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RAPID SEPARATION OF URINARY ACIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Over a hundred acidic urinary constituents were separated within 30 min by using 5-μm octadecyl-silica columns and gradient elution with increasing acetonitrile concentration in dilute aqueous phosphoric acid solution at 70°. The column effluent was monitored with a UV detector at 280 nm or with a fluorescence detector at 260 nm excitation and 340 nm emission wavelengths. The high sensitivity and speed of analysis, the excellent reproducibility and adequate resolution obtained suggest that this technique may be useful to obtain metabolic profiles in routine clinical work.

INTRODUCTION

In the last decade various chromatographic techniques were developed to separate a large number of body fluid constituents concurrently. Most success has been attained by the use of gas chromatography to obtain metabolic profiles of various groups of analytically related metabolites [1—3] or fingerprints of volatile substances [4, 5] present in urine or other physiological fluids. The identification of the individual compounds is greatly facilitated by the tandem operation of the gas chromatograph with the mass spectrometer [6—8]. Gas chromatography, however, is limited by the requirement that the sample components be sufficiently volatile and the analysis of polar compounds necessitates an elaborate derivatization step prior to the chromatographic separation.

In order to overcome this shortcoming, Scott and collaborators [9, 10] employed ion-exchange chromatography and succeeded by using columns packed with anion-exchange resin to analyze over a hundred UV-absorbing components in human urine. The originally excessive time of separation could be drastically reduced from 24 to 14 h with the use of coupled anion- and cation-exchange columns [11]. Although the technique offers a powerful tool for the study of metabolic disorders, the analysis time is prohibitively

long to afford the routine application of ion-exchange chromatography for such multicomponent separations in the clinical field.

Our recent study [12] has demonstrated that polar biological substances can be rapidly separated by high-performance liquid chromatography on a non-polar stationary phase such as octadecyl-silica with aqueous eluents. In this type of chromatography, which is often referred to as "reversed-phase" chromatography, solute retention is governed by hydrophobic interactions with the hydrocarbonaceous functions of the bonded stationary phase [13]. Evidently, the magnitude of the hydrophobic interactions between closely related solutes and the non-polar stationary phase are sufficiently large and different to obtain satisfactory retardation and specificity even with biological compounds which are considered polar [14]. The practical merits of this technique stem from its relative simplicity as well as high reproducibility and efficiency with respect to that of ion-exchange chromatography under comparable conditions. Gradient elution with increasing concentration of a suitable organic solvent in the eluent facilitates the separation of a wide variety of substances on non-polar bonded phases in a convenient and reproducible fashion.

The advantages of this type of chromatography prompted us to investigate the separation of urinary constituents on the basis of their "hydrophobicity". In view of recent advances in urinary metabolic profiles of organic acids [15—17] for diagnostic purposes, our interest was focussed on the development of a rapid chromatographic method for the separation of substances present in the extract of acidified urine. The results illustrate that solvophobic chromatography [13] is a rapid and powerful method for the separation of a large number of biological substances present in physiological fluids.

EXPERIMENTAL

Apparatus

A Perkin-Elmer Model 601 high-pressure liquid chromatograph with an LC 55 variable wavelength UV detector (Perkin-Elmer, Norwalk, Conn., U.S.A.) and a Schoeffel FS-970 fluorescence detector (Schoeffel, Westwood, N.J. U.S.A.) was used in the gradient elution mode. The sample was introduced by using a Siemens high-pressure injection syringe with a 10-μl needle (ES Industries, Marlton, N.J., U.S.A.).

In all experiments a flow-rate of 2.0 ml/min and a concave gradient at curvature setting 55 were the most effective. The starting eluent was 0.1 *M* phosphate buffer, pH 2.1 (reagent grade H_3PO_4 and KH_2PO_4, Fisher, Pittsburgh, Pa., U.S.A.) and acetonitrile or methanol (Burdick and Jackson, Muskegon, Mich., U.S.A.) were used as gradient formers. The gradient run, at a 50-min setting for 100%, was terminated after approximately 30 min, when the organic solvent concentration reached about 35—40% (v/v). Subsequently the column was re-equilibrated with the aqueous eluent at 2.0 ml/min in 15 min. The column temperature was maintained at 70°.

