Fluorimetric Measurement of Tyrosine in Serum and Plasma

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Introduction

This fluorimetric procedure for determining tyrosine is that of Ambrose et al. (1), and is based on the fluorimetric method of Waalkes and Udenfriend (2). Studies of the latter procedure and of the various published colorimetric procedures for tyrosine (3–9) led to a simplified procedure in which the extraction step involving ethylene dichloride was eliminated without loss of specificity (1). In addition, the instability of the reaction mixture containing nitroso-naphthol, nitric acid, and sodium nitrite encountered in previous fluorimetric methods (2, 10, 11) has been corrected by use of a phosphoric acid reagent.

The following techniques are used to assay tyrosine in biological fluids: paper chromatography (12), thin-layer chromatography (13), electrophoresis (14), enzymatic procedures (15, 16), ion-exchange chromatography (17), colorimetry (3–9), and microbiological procedures (18). These methods are either difficult to perform, expensive, not quantitative, or less sensitive than the fluorimetric procedure (1). The manual fluorimetric procedure for tyrosine described here is sensitive, simple to perform, accurate, and precise, and the fluorophore is stable.

Materials and Methods

Reagents

Reagents ("Baker Analyzed"; J. T. Baker Chemical Co., Phillipsburg, N. J. 08865) are prepared in specially processed water (Continental Water Conditioning Corp., P.O. Box 26428, El Paso, Tex. 79926) as previously reported (19, 20), and are filtered through 0.45-µm (av pore size) filters (Millipore Corp., Bedford, Mass. 01730). Unless otherwise stated, all reagents are prepared monthly and placed in borosilicate bottles.

Nitric acid, 2.5 mol/liter. Dilute 156 ml of 16 mol/liter (70.7 g/dl) nitric acid with water to 1 liter. Filter and store at room temperature in a glass bottle.

Phosphoric acid reagent. Prepare an 8 mol/liter phosphoric acid solution by diluting 533 ml of 15 mol/liter phosphoric acid to 1 liter with water. Dissolve 100 g of sodium pyrophosphate (Na2HPO4·10 H2O) in 500 ml of the 8 mol/liter phosphoric acid and dilute to 1 liter with 8 mol/liter phosphoric acid. Filter the solution and store at room temperature in a glass bottle.

Ethanol. Filter ethanol (regular, not absolute, containing 5 ml of water per deciliter) and store at room temperature in a glass bottle.

1-Nitroso-2-naphthol-sodium nitrite reagent. Prepare the reagent immediately before use. Weigh 50 mg of 1-nitroso-2-naphthol (Aldrich Chemical Co., Milwaukee, Wis. 53233) and 345 mg of sodium nitrite (NaNO2) into a 50-ml beaker. Add 40 ml of NaOH (50 mmol/liter), stir until dissolved, and transfer quantitatively to a 100-ml volumetric flask. Adjust the volume to 100 ml with NaOH (50 mmol/liter). Shake the mixture vigorously, filter, and transfer the solution to a glass bottle. Cover the bottle with aluminum foil, because the reagent is light-sensitive.

1 Use of trade names is for identification only and does not constitute endorsement by CDC, PHS, DHEW, or by the AACC.
Nitric acid reagent. Add one volume of nitric acid (2.5 mol/liter) to two volumes of nitrosonaphthol-sodium nitrite reagent. Stir the mixture with a glass rod, cover, and allow to stand for 20 min. Then add 1.5 volumes of the phosphoric acid reagent and again stir the mixture. The nitric acid reagent is now ready for use. Prepare this mixture as needed, immediately before adding it to the tubes containing the reagent blanks, standards, and the protein-free supernatates from the serum or plasma samples.

Trichloroacetic acid (TCA), 0.6 mol/liter. Dissolve 98.10 g of TCA in water and dilute to 1 liter. Filter and store in a glass bottle at 4 °C.

TCA, 60 mmol/liter. Transfer 100 ml of TCA (0.6 mol/liter) to a 1-liter volumetric flask, dilute to volume with water, filter, and store in a glass bottle at 4 °C.

