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COMPARISON OF ISOLATION METHODS OF URINARY ORGANIC ACIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Four methods for extracting organic acids from human urine prior to analysis by high-performance liquid chromatography (HPLC) were compared. The methods were manual solvent extraction with ethyl acetate and diethyl ether, continuous solvent extraction, anion exchange with pyridinium acetate as the eluting solvent and anion exchange with hydrochloric acid as the eluting solvent. All four methods produced samples that could be analyzed by reversed-phase HPLC, but the continuous solvent extraction and anion exchange with pyridinium acetate methods gave the best reproducibilities (approximately 6% relative standard deviations). Pretreatment of the urine with barium hydroxide and hydroxylamine hydrochloride prior to anion exchange did not markedly alter the HPLC profiles.

INTRODUCTION

Although the technique of metabolic profiling has been utilized since the 1950's [1] and has been considerably refined as chromatographers have developed higher-resolution techniques (see refs. 2—4 for reviews), relatively few papers have appeared which have used metabolic profiling to obtain information which had not already been discovered by some other simpler technique. A major reason for this is that the metabolic profiling studies to date have been largely qualitative rather than quantitative [4], and therefore, pathological concentrations of affected metabolites in the contents of the biological fluid or tissue usually have had to be one or two orders of magnitude above normal to be distinguished. Usually, such major changes have already been discovered by other techniques.

However, one of the most analytically useful features of the types of biological materials that are typically studied by metabolic profiling (e.g., urine, serum, cerebral spinal fluid, animal and plant tissue extracts) is exactly that feature which has been least utilized; namely that there is relatively little qualitative variation from sample to sample. The major inter-sample differences are in

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the amounts of each substance present, not in the variety of substances present [5].

Thus, one of the apparent requirements for the development of any new metabolic profiling system would be that it would be able to provide quantitative, as well as qualitative, measurements of the substances present in a biological sample. For samples that must be extracted from a biological matrix and then analyzed chromatographically, the extraction procedure should be reproducible and, if possible, give recoveries near 100%; and the chromatographic procedure should be sensitive, reproducible and give high enough resolution to separate the components in the biological extract.

The techniques developed in this laboratory to achieve reproducible, high-resolution separations of human urinary acids by high-performance liquid chromatography (HPLC) have already described [6]. However, such techniques are almost useless without a quantitative procedure for extraction of the biological sample, or a fraction of it, from its matrix. Extraction techniques suitable for recovering organic acids from human urine, for example, have been shown to vary widely in their efficacy. The best documented comparison of techniques for extracting organic acids from human urine has been that of Thompson and Markey [7], in which manual solvent extraction, continuous solvent extraction, and anion-exchange techniques were examined. They concluded that "anion-exchange chromatography has been shown to be more effective for isolating organic acids from urine than solvent extraction", as judged by measuring the results from analysis by gas chromatography (GC) and gas chromatography—mass spectrometry (GC—MS) [7]. They noted, in particular, the superior reproducibility and the greatly enhanced recovery of polyhydroxy acids by anion exchange compared to solvent extraction methods. They also pointed out that the anion-exchange method was much more time consuming than the manual solvent extraction method.

Thompson and Markey [7] used DEAE-Sephadex anion exchange with pretreatment of the urine with barium hydroxide and hydroxylamine hydrochloride, elution of the column with pyridinium acetate, and subsequent derivatization of the extracted organic acids. Subsequent workers have suggested that barium hydroxide might precipitate some compounds of interest, particularly citric acid [8], and that several modifications of the anion-exchange method yielded more reproducible results [9]. Other techniques have also been proposed, including one based on using DEAE-Sephadex with hydrochloric acid as an eluent [10] and another using silica gel adsorption followed by extraction with organic solvents [11]. In addition, all of these techniques were developed to prepare samples for GC analysis, so it is not immediately obvious that they will be suitable for analysis of samples by HPLC; for example, the extracting solvents or eluting buffers may produce high backgrounds or spurious peaks on the various HPLC detectors.