It is noted that the use of syringe pumps for gradient elution with water—methanol and water—acetonitrile mixtures over 40% (v/v) can result in poor reproducibility due to changes in the viscosity and compressibility of the eluent with the solvent composition as discussed by Martin et al. [18] and

Abbott et al. [19]. We found that with the instrument used in this study good reproducibility of the retention values was obtained over the full solvent composition range when the retrace-gradient setting was used for re-equilibration. The results were also satisfactory when after completion of elution the shut-off valve of the pump for the organic solvent was closed and the pressure was raised to the starting value prior to re-equilibration.

Columns

In the course of this investigation various home-made and commercial columns were evaluated. All columns employed were made of 25 cm × 4.6 mm I.D. × 6.4 mm O.D. No. 316 stainless-steel tubing with zero dead-volume fittings. Octadecyl-silica was prepared from 10- and 5-μm Partisil (Whatman, Clifton, N.J., U.S.A.) with octadecyltrichlorosilane (Aldrich, Milwaukee, Wisc. U.S.A.) according to the literature [20]. Columns were packed with a home-made instrument equipped with a 50,000-p.s.i. Haskel reciprocating pump (Haskel Engineering & Supply Co., Burbank, Calif., U.S.A.) by using the isopycnic slurry method at 10,000 p.s.i. It has been found, that columns packed with 5-μm octadecyl-silica containing 16—18% (w/w) carbon yield the best separation under our conditions. Since recently such columns became commercially available, the results presented here were obtained with 5-μm LiChrosorb ODS columns of the above dimensions (Rainin Instruments, Boston, Mass., U.S.A.).

Samples

Urine samples have been obtained from Dr. P. Jatlow, School of Medicine, Yale University. A 5-ml volume of urine was saturated with NaCl, centrifuged and the pH of the supernatant was adjusted to 13 with 1 *M* NaOH by using a Model 26 pH meter (The London Co., Cleveland, Ohio, U.S.A.). First, it was extracted with 10 ml of ethylacetate (ACS grade; Mallinckrodt, St. Louis, Mo., U.S.A.) at 30° in a water-bath shaker (Eberbach, Ann Arbor, Mich., U.S.A.) for 15 min in order to remove neutral and basic organic compounds. After phase separation the pH of the aqueous phase was adjusted with concentrated HCl to pH 1 and the organic acids were extracted with another 10 ml of ethyl acetate as described above. The extract was then evaporated to dryness in a nitrogen stream and stored in the refrigerator. Prior to analysis the residue was dissolved in 0.5 ml of 1 *M* HCl.

The organic acids have been supplied by Sigma (St. Louis, Mo., U.S.A.) and by Aldrich.

RESULTS AND DISCUSSION

Our goal was to demonstrate that many low-molecular-weight constituents of physiological fluids can be faster and more efficiently separated by recently introduced microparticular non-polar stationary phases than by ion-exchange resins employed conventionally. Preliminary studies with urine samples have been carried out by monitoring the column effluent of our chromatographic system with the UV detector at 210 nm as well as with the fluorometer at different excitation and emission wavelengths. The results suggested, that a further improvement of the separation efficiency and the method of detection

could yield chromatograms of urine with over 500 resolved peaks.

Since the number of urinary constituents [21] exceeds the peak capacity of the present system it behooved us to limit the number of sample components by using an extraction procedure prior to the chromatographic separation on the one hand and by setting the wavelength of the UV detector to 280 nm on the other. The extraction of acified urine with ethyl acetate has long been used for the isolation of the less hydrophilic urinary constituents with good analytical recovery [22]. As most substances having aromatic moieties strongly absorb light at 280 nm the method presented here is eminently suitable to obtain chromatographic profiles of urinary aromatic acids.