Primary stock standard and working standard. To prepare 50 mg/dl stock solution of L-tyrosine (Schwartz/Mann, Orangeburg, N. Y. 10962), weigh 50 mg of tyrosine into a 50-ml beaker and add 10 ml of TCA (0.6 mol/liter) plus 20 ml of Millipore-filtered water. Dissolve the tyrosine with the aid of a glass rod and transfer quantitatively to a 100-ml volumetric flask. Rinse the beaker and glass rod thoroughly with Millipore-filtered water several times and transfer the washings to the volumetric flask. Dilute to the 100-ml mark with Millipore-filtered water. To prepare a 1 mg/dl tyrosine working solution, transfer 1 ml of the stock solution to a 50-ml volumetric flask, and dilute to volume with TCA (0.6 mol/liter). Prepare the working standard fresh each week and store in a glass bottle at 4 °C.

Note: Long-tipped Corex (Mohr) pipets are used. This fluorometric procedure can be semi-automated by using all-glass “Repipets” (Labindustries, Berkeley, Calif. 94710) with ground-glass fittings.

Procedures

Preparation of serum and protein-free serum supernate. Clean the heel or finger of the patient with ethanol:water (70:30, by vol) and allow to air dry. Make the heel puncture with either a carbon “Rib-Back” blade No. 11 (Bard-Parker Co.) or a disposable “B-D Microlance” (Becton, Dickinson and Co.). Squeeze the area around the cut gently, and aspirate each drop of blood into a “Rasmussen Disposable Blood Collector” (Oakdale Co., P.O. Box 1111, South Bend, Ind. 46624) by mouth suction at the end of an attached rubber tube. Collect about 3 ml of blood into a 10 × 75 mm borosilicate tube. Allow the blood to coagulate and then centrifuge at 3000 × g or greater for 20 min. Transfer the clear serum with a Pasteur pipet into a borosilicate test tube, cap the tube with a Teflon-lined screwcap, and, if not immediately used, store at −20 °C. When blood is collected with the Rasmussen Disposable Blood Collector, the inlet is held firmly and closely to each drop of blood. Warning: a scraping motion must not be used because this leads to high amino acid values as a result of contamination from amino acids and protein on the skin. The proper techniques for collecting blood from a puncture wound of the heel, the finger, or the great toe are illustrated in a film entitled “Methods for Collecting Capillary Blood for Clinical Chemistry,”

Prepare the protein-free serum supernate (PFSS) by mixing the serum with an equal volume of Millipore-filtered TCA (0.6 mol/liter). Allow to stand at room temperature for 30 min and then centrifuge. Dilute the supernate fivefold with Millipore-filtered water to give a final 10-fold dilution in TCA (0.06 mol/liter). For sera containing more than 10 mg of tyrosine per deciliter, a further fivefold dilution is made in Millipore-filtered TCA (0.06 mol/liter) to yield a final 50-fold dilution.

Analytical procedure. 1. Add 0.5 ml of reagent blanks (TCA, 0.06 mol/liter, in duplicate) and of samples of protein-free serum supernate (in duplicate) to Ambrose cuvets (15 × 100 mm round cuvets with Teflon-lined screwcaps; Corning Glass Co., Corning, N. Y. 14830).

2. Add 0.1 to 0.5 ml of the 1 mg/dl tyrosine working standard (in duplicate) to other Ambrose cuvets. With TCA (60 mmol/liter), adjust the volume in each cuvet to a total of 0.5 ml.

3. Add 1.0 ml of the nitric acid reagent to each tube, close the cuvets with Teflon-lined screwcaps, and mix on a vortex-type mixer.

4. Heat the tubes in an 85 °C water bath for exactly 6 min, and cool in a 33 °C bath for 10 min.

5. Remove the tubes from the 33 °C bath and add 5 ml of ethanol to each tube.

6. Close the tubes with screwcaps, invert to mix the contents, and place in a 33 °C water bath for 30 min.

7. Measure the fluorescence in a Model 111 Fluorometer (constant-temperature door, 33 °C; G. K. Turner Associates, 2524 Pulgas Ave., Palo Alto, Calif. 94303), a Farrand Fluorometer (constant-temperature holder for Ambrose cuvets; Farrand Optical Co., Inc., 117 Wall St., Valhalla, N. Y. 10595), or a Model 10 Fluorometer (No. 10-030 holder for Ambrose cuvets; Turner Designs, 3132 Alexis Dr., Palo Alto, Calif. 94304). The fluorescence is stable for days. Steps 1–7 are performed in succession, without interruption. Before the fluorescence is read, remove extraneous fluorescent substances on the cuvet surface with laboratory tissues (“Kimwipes”) (19). These tissues are lint-free and will not contribute fluorescent lint residues to the cuvet surfaces.

