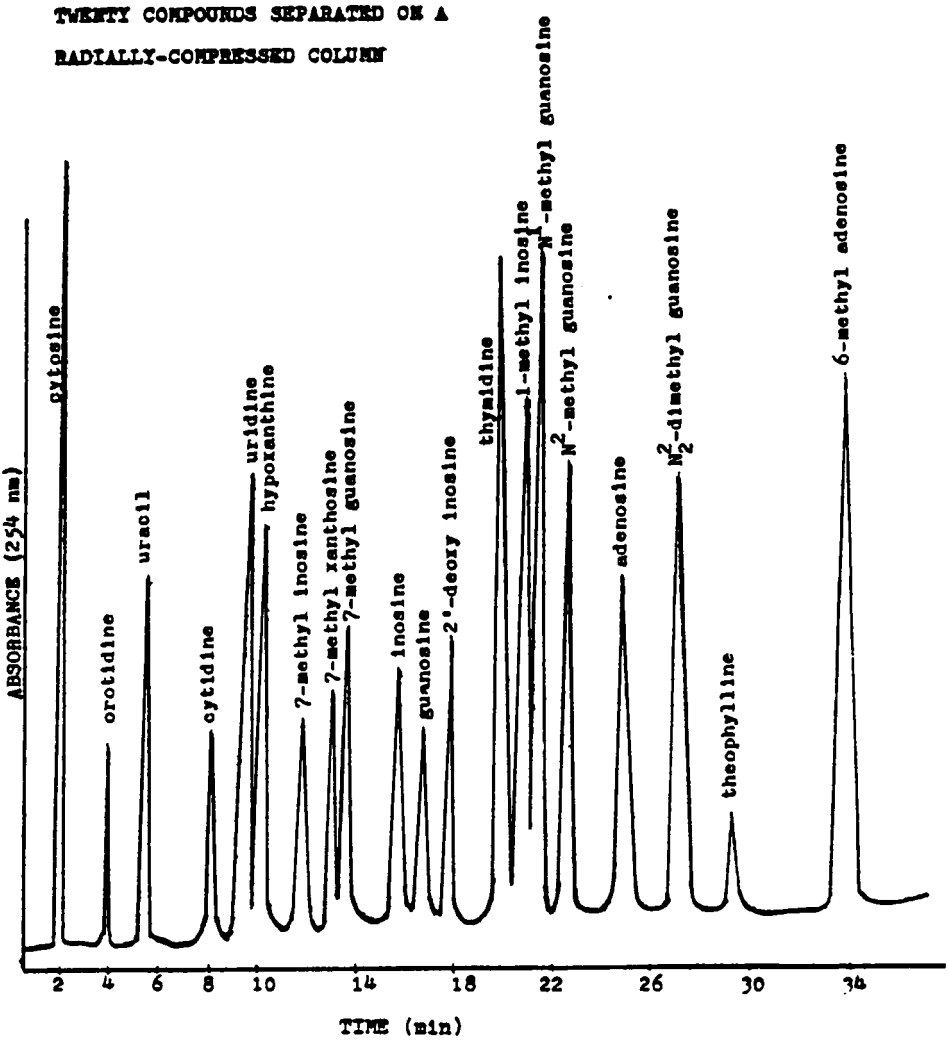

FIGURE 1.

Twenty low molecular weight, UV-absorbing compounds important to biological systems separated on a reversed-phase radially-compressed column. Injection volume: 50 µl. Conditions are listed in text.