With octadecyl-silica columns best results have been obtained, when the hydro—organic eluent is buffered in the neighbourhood of pH 2. In our experience, phosphoric acid buffer is particularly suitable for this purpose [12]. The use of low pH is required to retard the more hydrophilic acids on the nonpolar stationary phase. Earlier studies from our laboratory demonstrated that the capacity factors of unionized aromatic acids are 3 to 5 times higher than those of their conjugated bases [14]. The buffer not only maintains the eluent pH constant, thus enhances reproducibility, but also facilitates the rapid establishment of protonic equilibria both in the eluent and on the stationary phase [23]. It has been observed that asymmetric peaks are obtained in the absence of adequate buffering capacity, when both the acid solute and its conjugated base are present in the eluent.

Gradient elution with increasing organic solvent concentration in the eluent is also essential to obtain the peak capacity required for such multicomponent separations [24]. Although both acetonitrile and methanol yield suitable eluent gradients, the use of water—acetonitrile mixtures is preferred because the strong association between methanol and water results in dramatical changes in the physical properties of the solvent such as viscosity and density with changing solvent composition and can impair the reproducibility of the results.

A typical chromatogram of urinary aromatic acids as obtained with the UV detector at 280 nm is shown in Fig. 1. It is seen that over a hundred substances can be separated within 30 min. The identification of the individual peaks remains a major problem and would require the use of the mass spectrometer or some other method. We separated twelve aromatic acids of urinary significance under the same conditions and the chromatogram is shown in Fig. 2. Their retention values have been used for the tentative identification of the corresponding mandelic, phenylacetic, indoleacetic and cinnamic acid derivatives. The chromatogram of the urine extract spiked with these acids in Fig. 3 shows that the retention times of the marker peaks are the same as those of the pure substances in Fig. 2. This also supports our observation that the method is highly reproducible as long as the column properties remain unchanged (see below).

According to our experience the sensitivity of the UV detector can be increased by a factor of one hundred under the conditions used in these studies before the baseline noise interferes with data evaluation. Experiments with varying amounts of added aromatic acids such as *p*-hydroxymandelic acid or homovanillic acid showed, that the peak heights are linearly dependent on the