Micromodification. The following modifications may be used with micropipets (Drummond “Microcaps,” Eppendorf and Oxford micropipets, or the Micromedics pipet):

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2 Genetic Chemistry Laboratory, Center for Disease Control, Atlanta, Ga. 30333.
1. To 100 µl of serum or plasma, add 100 µl of Millipore-filtered TCA (0.6 mol/liter). Mix with a vortex-type mixer, allow to stand at room temperature for 30 min, and centrifuge at 15,000 × g for 5 min.

2. Remove the supernate (1:2 protein-free serum supernate) and use 50 µl of each sample for the tyrosine analysis. Use 50 µl of a working standard of an L-tyrosine solution (5 mg/dl) of Millipore-filtered TCA (0.3 mol/liter), and a reagent blank of 50 µl of the TCA solution only.

3. Prepare a standard graph by adjusting 10-µl increments (from 10 to 50 µl) of working standard in Ambrose cuvets to 50 µl with TCA (0.3 mol/liter).

4. Follow steps 3–7 in the procedure above.

Fluorometer settings. 1. Use a IX aperture with a Blue Lamp, T-5 Envelope (Turner No. 110–853), a primary filter combination of No. 2A + 47B (436 nm, activation wavelength), and a No. 16 (>535 nm, emission wavelength) secondary filter.

2. Use a constant-temperature door (33 °C) with a high-sensitivity kit (Turner No. 110-655).

3. If necessary, include a 10% neutral-density filter or other suitable percent light-transmission filter (depending on the sensitivity of the fluorometer) with the secondary filter, so that a reading of 50 to 70 dial divisions is obtained with the most concentrated standard.

4. Adjust the zero on the fluorometer with the black blank rod (dummy cuvet) furnished with the instrument. Always read the reagent blanks in duplicate.

5. Subtract the average blank value from each standard and sample value. Prepare a standard graph by plotting all duplicate standard values and determining the line of best fit that passes through the origin.

6. Calculate tyrosine concentration of the samples (in mg/dl) by multiplying the value obtained from the standard graph by a factor of 10 or 50 (depending on the dilution).

Note: Any increase in blank value indicates that fluorescent contaminants have been introduced into reagents, and that these solutions should be replaced. To avoid methodological complications, replace all reagents and standards every 30 days.

Discussion

Nature of the Reaction(s)

The complete mechanism of the reaction between 1-nitrosod-2-naphthol and tyrosine in which the fluorophore is formed is not known. At least three reaction steps precede fluorophore formation. All phenol compounds having a free position ortho to the hydroxyl group condense with nitrosonaphthol, with the elimination of a molecule of water (4, 9). The condensation derivative is then converted (probably by oxidation) to a fluorescent compound (2, 4). In the present procedure, the tedious extraction of blood samples with ethylene dichloride, a step stipulated in previous methods, has been eliminated (1).

Sera assayed with and without the extraction procedure and with an amino acid analyzer (Beckman Instruments Corp., Fullerton, Calif. 92634) gave identical results with all three procedures (1).

The instability of the mixture of reagents (nitrosonaphthol, nitric acid, and sodium nitrite) has posed a serious problem with the fluorometric method for tyrosine (2, 10, 11). Investigation (1) of this problem revealed that adding nitric acid to a mixture of nitrosonaphthol and sodium nitrite produced a reaction that reached maximum intensity in 15-20 min and was followed by a secondary reaction(s) that prevented formation of a fluorophore between nitrosonaphthol and tyrosine. When tyrosine is present, this initial reaction can lead to a fluorescent chromophore, which was stabilized by adding the phosphoric acid reagent. The phosphoric acid reagent either prevents or minimizes the secondary reaction(s) that interferes with the condensation between tyrosine and the active form of the nitrosonaphthol derivative.

Normal Values, and Sources of Error in Their Determination

The normal mean value obtained with this procedure for tyrosine in serum from fasting persons (27 individuals ranging in age from 5 days to 45 years) was 1.5 mg/dl for 54 determinations, with a between-duplicate standard deviation of 0.3 mg/dl (1). Phillips (11) noted that hemolyzed whole blood specimens yield substantially increased tyrosine values. Tyrosine values are increased in serum or plasma stored at 3 °C or above (11). Possible sources contributing to increased "tyrosine" concentrations in whole blood, serum, or plasma are hydrolysis of denatured protein, or of peptides containing tyrosine, and the formation of tyramine. Tyrosine concentrations of serum or plasma are stable at −20 °C for six months (11, 21); therefore pooled plasma or serum stored at −20 °C in 2-ml aliquots may be used as a control.

