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Multicomponent Analysis of Metabolites of Low Volatility in Biological Fluids by Field Ionization Mass Spectrometry

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Abstract □ An improved mass spectrometric method for multicomponent analysis of metabolites in urine, well-suited for clinical biochemistry, is described. The method involves solvent elution of the metabolites from an adsorbent and the concentration of the eluate on a microadsorption column. This is administered by a direct inlet probe into the ionizing source of field ionization mass spectrometry (FIMS), which yield a molecular weight profile of the metabolites. The procedure provides rapidly (within one hour) reproducible profiles from a small volume of urine. The optimization of the sampling technique and the reproducibility are discussed.

Keywords □ Multicomponent analysis, Field ionization mass spectrometry, Metabolic profile, Molecular weight profile, Fractionating column chromatography.

The number of metabolites occurring in biological fluids is likely to exceed several thousands, and therefore, the complete characterization of the mixture is virtually impossible. With the advent of high resolution chromatographic methods, however, the possibility of carrying out multicomponent analysis led directly to the concept of metabolic profile analysis.

Extensive research efforts have been made to identify the organic metabolites present in body fluids[1-5]. The use of chromatographic and mass spectrometric techniques provides highly valuable information. The objective of those works has been to study metabolic pathways and to develop effective diagnostic methods. The characteristic fragmentation patterns of individual compounds produced by electron impact mass spectrometry (EIMS) have been widely used for the identification of single components separated by gas liquid chromatography. Unlike EIMS, field ionization mass spectrometry (FIMS) produces molecular ions almost exclusively[6]. It provides a molecular weight profile, thus allowing the analysis of multicomponent mixtures.

FIMS technique was successfully applied to the study of urine metabolites in subjects suffering from acute infectious hepatitis[7], viral infections[8], and in mice with sarcoma I transplanted[9], opening up the possibility of using FIMS for routine screening purposes in the biomedical field. The type of field ionization sources, its geometry, and ion optics have continuously been modified and refined to achieve these purposes [6,10,11].

Prior to the analysis by FIMS, a certain fraction containing meaningful biochemical information has to be reproducibly isolated from the biological fluid. The chosen isolation method should fulfill the requirement of simplicity, rapidity and reliability.

The isolation proceduie using XAD-2 resin[7] requires several pH adjustment steps and trea-

tment with urease overnight, thus making it rather lengthy and complicated. Furthermore, two consecutive concentration steps after isolation are required for this technique. A simpler sampling method employing fractionating column chromatography was developed, thus allowing the isolation of organic substances from the biological matrix in a single step. A similar column chromatographic technique has been applied to the sampling of urinary metabolites[8]. These techniques are suitable for the analysis of metabolites of intermediate volatility (10^{-4} Torr at room temperature to 10^{-9} Torr at 200°C).

The present paper described a rapid sample preparation method for the organic metabolites in urine, and their analysis by FIMS in the molecular weight range 100 to 300 amu. An investigation of the experimental variables was made to optimize the method and to minimize its overall variance. The reproducibility of the methodology seems to be sufficient for diagnostic metabolic profile analysis.

EXPERIMENTAL METHODS

Materials

Urine samples were obtained from healthy volunteers and Were stored frozen (−17°C). Methylene chloride, reagent grade, (Mallinckrodt Chemical Works, St. Louis, Mo., USA) was distilled over phosphorous pentoxide (Baker Chemical Co., Phillipsburg, N.J., USA). Diethylether, anhydrous, (Fisher Scientific Co., Pittsburgh, Pa., USA), sodium chloride (Baker Chemical Co., USA), Chromosorb P, 80/100 mesh, acid washed, (Supelco Inc., Bellefonte, Pa., USA), alumina F-1, chromatographic grade, 45/60 mesh, (Applied Science Laboratories Inc., State College, a., PUSA), and Porasil

