Automatic, High-Resolution Analysis of Urine for Its Ultraviolet-Absorbing Constituents.* (32043)

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Over 300 compounds have been reported in human urine(1-4). These include inorganic compounds, organic acids, sugars, amino acids, purines and related compounds, enzymes, hormones, vitamins and their metabolites, estrogens, and many other organic compounds. The quantities of these constituents found in individual urine samples represent a wealth of information that can be used to evaluate body functions. Such quantitative data have been collected on a limited basis for many years and, as a result, many molecular constituents have been shown to have pathological significance. For example, a high uric acid content is associated with gout(5) and some leukemias (6); a high urocanic acid content is sometimes found in conjunction with asthma(7); large amounts of phenolic and indole amines appear to be excreted by schizophrenics(8), etc.

The Molecular Anatomy (MAN) program at Oak Ridge National Laboratory(9) is concerned with the description, at the molecular level, of the structure and organization of cells and tissue. One of the aims of this program is to develop high-resolution, automated systems for the analysis of low-molecular-weight substances found either free in cells or as constituents of macro molecules. A logical extension of this work is the further development of such analytical systems for use with human body fluids. This laboratory is now developing an automatic, high resolution analytical system for quantitatively determining the molecular constituents of human urine.

The approach is to modify and expand the capabilities of an existing nucleotide analyzer (10) for use as a urine analyzer. Initial results indicate that this concept is feasible; *i.e.*, a high-pressure, high-resolution modification of the nucleotide analyzer has resolved over 100 chromatographic peaks of ultraviolet-absorbing constituents from a 2-ml urine sample. Twelve of these peaks have been tentatively identified. Significant differences between urine chromatograms of normal subjects, leukemics, and schizophrenics have also been detected.

In this paper we describe the (a) development of a high-pressure, high-resolution chromatographic system, (b) evaluation of the system for analyzing human urine, and (c) identification of some of the chromatographic peaks observed.

Developmental studies. Although the development of an automatic urine analyzer could be directed toward measuring either one or a few molecular constituents that are associated with a single disease or abnormality, a more general approach will ultimately prove to be more useful. A general approach will allow one to determine whether there are substances interfering with the analysis of other urinary constituents. Such interferences appear to be among the major causes of inaccurate results in the analysis of specific urinary constituents.

The primary goal of the development effort it to devise a system which will allow quantitative determination of a large number (greater than 100) of the molecular constituents of human urine with a reasonable accuracy. Further, this system will be highly automated so that only a few minutes of operator time is needed per sample. Finally, the data from this system will be reduced to a form that is readily usable (a tabulation showing the amount of each constituent in milligrams). Results obtained in our work thus far indicate that the prospects for attaining these objectives are good.

Experimental system. The experimental portion of this program has involved modifying an existing nucleotide analyzer originally

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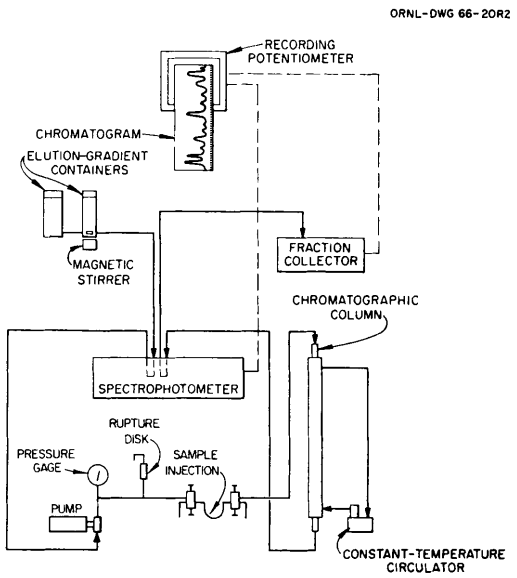

FIG. 1. High resolution urine analyzer for the ultraviolet-absorbing constituents of urine.

developed by Anderson and co-workers(10). This modified system is composed of a heated, high-pressure, anion exchange column (typically 0.62 cm ID × 200 cm, filled with Dowex 1-X8 resin, and operating at 40°C) as the separations system and a recording ultraviolet spectrophotometer that operates alternately at 2 to 4 wavelengths as the detector (Fig. 1). There are also provisions for volumetric measurement and collection of the column effluent.

Urine samples are prepared from a 24-hour composite that has been refrigerated during collection. They are acidified to a pH of 4.4 (with acetic acid) and filtered through a membrane filter having a pore size of 0.2 μ.

