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USE OF SEQUENTIAL COLUMNS OF MICRORETICULAR AND PELLICULAR ION-EXCHANGE RESINS IN THE HIGH-RESOLUTION SEPARATION OF COMPLEX BIOCHEMICAL MIXTURES*

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SUMMARY

The high-resolution separation of metabolites in physiologic fluids can be achieved by high-pressure ion-exchange chromatographic systems that use very-small-diameter microreticular resins. The resin phase contributes to diffusional resistance that results in a relatively slow separation process. Conversely, the recently developed pellicular resins, which have only an active outer film, achieve relatively rapid separation but at the expense of a severe reduction in capacity that makes high-resolution separations of complex mixtures very difficult.

An interesting concept is to combine these two types of ion-exchange resins by using sequential columns. The first column, containing microreticular resin, would contribute the necessary capacity for a preliminary separation. Subsequently, the second column, containing pellicular resin, would provide a rapid, final separation. Preliminary results from anion-exchange chromatographic systems indicate that this technique will be useful in the separation of ultraviolet-absorbing constituents of physiologic fluids.

INTRODUCTION

Several high-resolution analytical systems using high-pressure liquid chromatography (LC) have been developed for the analysis of various physiologic fluids[1–7], some of which contain literally hundreds of constituents. In this context the term "high-resolution analysis" has been chosen to describe an analysis in which a large number, or all, of the constituents of a complex sample mixture are separated and quantified. These LC systems require a relatively long separation time (measured in many hours), resulting in a low sample throughput rate.

Recently several operational options have been investigated in an effort to increase sample throughput in systems using high-pressure ion-exchange separations.

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These have included: (a) decreasing the stationary phase particle size to increase the rate of mass transport and thus to increase the rate of separation[2–4,6,7]; (b) use of coupled columns of anion-exchange resin followed by cation-exchange resin[8]; and (c) use of two or more parallel columns to give several simultaneous analyses[9,10].

An extension of the option listed in (b) above involves combining other types of separation media in sequential columns. An approach somewhat similar to that suggested by Snyder[11] is currently being considered and the preliminary data look very promising. In this case, sequential columns of microreticular (homogeneous matrix of small pores) anion-exchange resin are being used in combination with pellicular (solid core surrounded by an active film) anion-exchange resin for the separation of the ultraviolet (UV)-absorbing constituents of body fluids. Separation times can be significantly reduced compared with that required by the original system, which used only microreticular resin.

CONCEPTS

All of the discussion will center around the separation of the UV-absorbing constituents of body fluids as demonstrated by the previously developed UV analyzer[2,3,7]. This system has previously been characterized by relatively long columns of very small microreticular anion-exchange resin beads, and operates at high pressures to yield over 100 detectable chromatographic peaks from a single sample. Although very small ion-exchange resin beads are used, the controlling mechanism for separation is still solid-phase mass transport and the small particles in columns up to 150 cm long contribute to flow resistance that limits the eluent flow-rate even when pressures as high as 4000 p.s.i. are used. Unfortuantely, this separation is characterized by a relatively long run time.

Pellicular resin

The introduction of pellicular resins[12] and their subsequent development allow consideration of somewhat different concepts. With this material two primary factors have to be considered:

(1) The sorption medium is contained only in an outer, active film having no access to the solid center core; therefore, sorption resistance by solid-phase mass transport is minimal.

(2) On the other hand, the lack of active material throughout the stationary phase results in extremely low sorption capacity. Thus, one has a trade-off of speed *vs.* capacity.

In instances where relatively simple mixtures (small numbers of solutes) are involved or where the detector sensitivity is sufficiently high to require minimal sample size, the lack of sorption capacity does not present a problem and pellicular resin can be used successfully to provide a rapid high-resolution separation. However, for complex mixtures or for detection systems that require relatively large amounts of each of the solutes for quantitation, the sorption capacities of the pellicular resins are too limited to provide even a useful separation. This is certainly the case in the separation of physiologic fluids containing hundreds of solutes, especially urine, where current column monitoring systems require at least an 80-μl sample for good quantitative results.

Coupled microreticular and pellicular resins

An interesting concept is to combine the most useful features of the two different kinds of sorption media, the capacity of the microreticular resin and the speed of the pellicular resin, into a single separation scheme. Such a scheme would include coupling in series a column containing a small amount of microreticular resin followed by a column containing a large amount of pellicular resin, with the eluate of the first progressing directly to the second. The first column would provide a preliminary, but somewhat crude, separation that would be followed by a more rapid, final separation in the second column.

