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AMNIOTIC FLUID URIC ACID LEVELS DETERMINED BY REVERSED-PHASE LIQUID CHROMATOGRAPHY WITH SPECTROPHOTOMETRIC AND ELECTROCHEMICAL DETECTION

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SUMMARY

A rapid and precise, reversed-phase high-performance liquid chromatographic method for amniotic fluid uric acid is described. Detection of uric acid and other naturally occurring constituents is based on UV absorption at a wavelength of 280 nm and direct electrochemical oxidation at a potential of +0.800 V. The total analysis time is short (20 min) and the assay requires only filtration of the samples.

Uric acid levels were determined in 14 samples of amniotic fluid obtained during the 15th to 24th week of gestation. Results ranged from 0.897 to 4.39 mg per 100 ml of amniotic fluid.

INTRODUCTION

Amniotic fluid, a transudate from maternal serum across the placenta, fetal membrane and umbilical cord, is a dynamic system. While its composition is similar to maternal serum early in pregnancy, after the 14th week when the fetal kidneys begin to function it resembles neonatal urine.

The determination of age and maturity of the fetus by analyzing amniotic

fluid constituents is a subject of considerable interest. The knowledge of the exact composition of amniotic fluid is useful in elucidating the nature of solute transfer between the amniotic fluid, fetus, and mother, and in establishing the influence of fetal urinary excretion on the amniotic fluid composition.

A systematic study of the metabolic profile of organic acids in amniotic fluid was also reported at different gestational ages [1]. Marked similarities have been found between the organic acid profiles of amniotic fluid and urinary levels in newborns.

Moreover, a number of fetal abnormalities have been reflected by biochemically determined abnormalities in amniotic fluid [2]. Chemical study of amniotic fluid may also even reflect stress upon the fetus [3].

In the search for an indicator of fetal maturity, creatinine has been studied by several investigators [4-6]. Its levels have been found to parallel gestational age up to the 36th week [5]. Several attempts have also been made to correlate the levels of uric acid, the end-product of purine metabolism, with gestational age. This is still a subject of considerable controversy since several investigators report widely scattered levels [7], while others believe that uric acid is useful and possesses a predictive accuracy of up to 79% [8].

Clinical analysis of uric acid is currently being performed either on the basis of its reducing ability or by enzymatic degradation. The most common method is based on the reduction of phosphotungstic acid and subsequent spectrophotometric measurement of "tungsten blue" monitored at 700 nm. Due to interference from other reducing compounds, this assay is nonspecific and usually gives rise to falsely elevated results [9, 10].

Enzymatic degradation of uric acid with uricase with direct monitoring [11] or coupled with peroxidase [12] and/or catalase [13] gives improved specificity. However, the use of this method is severely compromised by enzyme inhibition and the need for highly pure substances. Alternatively, uric acid can also be analyzed by electrochemical measurement of the oxygen consumed during enzymatic degradation [14].

In an effort to develop a definitive method which would be reliable, selective and rapid for clinical testing of uric acid, high-performance liquid chromatographic (HPLC) analyses coupled with spectrophotometric [15] or electrochemical detection have been developed [16, 17]. The widespread use of this technique has been catalyzed by recent development of reversed-phase, micro-particulate, chemically-bonded packings. These packing materials offer several advantages over ion-exchange in terms of column life, simplicity of operation and ability to analyze simultaneously compounds of a wide polarity range.

The inherently higher sensitivity of electrochemical detection compared to spectrophotometric detection has been the subject of several publications [16, 17]. It should be noted that the presence of high concentrations of oxidizable compounds in biological fluids such as urine and serum makes electrochemical detection less selective than UV absorption. This problem is usually overcome by means of simple extraction procedures which allow separation of certain classes of compounds.

Since the levels of uric acid in amniotic fluid are relatively high compared to other naturally occurring compounds, electrochemical and spectrophotometric detection are equally successful. On the other hand, electrochemical detection

is considerably more sensitive and permits use of appreciably smaller quantities of biological substrate.

Reported in this paper is the determination of uric acid in samples of amniotic fluid analyzed by reversed-phase liquid chromatography coupled with spectrophotometric, fluorometric, and electrochemical detection.

