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# COMPARATIVE SERUM AND URINE ANALYSES BY DUAL-DETECTOR ANION-EXCHANGE CHROMATOGRAPHY

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## SUMMARY

A high-pressure anion-exchange chromatographic system has been modified to provide measurement of large numbers of molecular constituents in serum and for direct comparison to similar measurements in urine. Operating parameters have been adopted which greatly extend the range of elution for strongly retained anionic constituents and limit resolution of early-eluting basic and neutral compounds which were of less interest in this study. Dual monitoring by UV absorption and fluorescence produced by cerate oxidation provides both sensitive and wide-ranging detection capability.

Comparative serum and urine chromatograms for a clinically normal subject, a subject after ingesting the drug 4-hydroxyacetanilide, and an infant suffering from extreme acidosis, illustrate the potential usefulness of this analysis in studying the origin, transport, *in vivo* reactions, and disposition of metabolites.

## INTRODUCTION

Comparative analysis of various body fluids for large numbers of molecular constituents could be useful in studying the origin, transport, *in vivo* reactions, and disposition of those compounds. Chromatographic techniques make such measurements possible for steroids[1] and indoles[2,3], and because of the multicomponent capability appear suitable for further developments of this type. Impediments to advances in multicomponent determination have been the low concentration of many molecular species and the small volumes of samples that may be available.

An early study[4] at this laboratory demonstrated the separation of constituents of blood serum by high-pressure anion-exchange chromatography. However, detection and quantitation were limited to those constituents with relatively large absorptivity in the UV by the UV photometer used as the column monitor. More recently, a sensitive cerate oxidative detector responsive to additional compounds has been developed and applied to an anion-exchange chromatographic system where elution range has been extended to include the more anionic organic acids[5,6]. Using this

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system, we found that analyses of blood serum could be carried out with much greater sensitivity than achieved in the earlier work[4], and more anionic constituents could be resolved and sensitively detected. System design parameters and operating conditions were also modified to obtain better resolution and sensitivity for blood serum constituents, with the objective of reducing the time per analysis from two days to one day. Modifications were also made to extend the column elution range to include the strongly retained sulfate conjugates of drug metabolites and improve the resolution of other late-eluting compounds. Attaining the competing goals of a shorter analysis time and extended range of compounds eluted resulted in some sacrifice in the resolution of early-eluting compounds.

The modified chromatographic system is described, and the results obtained from reference compounds and pairs of blood serum and urine samples are shown.

## CHROMATOGRAPHIC SYSTEM

The chromatographic system shown schematically in Fig. 1 is similar to that described previously[6] for aromatic acids, but differs in column length, column temperature, buffer composition, and sample size. The separation column is 100 × 0.22 cm I.D., loaded with A-27 anion-exchange resin (Bio-Rad batch No. 9425), 10

