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Note

Aromatic amino acids in amniotic fluid samples analyzed by reversed-phase liquid chromatography with spectrophotometric detection

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The importance of prenatal diagnosis of diseases caused by aberrations in amino acid metabolism cannot be overestimated. Disorders such as tyrosinosis, phenylketonuria, and neonatal hypertyrosinemia are manifested in abnormal serum levels of tyrosine. Since the treatment requires a low amino acid diet in early infancy [1, 2], it is essential to monitor these compounds rapidly and sensitively.

In addition, in disorders in the metabolism of tryptophan, a precursor of serotonin, excretion of abnormal amounts of its metabolites has been observed in a number of conditions, such as bladder cancer [3], breast cancer [4, 5], Hodgkin's disease [6], vitamin B_6 deficiency [7], depression [8, 9] and migraines [10].

However, while current research has focused mainly on the analysis of catecholamine metabolites [11] in amniotic fluid, no efforts have been made to devise simple and routine analytical methods for the assessment of aromatic amino acid levels in normal subjects and patients with metabolic disorders.

Paper chromatography [12] does not possess the reproducibility necessary for quantitative analysis of trace levels of aromatic amino acids in amniotic fluid samples. Gas—liquid chromatography [13], although rapid and highly sensitive, requires elaborate derivatization procedures, which makes it unsuitable for routine clinical use.

Therefore, we have investigated the use of reversed-phase liquid chromatography in the analysis of tyrosine and tryptophan in amniotic fluid samples.

EXPERIMENTAL

Apparatus

A Model 6000A solvent delivery system, Model U6K universal liquid chromatograph injector, and a Model 660 solvent programmer (Waters Assoc. Milford, Mass., U.S.A.) were used in all determinations. The UV detection system consisted of two Model SF 770 Spectroflow monitors from Kratos Inc., Schoeffel Instrument Division (Westwood, N.J., U.S.A.).

The column was a stainless-steel μBondapak C_{18} (10-μm particle size), obtained from Waters Assoc.

Reagents

The reagents used were all of highest purity. Reference compounds were purchased from Sigma (St. Louis, Mo., U.S.A.), spectral grade acetonitrile from Burdick and Jackson Labs. (Muskegon, Mich., U.S.A.), and potassium dihydrogen phosphate from Mallinckrodt (St. Louis, Mo., U.S.A.). Reference solutions of tyrosine and tryptophan were prepared at 1.0 m*M* concentration and kept frozen when not in use.

Chromatographic conditions

The low-strength eluent was a 0.1 *M* solution of potassium dihydrogen phosphate, pH of 2.50, and the high-strength eluent acetonitrile—water (3:2, v/v). A linear gradient from 0% to 80% of high-strength eluent in 45 min was used. The low-strength eluent was always filtered through a Millipore membrane filter (Millipore, Bedford, Mass., U.S.A.), Type GS with a pore size of 0.22 μm, and the high-strength eluent was regularly degassed under vacuum. The flow-rate was 1.4 ml/min, and the temperature was ambient at all times.

Samples

Amniotic fluid samples were obtained by transabdominal amniocentesis from subjects who were tested for chromosomal abnormalities and fetal neural tube defects in a genetic testing center. Samples were obtained from the 16th to 24th week of gestation, and they were kept frozen at —10° until analyzed.

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

Peak identification

Initial identification of the peaks of interest was based on retention behavior and co-chromatography with the reference compounds. Since the high-performance liquid chromatographic effluents were simultaneously monitored at two wavelengths, peak height ratios were computed for the peaks in amniotic fluid samples and compared with those of the reference compounds. In addition, stopped-flow UV spectra were also obtained. Although these spectra characteristically lack in fine structure, this method has nevertheless proven to be a powerful tool for peak identification [14].

RESULTS

Prior to the analysis of amniotic fluid samples, the chromatographic conditions were optimized for the separation of tryptophan and tyrosine. Fig. 1 illustrates the separation of the reference compounds, detected at 235 nm and 285 nm. The low wavelength of 235 nm was necessary for monitoring the creatinine content in amniotic fluid samples.

Twelve samples of amniotic fluid were then analyzed using the described analytical technique. A typical chromatogram of a sample of amniotic fluid, monitored at 235 nm and 285 nm, is shown in Fig. 2.

The identitiy of chromatographic peaks was deduced from evidence accumulated from the retention behavior, co-chromatography with the reference compounds, peak height ratios, and stopped-flow UV spectra. A comparison of the spectra of chromatographic peaks in the amniotic fluid with those of the reference compounds is shown in Fig. 3.

Since the concentration of amino acids in body fluids varies with water

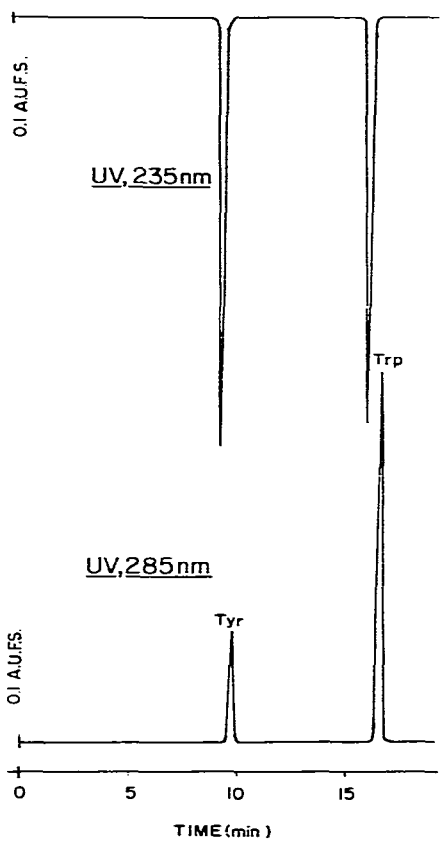

Fig. 1. A chromatogram of a synthetic mixture of tyrosine (Tyr) and tryptophan (Trp), detected at 235 nm and 285 nm. Amount injected: 3 nmoles each. Chromatographic conditions: column, μBondapak C_{18}; low-strength eluent 0.1 *M* KH_2PO_4, pH 2.50; high-strength eluent acetonitrile—water (3:2, v/v); gradient, linear, from 0% to 80% of high-strength eluent in 20 min; flow-rate, 1.4 ml/min; temperature, ambient.