Sources and Stability of L-Tyrosine

L-Tyrosine from various sources (Schwarz/Mann, Calbiochem, Pierce Chemical, Sigma, and Nutritional Biochemicals) yielded indistinguishable calibration graphs, with fluorescence linearly proportional to tyrosine concentration. The precision of the method and the stability of tyrosine in TCA (60 mmol/liter) during a 35-day period at 4 °C is shown in Figure 1. At each concentration the numbers indicate the mean fluorescent intensity and standard deviation for six determinations. The fluorophore is stable for several days at 33 °C.

Results for 47 determinations of various serum tyrosine concentrations by the present procedure were compared with those obtained with an amino acid analyzer (Beckman). The mean results for the former procedure were 104.4% (SD, ±4.8%) of those obtained with the latter (1).
Specificity

Concentrations of interfering compounds tested were 1.0–5.0 mg/dl, and the fluorescence intensity at the 1.0 mg/dl concentration (10 mg/dl in serum) is expressed as relative percent fluorescence compared to an equivalent weight of tyrosine. None of the compounds that interfere in the tyrosine procedure is present in significant concentrations in the blood of newborns (2, 22–26). Interfering compounds (1) include: tyramine, 193.8%; 3-hydroxyphenylactic acid, 171.7%; 3-hydroxyphenylacetic acid, 167.0%; 3-hydroxyindole acetic acid, 21.4%; 3-hydroxyphenylpyruvic acid, 20.1%; 3-m-tyrosine, 16.1%; 3-o-tyrosine, 9.6%; 3-hydroxyphenylacetic acid, 9.5%; 3-hydroxyphenylactic acid, 8.3%; DL-5-hydroxytryptophan, 5.9%; and L-tryptophan, 1.8%.

Tyramine yields a higher fluorescence value than tyrosine. Thus the increased tyrosine values found in hemolyzed whole blood or serum stored at room temperature and at 3–5 °C could be explained by enzymatic (or) nonenzymatic decarboxylation of tyrosine to tyramine.

The following 38 compounds give no detectable fluorescence in the tyrosine method (1): L-alanine, L-arginine, DL-3-amino-3-butyric acid, L-asparagine, L-aspartic acid, β-alanine, DL-β-amino-isobutyric acid, L-citrulline, creatinine, creatine, L-cysteine, δ-amino-γ-valeric acid, diiodotyrosine, L-ethionine, L-glutamic acid, L-glutamine, glycine, histamine, L-histidine, L-homocitrulline, DL-homocystine, DL-homocysteic acid, L-homoerine, L-isoleucine, L-kyanurenine, L-leucine, L-lysine, L-methionine, DL-norleucine, DL-norvaline, L-ornithine, L-phenylalanine, L-proline, L-serine, taurine, L-threonine, tryptamine, and L-valine. These compounds were tested at a concentration of 1.0–5.0 mg/dl (5–25 μg per tube), which is equivalent to a concentration of 10–50 mg/dl (100–500 μg/ml) in serum or plasma.

Clinical Application

Determination of the tyrosine concentration in serum or plasma in addition to the phenylalanine concentration aids in confirming phenylketonuria (1, 21, 27). In phenylketonuria, high serum phenylalanine concentrations (>20 mg/dl) are accompanied by an unchanged (or phenylalanine loading) serum tyrosine concentration that is less than 3–4 mg/dl, urinary phenylalanine concentrations greater than 10 mg/dl, and the presence of o-hydroxyphenylacetic acid in the urine (27).

Serum phenylalanine concentrations of 1.85 mg/dl (SD, ±0.69 mg/dl) have been reported in normal term newborns. The corresponding tyrosine concentration is 3.21 mg/dl (SD, ±1.53 mg/dl) (28).

Abnormally high phenylalanine and tyrosine concentrations can result from high protein intake (29–35). Levine et al. (29) described the presence of large amounts of tyrosine and its derivatives in the urine of premature infants on high-protein diets. Because administration of vitamin C prevented or eradicated the tyrosyluria, these workers believed that the disorder was related to ascorbic acid deficiency (29, 30). Others (13, 28, 31) reported abnormally high concentrations of tyrosine in the serum of premature babies. Some of the infants maintained high tyrosine concentrations even after they had been given supplemental dietary ascorbic acid. Mathews and Partington (32) found that 12 of 40 premature infants developed marked, but transient, increases in plasma tyrosine (>20 mg/dl). Premature infants whose daily protein intake was less than 5.0 g/kg of body weight had normal plasma tyrosine values. As the protein intake was increased, about a third of the premature infants developed very high plasma tyrosine concentrations (>20 mg/dl), and the rest of the study group of infants had higher than normal plasma tyrosine concentrations, but below 10 mg/dl. Adding ascorbic acid to the diet did not influence the plasma tyrosine concentrations of infants whose daily protein intake was greater than 5.0 g/kg.

