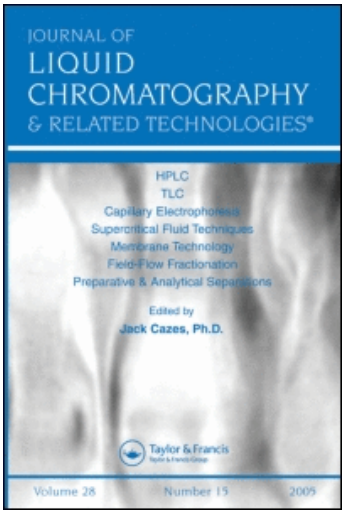

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Sample Preparation Techniques Prior to HPLC Analysis of Serum Nucleosides and Their Bases

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SAMPLE PREPARATION TECHNIQUES PRIOR TO HPLC ANALYSIS OF SERUM NUCLEOSIDES AND THEIR BASES

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ABSTRACT

Techniques for sample preparation and handling of serum prior to analysis for nucleoside and bases were evaluated. Efficiency data are reported for the trichloracetic acid (TCA), ammonium sulfate, ultrafiltration and pre-column concentration sample preparation techniques. Maximum recoveries for most of the compounds were obtained using the ultrafiltration, ammonium sulfate and pre-column concentration techniques. Poorer recovery was observed using the TCA/Freon-amine methods. Theophylline and tryptophan exhibited pH dependent recoveries using the ultrafiltration, ammonium sulfate and pre-column techniques.

INTRODUCTION

In the last several years there have been tremendous advances in the development of methods for the analysis by high performance liquid chromatography (HPLC) of the nucleosides and bases in serum and other biological fluids. While both cation and anion-exchange columns have been used (1-7), the analysis of picomole quantities of nucleosides and bases in complex sample matrices was made possible (8-11) by recent improvements in reversed-phase

chemically-bonded microparticle packing materials. However, concomitant improvements in the procedures used to prepare samples prior to analysis by HPLC have not been made. Consequently, there exists a need for sample preparation techniques which are rapid, reproducible and compatible with the high sensitivity of HPLC analyses.

The major requirement in the preparation of biological samples prior to analysis by HPLC is the removal of proteinaceous material from the sample. This is necessary both to eliminate interferences and to prolong column life. Several methods used to deproteinate biological samples are presented in Table 1. The use of strong acids, such as perchloro- or trichloroacetic acids (PCA or TCA), have classically been widely used techniques. In this case, a combination of low pH and high ionic strength cause precipitation of the macromolecules from the solution. With the TCA and PCA methods it is sometimes necessary to remove the acids and

TABLE 1

Deproteinization Methods

Principle	Example
Dielectric change	Acetonitrile or Ethanol
Temperature change	Boiling water bath
Specific complexation or adsorption	Borate gels, pre-column concentration
pH change	TCA and PCA
Ionic strength	Ammonium sulfate
Filtration	Ultrafiltration with membranes

their salts from the sample. Recently, the extraction of the acid from the sample with a water-insoluble amine dissolved in Freon was reported by Khym (12) and was found to be compatible with microparticle, chemically-bonded anion exchange columns (13). However, for nucleosides and bases the efficiency of extraction from serum was not determined.

Several other methods are available for the deproteinization of biological samples. Proteins may be precipitated by decreasing the dielectric constant of the solution with the addition of an organic reagent such as ethanol or acetonitrile. Precipitation of proteins can also be accomplished by increasing the ionic strength, e.g. the addition of a high concentration of a salt such as ammonium sulfate to the solution. Another less selective approach is to denature the proteins by heat.

A different approach taken by Gehrke and his group is the selective removal of nucleosides from biological samples by specific complexation with a borate-gel polymer (9,14).

A variation of this technique is the use of a small cartridge packed with a relatively non-selective stationary phase. The sample is injected by a syringe into the pre-column. Compounds which are compatible with the stationary phase are retained under specific conditions while proteins and undesired compounds are washed through the column. The compounds of interest are then eluted with a suitable solvent.

Since our research involved not only nucleosides but also bases and several amino acids, the selective removal of the

nucleosides alone was not compatible with our work. Of the other techniques available, heating of the sample was not chosen because of the gel-like consistency of serum after denaturation and also because of the thermal lability of many of the compounds of interest. The use of an organic solvent to precipitate proteins also was not investigated in detail because of the low solubility of several of the nucleosides and bases in many organic solvents.