The urine chromatogram, which shows the absorbance of the column effluent as a function of time, is developed by introducing a urine sample (0.5 to 2 ml) into the eluent stream just ahead of the ion exchange column by use of high-pressure valves, and eluting it through the column with an ammonium acetate-acetic acid buffer solution ranging from 0.015 to 6 *M*, at a pH of 4.4 and a flow rate of 30 to 60 ml/hr. In the usual chromatogram, 80 to 100 peaks are resolved in about 48 hours (Fig 2).

Higher resolution can be obtained by using very small anion exchange resin beads in the separation column; 5-10-μ-diam resin resolves about 50% more chromatographic peaks from the same urine than does 20-40-μ-diam resin. The small-diameter resin is separated from commercial batches of resin by a continuous water elutriation system.

Use of the small resin, however, necessitates operation at higher column pressures to maintain a comparable eluent flow rate. Therefore, stainless steel columns have been designed and are now routinely used in the pressure range of 2000 to 4000 psi.

Results. Chromatographic peaks for runs made at the same conditions (Fig. 2) are reproducible within 0.3 hour and more than 100 urine samples have been run on a single column without loss of resolution. Thus the system demonstrates good reproducibility and good resolution, both of which are mandatory for an automatic system.

Urine constituents are being tentatively identified by determining the position and the absorbance ratio, at 2 or more different wavelengths, of the chromatographic peaks of known chemicals and comparing them with unknown peaks in the urine chromatogram. Standard mixtures of known chemicals are also being combined with urine samples to enlarge suspected chromatographic peaks. Chromatographic position and absorbance ratios give two independent means of comparison, both of which should be characteristic of specific urinary constituents or small groups of constituents.

Twelve urinary constituents, many of which have pathological significance in various diseases, have been tentatively identified by this method (Fig. 2). These are:

creatinine	uric acid
tryptamine	hippuric acid
ergothioneine	p-aminobenzoic acid
hypoxanthine	p-hydroxybenzoic acid
xanthine	p-hydroxyphenylacetic acid
urocanic acid	kynurenic acid

More definite identification will be made by isolating the unknown from a colum effluent fraction and applying the classical methods of organic chemistry, as well as those reported by Cohn for biochemical identification (11). The more recent computational methods, in which complex mixtures are resolved