Hence, this work was undertaken to compare some of the currently available methods for extracting human urinary organic acids in terms of their efficiency, reproducibility and suitability for HPLC-based quantitative metabolic profiling.

EXPERIMENTAL

Preparation of urine samples

The urine samples used in this study were collected from an apparently healthy adult male. A 24-h sample of 1.3 l was kept at 4°C during collection, divided into aliquots using small plastic vials, and stored at −80°C until used. Samples were then prepared by the following methods. All glassware used in each method for holding the sample was silanized, except for the chromatography columns. All reagents were reagent-grade or better; deionized water passed through a Millipore Milli-Q water purifying system (Millipore, Bedford, MA, U.S.A.) and having a resistance of over 10 MΩ cm was used throughout. A 2.00-ml sample of urine was extracted by each method. The sample preparation methods are compared in Fig. 1.

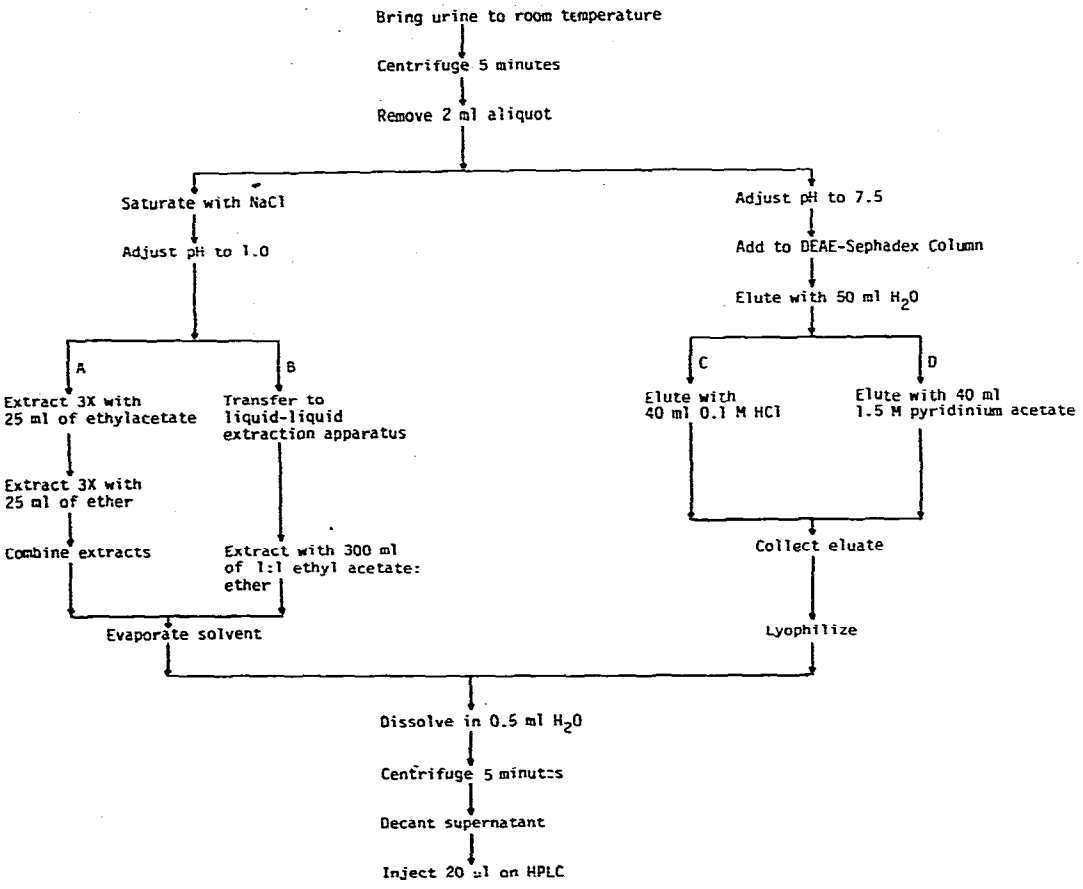

Fig. 1. Comparison of extraction schemes. (A) Manual solvent extraction; (B) continuous solvent extraction; (C) anion exchange with hydrochloric acid as the eluting solvent; (D) anion exchange with pyridinium acetate as the eluting solvent.