Another method of protein precipitation, the Iomogi technique ($ZnSO_4$-$Br(OH)_2$) was considered, but not used, since this technique removes uric acid as well as the serum proteins.

Therefore, the procedures which were investigated for sample preparation prior to HPLC analysis were; TCA - amine-freon, ammonium sulfate, ultrafiltration and pre-column sample concentration. They were evaluated for their efficiency of recovery of added standards from the serum matrix, and for their general suitability with respect to interferences, ease of use, cost and speed.

EXPERIMENTAL

Apparatus

A Waters ALC 202 Liquid Chromatograph (Water's Associates, Milford, MA) with a Model 440 absorbance detector, and a Model 660 solvent programmer, was used. Quantitation of the nucleosides and bases was at 254 nm using a Hewlett-Packard Model 3380-A integrator (Hewlett-Packard, Avondale, PA). Simultaneous detection at 280 nm was used with a dual-pen recorder to obtain peak-height ratios as an aid for the confirmation of peak identities.

For the ultrafiltration studies centrifuge cones (Amicon Corp., Lexington, MA) Type CF 25 were used. These cones had a pore size retaining compounds with a nominal molecular weight of greater than 25,000. Sep-Pak(R) cartridges were obtained from Waters Associates. These mini-columns were pre-packed with pellicular C_{18} packing material.

All centrifugations were performed on a Dynac centrifuge, (Clay, Adams, Parsippany, NJ) with a maximum force of 1145 relative centrifugal force (RCF). Samples were vortexed on a Vortex-Genie K 550-G (Scientific Industries Inc., Springfield, MA). Pipettings were accomplished using Eppendorf pipettes of the appropriate volumes (Brinkman Instruments, Westbury, NY).

Columns

Pre-packed analytical columns from Waters Associates were used. These 4.6 mm x 30 cm stainless steel columns were packed with μBondapak C_{18} packing material (10 μm in diameter). A 4.6 mm x 5 cm stainless steel pre-column packed with pellicular C_{18} material (Whatman Inc., Clifton, NJ) was used to protect the analytical column.

Chemicals and Supplies

The mobile phase for the liquid chromatography was prepared from reagent grade potassium dihydrogen phosphate (Mallinckrodt Chemical Works, St. Louis, MO), and from spectral grade methanol (Burdick and Jackson, Muskegon, MI). Distilled deionized water was used throughout. Prior to use, aqueous buffers were degassed by an aspirator vacuum. A stream of He (60 ml/min for 30 sec) was

bubbled through the methanol/water solutions instead of a vacuum in order to avoid changes in the composition of these solutions. The tri-n-octyl amine was purchased from ICN Pharmaceuticals, Inc., Plainview, NY and the Freon(R) from Matheson Gas Products, East Rutherford, NJ.

The nucleoside and base standards were purchased from Sigma Chemical Co., St. Louis, MO, and were of the highest purity grades available.

Serum Collection and Storage

Extraction studies were carried out on single lots of pooled human serum in order to eliminate any sampling variances. All sample preparations were carried out on the same day, and the extracts were immediately frozen. The prepared samples were chromatographed within 48 hours.

Chromatographic Conditions

A gradient elution mode was used, with an initial eluent of 0.02 mol/L KH_2PO_4, pH 5.6 (adjusted with KOH), and a high strength eluent of 60% MeOH/water (v/v). A linear gradient of slope 0.69% organic/min was used. The flow rate was 1.5 ml/min. All analyses were carried out at ambient temperature.

Sample Preparation Techniques

TCA-amine-Freon Method

The TCA-amine-Freon(R) method used was first reported by Khym (12), and optimized for nucleotides and nucleosides by Van Haverbeke and Brown (13). One ml of pooled serum and 1 ml of a standard 1 mM solution of the nucleosides and bases of interest

were vortexed vigorously for 2 minutes with 2.00 ml of 12% TCA. After centrifugation for 6 minutes at 1145 RCF, the supernatant was filtered through a Millipore Type GS membrane filter (0.22 micrometer pore size). A 1.00 ml aliquot of this filtrate was then vortexed for 2 minutes with an equal volume of 0.5 M tri-N-octyl amine dissolved in Freon(R). After a brief, low speed centrifugation, the upper, aqueous layer was withdrawn and immediately frozen.

Ammonium Sulfate

Standard solutions were prepared by dissolving weighed amounts of the standards in 0.01 mol/L KH_2PO_4/Na_2HPO_4, pH 6.88. 1.00 ml of a standard solution of the nucleosides was added to 4.00 ml of serum thus producing a 20% dilution of the serum matrix. The concentration of the standards was approximately 2×10^{-5} M in the diluted serum.