Operating parameters

To a degree, the coupling of two widely different sorbent materials with a common eluent results in a mismatch that must be circumvented. That is, conditions that will result in elution of the solutes from the microreticular resin are not necessarily optimal for the separation process using pellicular resin. This is especially true when relatively complex separations are required for a mixture of solutes possessing a wide range of distribution coefficients. For example, in the separation of the UV-absorbing constituents of urine by microreticular anion-exchange chromatography, the distribution coefficients can vary from values near unity to values several orders of magnitude higher in the presence of the initial eluent. A useful separation will require the use of gradient elution, in which the eluent (acetate buffer, pH 4.4) concentration is increased from 0.015 *M* to 6 *M* and the column temperature is varied from 25 to 60°. Both the higher buffer concentration and the higher temperature reduce the distribution coefficients of the solutes.

The increase in buffer concentration and temperature allow the more strongly sorbed species to be ultimately eluted from relatively long columns as detectable, relatively narrow chromatographic peaks. Unfortunately, the apparent or effective distribution coefficients for urinary constituents separated on pellicular resins are much smaller than those for microreticular separations; hence changes in the eluent concentration affect the distribution coefficients to a greater extent. It is conceivable, then, that the increased eluent concentration necessary to promote earlier elution of the strongly sorbed species on microreticular resin could essentially destroy resolution on the pellicular resin since all of the distribution coefficients would be reduced to very low values. For sequential column operation relatively small amounts of microreticular resin should be used so that a less severe buffer gradient will be required for rapid elution. Increasing the operating temperature is an effective means for increasing the rate of elution from the microreticular column, since the pellicular column that follows can easily be cooled and operated at a lower temperature.

DESCRIPTION OF THE SYSTEM

The UV analyzer, as modified for this study, was an automated, gradient-elution LC system capable of operation at pressures as high as 4000 p.s.i.[2,3,7]. It included the following major components (see Fig. 1): two vertical and connected stainless-steel, closed tubular columns packed with particles of solid ion-exchange material; an eluent storage and gradient-elution preparation reservoir connected to a high-pressure eluent pump; a sample injection valve for introducing 10- to 100-μl

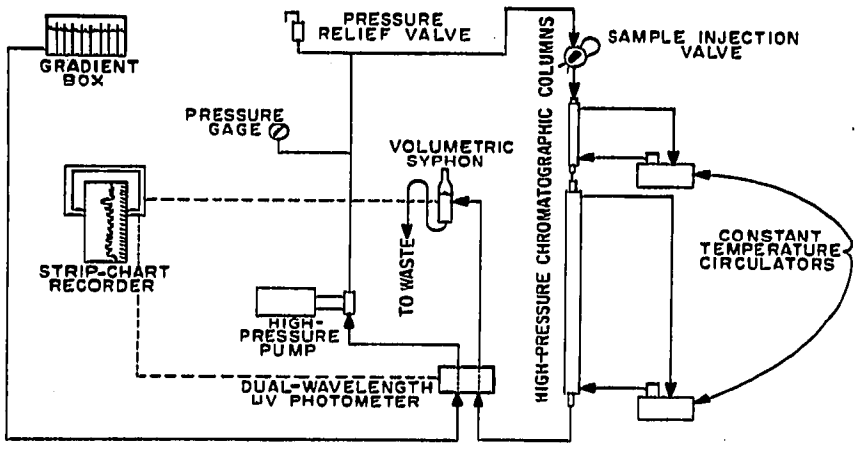

Fig. 1. Coupled-column chromatography with sequential columns of microreticular and pellicular anion-exchange resins for the analysis of the UV-absorbing constituents in physiologic fluids.

samples; and a dual-channel flow photometer connected to the column for detecting and quantifying separated constituents. Readings of the eluate absorbance at two wavelengths were recorded on a conventional strip-chart recorder as a function of time.

