Increased Excretion of Histidyl-L-Proline Diketopiperazine by Infants Receiving Pregestimil and Nutramigen Formulas

R. August Roesel, Paul R. Blankenship, Elaine B. Mobley, and Margaret E. Coryell

Histidyl-L-proline diketopiperazine is excreted in increased amounts by infants receiving Nutramigen or Pregestimil. When these formulas are discontinued, its excretion becomes undetectable. The compound was isolated from Nutramigen and Pregestimil, as well as from the urine of the infants receiving these formulas, and was identified by comparison with authentic histidyl-L-proline diketopiperazine standard in various chromatographic and electrophoretic systems. A neuropeptide widely distributed in the brain and gut and having a variety of biological functions, histidyl-L-proline diketopiperazine may have as-yet-undetermined effects on infants who are receiving these formulas.

Materials and Methods

Analytical-grade solvents and reagents were purchased from Fisher Chemical Co., Springfield, NJ 07081, unless stated otherwise. To quantify amino acids, we used an automated analyzer (Model 120C; Beckman Instruments, Palo Alto, CA 94303) with lithium citrate buffers.

Isolation of the unknown from urine. To isolate the unknown from the patient’s urine, we streaked 10 mL of urine on four 46 × 57 cm sheets of Whatman 3MM paper (Whatman Lab. Products, Clifton, NJ). We subjected the sheets to electrophoresis in a ET 48 system (Servonuclear Corp., Long Island, NY) at 3000 V for 45 min, in a pH 1.9 formic acid/acetic acid/water (32/90/88, by vol) mixture. We cut 1-cm stripes and stained them with diazotized sulfanilic acid to establish the location of the unidentified compound. We then eluted the unknown from the corresponding area of the unstained paper with water. After removing the solvents from the eluates under reduced pressure, we dissolved the residue in 6 mL of water and applied this to three 22 × 42 cm sheets of Whatman 3MM paper. We placed the sheets in chromatography tanks and developed them overnight in butanol/acetic acid/water (120/30/60, by vol). We again located the unknown by staining narrow strips of the chromatograms with diazotized sulfanilic acid and eluted the corresponding unstained areas of the rest of the chromatogram. This eluate was evaporated under reduced pressure and the residue was reconstituted to 0.5 mL with water.

Isolation of the unknown from infant formulas. We investigated the infant formulas Nursoy, SMA (Wyeth Laboratories, Philadelphia, PA 19101); Similac, Similac with Whey, Similac Isomil (Ross Laboratories, Columbus, OH 43216); Enfamil, ProSobee, Nutramigen, and Pregestimil (Mead Johnson Laboratories, Evansville, IN 47721). We centrifuged 50 mL of formula at 2200 × g for 30 min to remove the solid materials, then deproteinized the supernate by centrifugation in an ultrafiltration membrane cone (No. CF 25; Amicon Corp., Danvers, MA 01923) for 45 min at 900 × g. To desalt the deproteinized material, we passed it through a 1.2 × 20 cm column of AG 50W-X4, 100–200 mesh resin (H+ form) (2). We concentrated the desalted solution by evaporation to 10 mL under reduced pressure and isolated the unknown as described above for urine.

Synthesis of histidyl-L-proline diketopiperazine. Authentic histidyl-L-proline diketopiperazine was synthesized from the dipeptide L-histidyl-L-proline (Sigma Chemical Co., St. Louis, MO 63178) by heating in 8 mol/L acetic acid at 90 °C for 2 h (3). The resulting diketopiperazine was separated from small amounts of several unidentified imidazole contaminants by high-voltage electrophoresis as described above.

Results

Figure 2 indicates the location of the unknown compound after electrophoresis—chromatography of a sample of urine from an infant receiving Nutramigen. The compound, not
stained by ninhydrin, was stained by diazotized sulfanilic acid. Of the infant formulas investigated, only Nutramigen and Pregestimil contained this compound.

The \( R_f \) values obtained in different chromatographic systems (butanol/acetic acid/water, 120/30/50, by vol, \( R_f \) 53; methanol/water/pyridine, 20/5/1, by vol, \( R_f \) 70; ethanol/water/ammonium hydroxide, 18/1/1, by vol, \( R_f \) 43; acetonitrile/formic acid/water, 40/1/8, by vol, \( R_f \) 56; and isopropanol/ammonium hydroxide/water, 8/1/2, by vol, \( R_f \) 67) indicated that the unknown isolated from the Pregestimil and Nutramigen formulas was the same compound as that isolated from the urine of patients receiving those same formulas, and was identical to authentic histidyl-L-proline diketopiperazine.

Electrophoresis of authentic histidyl-L-proline diketopiperazine and of the isolated unknowns in the formic/acetic acid buffer at 53 V/cm yielded only one spot (13.5 cm from the application point) in each case, further confirming the unknown to be the same as the authentic standard.

Furthermore, electrophoresis–chromatography of the authentic standard with each isolated unknown (Pregestimil formula, Nutramigen formula, urines from patients receiving Pregestimil and Nutramigen) yielded only one diazotized sulfanilic acid-staining spot on the chromatogram, indicating complete overlap between the standard and the unknown. Analysis for amino acids in the unknown and histidyl-L-proline diketopiperazine, after hydrolysis in 6 mol/L HCl for 2 h at 120 °C yielded only histidine and proline, in equal proportions.

Discussion

From these results we conclude that the unknown isolated from the urine of patients receiving Nutramigen and Pregestimil is the same as that isolated from the respective formulas, and that it is histidyl-L-proline diketopiperazine. When the formulas were discontinued, the patients no longer excreted detectable amounts of the compound.