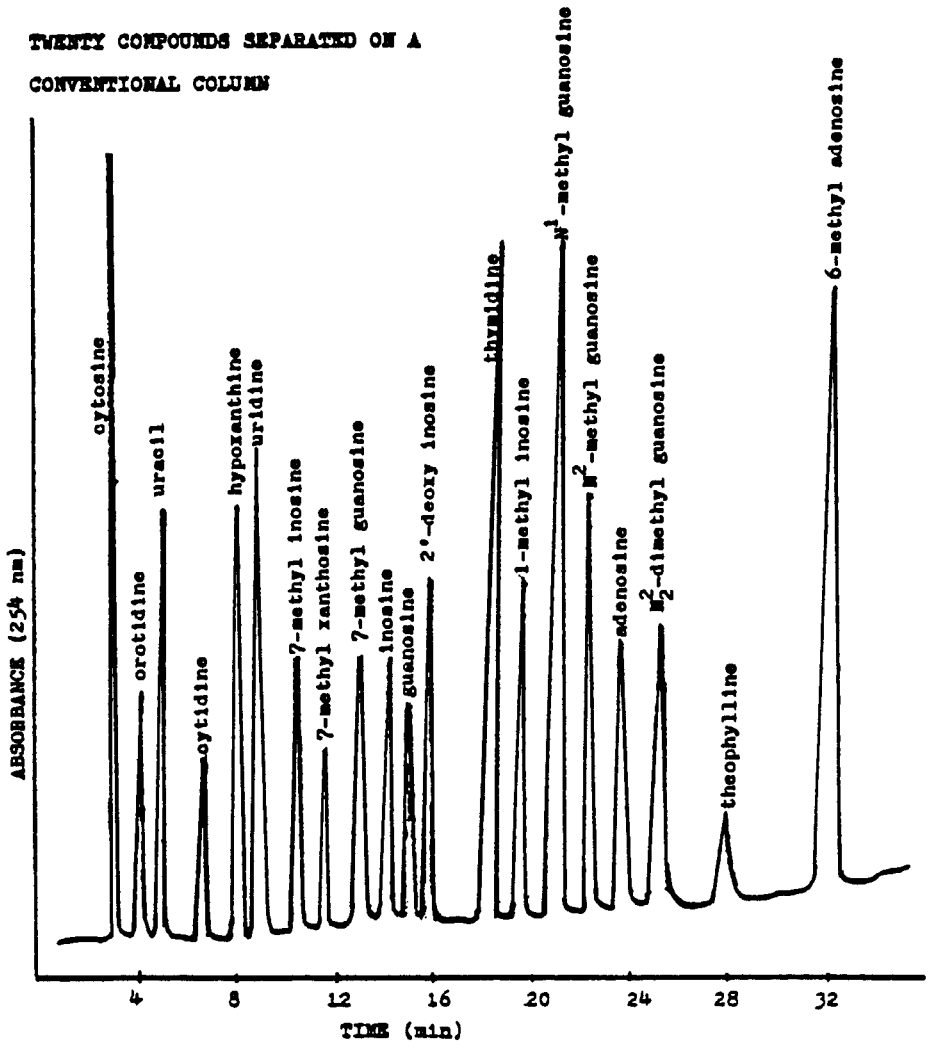

FIGURE 2.

Identical conditions separation of twenty biologically important compounds using a conventional rigid-walled reversed-phase column. Injection volume: 50 µl.

rates are used with the rigid-walled columns did not occur. Figure 3 illustrates the gradient separation of some biologically important compounds on the radially-compressed column with a 3.0 ml/min flow-rate; all other parameters remained constant.

With the radially-compressed column, retention times and peak widths (shapes) remained constantly within 1% for 150 analyses conducted over a period of several months time. However, variations in efficiency were noted from one flexible-walled col-