EXPERIMENTAL

Apparatus

A Model 6000 A Solvent Delivery System, Model U6K Universal Injector and Model 660 Solvent Programmer, from Waters Assoc. (Milford, Mass., U.S.A.) were used in all determinations. A Model SF 770 Spectroflow Monitor (Kratos Inc., Schoeffel Instrument Division, Westwood, N.J., U.S.A.), with a deuterium lamp and a 8- μ l cell volume was used for monitoring column effluents. This detector was also equipped with a 339A Wavelength Drive and MM 700 Memory Module which were used for obtaining stopped-flow UV spectra.

In addition, a Metrohm/Brinkmann Voltametric/Amperometric Detector Model E 611 with an EA 1096 Detector Cell (Brinkmann, Westbury, N.Y. U.S.A.), operating on a three-electrode potentiostatic system, was used in line with the spectrophotometric and fluorometric detectors. The electrochemical detector employs a glassy carbon electrode as the indicating electrode and an Ag/AgCl electrode as reference.

Stainless-steel columns (30 cm \times 4.6 mm I.D.) were prepacked at the factory with 10- μ m, totally-porous silica support, utilizing an octadecyl (C₁₈) chemically-bonded stationary phase (Waters Assoc.).

Reagents and materials

All reference compounds, purchased from Sigma (St. Louis, Mo., U.S.A.), were of the highest purity (ACS Certified). Uricase (E.C. 1.7.3.3) was also obtained from Sigma. Potassium dihydrogen phosphate was purchased from Mallinckrodt (St. Louis, Mo., U.S.A.) and acetonitrile, distilled in glass, from Burdick & Jackson (Muskegon, Mich., U.S.A.).

Solutions of reference compounds were prepared in distilled-deionized water and were kept frozen when not in use.

Chromatographic conditions

For the analysis of amniotic fluid constituents, a gradient elution mode of reversed-phase liquid chromatography was used. The low-strength eluent was 0.1 M KH₂PO₄ (pH 2.50) and the high-strength eluent was a 60:40 (v/v) mixture of acetonitrile and distilled water. The low-concentration eluent was always filtered through a Millipore membrane filter (Millipore, Bedford, Mass., U.S.A.), type HA, pore size 0.22 μ m. The high-strength eluent was degassed prior to use.

A 30-min linear gradient from 0 to 100% of the high-strength eluent was used. The flow-rate was 1.2 ml/min, and the temperature was ambient in all cases. Chromatographic peaks were monitored spectrophotometrically at 280 nm, fluorometrically with an excitation wavelength of 285 nm and emission

cut-off filter of 320 nm, and electrochemically at a potential of +0.800 V.

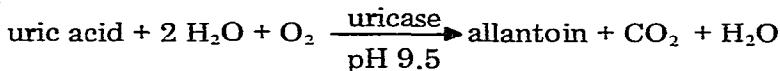
Samples

Amniotic fluid samples were obtained by transabdominal amniocentesis from subjects who were undergoing testing for chromosomal abnormalities and fetal neural tube defects in a genetic testing center. The samples proved to represent a group of women each of whom later gave birth to normal children. Samples were obtained from the 16th to 24th week of pregnancy and they were kept frozen at -20° until analyzed.

Prior to chromatography, samples were filtered through Millipore Membrane Filters, Type HA, pore size 0.22 µm, to remove the particulate matter.

Peak identification

Peak identity was confirmed using several identification methods. Tentative identification was performed on the basis of retention behavior and co-chromatography with the reference compound. In addition, stopped-flow UV spectra were obtained for the reference solution and the peaks in amniotic fluid. This technique has proven to be a simple and elegant method of peak identification [18]. Furthermore, the identity of the uric acid peak was also tested using an enzyme peak-shift reaction with uricase which reacts according to the reaction:



In carrying out the enzymatic reaction, an aliquot of the sample was first buffered to a required pH and then incubated with the enzyme. Since the reaction product, allantoin, does not absorb at 280 nm, the disappearance of the substrate peak was taken as an indication of the peak identity.