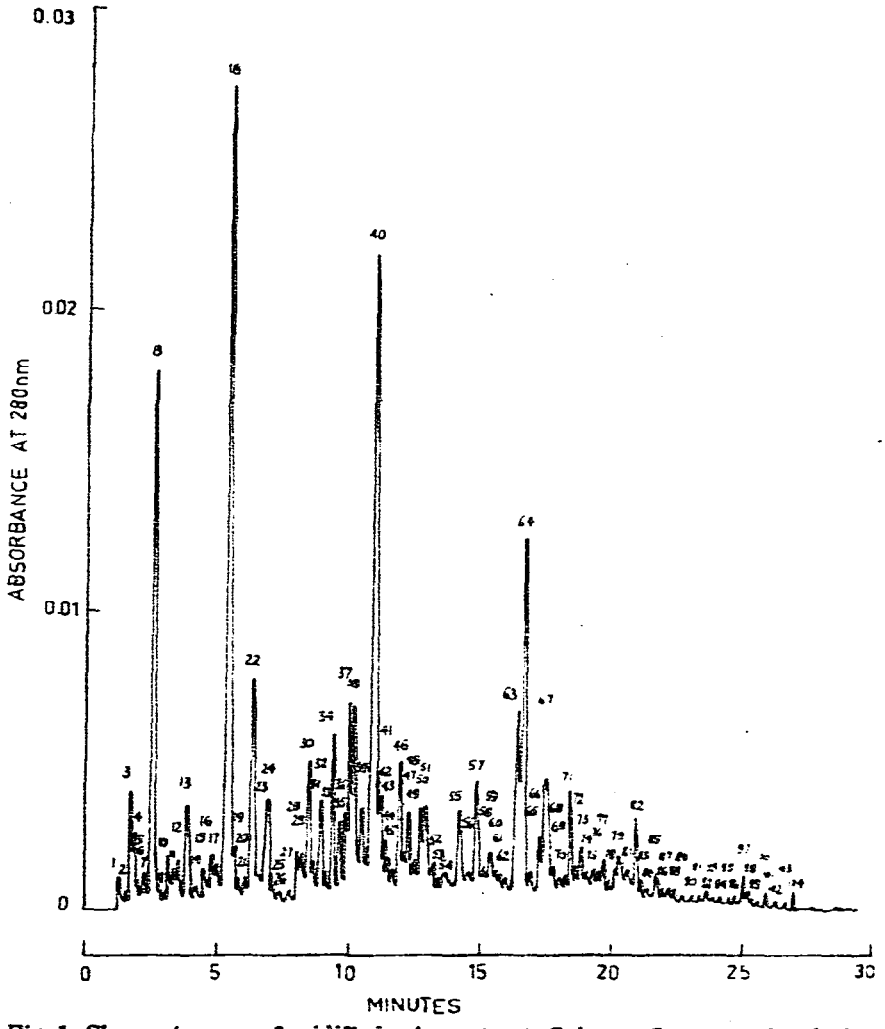

Fig. 1. Chromatogram of acidified urine extract. Column: 5-μm octadecyl-silica, 25 cm × 4.6 mm I.D.; temperature, 70°; flow-rate, 2.0 ml/min. Gradient elution from 0.1 *M* phosphate buffer, pH 2.1, with acetonitrile to about 40% (v/v) organic solvent concentration. Sample size, 10 μl containing the extract of 100 μl of urine.

urinary concentration of these components in the range of 0—200 mg/l.

The use of sensitive fluorescence detectors offers an alternative to obtain urinary profiles as shown in Fig. 4. Some of the peaks have been tentatively identified by the retention values of added marker substances. In general, the metabolites of tryptophane and many of the acids containing hydroxyphenyl moieties have been found to have strong fluorescence under the conditions employed. As the oxidation products of catecholamines very strongly fluoresce and they can efficiently be separated by reversed-phase chromatography [12], this method combined with a simple pre-column oxidation procedure [25] may offer an opportunity for the quantitative analysis of