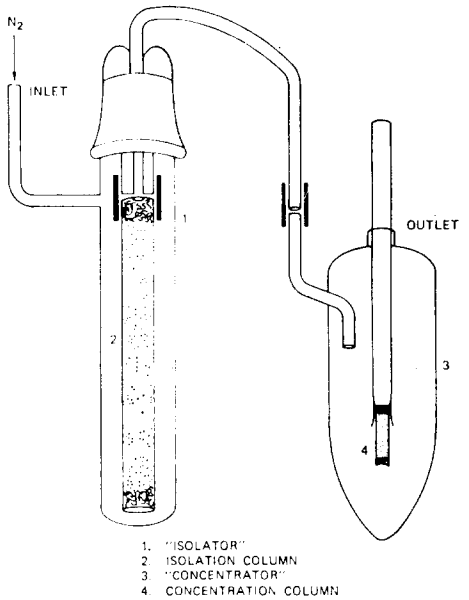

Fig. 1: Sampling system.

A, C, and E, 80/100 mesh, (Waters Associates, Inc., Milford, Massachusetts, USA) were used.

Sample Preparation

The urinary organic metabolites were isolated and concentrated by the sampling system illustrated in Figure 1. This system consists of two custom-made parts: an "isolator" and a "concentrator". The isolator is made from a commercially available 19/20 standard tapered pyrex glass joint. The isolation column is a glass tube (135.0mm×8.0mm O.D., 6.2mm I.D.) with one end rounded. It was firmly packed with an adequate amount of adsorbent and its ends were plugged with glass wool. Before and after the packing, both the adsorbent and glass wool were carefully washed sequentially with methanol, dichloromethane and ether, and then baked out in a vacuum oven (200°C, 10^{-1} Torr) overnight. It was then stored in a tube with a Teflon-lined screw cap. The concentration column is a glass capillary (18.0mm×2.0mm O.D., 1.4mm I.D.) of the same size as that of the solid sam-

ple holder of mass spectrometer. The concentration column was prepared by tightly packing 15 mg of Chromosorb P into the capillary and plugging the ends with glass wool. Prior to packing, both the adsorbet and glass wool were washed and baked in the same manner described above. After packing, the concentration columns were rebaked under vacuum for 48 hours. The preparation of columns takes an average of 30 min/column when carried out in batches.

One ml of urine sample and 0.3 g of sodium chloride are placed in the outer tube of the isolator. A drop of concentrated HCl and a drop of 6 N NaOH were added to make acidic or basic urine samples.

After vortex mixing, the tube is fitted to the jacket of the isolator, the inlet and the outlet of which are attached to an isolation column and a concentrator, respectively. Gas tight connections between two glass tubes are achieved using shrinkable Teflon sleeves. The urine sa mple is then loaded onto the isolation column by applying a positive N_2 pressure. After the sample was adsorbed on the columm, a 5 ml portion of solvent is then introduced into the tube through the inlet, which is then reconnected to the nitrogen line. The solvent is forced upward into the column, eluting the organic metabolites.

The eluate is then collected in the concentrator, followed by evaporation of the solvent to dryness by purging with nitrogen gas at room temperature. A 50 μl of the solvent is added to the concentrator to dissolve the dried sample. A concentration column attached to a Teflon tubing (11.0mm×4.0mm O.D.) is then introduced into the concentrator outlet and dipped into the soution for a short time to wet only one-third of the column. The solvent is flushed off the column with nitrogen. The wetting and drying procedures are repeated until the total sample is transferred onto the column. After final solvent removal, the concentration column is disconnected and stored in a clean vial.

The sample was either analyzed immediately after sampling or stored in a freezer (−17°C) ready for mass spectrometric analysis. Sample blanks were prepared by the same procedure, except for the addition of urine sample.

Mass Spectrometric Analysis

A multiscanning field ionization quadrupole mass spectrometer previously described[7] was used in the semiquantitative study of the sampling methods. The temperature of the sample probe was manually increased from ambient to 160°C for 30 min and held at 160°C for 10 min, while the ionization source was maintained at 220°C. The mass analyzer was scanned over the mass range of m/z 60 to 260 at a rate of 16 sec/scan.