Columns

The chromatographic columns were fabricated from nominal 1/8-in. O.D., Type 316 seamless, stainless-steel tubing with a 0.22-cm I.D. The reference separation was performed on a 150 cm long column that contained microreticular anion-exchange resin (12- to 15 μm-diameter Aminex A-27, Bio-Rad Lab., Richmond, Calif., U.S.A.). In the separations with sequential columns, column lengths varying from 25 to 150 cm were used; however, the best results were obtained with a system comprised of a 50-cm column of Aminex A-27 resin coupled to a 150-cm column containing pellicular resin. Several different types of pellicular resins were used with somewhat comparable results, but the results reported here are for Pellionex AS (H. Reeve Angel, Clifton, N.J., U.S.A.). This material, characterized by a very thin film of active anion-exchange resin on a solid spherical core, had an average particle size of about 45 μm diameter. The columns were enclosed in stainless-steel jackets containing a circulating heating–cooling fluid that was temperature-controlled.

Eluent system

A nine-chamber eluent storage and gradient-elution preparation box was used (Phoenix, Gardiner, N.Y., U.S.A.) with a high-pressure positive-displacement pump (Microflow Pulsafeeder, LS-30, Lapp Insulator Co., Leroy, N.Y., U.S.A.). The eluent was an ammonium acetate–acetic acid aqueous buffer (pH 4.4) that increased in acetate concentration from 0.015 *M* to 6 *M*. Operating pressures up to 4000 p.s.i. were used.

Sample introduction

The six-port valve used for sample injection was similar to a previous design[13] except that it had been modified for sample sizes in the range of 10 to 100 μl. It

operates by allowing an external sample loop to be filled at ambient pressure; then, after the internal connecting ports have been oriented by turning the valve handle, the sample loop becomes part of the eluent stream and the sample is injected. The smaller samples were introduced by leaving the valve in the sample-inject orientation just long enough for the desired sample volume to be flushed out of the sample loop.

Detection system

The column monitor was a small, dual-beam UV photometer operating at 254 and 280 nm[14]. The vertical flow cuvet had an effective path length of 2.8 mm, and the photometer was operated with a gain of five times in transmittance, *i.e.* 80–100% transmittance. Detector outputs for each wavelength were recorded on a dual-pen strip-chart recorder.

Operation

The eluent flow-rates were adjusted to 8.0 ± 0.1 ml/h and 12.0 ± 0.1 ml/h at the beginning of each run for the single 150-cm microreticular column system and the sequential column system, respectively. Each run was initiated by introduction of a sample, at which point the eluent buffer concentration gradient was begun. The column temperatures were maintianed initially at ambient (25° ± 1°), increasing to 60° at 3 h for the single microreticular resin column and to 60° and 40°, respectively, at 1 h for the coupled microreticular and pellicular resin columns.

RESULTS AND DISCUSSION

The goal of this study was to achieve a rapid separation and high resolution of at least the major UV-absorbing constituents of physiologic fluids. As a reference, a typical separation of 80 μl of urine on a 150-cm microreticular resin column requires about 24 h and over 100 chromatographic peaks can be detected when the column eluate is monitored for absorption at both 254 and 280 nm (see Fig. 2). More than three-quarters of the typical UV chromatographic peaks from the anion-exchange separation of normal urine have been identified[7]. In evaluating the different modes of operation, some of these identified peaks have been labeled for use as indicators for the effectiveness of the separation. Chromatographic positions of these indicator peaks on pellicular resin separations were established by absorbance ratios at the two wavelengths (254 and 280 nm).

As shown by the results presented here, the combined use of microreticular and pellicular anion-exchange resins in sequential columns does provide a unique separation. The concept is relatively straightforward, although nearly all of the operating parameters affect the separation. Some of these parameters were investigated in this study; on the other hand, most were arbitrary choices, and this must be taken into consideration when the results are evaluated.

Properties of pellicular resins

In preliminary tests, four different types of pellicular resins were found to have at least the general properties indicated by the data reported here for Pellionex AS resin. All of these materials had very low capacities when compared with the microreticular resins (a factor of 200 to 500 less), and the cyclic operating conditions of the