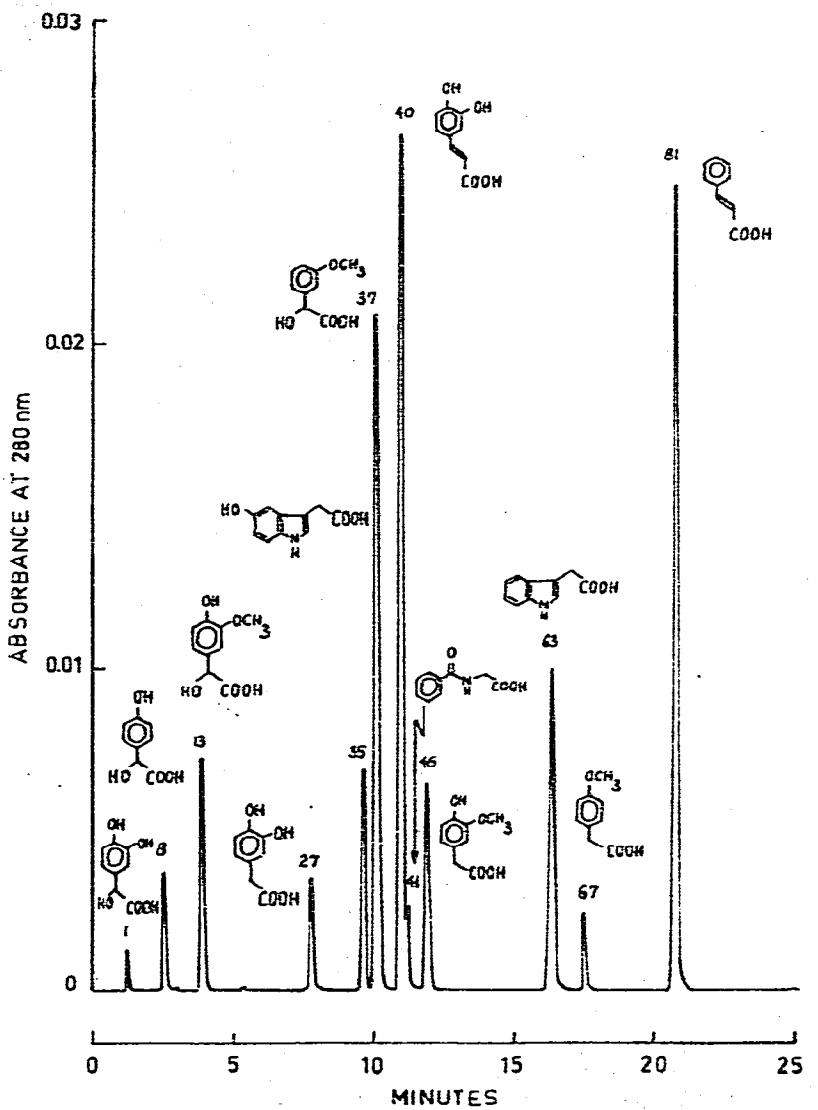

Fig. 2. Chromatogram of aromatic acids. Column and elution conditions as in Fig. 1. The peak numbers correspond to those shown in Figs. 1 and 3. Sample, 10 μl of an aqueous solution containing 1 μg of each compound.

urinary catecholamine derivatives at the femtomol level. When the effluent is monitored by the fluorescence detector at 260 nm excitation and 340 nm emission wavelength, 1-hydroxy-2-naphthoic acid is an appropriate internal standard.

It has been observed, that the elution pattern of the urinary acids can change with sample storage. Therefore, the reproducibility of the results may depend upon the strict adherence to an exact protocol for the handling of the urine samples. According to our experience, the dry extract can be stored in