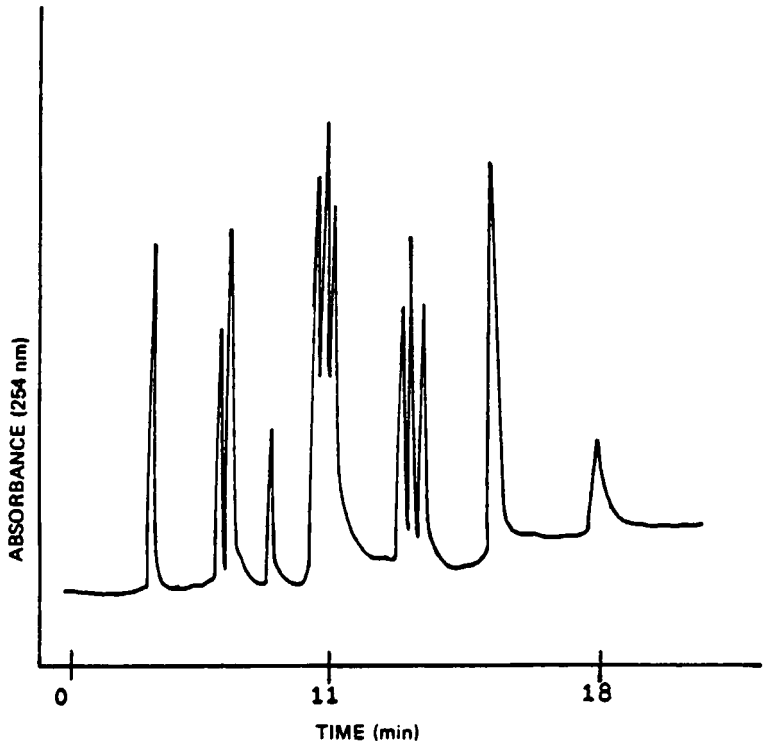

FIGURE 3.

Separation of some important serum compounds on a radially-compressed column using increased flow-rate. Conditions in text; Flow-Rate: 3.0 ml/min. Peaks from left to right: uracil, uridine, hypoxanthine, 7-methyl xanthosine, inosine, guanosine, 2'-deoxy inosine, 1-methyl inosine, N^1-methyl guanosine, N^2-methyl guanosine, N^2-dimethyl guanosine, and theophylline.

umn to another. Therefore, conditions had to be adjusted for maximum efficiency with each column.

The radial compression chamber is equipped with three pistons which force fluid into a Viton ^{R} jacket that compresses the flexible-walled column. The chromatographer has the option of exerting either 2 or 3 pistons compression pressure upon these flexible-walled columns. The maximum amount of column compressibility depends on the amount of packing material contained within the column and the rigidity of the column walls. Each column was found to have different compressibilities under the same number of applied pistons. This would account for the variations noted from one column to another.

The effect of compression pressure upon the retention times and peak widths of some biologically important compounds was observed. Figure 4 illustrates the relationship of the retention times of the bases uracil, and hypoxanthine, and the nucleosides 7-methyl xanthosine, inosine, guanosine, and N^2_2-dimethyl guanosine with the number of pistons used to exert the radial compression. Three pistons exerted an estimated 320 kg/cm^2 pressure and two pistons 200 kg/cm^2 pressure upon the column used in this study. The more tightly compressed column gave shorter retention times and narrower peak widths (Figure 5).

Since the radially-compressed columns utilize a packing material with lower carbon loading per gram, but higher loading per unit area, parameters affecting capacity and resolution of nucleosides and bases were investigated.

The effect of changing methanol concentration in the mobile phase upon the retention times of some nucleosides and bases was investigated for these columns. Figure 6 illustrates the relationship between retention time and gradient slope (percent methanol/min, at constant flow-rate) for selected compounds. Each point represents triplicate values with less than 1% relative standard deviations. These values were obtained by holding constant a 1.5 ml/min flow-rate and changing the time required to