Manual solvent extraction

The method of Horning et al. [12] was used, with slight modification. Urine samples were saturated with sodium chloride and the pH adusted to 1.0 with

6 *M* hydrochloric acid. It was extracted six times in a 125-ml silanized separatory funnel: three times with 25-ml portions of redistilled ethyl acetate (Fisher Scientific, Pittsburgh, PA, U.S.A.), followed by three 25-ml portions of diethyl ether (Mallinckrodt, St. Louis, MO, U.S.A.). The combined extracts were evaporated in a rotary evaporator in a silanized 250-ml round-bottom flask, and then transferred, with washing, to a 10-ml silanized flask, where the final solution was evaporated to dryness and reconstituted in 0.5 ml water. The extract was centrifuged for 5 min in a table-top centrifuge to remove solid material, and the supernatant was frozen at −80°C in a silanized test tube until analyzed.

Continuous solvent extraction

Continuous solvent extraction was performed by a modification of the method described by Thompson and Markey [7]. The sample was prepared similar to that for manual solvent extraction, and then extracted with 300 ml of diethyl ether—ethyl acetate (1:1, v/v) in a micro liquid—liquid extraction apparatus (Pyrex) for 5 h. The solvents were evaporated; the residue was reconstituted in 0.5 ml water and then further treated as described above.

Anion exchange with pyridinium acetate as the eluting solvent

The method of Thompson and Markey [7], as modified by Gates et al. [9], was further modified as follows: DEAE-Sephadex, swollen in 1.0 *M* pyridinium acetate, was equilibrated in 0.5 *M* pyridinium acetate and packed into a 1 cm I.D. chromatography column (Kontes, Vineland, NJ, U.S.A.), to a height of 8 cm. A 50-ml aliquot of 0.5 *M* pyridinium acetate was passed through the column. The urine to be analyzed was warmed to room temperature, shaken, and centrifuged 5 min in a table-top centrifuge. The supernatant was decanted and adjusted to pH 7.5 with solid $NaHCO_3$. A 2.00-ml aliquot of the urine was added to the column, which was eluted with 50 ml water; this eluate was discarded. The urinary acids were eluted with 40 ml of 1.5 *M* pyridinium acetate. The eluted sample was frozen and partially lyophilized in a 250-ml round-bottom flask, then transferred to a 50-ml conical centrifuge tube with three water washes, and completely lyophilized. The residue was reconstituted in 0.5 ml of water and centrifuged for 5 min. The supernatant was stored at −80°C until analyzed on the high-performance liquid chromatograph.

Anion exchange with hydrochloric acid as the eluting solvent

The procedure of Horrocks et al. [10] was used. This involved using essentially the same procedure as that described for anion exchange with pyridinium acetate as the eluting solvent, except that the DEAE-Sephadex-A25 was swollen in water instead of pyridinium acetate, and the acidic metabolites were eluted with 40 ml 0.1 *M* hydrochloric acid.

Anion exchange with sample pretreatment

Samples were prepared by anion exchange with pyridinium acetate as the eluting solvent, as described, except that the samples were pretreated with barium hydroxide and hydroxylamine hydrochloride, as described by Thompson and Markey [7]. A 2.0-ml aliquot of urine was treated with 6.0 ml of 0.1 *M* barium hydroxide (Mallinckrodt), centrifuged, and the supernatant

treated with 400 μl of a 75 g/l solution of hydroxylamine hydrochloride (Sigma, St. Louis, MO, U.S.A.). After heating at 60°C for 30 min, the sample was cooled and the pH adjusted to 7.5 with 2 *M* hydrochloric acid. The samples were then added to the column and further prepared as described above.