A 35-cm, 60° magnetic sector multiscanning field ionization mass spectrometer (Fig. 2) was employed in the latter stages of the investigation to study the reproducibility of

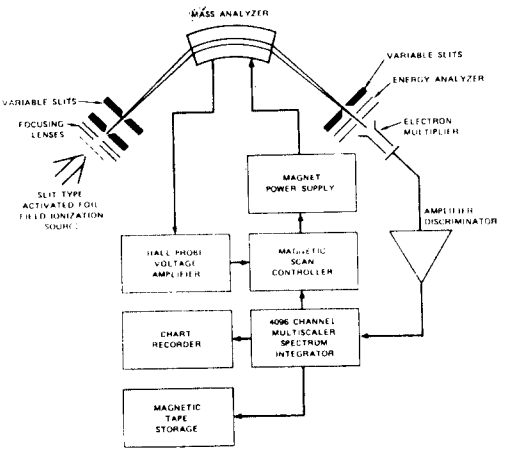

Fig. 2: Schematic diagram of a multiscanning field ionization mass spectrometer and data acquisition system.

the analytical procedure. The ionization source was glass-lined cobalt-activated stainless steel foil[11] which was assembled in a geometry of the slit type[10].

The sample probe was inserted at −37°C and heated to 180°C over a period of 40 min by a temperature programmer. The source temperature was manually adjusted from 100°C to 200°C during the sample run, keeping it always at a higher temperature than that of the sample probe. The mass range of m/z 90 to 304 was scanned in 16 sec.

Computer Analysis of Data

After smoothing by the least-square procedure of Savitzky and Golay[12], the multiscanned data were processed as described earlier[7], with the exception that the spectra were normalized to unit area for all but the five largest peaks. The reproducibility of the entire analytical procedure was evaluated by computing variances for the normalized peak areas at each mass number.

RESULTS AND DISCUSSION

The sampling device described here provides both isolation and concentration of organic substances from urine matrix in less than one hour. The concentrator (Fig. 1) permits both concentration and complete transfer of samples about ten times faster than the previously used capillary evaporation method[7]. The efficiencies of these two methods for identical samples are compared in Figure 3. It can be seen that the two profiles are remarkably identical, but the new method provides much better recovery. It has been demonstrated that the concentration