For the ammonium sulfate technique, an equal volume of saturated ammonium sulfate (25°C) was added to the serum-standard mixture. After 2 minutes of vortexing, the samples were centrifuged at 1145 RCF for 10 minutes and the supernatant fluid filtered through a Millipore membrane filter, Type GS (Millipore Corp., Bedford, MA). The filtered samples were immediately frozen in plastic vials.

Ultrafiltration

For the ultrafiltration method, the samples were filtered directly through the ultrafiltration membrane cones. Uniform centrifugation times (15 min) and speeds (500 RCF) were used to insure reproducibility.

Pre-Column Concentration

One ml aliquots of serum were passed through the Sep-Pak(R) cartridges followed by 1.0 ml of 0.02 mol/L KH_2PO_4, pH 5.6 to wash out the proteins and unretained compounds. 1.0 ml of 60% methanol-water (v/v) was then passed through the cartridges while the effluent was collected. Aliquots of the effluent were then injected directly into the HPLC.

Experimental Design

Model Compounds

For the initial TCA studies the nucleosides, xanthosine, inosine and guanosine were chosen as model compounds. Inosine and guanosine are endogenous to human serum, and preliminary studies indicated that the recovery of these compounds from serum was poor using the TCA techniques.

For the other techniques, recoveries from serum of added hypoxanthine, theobromine, theophylline, caffeine, and the amino acid, L-tryptophan were also evaluated. These compounds were chosen because of their diverse range of chemical functionalities and the possibility of their occurrence in human serum.

Statistics

In order to obtain statistically valid results, at least 5 independent samples were prepared for each of the methods, using the same lot of pooled serum. Three independent blanks were prepared simultaneously. The recoveries of the standards which had been added to the sera were calculated by first subtracting the average areas of the peaks in the blanks from the areas of the

samples with the added standards. These areas were then compared with those of the aqueous standard solutions which had been injected directly. The appropriate dilution factors and injection volumes were taken into account. All statistics are reported at the 95% confidence limit unless otherwise stated.

Calculations for Pre-Column Method

When using the pre-column method (Sep-Pak$^{(R)}$), it is useful to be able to determine approximately the conditions necessary for the elution or retention of the compounds of interest. This may be facilitated by the following approach. Since it has been shown previously that the ln k' is approximately linear with the concentration of organic solvent in the mobile phase (15), then it is possible to derive equation 1, where A is defined as m, m being the slope of the ln k' vs. % organic plot. The k'_o is the y intercept of the ln k' plot V_o for the sample cartridge is easily determined by weighing a cartridge dry and after being filled with water.

$$V_R = V_o \cdot (k'_o \cdot e^{-AC} + 1) \qquad \text{Eq. 1}$$

If the intercept and the slope of the plot of ln k' vs. organic concentration is obtained for the reversed-phase material, then one can use Eq. 1 to predict approximately the minimum volume necessary to elute a compound, or conversely, to predict how much solvent can be passed through the Sep-Pak without eluting a compound of interest. These values will represent the elution volume to the center of the solute band. Appropriate compensation must be made for band broadening.

RESULTS

TCA Method

For the nucleosides, guanosine, inosine and xanthosine, recoveries in the range of 65-70% were observed using 12% TCA. If the TCA concentration was decreased to 6%, lower recoveries averaging about 60% for xanthosine and inosine and 54% for guanosine, were obtained (Table 2).

Since it was postulated that the low recoveries of the nucleosides might be due to acid-base reactions with excess amine in the Freon(R), titration curves were obtained by monitoring the pH of the aqueous sample as the amine-solution was added. In this way, the equivalence point was found. For subsequent analyses, the exact volume of the amine solution needed to neutralize the TCA in the samples was added. In contrast to the recovery of nucleotides from serum (13), excess amine solution had no significant effect on the recovery of the nucleosides.

The solubility of the nucleosides in Freon(R) alone was next investigated. Using standard solutions of these nucleosides, it was found that only 85% of each nucleoside was recovered from the aqueous solution after vigorously mixing with Freon(R). Thus, about half of the nucleoside loss can be attributed to solubility of the nucleosides into the Freon(R) itself. The other half of the loss was due either to co-precipitation with the proteins or adsorption of the nucleoside to the proteinaceous material.