Chromatography of samples

The chromatographic techniques developed by Mattiuz et al. [6] were used. A Beckman 322 microprocessor-controlled high-performance liquid chromatograph with dual Beckman 100A pumps was used. Sample injections were 20 μl onto a 25 cm × 4.7 mm Beckman Ultrasphere ODS (5 μm particle size) column. The column was kept at 50°C, and a guard column of 10 μm LiChrosorb RP-18, 7 cm × 2.2 mm, was always used. A Beckman 100-10 variable wavelength UV—visible detector was set at 280 or 210 nm, as noted. A Hewlett-Packard 3380 S integrator was used to determine peak areas.

The sample was eluted from the column with a non-linear phosphate—acetonitrile gradient. The phosphate buffer, 0.2 *M* and pH 2.1, was prepared from KH_2PO_4 (Mallinckrodt) and 85% phosphoric acid (Fisher). Both solvents were filtered (Millipore) and the phosphate buffer was degassed for 15 min before using. The flow-rate was 2.0 ml/min. The column performance was checked daily, and matching sets of studies were run on the same day.

Statistical analysis

Standard statistical analyses were performed, except that data were analyzed using either normalized or unnormalized values. Normalization was achieved by summing selected peak areas and expressing each area as a percent of the sum [13, 14].

RESULTS AND DISCUSSION

Method precision

Five injections of a single manual solvent extract of urine were analyzed to obtain a measure of the chromatographic and integrator precision. For 28 major peaks, the mean and median of the relative standard deviations for each peak were 5.8% and 4.4%, respectively. If the data were normalized, the mean and median precisions were 5.0% and 3.5%, respectively, for the same data.

By comparison, the relative standard deviations for three samples by each of the four methods are shown in Table I. In each case, identical conditions were used, and each of the 12 samples was carried through the isolation procedure independently of the others.

Data were normalized because it was apparent that almost all components in a given sample were occasionally higher or lower than those in other samples; differences in dilution or amount of sample injected might give rise to such variations, for example. This was especially noticeable in the continuous solvent extraction data. Since metabolic profiling data are often normalized to reduce the importance of differences arising from levels of fluid consumption by the subjects [13, 14], comparison of the precision of normalized data may be more meaningful than that for unnormalized data.

Assuming that the variance introduced during the extraction procedure is

TABLE I

PRECISION OF EXTRACTION OF ORGANIC ACIDS BY FOUR METHODS

Based on triplicate analyses of the same urine by each method. Approximately 20 well-resolved peaks measured at 280 nm were used for comparison.

Method	Relative standard deviation (%)			
	Raw data		Normalized data	
	Mean	Median	Mean	Median
Manual solvent extraction	14.9	12.4	14.8	14.3
Continuous solvent extraction	10.4	10.5	6.6	5.9
Ion exchange/pyridinium acetate	10.8	6.2	10.8	6.0
Ion exchange/hydrochloric acid	16.4	18.0	14.6	9.7

TABLE II

REPRODUCIBILITY OF EXTRACTION METHODS

GC data are from Thompson and Markey [7], Table VI, 14 major components. HPLC data are based on 5 injections and 28 major components. Data are not normalized.

	Median percent relative standard deviations		
	Manual extraction	Continuous extraction	Ion exchange/ pyridinium acetate
GC	40	20	12
HPLC	12.4	10.5	6.2

independent of that from the chromatographic process; it is possible to calculate the mean percent standard deviation from the extraction procedure, P_E, from total mean percent standard deviation, P_T, and the contribution from the chromatographic process, P_C, as $P_T = \sqrt{P_E^2 + P_C^2}$.