In addition, the electrochemical oxidation reaction which presumably involves a loss of $2e^-$ and 2H^+ to form a bis-imine is specific enough to be a confirmatory test when used in combination with other methods.

Linearity of detector response and limits of detection

Detector responses were found to increase linearly with concentration over the entire working range. For the spectrochemical and electrochemical detectors, the limits of detection were in the nanogram and picogram range, respectively.

Quantitation

Chromatographic peaks were quantitated on the basis of peak heights and comparison with the reference compound response. Triplicate injections gave relative standard deviation of peak heights and retention times of 1.0% and 0.5%, respectively.

RESULTS AND DISCUSSION

The conditions for the gradient elution mode of reversed-phase HPLC were optimized in order to obtain the best analytical conditions for a rapid analysis of uric acid, while maintaining good resolution of other amniotic fluid

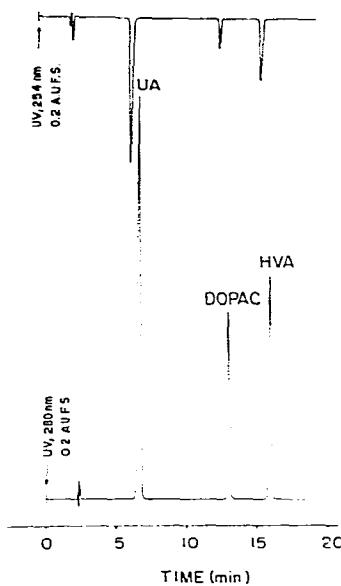


Fig. 1. Separation of a synthetic mixture of uric acid (UA), dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), detected at 280 nm and 254 nm. Chromatographic conditions: column, μ Bondapak C₁₈ (10 μ m average particle size); eluents, low strength 0.1 M KH_2PO_4 (pH 2.50), high strength acetonitrile-water (60:40, v/v); gradient, linear from 0 to 100% of the high-strength eluent in 30 min, flow-rate, 1.2 ml/min; temperature, ambient.

constituents. The separation of a synthetic mixture of some reference compounds detected at 280 nm and 254 nm is shown in Fig. 1. Simultaneous monitoring at two wavelengths aids in identification since the ratios of absorbance can be used to characterize solutes [19]. Furthermore, the use of stopped-flow UV scanning permits examination of the entire spectrum which, although lacking in fine structure, is nevertheless a powerful fingerprint of the chromophore. Fig. 2 illustrates the corrected stopped-flow UV spectra of the reference compounds.

Selective monitoring of the physiological levels of compounds in amniotic fluid was achieved by a combination of detection devices. In order to obtain

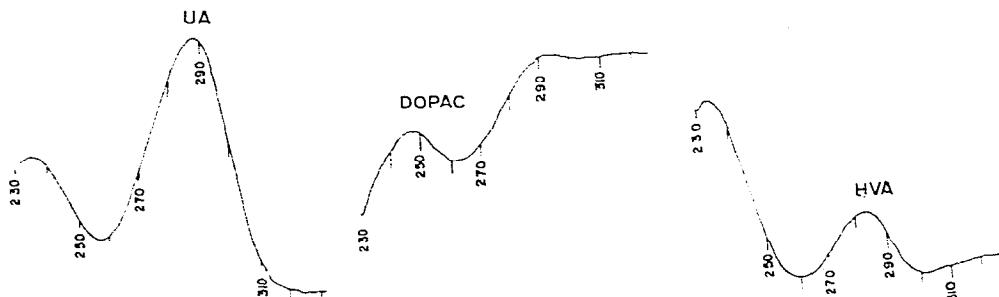


Fig. 2. Corrected stopped-flow UV spectra of the reference compounds shown in Fig. 1. Scanning rate: 100 nm/min.