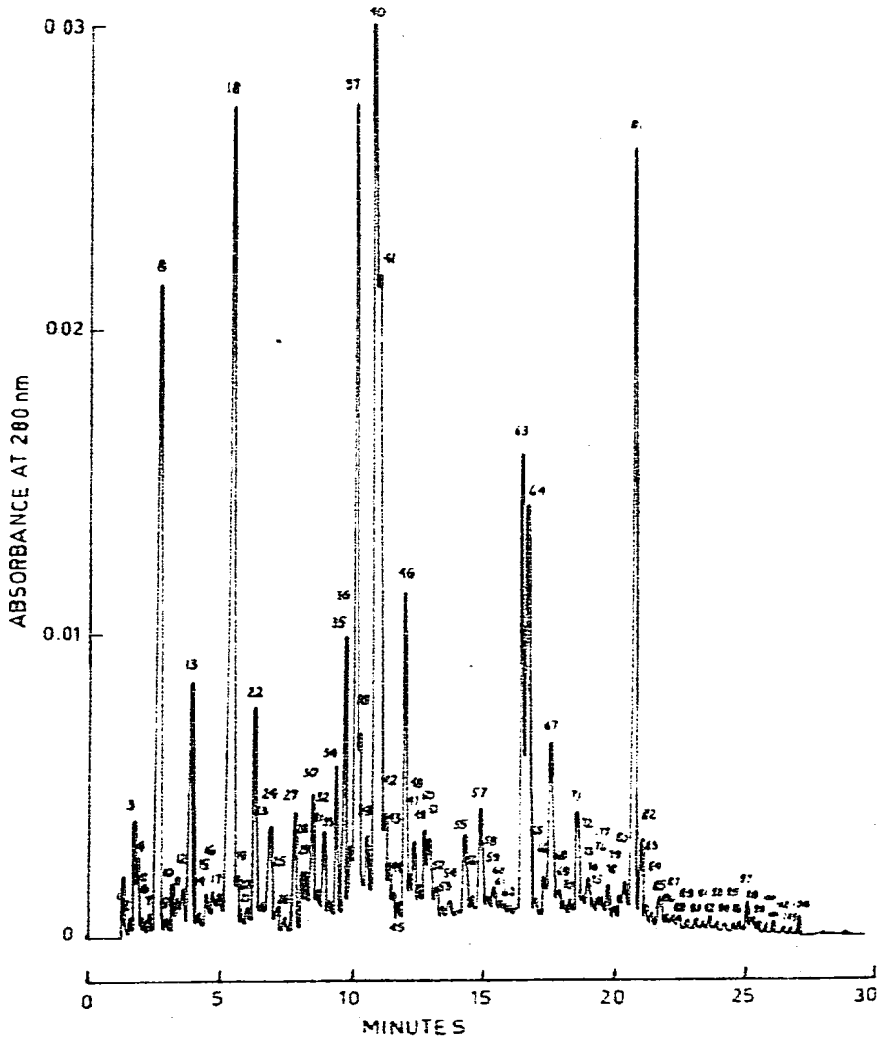

Fig. 3. Chromatogram of acidified urine extract with the added marker compounds shown in Fig. 2. Column and elution conditions as in Fig. 1.

the refrigerator over two months without changes in the chromatographic profile. It is noted, that the present method is eminently suited to follow chemical changes in the urine, which is exposed to the atmosphere at room temperature. At present little is known about these changes, which may alter the composition of the urine prior to the chromatographic analysis.

The retention times showed a slow decrease upon extensive use of a given column. This phenomenon is attributed to the slow hydrolysis of the octadecylsiloxane moiety on the surface of the stationary phase at 70°. Under such condition the useful life of a column packed with 5-μm octadecyl-silica containing 16—17% (w/w) carbon is approximately 300 h. We found, however, that the column can be easily rejuvenated by an in situ treatment with a dilute solution of octadecyltrichlorosilane in toluene [26].