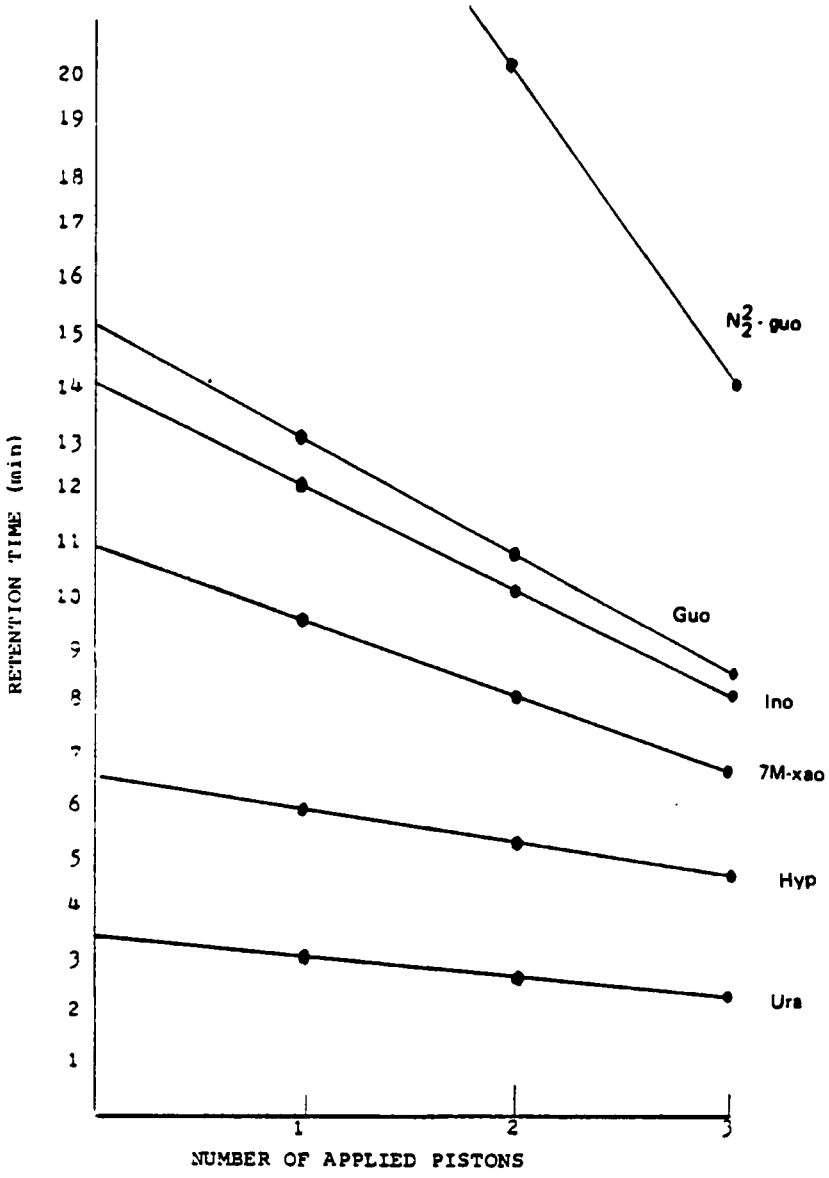

FIGURE 4.

Effect of radial-compression separation under different applied compression pressures. Flow-rate: 3.0 ml/min.