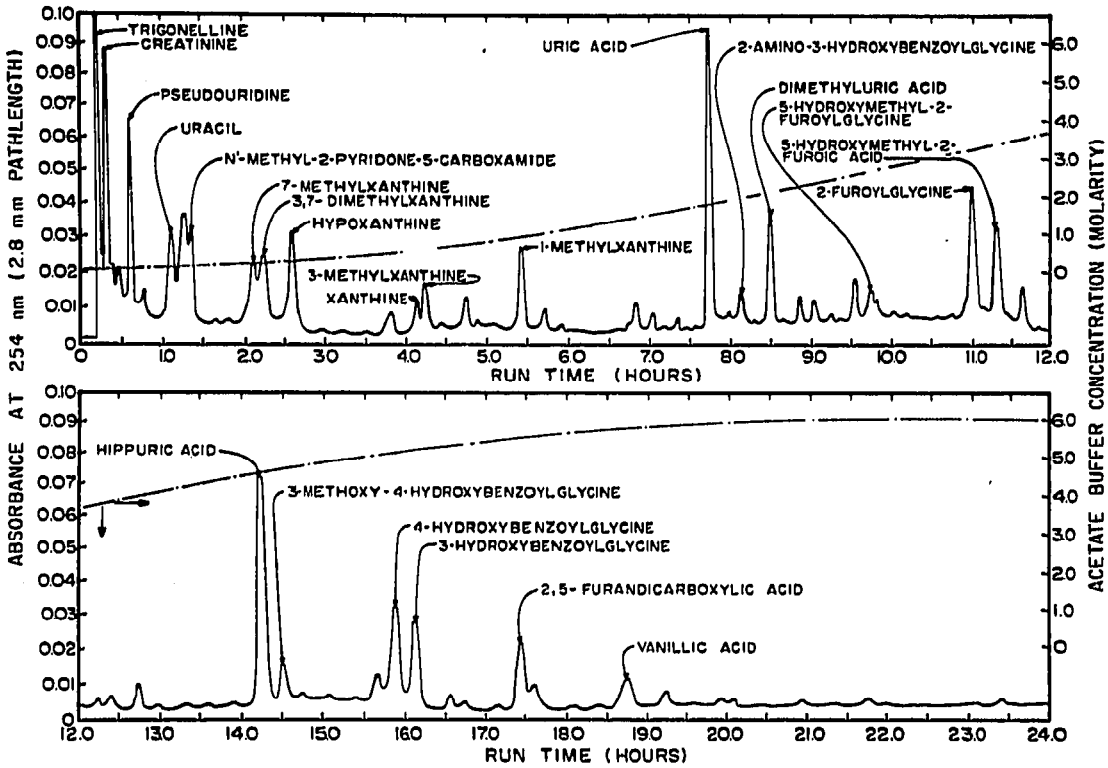

Fig. 2. Separation of the UV-absorbing constituents of an 80-μl sample of a reference urine on a 0.22 × 150 cm column of microreticular anion-exchange resin (Aminex A-27, 12- to 15-μm diameter). Eluent, acetate buffer (pH 4.4); average flow-rate, 8.0 ml/h; temperature, increasing from ambient to 60° at 3 h.

separation (changing buffer concentrations and temperature) apparently resulted in a further decrease in capacity. For example, the anion-exchange capacity of the Pellionex AS resin for Cl^- in 0.01 *N* HCl varied from 0.007 to 0.005 mequiv./ml of bed after ten and twenty analytical cycles, respectively. Some of the pellicular resins also tended to undergo physical degradation with apparent loss of the outer ion-exchange film, portions of which tended to plug the support filter at the bottom of the column. It should be noted that this operating cycle is very severe as compared with many other useful applications for pellicular resins. Nonetheless, interesting results were achieved using the sequential-column configuration.

Column configuration

Test were made in which the lengths of the individual columns (both microreticular and pellicular) were varied from 25 to 150 cm in increments of 25 cm. No apparent increase in resolution was achieved by connecting a 150-cm pellicular resin column in series to the initial 150-cm microreticular resin column; in fact, there was a noticeable increase in the peak bandwidths which tended to reduce resolution.

Using shorter individual microreticular resin columns tended to decrease the time of separation, resulting in much sharper chromatographic peaks but signifi-

cantly decreased resolution. However, when microreticular resin columns 50 cm or less in length preceded longer pellicular resin columns in a sequential mode of operation, there was a noticeable increase in resolution over that obtained with the individual short microreticular column, even at higher flow-rates. When the pellicular resin column preceded the short microreticular resin column, there was actually a decrease in the resolution as compared with the individual microreticular resin column.

The optimum column configuration for the selected size range and operating conditions was a 50-cm microreticular resin column followed by a 150-cm pellicular resin column.