TABLE 2

Recoveries of Compounds Added to Pooled Human Serum

CMPD.	TCA at: 6%	TCA at: 12%	$(NH_4)_2SO_4$	Ultrafiltration at: pH 7.8	Ultrafiltration at: pH 5.1	Pre-Column
Xao	58.8±6.2	66.4±1.0	97.4±7.7	99.3±2.5	74.8±4.2	**
Ino	59.4±7.7	65.9±2.7	95.6±7.6	98.9±2.2	97.7±1.7	101.±2.85
Guo	54.0±4.7	67.6±0.3	84.8±9.6	73.6±3.9	85.9±6.4	92.0±5.32
Trp	----	----	102.±12.6	12.1±2.6	96.2±3.7	43.3±2.15
Thp	----	----	82.7±5.1	40.7±5.0	85.5±4.7	91.2±4.8
Thb	----	----	72.1±7.5	81.3±3.2	83.2±4.3	90.2±5.4
Caf	----	----	88.9±6.7	83.0±9.1	87.7±4.5	92.8±5.7

[a]All ranges are reported at the 95% confidence level.

**Highly variable recovery, pH dependent.

Ammonium Sulfate and Ultrafiltration

Ammonium Sulfate

Because of the low recoveries of nucleosides obtained when the TCA-amine-Freon(R) method was used for sample preparation, alternative techniques were investigated. Table 2 summarizes the average recoveries of the added compounds when the ammonium sulfate method was used. High recoveries (73-100%) were obtained when using this method. In addition, the chromatograms of the serum samples showed few interferences for the compounds which elute rapidly.

Ultrafiltration

Ultrafiltration at pH 7.8 (the pH of the pooled serum) also showed excellent recoveries for most of the compounds studied with the exception of tryptophan and theophylline. The recovery of tryptophan averaged only 12.1% and theophylline 40.7%. The chromatograms of the sera processed by the ultrafiltration technique also did not contain the interfering compounds present in the chromatograms of samples prepared with TCA (Fig. 1). The dotted line in Figure 1 shows the interferences when the TCA method of protein removal was used. Samples processed using the ammonium sulfate method are qualitatively very similar to the ultrafiltration profile observed in Figure 1 except for lower tryptophan levels encountered with ultrafiltration (Table 2).

In order to determine if the lower ultrafiltration recoveries of tryptophan and theophylline were due to a pH dependent binding of these compounds to proteins, the experiments were repeated using

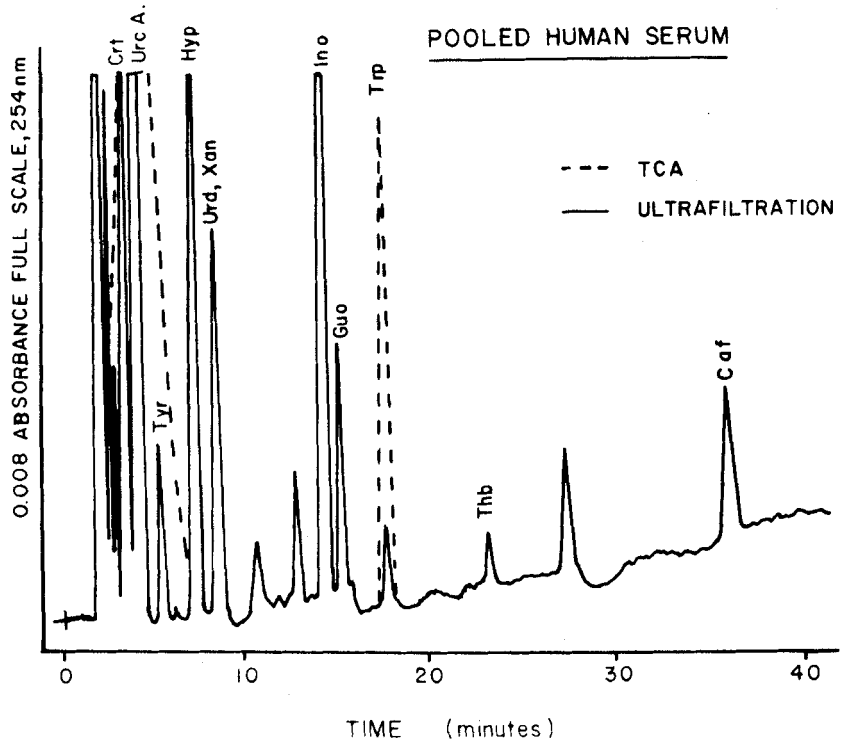

Figure 1. Separation of 80µl of human serum prepared by ultrafiltration and trichloroacetic acid (TCA) methods. Chromatographic conditions: column; reversed-phase (C_{18}) 10 µm particle diameter. Gradient: low strength, 0.02 mol/L KH_2PO_4, pH 5.6; high strength, 60% methanol/water (v/v). Gradient: 0.69%/min. linear (0-60% methanol in 87 min). Temperature: ambient, flow rate: 1.5 ml/min.