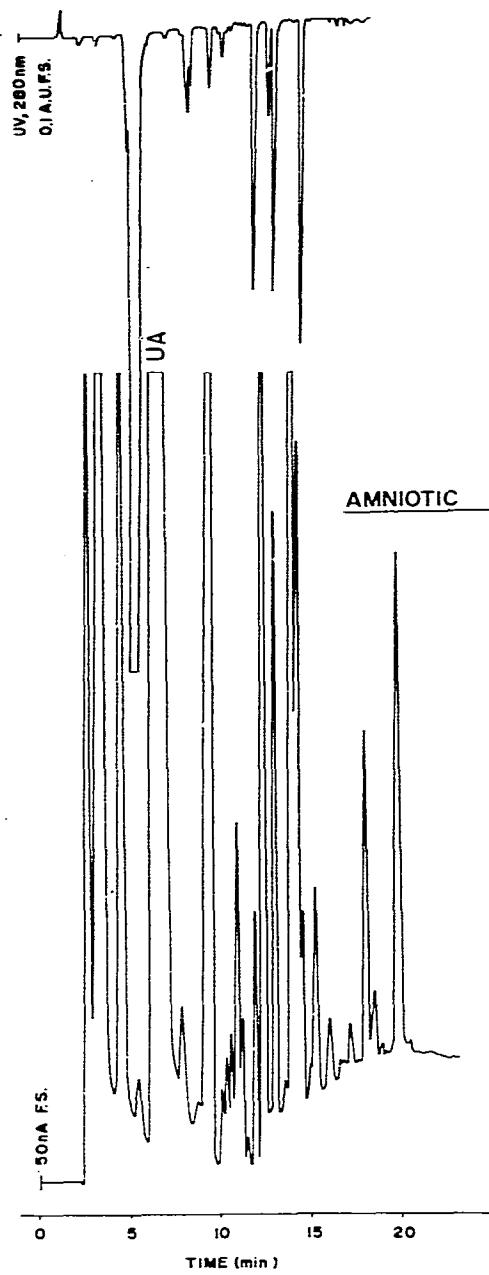


Fig. 3. Chromatogram of a sample of amniotic fluid detected amperometrically at +0.800 V and spectrophotometrically at 280 nm. Chromatographic conditions same as in Fig. 1. Volume of sample injected: 80 μ l.

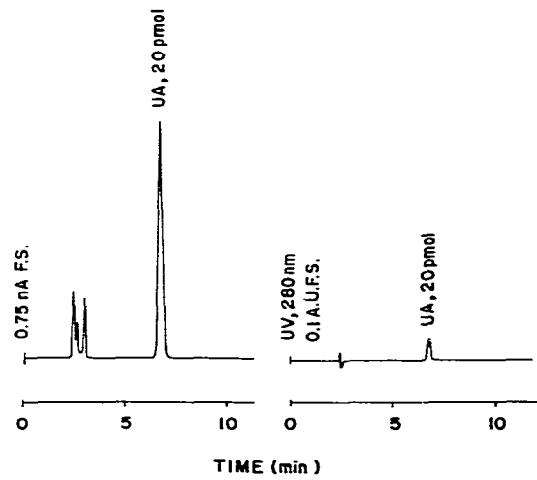


Fig. 4. Twenty picomoles of uric acid (UA) detected (left) amperometrically at +0.800 V, and (right) spectrophotometrically at 280 nm. Chromatographic conditions same as in Fig. 1.