Mild tyrosinemia (>4.5 mg/dl of plasma) occurs in 10% of term infants during the first week after birth, whereas severe tyrosinemia (>18.1 mg/dl plasma) occurs in 30% of premature infants (33).
Because phenylalanine and tyrosine concentrations are greatly increased in premature infants who are given high-protein diets [such as "Olac," (32-35)], the diagnosis of phenylketonuria cannot be based on a high serum phenylalanine value alone. Substantially supranormal phenylalanine concentrations usually are associated in prematurity with high tyrosine concentrations. Thus, in the case of the transient elevation of phenylalanine, a blood tyrosine assay normally may be used to rule phenylketonuria. Because prematurity with tyrosinemia may mask an actual case of phenylketonuria, weekly tyrosine and phenylalanine assays (with vitamin C supplements) probably should be done for 6–10 weeks.

Tyrosinemia (>4.5 mg/dl) is considered relatively harmless, and a decline in severe tyrosinemia (>18.1 mg/dl) parallels the maturation of the liver enzyme, (28, 33, 36). Furthermore, persistent tyrosinemia responds dramatically to ascorbic acid intake or to dietary protein reduction. Tyrosinemia can be readily differentiated from tyrosinosis (hereditary tyrosinemia), which does not respond to these measures and must be treated with a low-tyrosine diet.

Medes (37) described the first case of tyrosinosis in 1932. This syndrome is characterized by impaired tyrosine metabolism, nodular cirrhosis of the liver, and multiple renal-tubular defects (17). Initial characteristics of tyrosinosis in early childhood are vitamin D-resistant rickets and abdominal distension with hepatosplenomegaly. The biochemical pattern includes high concentrations of tyrosine in blood, and the abnormal excretion of tyrosine, p-hydroxyphenylpyruvic acid, p-hydroxyphenylactic acid, and p-hydroxyphenyllactic acid. The abnormality, transmitted by an autosomal recessive gene, is probably the result of the decreased activity of p-hydroxyphenylpyruvic acid oxidase (38, 39).

Aronsson et al. (17) reported the effect of long-term dietary treatment on a boy with tyrosinosis. Treatment by diet was begun when the boy was 22 months old and was continuing 3.5 years later when the report was made. Restriction of tyrosine and phenylalanine had marked effects on the renal tubular function; tubular reabsorption of phosphate, glucose, and amino acids was increased. Glucosuria disappeared completely, and phosphaturia was greatly diminished. There was a corresponding increase in the serum phosphate concentration and the rickets healed. The effect on liver size of dietary treatment was reversible, with the liver becoming larger with an increase in the intake of tyrosine and phenylalanine. The p-hydroxyphenylpyruvate hydroxylase [p-hydroxyphenylpyruvate, ascorbate:oxygen oxidoreductase (hydroxylating); EC 1.14.2.2] activity of the liver was still detectable, but its activity was greatly reduced.

Recently Menkes et al. (40) reported a delayed adverse effect attributable to high tyrosine concentrations in the serum of premature infants whose birth weight exceeded 2000 g. At birth, clinical abnormalities were not observed in premature infants with abnormally high tyrosine concentrations. In addition, no increased incidence of neurological abnormalities was apparent when the infants were tested at 15 months. However, a high incidence of intellectual impairment was noted in the area of perceptual function when these infants were tested seven to eight years later. No adverse effect on intellectual performance attributable to high tyrosine concentrations was noted in smaller premature infants. The authors concluded that the use of a high-protein formula contributed to the increased incidence of slight cerebral damage, perceptual problems, and learning disorders.

Because of the many phenol compounds in urine, this procedure is not specific for urine tyrosine. However, any values for phenol content of urine greater than 10 mg/dl should be further investigated (1).

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References

This paper will probably appear as a chapter in the forthcoming AACC volume, Selected Methods of Clinical Chemistry, and criticisms of it are invited from any reader (see Editorial, October 1973). Such criticisms should be addressed to the senior author, with a copy to the Chairman of the Committee on Selected Methods.

No reprints of this paper will be available.