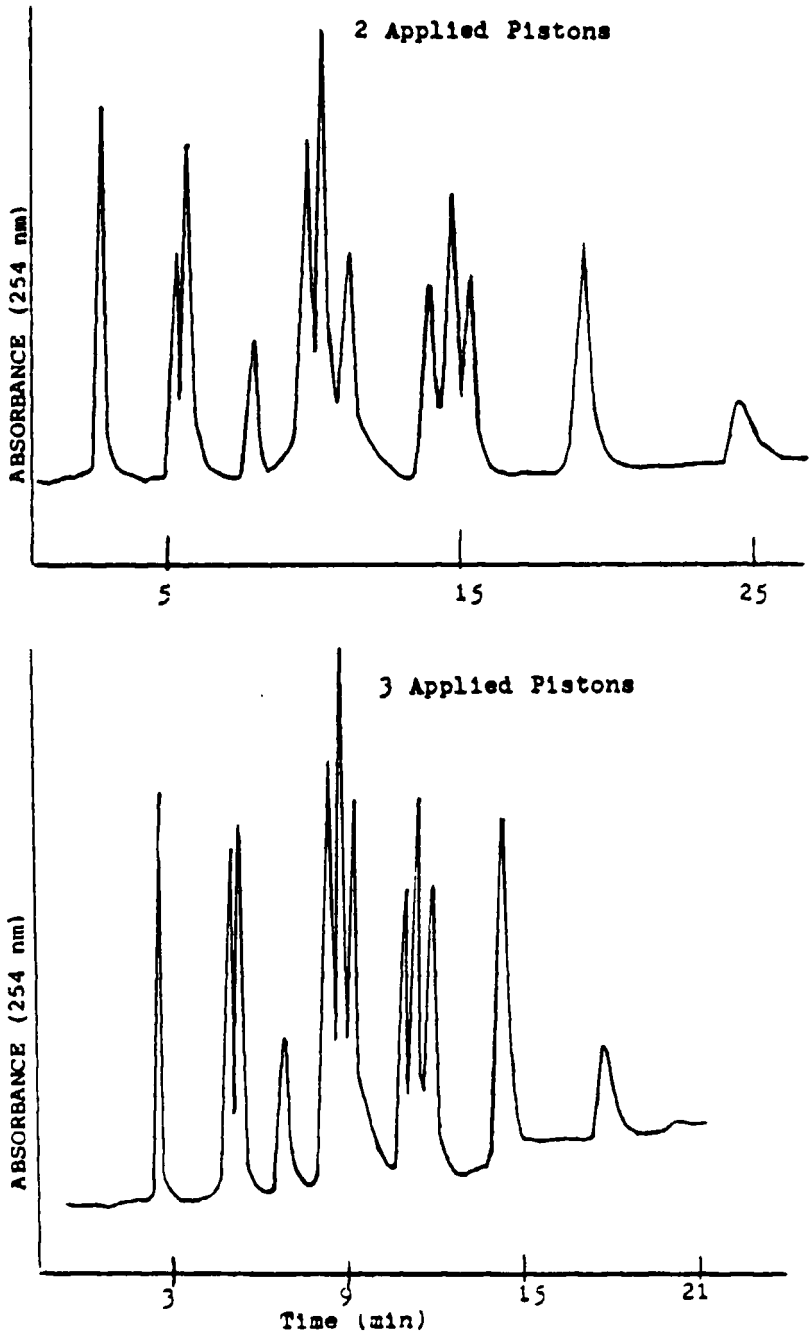

FIGURE 5.

Comparison of chromatograms obtained with a radially-compressed column under different compression pressures. Compounds from left to right are as listed in Figure 3. Conditions in text. Flow-Rate: 3.0 ml/min.