Thus, the mean normalized relative standard deviation of each extraction method, exclusive of any chromatographic contribution, would be 13.9%, 4.3%, 9.6%, and 13.7% for the manual solvent extraction method, continuous solvent extraction, ion-exchange/pyridinium acetate and ion-exchange/hydrochloric acid methods, respectively.

It is possible to compare these data to those of Thompson and Markey [7], however, only using unnormalized data for three of the methods. The comparison is shown in Table II. It should be noted that the same trend is present, although the numbers for the GC and HPLC reproducibilities are quite different. Thus, by either GC or HPLC, the ion-exchange/pyridinium acetate method gives the best precision on unnormalized data, and the manual solvent extraction method the poorest. The differences in the numbers between GC and HPLC probably are largely a function of the number and size of peaks chosen and the integrator used, rather than of differences in the absolute precision of each technique.

Relative recoveries

Recoveries of individual compounds can be judged from Fig. 2, where the amount of sample injected and the dilution of the sample are the same for each method, and the detector is set at 280 nm. Continuous solvent extraction clear-

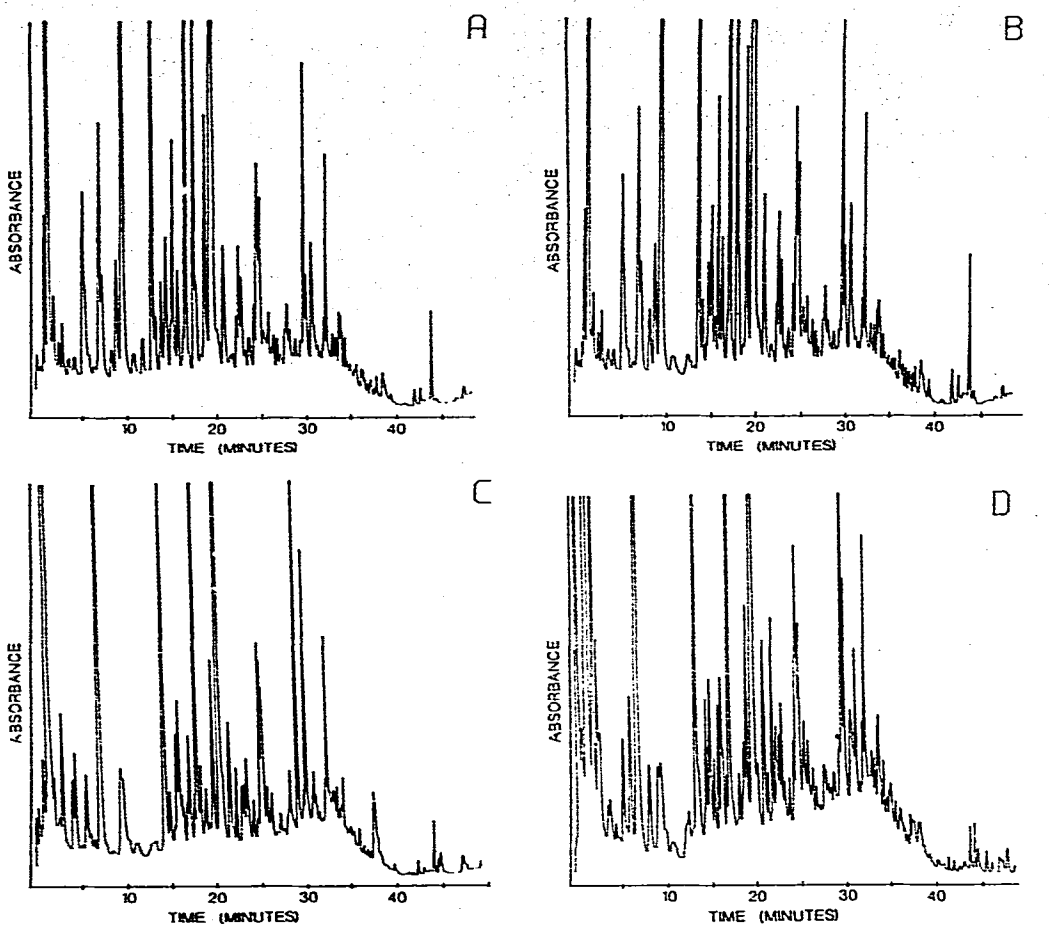

Fig. 2. Comparison of extraction methods at 280 nm. Each sample is derived from identical urine samples, and is diluted to the same final concentration. (A) Manual solvent extraction; (B) continuous solvent extraction; (C) anion exchange with hydrochloric acid as the eluting solvent; (D) anion exchange with pyridinium acetate as the eluting solvent. 0.05 a.u.f.s.

ly (Fig. 2B) gives better recoveries than manual solvent extraction (Fig. 2A) of virtually all peaks. On the other hand, many of the components obtained using the anion-exchange/pyridinium acetate method (Fig. 2D) are missing from the anion-exchange/hydrochloric acid chromatogram (Fig. 2C). Comparison of the anion-exchange/pyridinium acetate and continuous solvent extraction is more difficult. Most of the peaks which appear in both chromatograms are of approximately equal size; however, a number of peaks are unique to each method.

An additional complicating factor is that detection at 280 nm is biased toward aromatic substances; hence, a comparison at 210 nm, which includes some aliphatic substances as well, may be useful. This is shown in Fig. 3. Basically the same pattern is observed, with recoveries generally best for the continuous extraction and anion-exchange/pyridinium acetate methods. However, at 210 nm procedural blanks for each method showed several major peaks. Both solvent extraction methods have several major artifactual peaks that