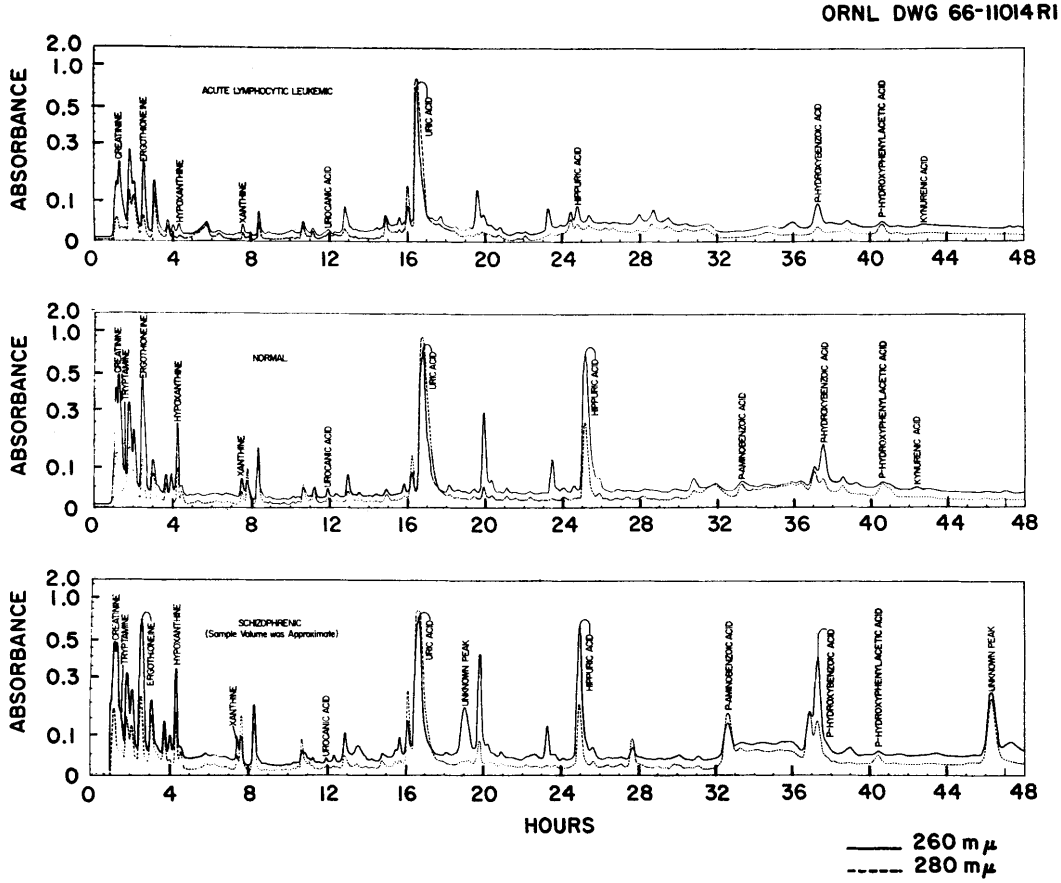

FIG. 2. Typical chromatograms from the urine analyzer showing reproducibility and comparison between a normal chromatogram and chromatograms from typical acute lymphocytic leukemics and schizophrenics. Run conditions: column, 0.62 cm I. D. × 200 cm stainless steel with 5-10 μ diam Dowex 1-×8; urine sample, equivalent to 0.05 kg of body weight for a 24 hr-collection period; temperature, 40°C; pressure, 1000-2200 psig; eluent, ammonium acetate-acetic acid buffer, at a pH of 4.4, varying in concentration from 0.015 to 6 *M;* average flow rate of eluent, 28 ml/hr.

by using linear programming and least-squares techniques on physical properties such as ultraviolet spectra, will also be used (12).

Several pathological urine samples have been run to determine if the system is capable of differentiating between normal and abnormal urine. Significant differences have been observed between normal samples and those from subjects with acute lymphocytic leukemia‡ and schizophrenia§ (Fig. 2). The urine from 4 such leukemics showed a notable decrease in hippuric acid content as well as an increase in the ratio of uric acid to xanthine and hypoxanthine. The urine chromatograms from three schizophrenics had two very large unknown chromatographic peaks which we have not previously seen in normal urine.

Discussion. The experimental system in its present form is suitable for some types of biochemical and medical research. However, the total analysis time (approximately 48 hours) may be prohibitive for extensive general clinical use. The analysis time has

‡ Urine samples from subjects with leukemia were obtained from Dr. C. L. Edwards, the Medical Division of Oak Ridge Associated Universities, Oak Ridge, Tenn.

§ Urine samples from subjects with schizophrenia were obtained from Dr. C. F. Mynatt, Eastern State Psychiatric Hospital, Knoxville, Tenn.

already been reduced by about 8 hours from that originally required, and a further decrease is anticipated by using higher-pressure systems and optimizing operating parameters such as temperature and elution gradient.

The capability of the system we have described is limited to the analysis of ultraviolet-absorbing constituents of urine; however, it could be extended by incorporation of additional detection systems. For example, if the ninhydrin colorimetric detection system(13) and a carbohydrate colorimetric technique recently developed by Green(14) could be integrated into the present analyzer (while using the same separation system), the resulting instrument would be able to detect a much larger number of compounds.

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Effect of Melatonin on Thyroid Hormone Secretion Rate and Feed Consumption of Female Rats.* (32044)

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The physiological role of the pineal gland has long been a mystery, but numerous reports have appeared during the last ten years regarding the status of this gland. According to Wurtman *et al*(1) the pineal is an endocrine gland and secretes the hormone melatonin which affects the reproductive system, primarily in females. Pinealectomy in the immature rat produced ovarian hypertrophy (2,3) while administration of pineal extracts caused ovarian atrophy(3,4). It was then shown by Wurtman *et al*(5) that the effects produced by pinealectomy were similar to those produced by exposure to constant light, but these were not additive. The ability of the rat pineal gland to synthesize melatonin was reported to be markedly reduced by exposure to light(6) and enhanced by constant darkness(7,8). It would thus appear that melatonin depressed the precocious development of the ovary; and light, by depressing melatonin secretion, stimulates the gonadotrophic hormones and ovarian development.

The possible relation of the pineal gland and melatonin to other endocrine gland functions requires study. Evidence indicating a relation to thyroid gland weight(9), adrenal gland(8,10,11,12,13,14) and the pituitary gland(3,15) has been presented. Kitay and Altschule(16) summarized the literature on the relation of pinealectomy to the thyroid gland citing references indicating hypertrophy or no change. Malm *et al*(17) reported that pinealectomy stimulated the growth of rats

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