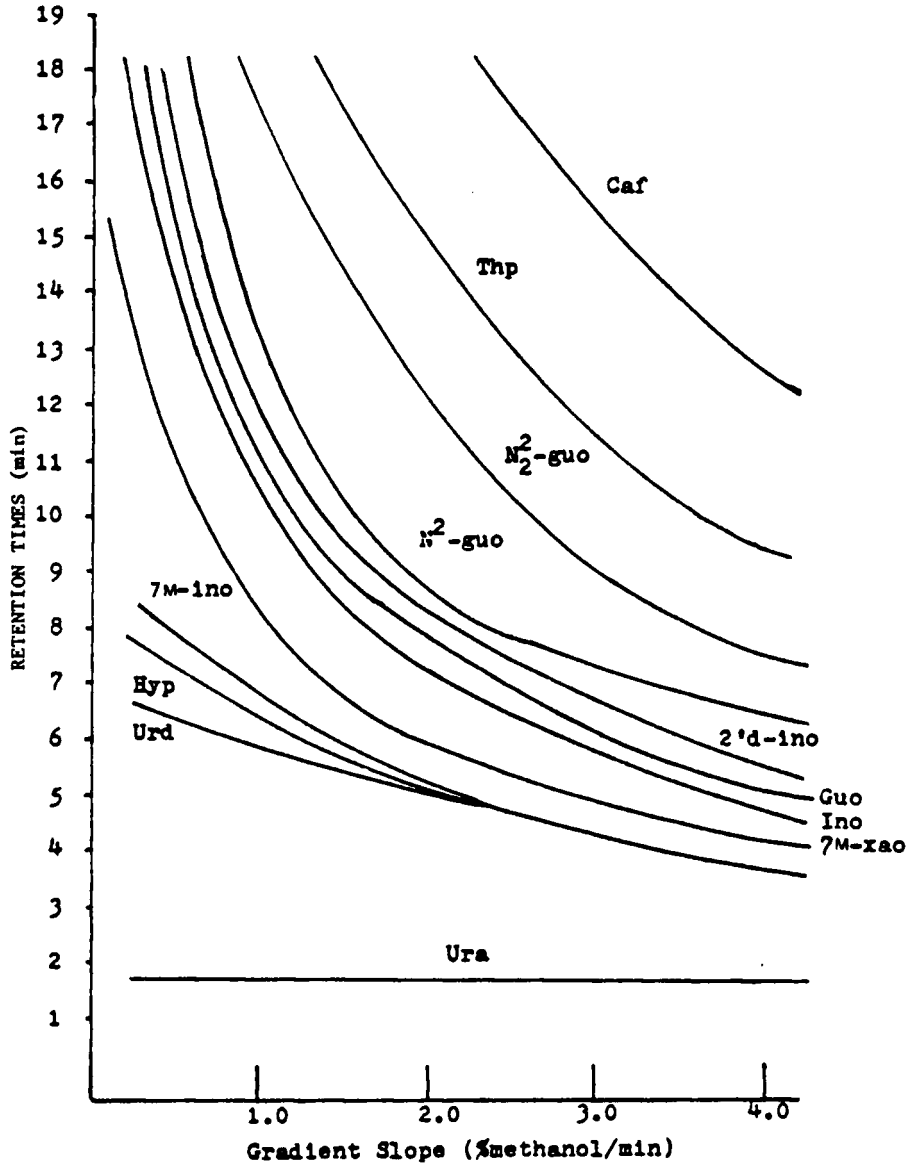

FIGURE 6.

Effect of increasing methanol concentration on the radial-compression separation of caffeine (Caf), theophylline (Thp), N^1-dimethyl guanosine (N^1-guo), N^2-methyl guanosine (N^2-guo), 2'-deoxy inosine (2'd-ino), guanosine (Guo), inosine (Ino), 7-methyl xanthosine (7M-xao), 7-methyl inosine (7M-ino), hypoxanthine (Hyp), uridine (Urd), and uracil (Ura). Conditions listed in text.

reach 100% of the final eluent (3:2 methanol-water) of the linear gradient. Increasing the percent change of methanol in the mobile phase had no effect on the compound uracil. However, retention times of the other compounds decreased dramatically.

The effects of pH of the initial eluent on the separations were also investigated for the radially-compressed columns. In Figure 7 retention times are plotted versus pH of the initial eluent in a gradient separation of selected compounds. To observe the effect of pH changes, the buffer concentration was held constant and the pH adjusted through the range 2.7 to 6.7 by addition of dilute KOH or H_3PO_4. Changing the pH of the initial eluent had little affect on the retention times of the selected compounds separated with gradient elution.

Based on these data, a rapid and efficient separation of the low molecular weight, UV-absorbing constituents of human sera was obtained. A sample from a person with no known diseases and on no medications was analyzed in less than 20 minutes using the radially-compressed column with a flow-rate of 3.0 ml/min and a gradient slope of 1.45% methanol/minute (Figure 8).