serum adjusted to a pH of 5.1. The data in Table 2 show that recoveries of 96.2% (trp) and 85.5% (thp) were obtained at this lower pH value. This observation is in agreement with those of other researchers (16) who have found that tryptophan is reversibly bound to serum albumin, probably through the carboxylic group (17).

In order to determine if binding to the filter cones was responsible for the low recoveries of tryptophan and theophylline,

standard aqueous solutions (10^{-5}moles/L) of the nucleosides and bases at 7.8 were passed through the cones. Recoveries of greater than 98% were observed for all compounds except theophylline. The recovery for theophylline averaged 80.0%. Thus, 20% of the 59.3% of the theophylline lost during ultrafiltration was adsorbed onto the cones. The rest of the loss might be due to protein binding. This observation could be of importance in the clinical assays for theophylline which employ ultrafiltration as the sample preparation technique.

Pre-Column Concentration Method

Sera were processed by the pre-column concentration method (Sep-Pak$^{(R)}$) as described in the Experimental Section. In order to determine the approximate minimum and maximum elution volumes necessary to isolate the compounds of interest without loss, Eq. 1 was used in conjunction with data obtained previously on chemically-similar stationary phases. Curves of elution volumes calculated from Eq. 1 were plotted against % methanol in the eluent. Figure 2 shows that approximately 80 ml of aqueous buffer can be passed through the cartridge before loss of caffeine will occur. Conversely, only about 0.5 ml of 60% methanol will be needed to quantitatively elute the caffeine for sample collection.

The recoveries obtained for the pre-column technique are similar to those obtained for the ultrafiltration at pH 5.1 and for the ammonium sulfate techniques (Table 2). However, it is interesting to note that tryptophan showed an intermediate recovery (43%) while theophylline was recovered in high yield

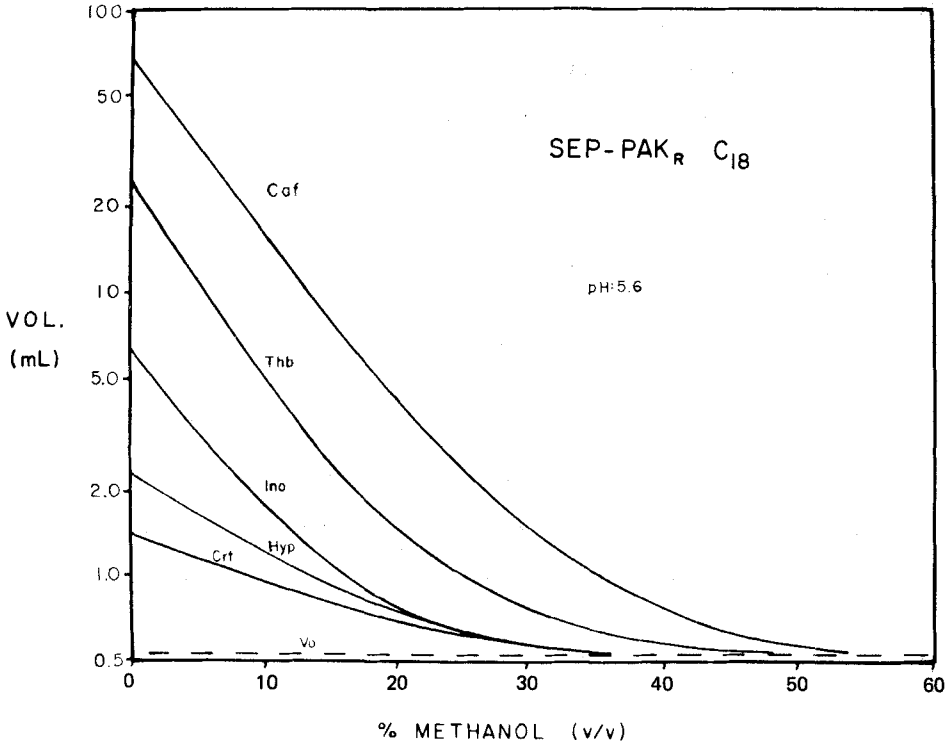

Figure 2. Calculated volumes ofr elution of some of the nucleosides and bases from a small cartridge pre-column packed with reversed-phase (C_{18}) pellicular packing material. Curves represent theoretical values calculated from retention data obtained on an analytical (30 cm) reversed-phase column.