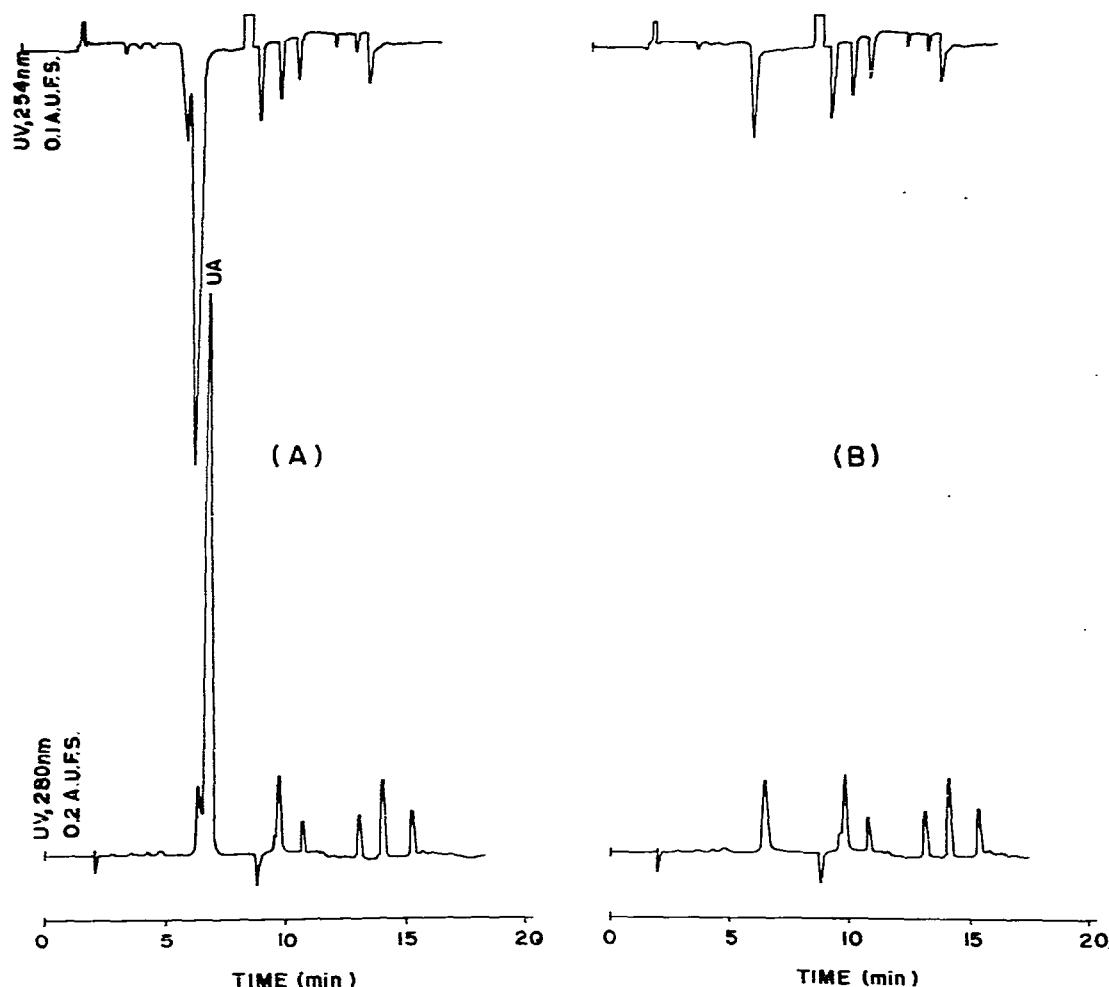


Fig. 5. (A) Chromatogram of a sample of amniotic fluid monitored spectrophotometrically at 280 nm and 254 nm. (B) Chromatogram of the same sample, incubated with uricase. Chromatographic conditions same as in Fig. 1. Volume of sample injected: 15 μ l.

the sensitivity necessary for the analysis of trace amounts of catecholamine metabolites, currently under study in our laboratories, HPLC effluents were monitored amperometrically at a potential of +0.800 V against the standard Ag/AgCl electrode, and spectrophotometrically at 280 nm. While this enhanced sensitivity is not crucial in the analysis of uric acid, it is mandatory in determining certain compounds for which spectrophotometric detection is not adequately sensitive. Fig. 3 shows a chromatogram of a sample of amniotic fluid monitored amperometrically and spectrophotometrically. The inherently higher sensitivity afforded by electrochemical detection is illustrated in Fig. 4.

The specificity of fluorometric detection is well known and has been discussed in literature [19, 20]. Monitoring native fluorescence of compounds in complex biological mixtures matrices is particularly advantageous since high sensitivity can be achieved and interference eliminated or reduced.