DISCUSSION

The efficiencies and reproducibilities of the radially-compressed columns used in this study were excellent. One radially-compressed column was used for approximately 500 analyses and there was less than 5% variability in retention times. Frequent and rapid solvent changeovers did not cause the radially-compressed columns to void. Equilibration times were significantly more rapid than with the rigid-walled columns because high flow-rates could be used. In addition, reversing the direction of flow through the column caused no ill-effects. These columns were readily regenerated after deliberate fouling of the column with the injection of high molecular weight materials. The use of greater flow-rates enabled more rapid regenerations when desired. Finally, improper handling, such as severe shocks, sonication, or

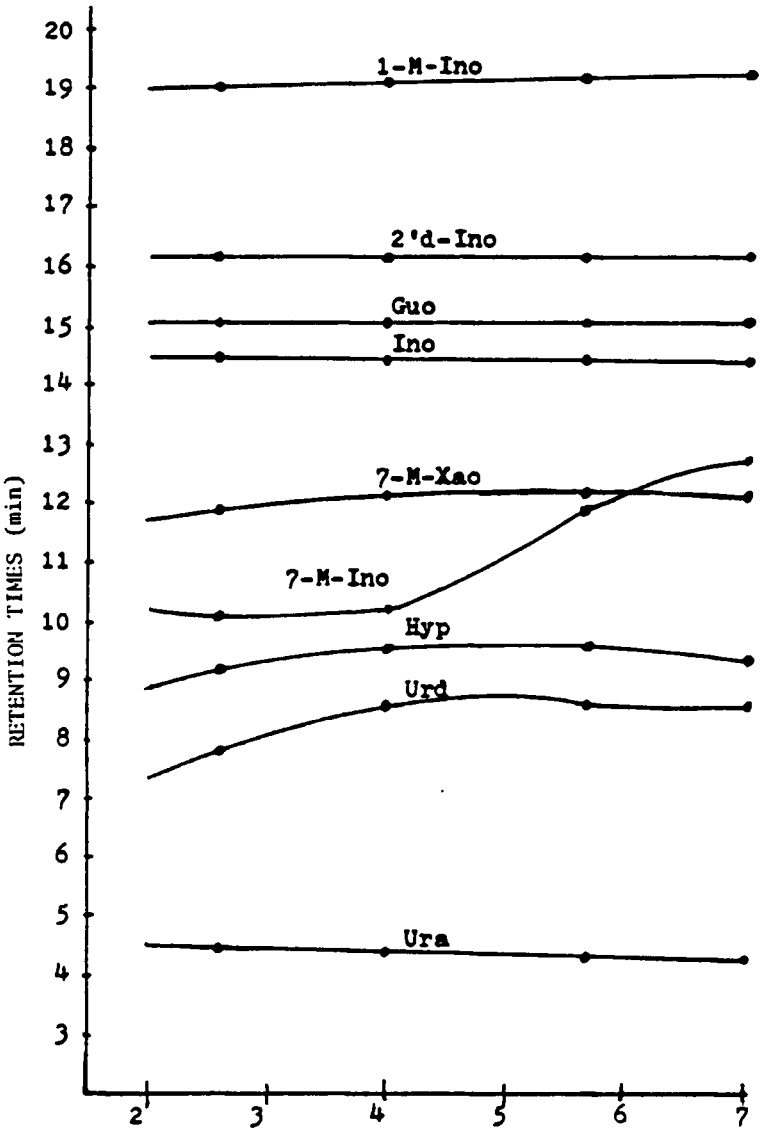

FIGURE 7.

Effect of different pH of the initial eluent in a gradient separation on a radially-compressed column. Conditions in text. Compounds: 1-methyl inosine (1-M-Ino), 2'-deoxy inosine (2'd-Ino), guanosine (Guo), inosine (Ino), 7-methyl xanthosine (7-M-Xao), 7-methyl inosine (7-M-Ino), hypoxanthine (Hyp), uridine (Urd), and uracil (Ura).