Separation on pellicular resin alone

Although many simple biochemical mixtures (*e.g.*, the common nucleotides or nucleosides) have been effectively separated on pellicular resins, complex mixtures such as urine present a more difficult task. However, consideration of the separation of urinary constituents helps give an insight into the effects of some parameters in the sequential-column operation. Operating conditions were those chosen for optimum separation of a 40-μl urine sample on a 50-cm microreticular resin column.

Even though the pellicular resins have very low capacities, some of the strongly sorbed components in urine still require relatively high concentrations of the acetate buffer for elution. As shown in Fig. 3A, more than 25 chromatographic peaks were separated from 2.5 μl of a reference urine (diluted to 10 μl) in 5 h while using a standard eluent buffer concentration gradient. When the sample size was increased to 10 μl (undiluted), some additional peaks were detected; however, the main effect was to contract severely the front part of the chromatogram with loss of resolution (Fig. 3B). This increase in solute concentration obviously overloads the resin, causing a significant decrease in retention volumes. An increase in the sample volume from 10 μl to 80 μl by additional dilution of the sample also affects the separation, primarily in the front end of the chromatogram where some band-spreading is evident (Fig. 3C). There is no apparent effect on elution volumes. This dilution effect is representative of what occurs in the preliminary separation by the microreticular resin in the coupled-column system. That is, small fractions of the original urine sample are significantly diluted and sequentially introduced to the pellicular resin column.

Sequential column operation

Decreasing the urine sample size from 80 μl to 40 μl gave a better separation for the sequential-column system; however, additional decreases in sample size were counter-productive since the small chromatographic peaks were no longer detected. As expected, a system consisting simply of a shorter microreticular column (*e.g.*, 50 cm long) gave a much more rapid separation but less satisfactory resolution than the regular 150-cm microreticular resin column (Fig. 4A). The addition of a 150-cm pellicular resin column in sequence did not greatly affect the length of time for the separation but did result in additional resolution, especially in the early portion of the chromatogram (Fig. 4B).

This more rapid separation with a somewhat smaller sample does have utility as a screening tool since most of the expected major chromatographic peaks can be identified and quantified. For example, when one uses this analytical system to compare normal urine with the urine from a cancer patient undergoing *allo*purinol therapy