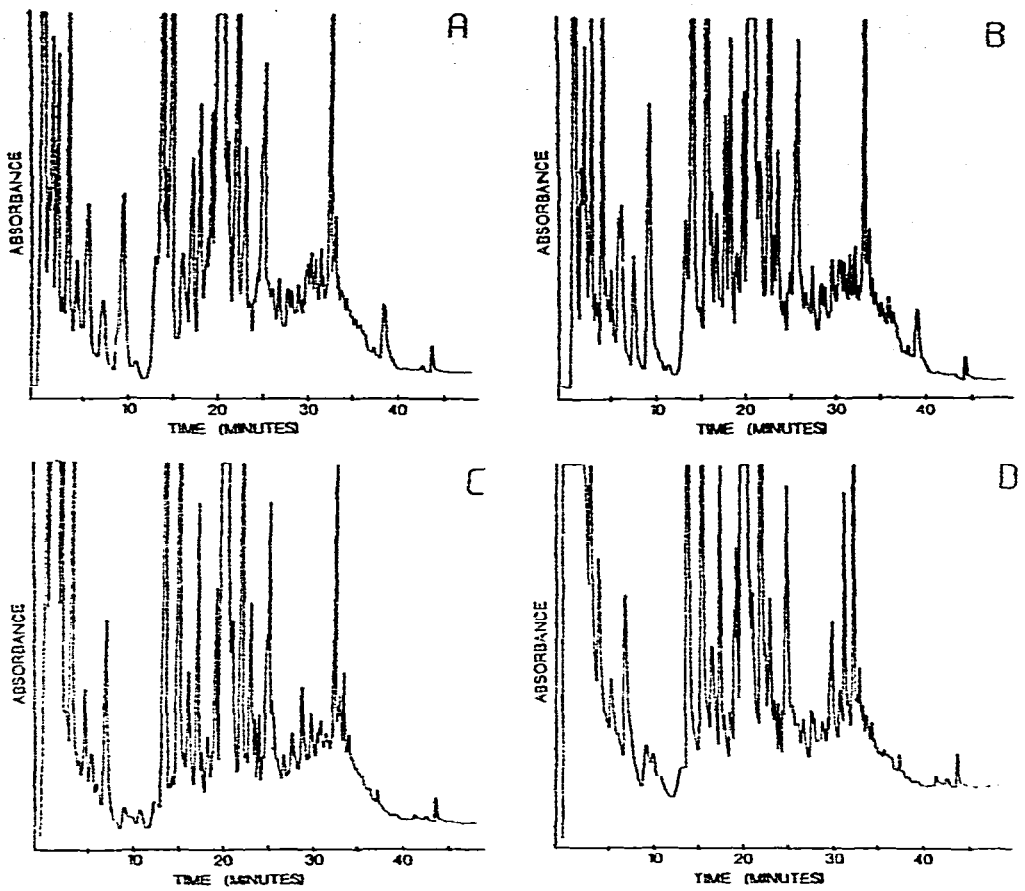

Fig. 3. Comparison of extraction methods at 210 nm. The samples are the same as those in Fig. 2.

would interfere with analyses at 210 nm; these are presumably from the solvents and might be eliminated by using more highly purified diethyl ether and ethyl acetate.

Thompson and Markey [7] have pointed out that several of the compounds unique to the solvent extraction method are not, in fact, acids (e.g., urea and several sugar alcohols). Hence, it is not surprising that they do not appear in the acid fraction of the anion-exchange methods. A number of acids, particularly the polyhydroxy acids, are not recovered by solvent extraction methods [7]. Therefore, the choice between these two types of methods may depend upon the types of compounds that are of interest to the investigator, as well as the recovery and reproducibility obtained with each.