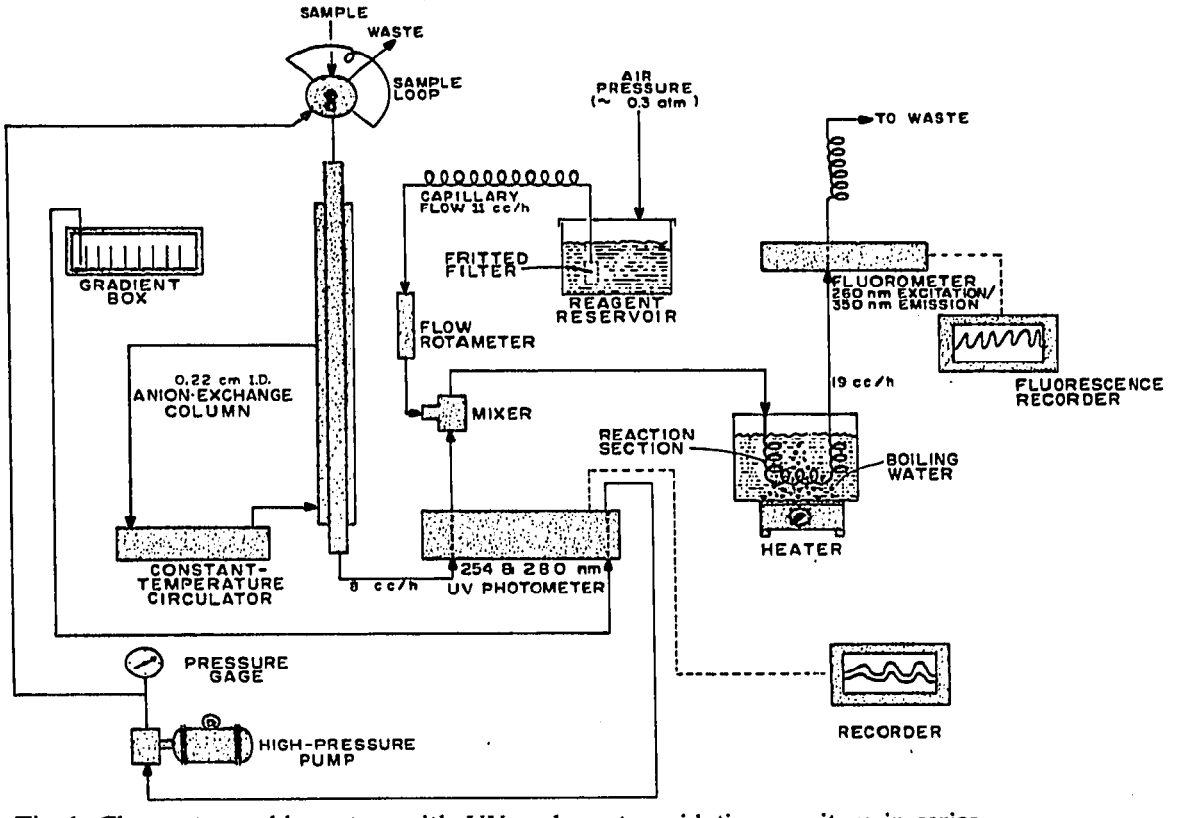


Fig. 1. Chromatographic system with UV and cerate oxidative monitors in series.

to 12 $\mu$ in size. Prior to each analysis, the column is equilibrated with 0.015 *M* sodium acetate–acetic acid buffer (pH 4.4). During analysis, the molecular constituents are eluted from the column with a concentration gradient of pH 4.4 sodium acetate–acetic acid buffer provided by a nine-chamber box initially containing 25 g of buffer per chamber (0.015 *M* in chambers 1 and 2, 4.0 *M* in chambers 3 and 4, and 6.0 *M* in chambers 5, 6, 7, 8, and 9). A liquid flow-rate of about 8.5 ml/h through the column is attained with a high-pressure diaphragm pump (Lapp Pulsafeeder, Lapp Insulator Company, LeRoy, N.Y., U.S.A.) at a pressure of about 130 atm.

The body fluid samples are introduced through a high-pressure valve using a 0.144-ml sample loop for urine and a 0.772-ml loop for blood serum samples. The urines were pressure-filtered previously through 0.20-$\mu$ membranes, and the serums are filtered through dialysis tubing.

For the first 80 min the column is at room temperature, and for the remainder of the 21 h run it is at 70°. The higher temperature, the steeper gradient, and shorter column provide in 12 h of operation a resolution for urine samples comparable to that achieved previously[7] in 20 h. The additional 9 h of elution permit determination of the more strongly sorbed constituents not eluted under previous operating conditions[4,7].

The column eluent flows through the UV photometer. An electronic gain of 2X (50 to 100% transmittance) is used for urine samples and 5X (80 to 100% transmittance) for serum samples. The eluent is then mixed with a ceric sulfate reagent stream flow-rate of 11 ml/h. The reagent contains $5.0 \times 10^{-4}$ and 1.5 mole/l of cerium (IV) and sulfuric acid, respectively, and is stabilized with 20 mg/l of sodium bismuthate. The increase in cerium(IV) and decrease in sulfuric acid concentrations over those used before[5] further decreased peak distortion and base-line noise. The mixed streams pass through a reaction coil in a boiling-water bath and then through a modified fluorometer[6]. The electronic gain of the fluorometer is adjusted to be 2.5 times greater for analysis of serum than of urine samples. The change in sensitivity achieved through sample loop sizes and UV photometer and fluorometer settings results in urine and serum chromatograms with peaks of comparable size.