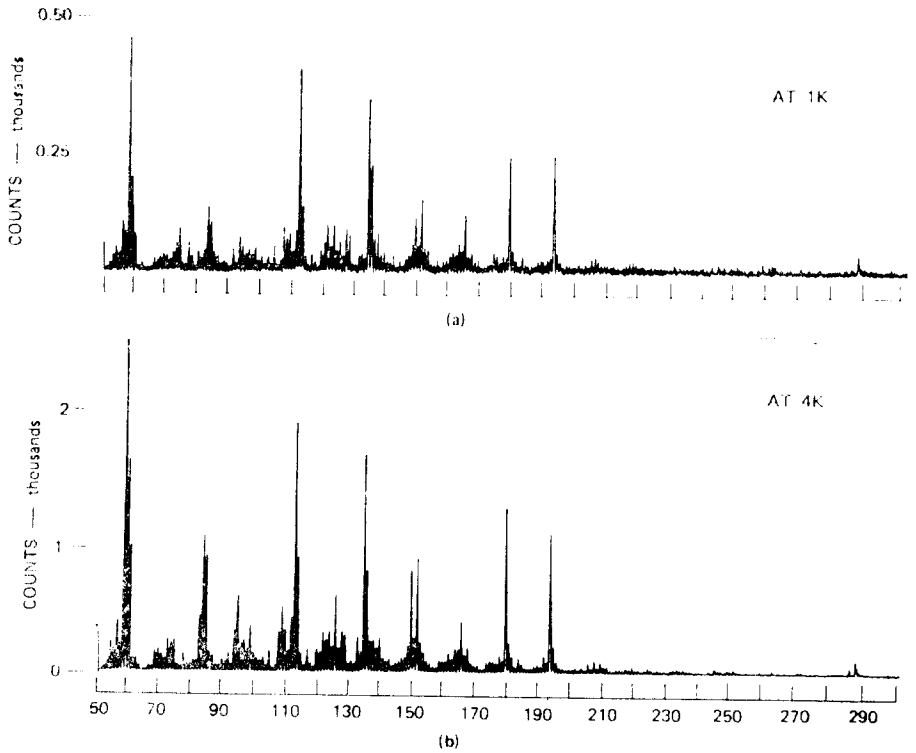

Fig. 3: Molecular mass profiles of neutral metabolites in a normal urine sample, using (a) old method and (b) column concentration method.

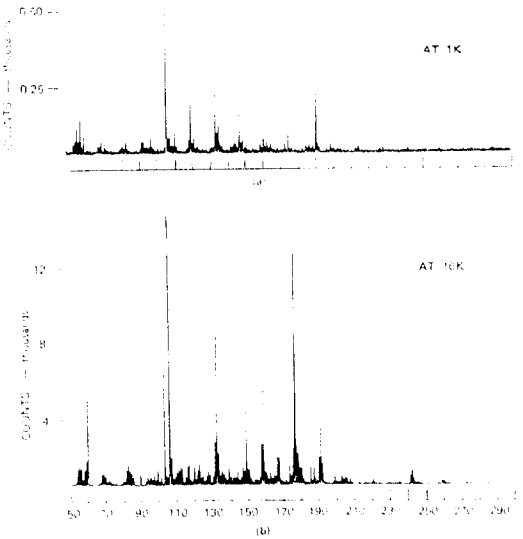

Fig. 4: Mass profiles of acidic urine-ether, using (a) alumina and (b) Chromosorb P.

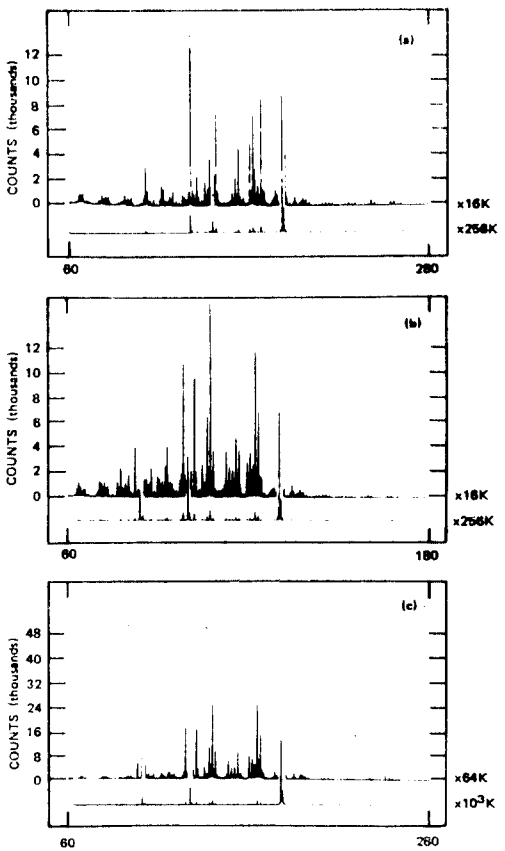

Fig. 5: Mass profiles of acidic urine-ether, using (a) Porasil A, (b) Porasil C, and (c) Porasil E.

columns do not introduce significant artifacts, irreversible pressure increase during mass spectrometric analysis. Adsorbents other than Chromosorb P, such as microglass beads, Anachrome Q, or porous glass have been tested as concentrating agents, but Chromosrb P was found to be superior both in adsorption and recovery. The optimization of the sampling method required a thorough investigation of three variables: type of adsorbent, the eluting solvent, and the pretreatment of urine. The proper adsorbent of isolation columns should be nonextractable by the eluting solvent, but should retain the water, urea and inorganic salts, as well as biopolymers,if present, while allowing organic substances of interest to be eluted. Thus, inorganic hydrophilic adsorbents are the most suitable column materials for this purpose. The solvent should preferably have high volatility, high solvent power, water immiscibility, and long-term stability. Alumina,