One variant of the anion-exchange method was also investigated: the use of the barium hydroxide and hydroxylamine treatments suggested by Thompson and Markey [7]. As shown in Fig. 4, treatment with these two substances did not markedly alter the profile, although recoveries of a few compounds appear to be slightly reduced. This is not unexpected, since phosphoric acid, the major component precipitated by barium hydroxide [7], is not detected at either 280 or 210 nm, and in fact is used in the chromatographic buffer. Citric acid, the

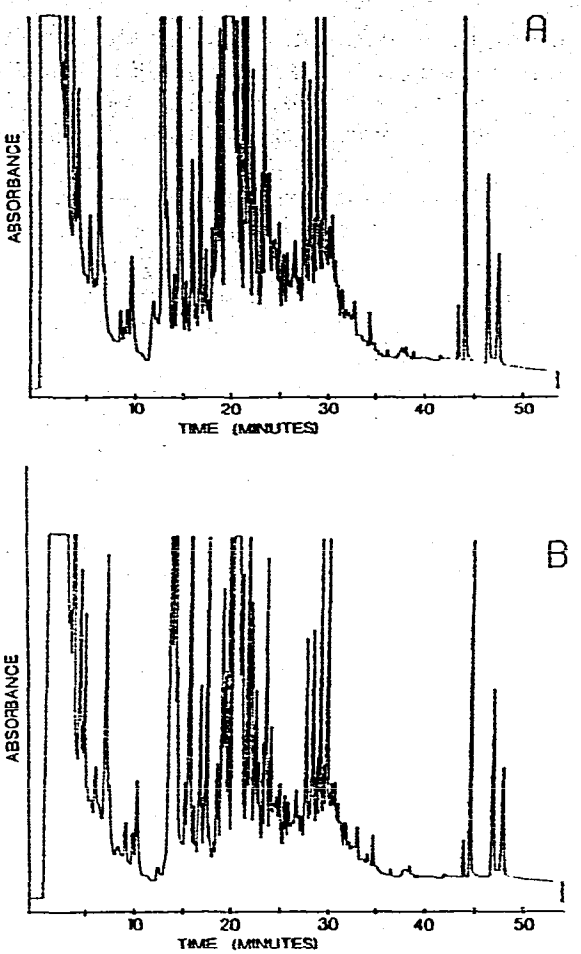

Fig. 4. Effect of barium hydroxide precipitation. The samples were treated identically, using the anion-exchange/pyridinium acetate procedure, except that B was treated with barium hydroxide and hydroxylamine hydrochloride prior to the anion-exchange separation.

other major component precipitated [8], is not detectable at 280 nm. The hydroxylamine hydrochloride only reacts with ketones, which are known to be almost completely absent from the acid fraction of normal urines [13].