A series of analytical runs was made with (i) a mixture of reference compounds, (ii) a pair of fasting serum and urine samples from a clinically normal 58-year-old male before drug ingestion, (iii) a pair of fasting serum and urine samples from the same individual taken 3 h after ingestion of 1950 mg of 4-hydroxyacetanilide, and (iv) a pair of serum and urine samples from a 1-week-old infant suffering from extreme acidosis.

## RESULTS

The peaks obtained for the reference compounds, as shown in the chromatogram of Fig. 2, indicate that the limits of detection are near 0.02 $\mu$g/ml for *p*-cresol; 0.05 $\mu$g/ml for hypoxanthine; 0.1 $\mu$g/ml for trigonelline, 7-methylxanthine, 4-hydroxyacetanilide, uric acid, 2-furoylglycine, 5-hydroxymethyl-2-furoic acid, homovanillic acid, 4-hydroxyphenylacetic acid, and 2,5-furandicarboxylic acid; 0.5 $\mu$g/ml for hippuric acid; and 5.0 $\mu$g/ml for creatinine.

The chromatographic comparison of serum and urine samples from a clinically normal subject shows that serum, though measured with a greater than 13-fold increase in sensitivity ($5.36 \times 2.5$ to account for the difference in sample size and in-

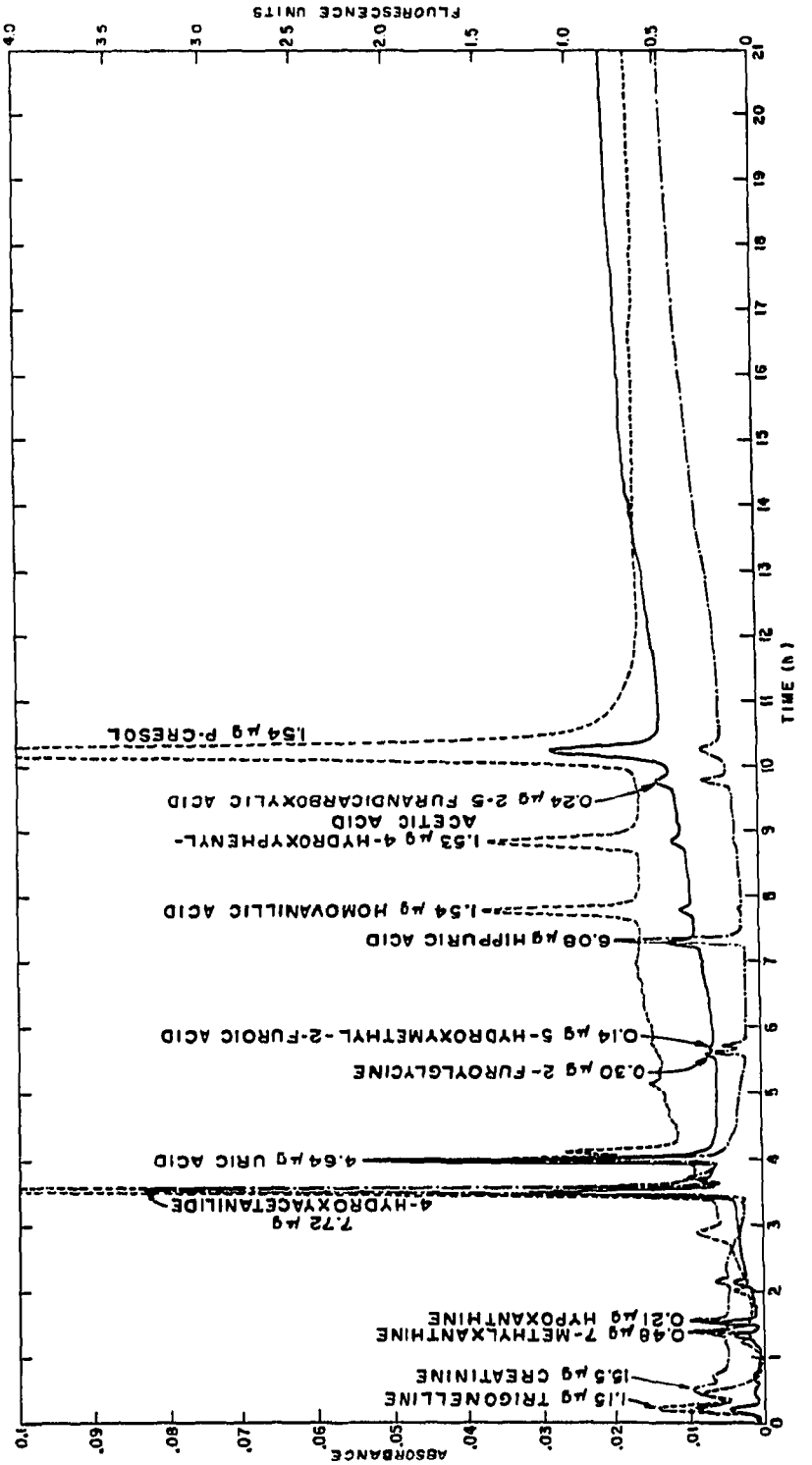


Fig. 2. Chromatogram of reference compounds (0.77-ml sample). – – –, Fluorescence; ·····, 254 nm absorbance; ———, 280 nm absorbance.