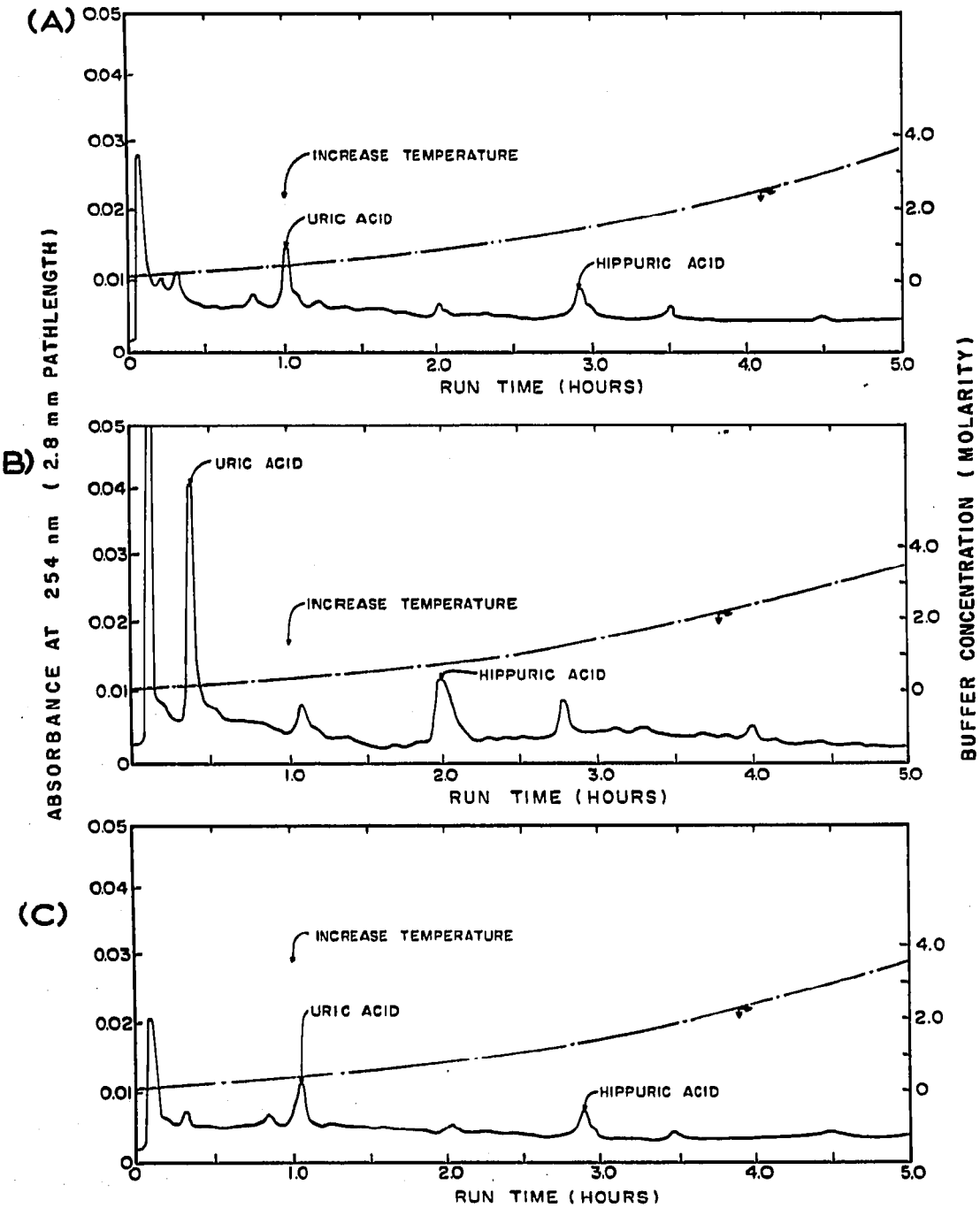

Fig. 3. Effects of solute concentration and sample size on the separation of UV-absorbing constituents of urine on a 0.22 × 150 cm column of Pellionex AS pellicular anion-exchange resin. Eluent, acetate buffer (pH 4.4); average flow-rate, 12.0 ml/h; temperature, increasing from ambient to 40° at 1 h. Samples: (A) 2.5 μl reference urine diluted to 10 μl; (B) 10 μl reference urine; (C) 2.5 μl reference urine diluted to 80 μl.