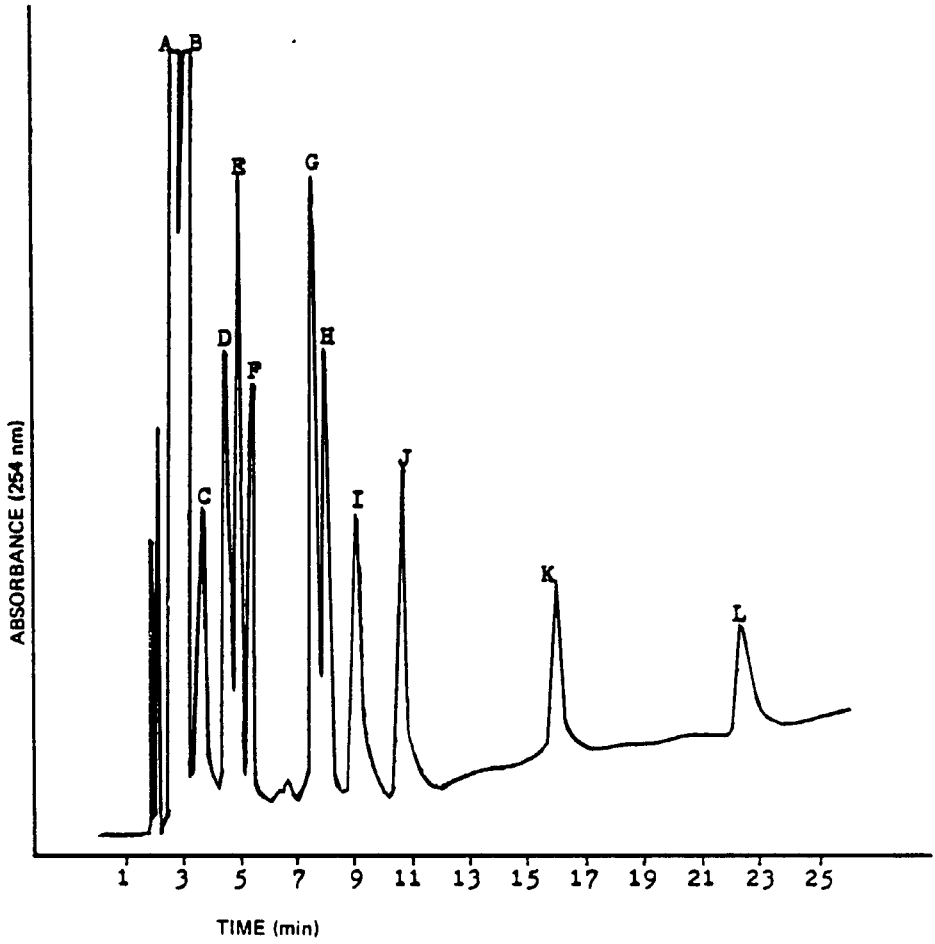

FIGURE 8.

Separation of human serum on a radially-compressed column. Conditions: Initial eluent - 0.02 M KH_2PO_4, pH 5.7; Final eluent - 60% Methanol-Water (v/v); Programming - 0 to 60% in 25 minutes; Flow-rate - 3.0 ml/min; Applied compression pressure - 3 pistons. Identifications: creatinine (A), uric acid (B), tyrosine (C), uridine (D), hypoxanthine (E), xanthine (F), inosine (G), guanosine (H), tryptophan (I), theobromine (J), theophylline (K), and caffeine (L).

drying out, did not cause any reduction of efficiency of these columns.

One problem noted with a radially-compressed column was the change of shape in the column walls, after prolonged use under a maximum pressure of 300 kg/cm^2. Subsequent use of this column showed that the compressibility of the column had decreased, thus causing a reduction in efficiency. Although another column developed a seepage of eluents through the heat-sealed fittings after several months use, the seepage was cured by resealing the end-fittings with heat.

It is important to stress that the radially-compressed columns used were prototypes and as with all prototypes, improvements can be expected in the final product.

These columns have great potential where analysis time is a major factor. Since the time per analysis can be reduced by 50%, these columns can be valuable wherever the analysis of large numbers of samples is required.

In addition, the long life of these columns, as well as the reproducibility obtained over extended usage, make them suitable for use in the clinical laboratory or in large scale biomedical investigations.

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