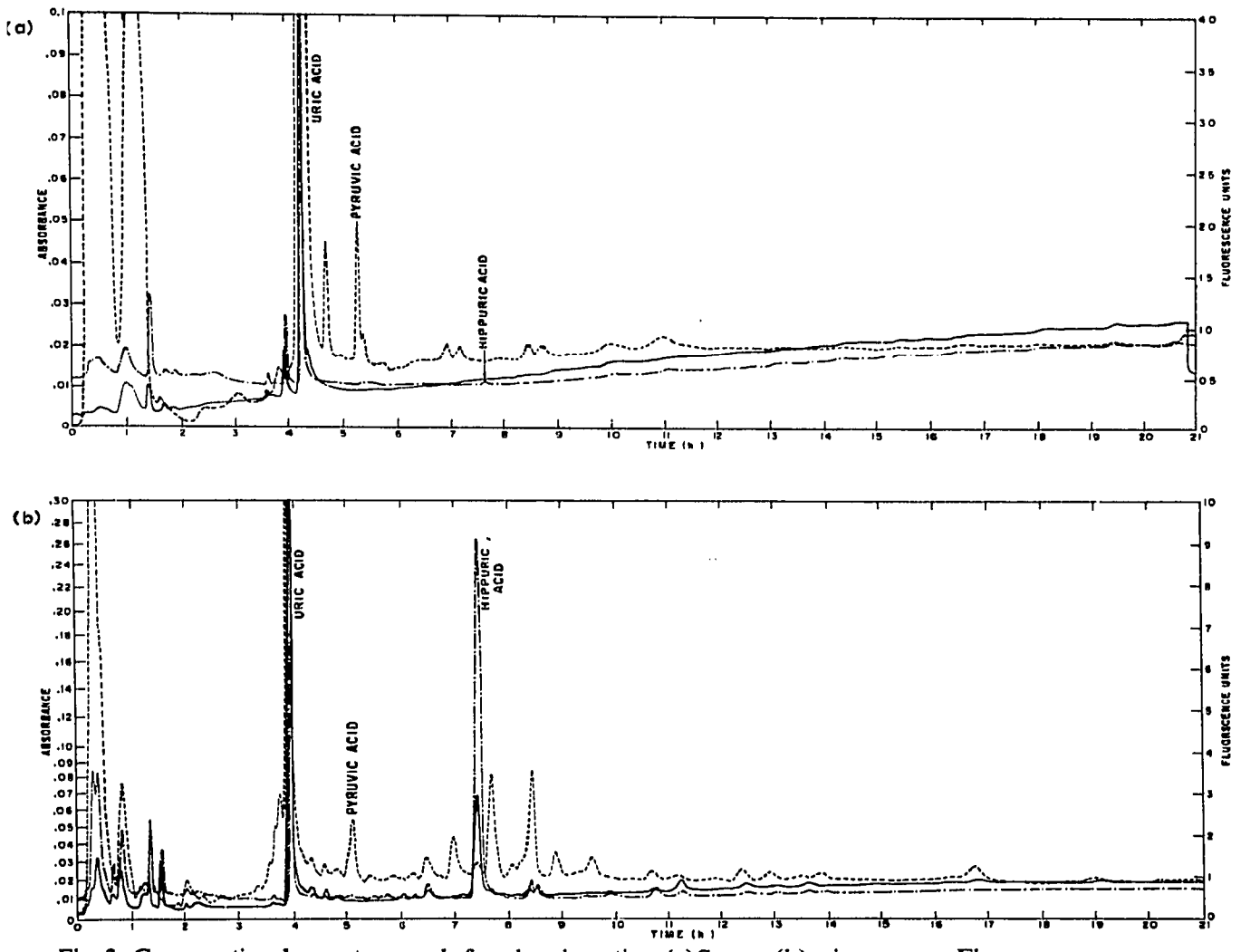


Fig. 3. Comparative chromatograms before drug ingestion. (a) Serum; (b) urine. — — —, Fluorescence; -·-·-, 254 nm absorbance; ———, 280 nm absorbance.

strument attenuation), results in fewer chromatographic peaks than resulted from the urine sample (Fig. 3). Strong response of the cerate detector to some compounds in serum was observed (*e.g.*, the compound eluting at 1.2 h tentatively identified as tyrosine, and the compound co-eluting with uric acid at 4.1 h tentatively identified as lactic acid). The presence of the UV-absorbing peak at 7.5 h in the serum (tentatively identified as hippuric acid) is unusual since we have not found that peak to occur in other serums analyzed.

The chromatogram for samples from the same subject taken 3 h after the drug ingestion shows (Fig. 4) the presence of seven urinary metabolites of 4-hydroxyacetanilide and at least four metabolites in the comparative serum. The measurement of these metabolites during a 24-h test period and their biological significance are