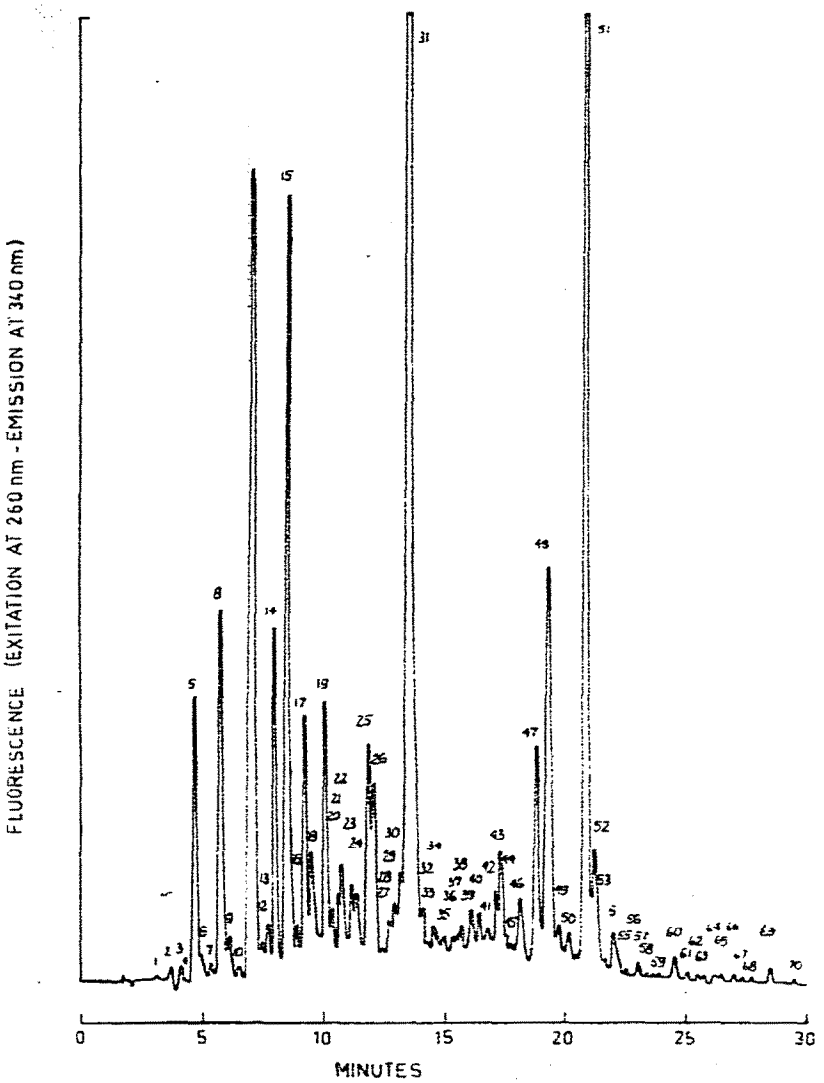

Fig. 4. Chromatogram of urine extract under conditions similar to those given in Fig. 1, but after the column was used for about 300 h. The fluorescence detector was set for 260 nm excitation and 340 nm emission wavelengths. The sensitivity and the time constant were 1 μA and 0.5 sec, respectively.

The thermodynamic basis of retention in this type of chromatography is, albeit very complex, fairly well understood [13, 14]. As a rule of thumb, the capacity factor increases with the non-polar surface area of the solutes, but polar and in particular ionized groups, which strongly interact with the aqeous eluent, reduce solute retention. The increase in eluent strength with increasing organic solvent concentration is mainly attributed to the reduction of the effective surface tension of the eluent. As seen in Fig. 2 the elution order of the

solutes corresponds to the qualitative statement regarding the relationship between the molecular structure and retention.

The clinical significance of new "high-resolution" techniques for the analysis of urinary constituents has been well recognized [27—29]. There is ample evidence, that many of the substances may have pathological importance. Gas chromatographic profiling and concomitant mass spectrometric analysis have been particularly useful for the characterization of inborn errors of metabolism [30]. About 50 diseases have already been found to give rise to high concentration of acidic metabolites in urine and for instance the excess amount of certain acidic metabolites has been related to such diseases as isovaleric aciduria, maple-syrup urine disease and glutaric aciduria [31—33]. The present technique could serve as a rapid method for the establishment of the relationship between the urinary level of individual aromatic acids and certain disease states. It is noted, that many of these acids are produced by the abnormal metabolism of phenylalanine [34]. The extinction coefficient of aliphatic acids, with the exception of keto acids, is usually very low at 280 nm, therefore, their analysis has to be carried out at a detector wavelength of 215 nm and below. The retention times of the short-chain aliphatic acids is relatively small under the conditions used and their separation may require lower column temperature and isocratic elution. Preliminary results indicate that closely related keto and hydroxy- acids, which cannot be separated by gas chromatography with packed columns [32], can be resolved by the present technique due to their different hydrophobicities.

Recently Chalmers et al. [22, 35, 36] have investigated urinary acids in man and established the quantitative ranges and frequency distribution patterns of excretion for a number of acids in a normal population. An important finding of these studies is that whereas the excretion patterns of acids widely differ from individual to individual, even extreme dietary alterations produce only small changes in the individual excretion patterns under normal conditions. Consequently, changes in the urinary acid profile of an individual may be used for early diagnosis of certain diseases.

High-pressure liquid chromatography appears to be an efficient tool for the rapid separation of the components of the physiological fluids. It can be used either for the determination of the physiological level of individual constituents having established clinical significance [37—39] or to obtain metabolic profiles by chromatographic fingerprinting. In each case it would be desirable to obtain positive identification of the peaks of interest and it is hoped, that the tandem operation of the mass spectrometer with the liquid chromatograph becomes practical to carry out this task. The present limitations notwithstanding, liquid chromatography has many advantages over gas chromatography and in view of the recent improvements of efficiency and sensitivity it can qualify as one of the "new high-resolution analytical systems, that are capable of separating and quantifying many of the individual constituents of a physiological sample" envisioned by Scott and Melville [21] and due to the high speed of analysis it "may be useful in the clinical laboratory for in-depth analysis".

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