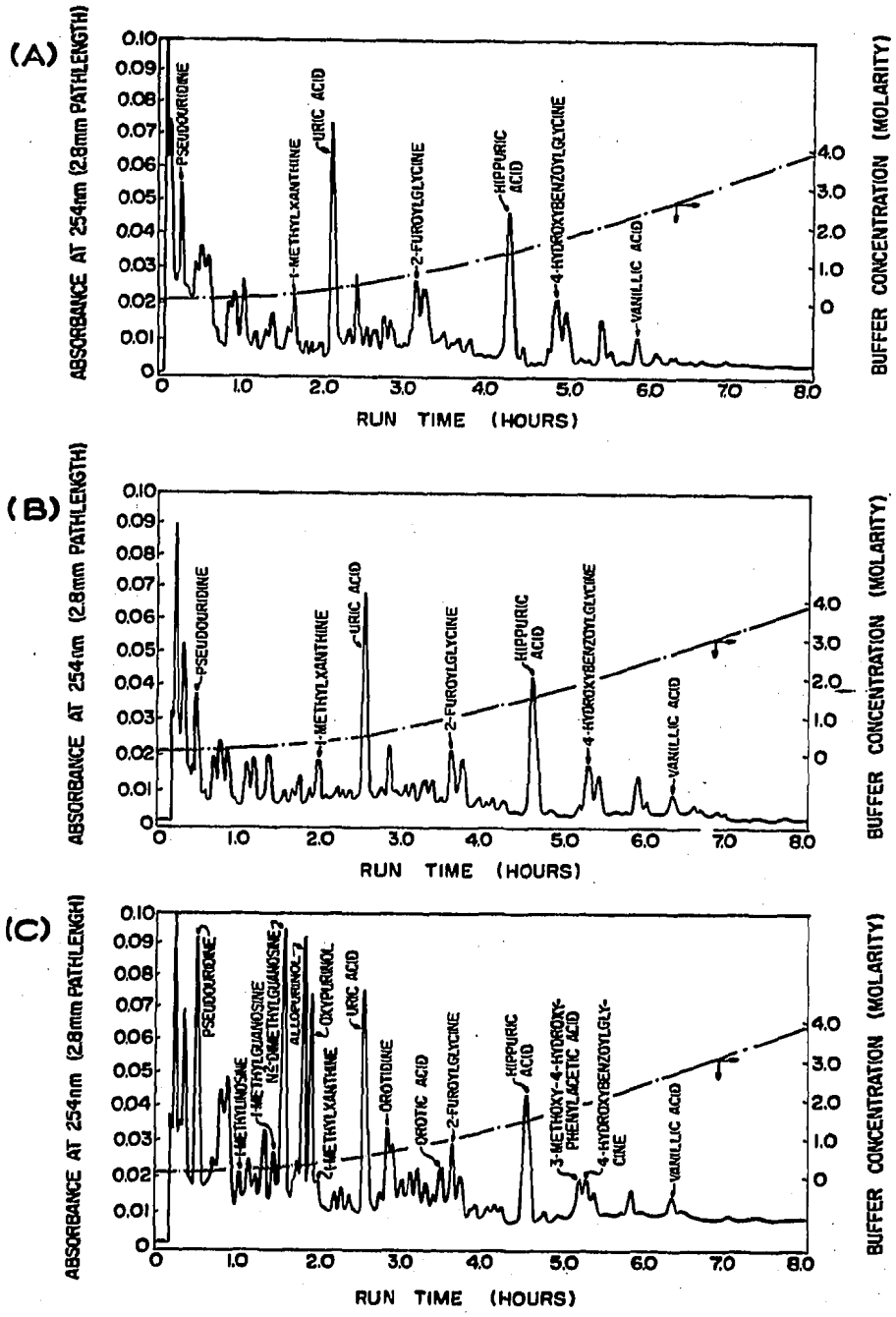

Fig. 4. Comparison of the separation of the UV-absorbing constituents of urine on a short, 50-cm, column (A) of microreticular anion-exchange resin (Aminex A-27, 12- to 15-μm diameter) and on sequential columns of microreticular (50 cm) and pellicular (Pellionex AS) (150 cm) resins (B and C). Eluent, acetate buffer (pH 4.4); average flow-rate, 12.0 ml/h; temperature, increasing from ambient to 60° and 40°, respectively, for the two columns at 1 h. Samples: (A and B) 40 μl normal reference urine; (C) 40 μl pathologic urine.

(Fig. 4C compared with Fig. 4B), increased excretion of several methylated compounds that may be indicative of the cancer is seen (1-methylinosine, 1-methylguanosine, and N^2-dimethylguanosine) as well as the drug (*allo*purinol), its major metabolite (oxypurinol), and the increased excretion of orotidine and orotic acid that is apparently caused by the drug[15].

CONCLUSIONS

The coupling of a short column containing small, microreticular ion-exchange beads in series with a longer column containing pellicular ion-exchange resin has been shown to produce a more rapid separation of the constituents of physiologic fluids than is observed for microreticular resin alone. In addition, the resolution is higher than that obtained with pellicular resin alone. Apparently the first column contributes capacity for a preliminary separation, subsequently allowing the pellicular resin column to provide a fast, final separation without loss of resolution due to overloading. Currently available pellicular resins have been shown to be useful in such a separation scheme; however, there is apparently some loss of capacity after many analytical cycles, part of which may be due to loss of some of the ion-exchange film.

The manufacturers of pellicular resins will undoubtedly continue to upgrade their products until such materials are relatively stable, even when subjected to severe chemical and physical changes such as those in the separation scheme described here. From our standpoint, it would also be desirable to have a pellicular resin with significantly greater capacity (*e.g.*, one-fiftieth of that of the microreticular resin) for separation of the components of complex physiologic fluid samples. This would allow use of larger samples without the risk of overloading the capacity of the pellicular resin. An increase in capacity could be achieved with current technology by increasing the thickness of the ion-exchange resin film or by decreasing the particle size. The latter would be relatively easy. In this laboratory, an alternative approach to pellicular resin manufacture is being investigated. In this approach, the inner pores of conventional microreticular resin are filled with an inert material which allows mass transport only through the micropores of an outer film of the resin. Preliminary results with this "corefilled" ion-exchange resin look promising.

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