Perry et al. (4) had previously detected this compound in normal human urine, and had noted markedly increased amounts of the diketopiperazine of histidylproline in urines of phenylketonurics receiving Lophenalac as part of the diet. Histidyl-L-proline diketopiperazine was recovered from the Lofenalac formula as well. Nutramigen, Pregestimil, and Lofenalac formulas are protein hydrolysates. Evidently the diketopiperazine is formed during the preparation of the formulas, presumably during the heating or hydrolysis step (5). However, the dipeptide l-histidyl-L-proline will form the diketopiperazine spontaneously if allowed to sit for several days at room temperature.

Histidyl-L-proline diketopiperazine is also produced endogenously, being a metabolite of thyroprotein (thyrotropin-releasing hormone) (6). The effects of this diketopiperazine on the central nervous system or pituitary include influencing prolactin release and thermoregulation, depression of the central nervous system activity, stereotypic behavior, and changes in the concentrations of cyclic nucleotides (6). Ubiquitously distributed throughout the brains of rats (7) and primates (8), it also has a natriuretic effect (9), inhibits the opiate abstinence syndrome in mice (10), inhibits brain \((\text{Na}^+ + \text{K}^+)\)-ATPase (11), and reduces food intake (12). This pepetide is also reported to be present in greater concentrations in the plasma of hypothyroid patients than in that of normal and hyperthyroid individuals (13).

In addition to its various effects on the central nervous system, this cyclized peptide, which is found in high concentration throughout the rat gastrointestinal tract, may be involved in regulating rat gastrointestinal functions (14). Whether the ingestion of these formulas results in any alteration of metabolism in infants awaits elucidation.

References
Evaluation of a Direct Fluorometric Method for Determination of Serum Retinol

W. J. Drlakel, Jolene S. Hewett, and Mark M. Bashor

We evaluated the usefulness of a fluorometric method for determining serum retinol (Futterman et al., Invest Ophthal mol Vis Sci 1975:14:125–30) in which fluorescence (excitation 335 nm; emission 460 nm) of retinol is directly measured in unextracted, diluted serum. Using serum from 466 individual donors, we compared values so obtained with those by a "high-performance" liquid-chromatographic method. The correlation coefficient (r) was 0.74. When we compared fluorometric retinol values with retinol-binding protein values for the 466 samples, r was 0.71. About 1% of the 466 samples had markedly higher values by fluorometry than by chromatography, the result of positive interferences. For two serum pools, we obtained CVs of 1.56% (n = 57) and 1.79% (n = 57) in long-term precision studies lasting 60 days. Although the fluorometric method of Futterman et al. has not been widely adopted, we find that it is simple to perform and that results compare favorably with the chromatographic method in precision and accuracy. It is unique among the commonly used serum retinol methods in that the serum need not be extracted with organic solvents.

"High-performance" liquid chromatography (LC) has become the method of choice for measuring retinol in serum because of its specificity. In some areas of the world where vitamin A deficiency is most prevalent, simpler and less-expensive methods must be used. The fluorometric method for this assay described by Futterman et al. (1) is the simplest and least-expensive method available for retinol, but it does not seem to be in common use. Although published in 1975, it rarely is cited in the literature.

In the method of Futterman et al., retinol is measured directly in serum, without first extracting the serum with organic solvents as is required in other methods—whether LC, colorimetric, or fluorometric (2–5). The feasibility of the method depends on the relatively very high fluorescence of the retinol–retinol-binding protein (RBP) complex at the excitation wavelength of 335 nm, an order of magnitude higher than that produced by free retinol. Because the serum needs only to be diluted before the fluorescence is measured, reagent costs are minimal and no hood or special ventilation is needed because no volatile toxic organic solvents are involved.

Here we compare values for retinol in serum as measured by the method of Futterman et al. with values for corresponding samples obtained by an LC method, show the correlation between Futterman fluorometric values and RBP values, and examine the background fluorescence in sera from which the retinol–RBP complex has been selectively removed.

Materials and Methods

Materials

All-trans-retinol and all-trans-retinyl acetate, both in pure crystalline form, were from Sigma Chemical Co., St. Louis, MO 63178. Methanol and n-hexane ("HPLC" grade; Fisher Scientific Co., Atlanta, GA 30091) were used without further purification. Ascorbic acid was analytical grade. Quinine sulfate was from the National Bureau of Standards (Standard Reference Material No. 936). The radial immunodiffusion plates for RBP determination were from Calbiochem-Behring, La Jolla, CA 92037.

The serum samples were selected without conscious bias from samples that had been collected by the National Center for Health Statistics for the Hispanic Health and Nutrition Examination Survey (HANES) survey. The blood had been drawn in glass Vacutainer Tubes (Becton-Dickinson, Oxnard, CA 91065) containing no preservatives or anticoagulants. The serum samples were stored at −20 °C for four to six months before being assayed.

Procedures

LC retinol assay. This was done as described previously (6) except that, instead of extracting the serum with absolute ethanol, we used a 99.1 (by vol) mixture of absolute ethanol and a 100 g/L solution of ascorbic acid in water.

Fluorometric retinol analysis. Sample preparation and fluorescence measurement were essentially as described by Futterman et al. (1). Serum samples (0.1 mL) were diluted to 10 mL in 0.1 mol/L NaCl and fluorescence was measured, with use of a 10-nm slit width for both excitation and emission. The fluorescence spectrophotometer was set to read at 200 arbitrary (fluorescence) units, with a 100 µg/L