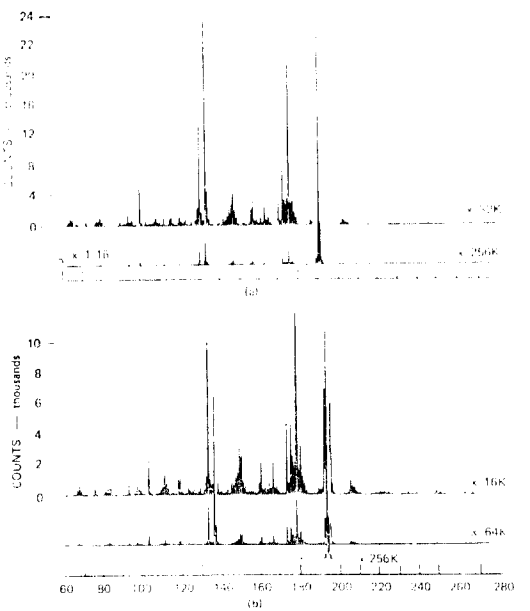

Fig. 6: Mass profiles of acidic urine-Chromorb P, using dichloromethane (a) a 10ml and (b) a 5ml.

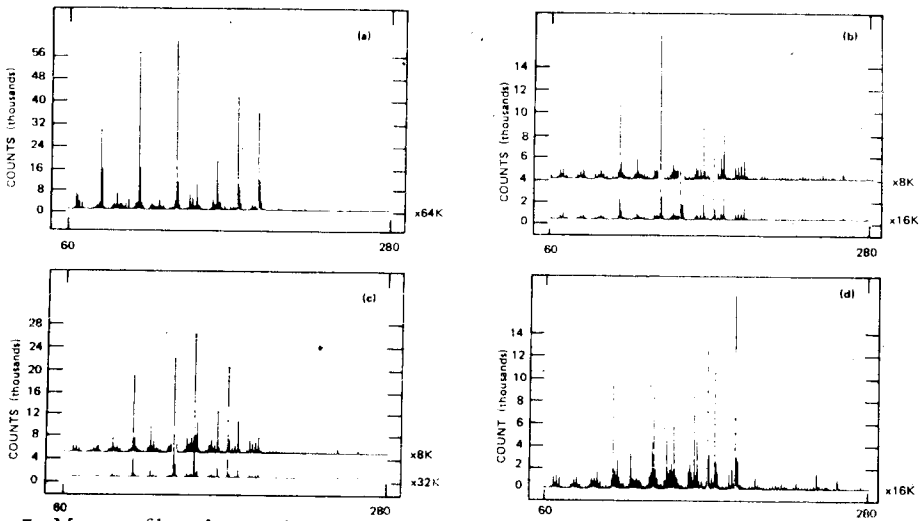

Fig. 7: Mass profiles of neutral urine-ether, using (a) Chromosorb P, (b) Porasil A, (c) Porasil C, and (d) Porasil E.