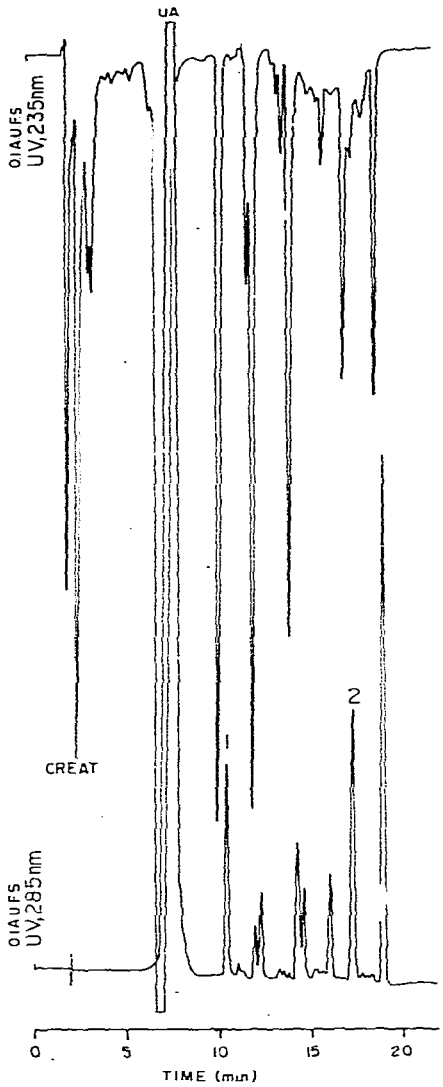

Fig. 2. Chromatogram of a sample of amniotic fluid. Volume injected: 100 µl. Chromatographic conditions as in Fig. 1.

intake, the levels of tryptophan and tyrosine were expressed in mg per mg of creatinine, which was measured simultaneously at the wavelength of 235 nm. The concentration range for tyrosine and tryptophan in 12 samples of amniotic fluid was 0.682—1.35 mg per mg of creatinine and 0.184—0.548 mg per mg of creatinine, respectively.

In conclusion, the described reversed-phase liquid chromatographic method for the rapid determination of tyrosine and tryptophan in amniotic fluid has great potential for the assessment of normal values of aromatic amino acids and early diagnosis of phenylketonuria, and other disorders involved in aberrations in the metabolism of aromatic amino acids.

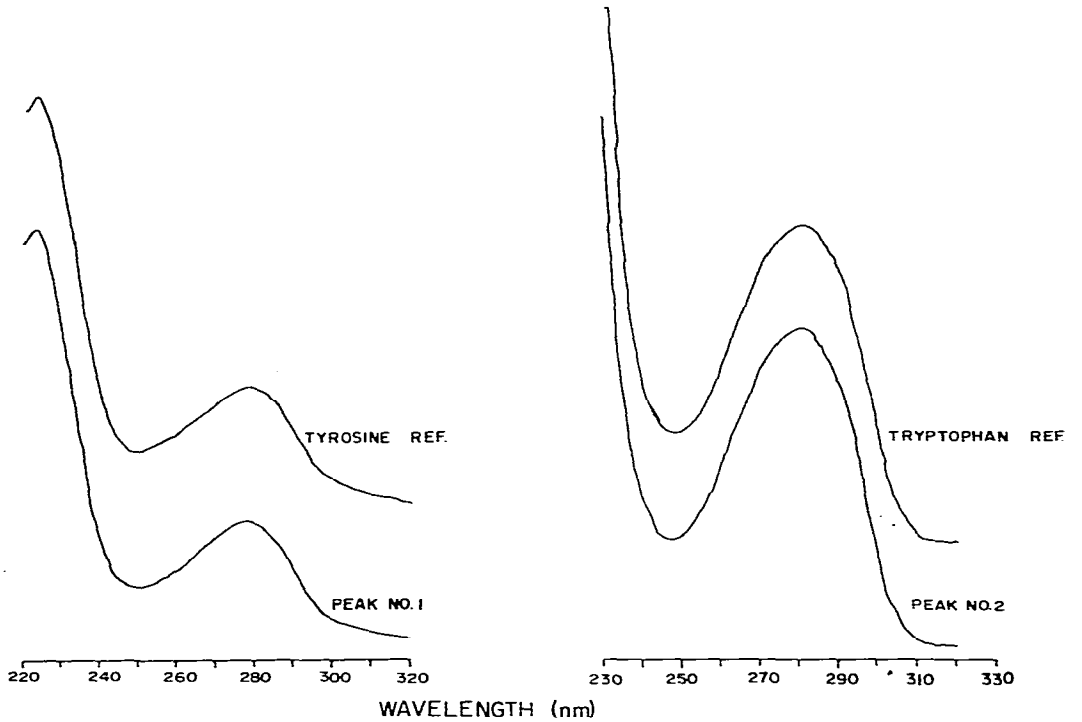

Fig. 3. Stopped-flow UV spectra of the reference compounds and peaks in amniotic fluid sample (scanning rate = 100 nm per min); absorbance, 0.1 a.u.f.s..

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