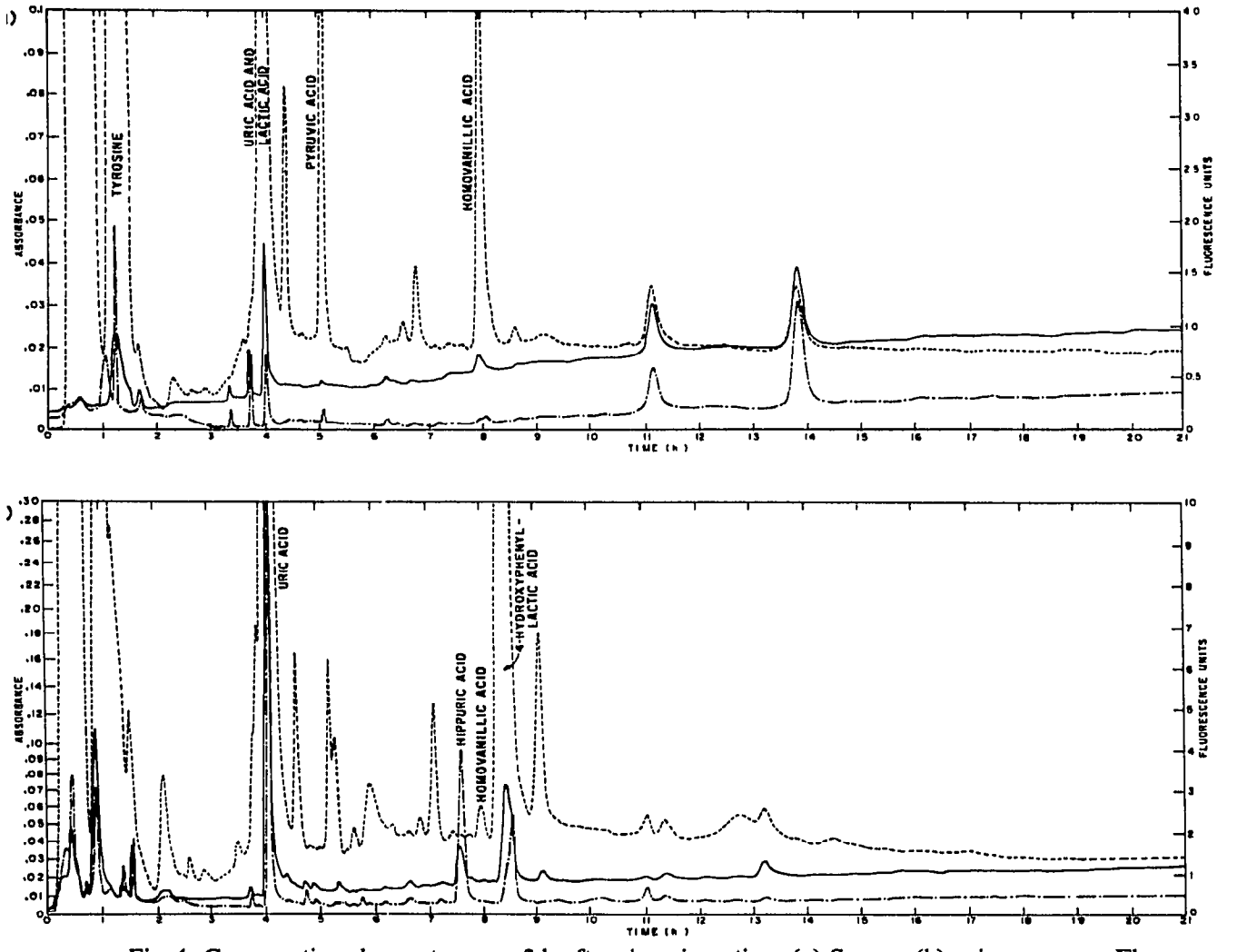


Fig. 4. Comparative chromatograms 3 h after drug ingestion. (a) Serum; (b) urine. — — —, Fluorescence; — · — · —, 254 nm absorbance; ———, 280 nm absorbance.

discussed elsewhere[8], as is the identification of these metabolites in body fluids[9].

The comparative serum and urine chromatograms for the infant suffering from extreme acidosis (Fig. 5) indicate the presence of large amounts of tyrosine in both fluids, unusually large amounts of lactic, pyruvic, and homovanillic acids in the serum, and unusually large amounts of 4-hydroxyphenyllactic acid in the urine. Additionally, two peaks at 11.2 and 13.8 h can be presumed to be due to uncommon aromatic acids.

## DISCUSSION AND CONCLUSIONS

The serum analyses provided by the apparatus described here are significantly superior in sensitivity to those resulting from previous similar analyses[4] and provide