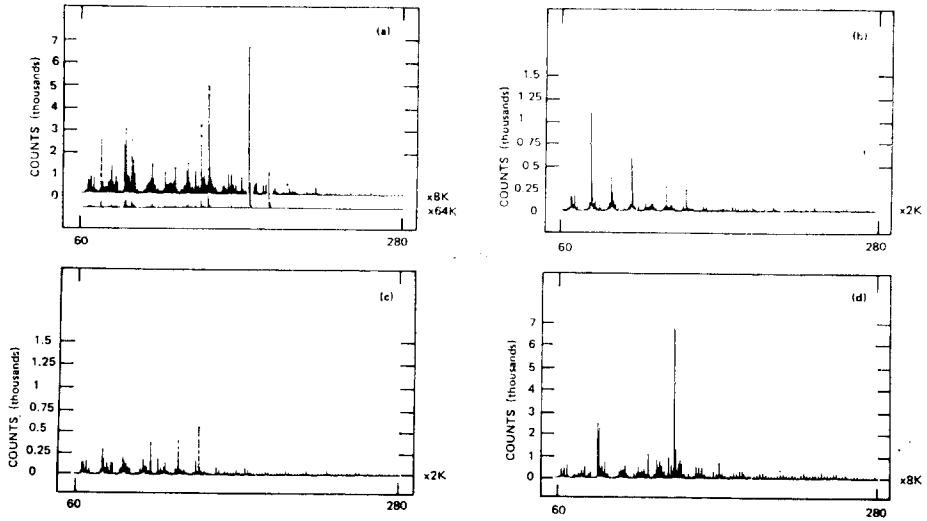

Fig. 8: Mass profiles of neutral urine-dichloromethane, using (a) Chromosorb P, (b) Porasil A, (c) Porasil C, and (d) Porasil E.

Chromosorb P, and Porasils were tested as adsorbents, and ether and dichloromethane as the eluting solvents.

The optimum amount of adsorbent depends on its loading capacity, the size of the sample, and the volume of eluting solvent. These parameters were experimentally tested and optimized: for a 1 ml of urine and a 5 ml of solvent, the amount of alumina, Porasil, and Chromosorb P were about 3.6 ml, 3.0 ml, and 2.7 ml, respectively.

Untreated urine, acidic urine (pH 1), and basic urine (pH 11), each saturated with NaCl, were examined. Of the different combinations of the variables studied the best results, both for acidic and untreated urines, were obtained

by combinations of ether-Chromosorb P, ether-Porasil and dichloromethane-Chromosorb P, as depicted in Figures 4 through 8. For basic urine, dichloromethane-Chromosorb P, was the only combination to give satisfactory yields as seen in figure 9. Alumina was found to be inadequate for any type of urine. From the standpoints of simplicity, stability, and cost, dichloromethane-Chromosorb P was selected as the preferred combination applicable under all conditions.

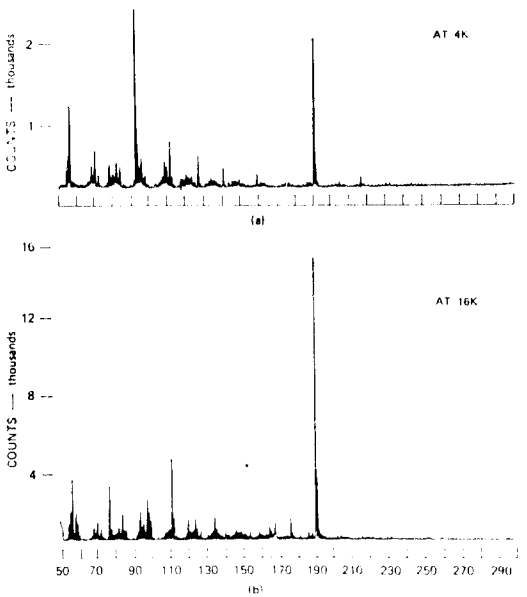

Fig. 9: Mass profiles of basic urine-dichloromethane, using (a) alumina and (b) Chromosrb P,

The reproducibility of the overall analytical procedure for acidic urine using the chosen combination is demonstrated in Figure 10.

The ionization efficiency of the multipoint field ionizer[7] was very significantly reduced following the analysis of organic metabolites, especially acidic samples. This was due to the deactivation of source caused by electronegative substrates, in particular carboxylic compounds. Therefore, the variation in performance of the ionizer provided the largest source of variance. To overcome this shortcoming, a new type of source with long-term stability was searched for. The glass-lined electrochemically activated foil