CONCLUSIONS

Several conclusions are possible from these studies. One important conclusion is that there does not appear to be any one extraction method which absolutely cannot be used with HPLC. Each method produces an extract which can be detected easily at 280 nm, with few interfering substances or method artifacts. However, special care must be taken to purify solvents if detection at 210 nm is anticipated, particularly for solvent extraction techniques. The one major artifact peak at either 280 or 210 nm from the anion-exchange/pyridinium acetate method is presumably from the pyridine left in the sample after

lyophilization, and, hence, probably cannot be eliminated. The only technique probably unsuitable for HPLC is the anion-exchange/hydrochloric acid method, because chloride salts are generally considered to be too harmful to the metal surfaces in the high-performance liquid chromatograph to be used for long periods of time.

Of the four major methods tested — manual solvent extraction, continuous solvent extraction, anion exchange/hydrochloric acid and anion exchange/pyridinium acetate — clearly the best recoveries and reproducibility were obtained with the continuous solvent extraction and anion-exchange/pyridinium acetate methods. In general, it would appear that these methods are roughly equal. Both methods are quite time consuming, although each could be automated to a considerable extent, if desired. Neither method is as easy as the manual solvent extraction method, which may still be the method of choice if great speed is desired and quantitative results are not required. It also does not appear to be useful for HPLC-based methods to perform a barium hydroxide precipitation.

Overall, therefore, we have concluded that in our own laboratory we will continue to use the anion-exchange/pyridinium acetate method as our primary method. Other workers may wish to choose one of the other methods; however, it should be emphasized that none of the methods will produce acceptable results without careful attention to the details of good analytical technique. It would also appear that there is still a considerable need for development of a quantitative technique more rapid than those described here.

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REFERENCES

1 R.J. Williams, Biochemical Individuality, The Basis for the Genetotropic Concept, University of Texas Press, Austin, 1956.
2 I.A. Politzer, B.J. Dowty and J.L. Laseter, Clin. Chem., 22 (1976) 1775.
3 E. Jellum, J. Chromatogr., 143 (1977) 427.
4 S.C. Gates and C.C. Sweeley, Clin. Chem., 24 (1978) 1663.
5 S.C. Gates, M.J. Smisko, C.L. Ashendel, N.D. Young, J.F. Holland and C.C. Sweeley, Anal. Chem., 50 (1978) 433.
6 E.L. Mattiuz, J.W. Webb and S.C. Gates, J. Liquid Chromatogr., submitted for publication.
7 J.A. Thompson and S.P. Markey, Anal. Chem., 47 (1975) 1313.
8 R.A. Chalmers, A.M. Lawson and R.W.E. Watts, Clin. Chem., 23 (1977) 903.
9 S.C. Gates, N. Dendramis and C.C. Sweeley, Clin. Chem., 24 (1978) 1674.
10 R.H. Horrocks, E.J. Hindle, P.A. Lawson, D.H. Orrell and A.J. Poole, Clin. Chim. Acta, 69 (1976) 93.
11 V.P. Williams, D.K. Ching and S.D. Cederbaum, Clin. Chem., 25 (1979) 1814.
12 M.G. Horning, K.L. Knox, C.E. Dalgliesh and E.C. Horning, Anal. Biochem., 17 (1966) 244.
13 S.C. Gates, C.C. Sweeley, W. Krivit, D. DeWitt and B.E. Blaisdell, Clin. Chem., 24 (1978) 1680.
14 H. Dirren, A.B. Robinson and L. Pauling, Clin. Chem., 21 (1975) 1970.