(91%). A possible explanation for this is that the tryptophan is essentially partitioned between 2 competing phases, that of the serum protein and of the hydrophobic packing material. When a slightly acidic (pH 5.6) eluent was used, an indeterminate amount of tryptophan was lost from the column before being bound to the stationary phase.

We were not able to determine accurately the recovery of Xao using the pre-column (cartridge) technique because of the critical

retention behavior of this compound in the pH region of its pK_a of 5.7 (8). The pH differential between the serum and the eluting buffer caused a variable recovery of the added xanthosine.

Efficiency of Protein Removal

The relative efficiencies of the protein removal methods were investigated (Table 3). Under the conditions used for the ammonium sulfate procedure, only 65% of the protein was removed from a serum pool with an average protein concentration of 56.5 mg/100 ml. The protein concentrations were measured by the Biuret method. The TCA, ultrafiltration and pre-column techniques removed essentially all of the protein. The protein remaining in the ammonium sulfate samples did not appear to affect column lifetimes, although no controlled experiments were designed to prove this.

TABLE 3

Efficiency of Deproteinization Methods

Method	% Efficiency[a]
TCA	100%
Ammonium Sulfate	(65.5±2.1)%[b]
Ultrafiltration	100%
Pre-column Concentration	100%[c]

[a]Biuret method or protein assay

[b]95% confidence level.

[c]Dependent upon elution conditions

DISCUSSION

Ease of use and qualitative differences can often be of more importance in a sample preparation technique than the absolute recoveries. From Fig. 1 it is obvious the TCA method precludes the accurate analysis of compounds eluting within the first 5-7 minutes. The ammonium sulfate method reduces these interferences and yields essentially quantitative recovery of tryptophan and theophylline. The ultrafiltration technique produces considerably cleaner chromatograms then both the TCA and the ammonium sulfate methods in the first 5 minutes of the chromatogram. In addition, the sample is not diluted, as is the case with the other procedures. The Sep-Pak(R) technique is unique in that compounds of interest are selectively retained. However, compounds eluting early on a reversed-phase system, such as creatinine and uric acid, may be partially lost. It should be noted that graphs such as presented in Fig. 2 should be used as approximations only.

In summary, it appears that the TCA method is the least suitable for the analysis of serum nucleosides and bases, in terms both of recoveries and of UV absorbing compounds which may interfere with the reversed-phase HPLC analysis of nucleosides and bases. The ammonium sulfate method has the advantage of being chemically mild and of producing essentially 100% recoveries of tryptophan and theophylline. However, there is the possibility of sample contamination from the added salts. In addition if a saturated solution of ammonium sulfate is used (as in this study)

rather than by the addition of large amounts of solid ammonium sulfate, the detection limits are raised due to sample dilution.

The pre-column concentration method has the advantage of concentrating the solutes of interest by selective sample adsorption, thus offering fewer interferences in the prepared samples. The technique is rapid and requires no accurate volume measurements if an internal standard is used. However, some compounds in the serum which are eluted early on a reversed-phase system (such as creatinine and uric acid) may be lost.

The ultrafiltration method offers the advantages of freedom from chemical interferences, no sample dilution and lack of interferences present in the reversed-phase analysis of serum nucleosides and bases. Bound components will not be recovered unless proper care is taken to adjust the pH of the sample. An advantage of using the ultrafiltration cones is that multiple samples can be prepared rapidly and efficiently, producing the greatest throughput per hour of all of the methods investigated.

In conclusion, each of the methods discussed has certain advantages and disadvantages depending upon the problem at hand. For the routine analysis of nucleosides, bases and other low-molecular weight, UV-absorbing compounds in serum, we have found either the pre-column cartridge or the ultrafiltration method to be the most useful for the comparison of serum profiles of normal subjects and cancer patients. However, in work involving tissues or other body fluids, all techniques should be examined and optimized for each problem to obtain the best results.

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LIST OF ABBREVIATIONS

Xao	Xanthosine	Thp	Theophylline
Ino	Inosine	Caf	Caffeine
Guo	Guanosine	Crt	Creatinine
Trp	Tryptophan	Thb	Theobromine

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