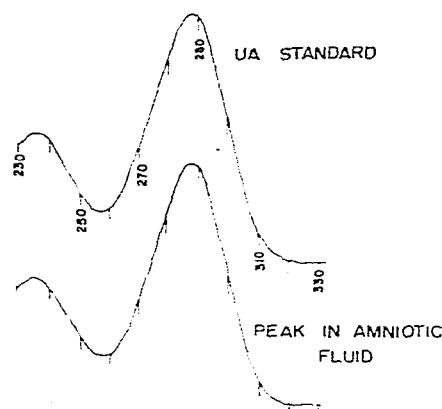


Fig. 6. Corrected stopped-flow UV spectra of uric acid (UA) reference solution and the peak in amniotic fluid with the same retention time. Scanning conditions same as in Fig. 2.

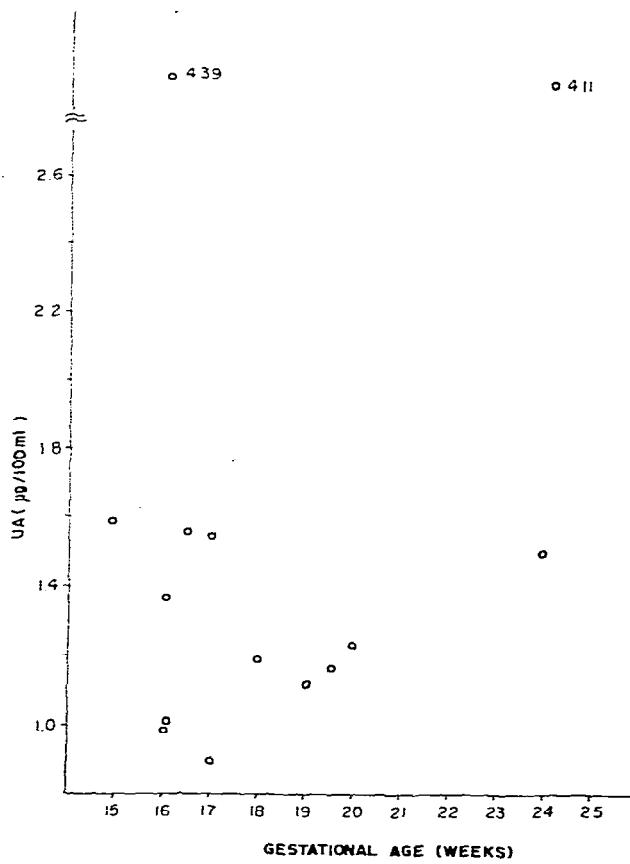


Fig. 7. Uric acid (UA) concentrations in amniotic fluid versus gestational age.

TABLE I

QUANTITATION OF URIC ACID IN AMNIOTIC FLUID SAMPLES

No. of samples	Gestational age (weeks)	Uric acid (mg per 100 ml)
2	24	4.110, 1.495
1	20	1.298
1	19.5	1.350
1	19	1.120
1	18	1.390
2	17	1.560, 0.897
1	16.5	1.580
4	16	1.378, 1.020, 4.390, 0.950
1	15	1.490

The described analytical conditions were then tested in the analysis of uric acid in amniotic fluid. Fig. 5A illustrates a typical chromatogram of a sample of amniotic fluid, monitored at 280 nm and 254 nm.

The identity of the peak with retention time of uric acid was first confirmed by an enzymatic peak-shift reaction with uricase. The chromatogram of the incubated mixture is shown in Fig. 5B. The disappearance of the peak under study confirms its identity. It should be pointed out that enzymatic peak-shift reactions also serve to "unmask" the chromatogram to prove that impurities are hidden under the major peaks [19, 21]. Furthermore, stopped-flow UV spectra were obtained for the uric acid reference compound and the peaks in amniotic fluid with the same retention time. Fig. 6 illustrates the agreement between the two spectra.

In an effort to establish the relationship between uric acid levels in amniotic fluid and the gestational age, 14 samples were analyzed using the described liquid chromatographic method. Quantitative results are given in Table I.

A scattergram (Fig. 7) depicts graphically uric acid levels as a function of gestational age. No apparent relationship exists between the uric acid content and fetal maturity for the samples analyzed. Whether or not this is due to the relatively narrow spread of gestational ages necessitates further follow-up. Current effort is being directed at identification and quantitation of other amniotic fluid constituents in a larger sample population to determine if any other compounds could be used as indicators of fetal maturity [22].

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