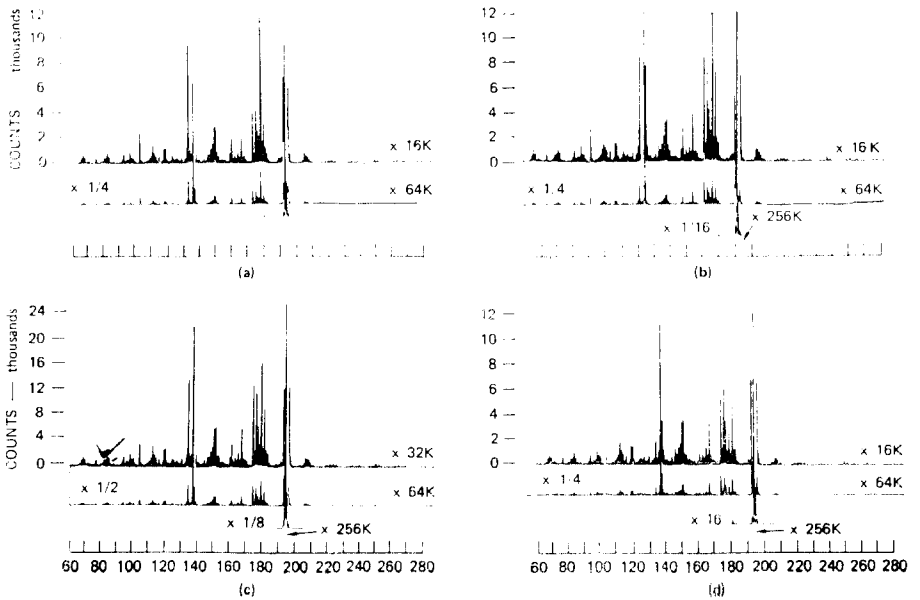

Fig. 10: Comparison of four different experiments under the same experimental conditions using acidic urine-5ml dichloromethane-Chromosorb P.

slit source[10,11] was found to be well suited for this purpose.

The reproducibility of the urinary mass spectra was evaluated. Knowledge of the error components is necessary for optimizing the method. During the early stages of the investigation when the multipoint field ionization source and the quadrupole analyzer were used, average coefficient of variation was about 22 to 28%, when one urine sample was analyzed ten times, and each time the whole extraction and concentration procedures were repeated, The same variance was observed, however, when the extracts from a number of extractions were pooled and then analyzed repeatedly on the mass spectrometer. It was concluded, therefore, that the mass spectrometric analysis is by far the major contributor to the variance. The sector magnet mass spectrometer with a new type of field ionizer was therefore used in the later stages of this investigation.

Ten replicate samples of neutral metabolites from the same urine were prepared using dichloromethane-Chromosorb P combination and then analyzed using the glass-lined foil source described above. As the sample itself is quite volatile in the high vacuum, a cryogenic loading procedure was employed to avoid the loss of more volatile constituents during the pumping-out procedure. The variance was analyzed by computer, which provided the coefficients of variation of 212 peaks in the spectrum and the average variance. An average coefficient of variation of 13.4 % was obtained for constituents with molecular weight higher than 110 amu. The constituents of lower than 110 amu had significantly higher variance due to their higher volatility, the reproducibility of which seems not to be sufficiently controlled by our precooling and temperature programming procedures. The less volatile constituents showed, however, less dependence of variance on molecular weight (or volatility), indicating minimal systematic error.

CONCLUSION

The multicomponent anaytical technique described in this paper is quite promising when aimed at diagnosing a metabolic disorder on the basis of a characteristic pattern of a subset of constituentes in its molecular weight profile. Although we wish to determine each constituent with maximum precision, say 1 %, this may not be necessary when the biological variances, in most cases, 10 % or higher. Since for diagnostic purposes we are looking for coincidential changes in a number of parameters, even a larger variance of the analytical technique is acceptable. It may be concluded, therefore, that the application of FIMS multicomponent analysis is now not limited by the level of effort required or by the precision of the technique. It will take, however, a number of careful clinical studies, such as the one on infectious hepatitis[7], which has not yet benefited from the later developments of the technique, to establish its usefulness for biomedical research and clinical diagnosis.

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