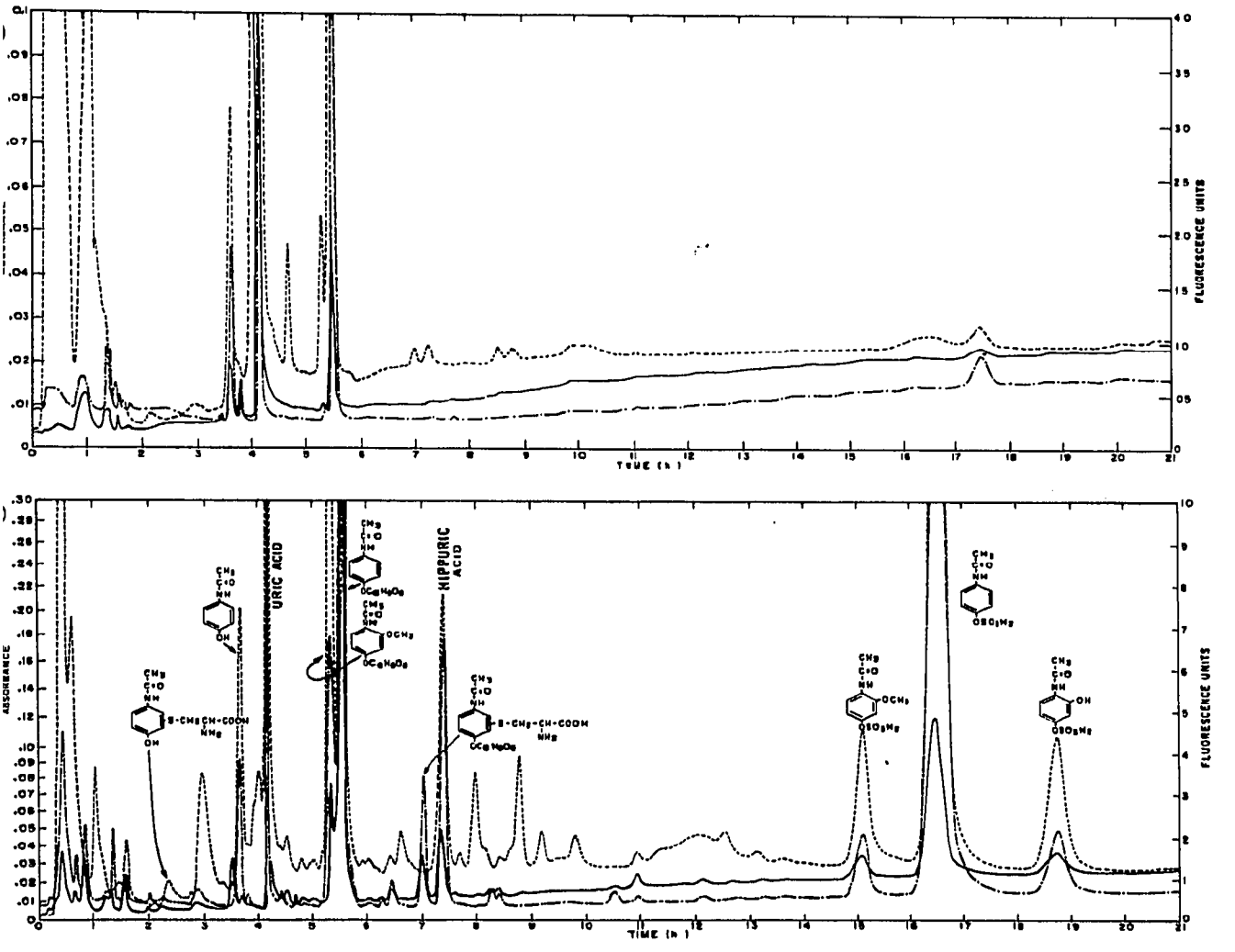


Fig. 5. Comparative chromatograms for 1-week-old child with extreme acidosis. (a) Serum; (b) urine. — — —, Fluorescence; -·-·-, 254 nm absorbance; ———, 280 nm absorbance.

better resolution, particularly for later-eluting compounds. The analytical time has been reduced to one-half of that required earlier[4], and the range of analysis has been extended to provide in 21 h what would have required over 60 h. The extended analysis permits determination of sulfate conjugates of drug metabolites and other highly anionic species not separated within the earlier reported elution periods[4,7]. At least thirty and fifty compounds, respectively, are detected in any one serum or urine and many additional compounds have been detected in different samples of serum and urine.

Steps which might chemically alter the compounds being determined are avoided in the procedure described here. Concentration of the sample, which might also increase salt content and cause shifts in elution position of the desired compounds, is

not necessary as a result of the improved sensitivity attained. The only sample pretreatment required is filtration of the samples to prevent accumulation of macromolecular compounds at the column entrance. Thus, as demonstrated for the samples involving ingestion of 4-hydroxyacetanilide, the various conjugates, as well as the free compounds, can be comparatively determined[9].

Clinically, the absence of high levels of diagnostically important compounds can be readily established, and the presence of some unexpected constituents discovered, as in the illustrative case of the infant suffering from extreme acidosis.

ACKNOWLEDGEMENT

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