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Note

Rapid method for the analysis of urinary pyrrole-2-carboxylic acid using reversed-phase high-performance liquid chromatography

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Pyrrole-2-carboxylic acid (P2C) is a trace metabolite produced as a degradation product of sialic acids [1] and an acid-catalyzed dehydration product of Δ^1-pyrolline-4-hydroxy-2-carboxylate in the metabolism of 4-hydroxyproline [2,3]. It has been demonstrated that active kidney extracts from hog [3] and rat [4,5] are capable of converting 4-hydroxyproline to P2C and a number of laboratories have reported the presence of endogenous P2C in human urine [6—9]. Excretion of the glycine amide of P2C, N-(pyrrole-2-carboxyl)glycine has been identified in humans with the disease hyperprolinemia Type II [10].

A quantitative method for the analysis of P2C in urine has been reported by Gerber et al. [8] and refined by Heacock and Adams [7]. These methods are based on extraction of P2C from the urine sample and reaction with Ehrlich's reagent (*p*-dimethylaminobenzaldehyde) to give a quinoid chromophore. The analyses are multistep, time-consuming processes involving extraction and distillation or column chromatography. Furthermore the method is susceptible to interference from other pyrrole derivatives with a free C—H group [11].

This paper describes a simple and rapid method for direct analysis of urinary P2C using reversed-phase high-performance liquid chromatography (HPLC). The method is used to determine endogenous P2C in 24-h urine samples from healthy subjects.

EXPERIMENTAL

Reagents

All solvents were HPLC grade. Buffer salts were certified A.C.S. grade

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purchased from Fisher Scientific (Fairlawn, NJ, U.S.A.). P2C was purchased from Sigma (St. Louis, MO, U.S.A.) and recrystallized before use.

Apparatus

The liquid chromatography apparatus was comprised of a Series 4 solvent delivery system, a Model LC-75 variable-wavelength UV—VIS detector, a Model 7125 Rheodyne injector and a Model LCI-100 laboratory computing integrator, all from Perkin-Elmer (Norwalk, CT, U.S.A.). A 25 cm × 4.6 mm I.D., 5-μm Zorbax C_8 column (Dupont Instruments, Wilmington, DE, U.S.A.) was used for all analyses.

Sample treatment

Urine samples were collected from each subject over a 24-h period. To a 20-ml aliquot from the 24-h samples was added 1.0 ml of standard P2C solution in distilled water as internal standard. Final concentrations of 0.02, 0.05, 0.10, 0.20, and 0.30 μg P2C per ml urine were used. Urine samples containing the internal standard (or water when no internal standard was added) were adjusted to pH 2.0 with 0.1 *M* hydrochloric acid. Urinary P2C from a 4.0-ml sample of the acidified urine was extracted with 6.0 ml of anhydrous diethyl ether by shaking for 3 min. Of the ether layer 4 ml were removed, evaporated to dryness under a stream of nitrogen and redissolved in 0.2 ml of 0.01 *M* phosphate buffer, pH 4.4.

HPLC conditions

Samples (3 μl) were applied to the column. The mobile phase was 0.01 *M* phosphate buffer, pH 4.4—acetonitrile (99:1). The separation was performed at room temperature at a flow-rate of 0.4 ml/min. Absorbance was measured at 263 nm.

RESULTS AND DISCUSSION

A number of researchers have demonstrated that P2C is produced during hydroxyproline catabolism in mammals [3—5]. Since hydroxyproline is found in greatest abundance in collagen, it has been suggested that P2C levels in urine may be a means of monitoring collagen metabolism. Indeed, Yamanishi et al. [6] have concluded that elevated urinary P2C levels may reflect increased collagen metabolism in the hyperthyroid condition of both humans and rats.

Recovery of P2C during the extraction procedure was determined by extracting aqueous P2C standard solutions and comparing peak areas with unextracted standards. Recovery (mean ± S.D.) was 84 ± 5%. Triplicate analyses for each standard were performed.

Since P2C was a constituent of the normal human urine extracts from our subjects, we used a series of internal standards in preparing the standard curve. The urine extracts, with and without 0.3 μg of P2C per ml of urine as internal standard were also chromatographed on Zorbax TMS and Zorbax NH_2 columns (Dupont Instruments). Although the separations were not as efficient as with the C_8 column described here, peak areas were consistent with those obtained with the C_8 column. Typical C_8 chromatograms are shown in Fig. 1.

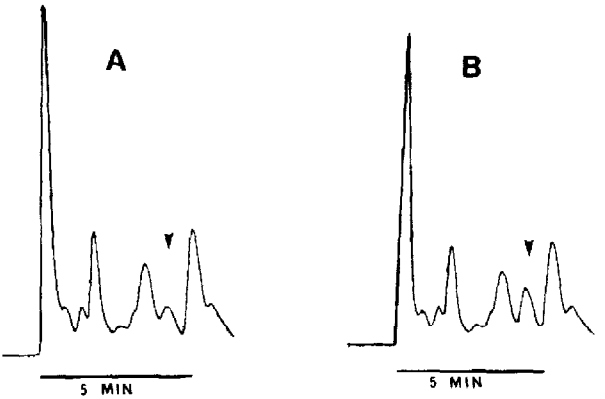

Fig. 1. HPLC profiles of an extract of human urine without P2C internal standard (A) and with 0.3 μg of P2C internal standard per ml of urine (B).

TABLE I

URINARY EXCRETION OF PYRROLE-2-CARBOXYLIC ACID

Subject	Age	Sex	Excretion of P2C (μmol per 24 h)
1	35	Male	0.704
2	20	Female	0.893
3	38	Male	2.02
4	30	Male	0.881
5	25	Male	0.792
6	26	Female	1.46
7	23	Male	0.718
Mean ± S.D.			1.07 ± 0.493

P2C peak area for the sample urine extract with no internal standard was subtracted from the peak area of the extracts containing the respective internal standards. A calibration curve for P2C concentrations ranging from 0.02 to 0.30 μg of P2C per ml of urine were prepared by plotting peak-area differences versus concentration. A linear relationship was obtained represented by the equation $y = 3587x + 71$. The correlation coefficient was 0.983. This procedure was used to determine 24-h P2C excretion in normal human urine. Seven subjects were used in the analysis and the results are shown in Table I. All analyses were performed in triplicate.

Mean 24-h P2C excretion in a sample has been determined by a number of laboratories and the results are compared in Table II. It is interesting to note that the mean P2C levels determined in this study are somewhat lower than those obtained in earlier analyses. Those previous levels have since been shown to be overestimated [7]. Our values are higher, though, than those determined using the modified method of Heacock and Adams [7] which involves additional isolation steps to purify the P2C prior to reaction with Ehrlich's reagent. The coefficient of variation for our study is in the same range as that observed in the other studies.

TABLE II

COMPARISON OF URINARY PYRROLE-2-CARBOXYLIC ACID EXCRETION FROM NORMAL SUBJECTS AS DETERMINED IN DIFFERENT STUDIES

Reference	Excretion of P2C (mean ± S.D.) (μmol per 24 h)	Coefficient of variation (%)
8	2.1 ± 0.96	45
7	2.4 ± 0.91	38
7	0.51 ± 0.31	61
9	2.5 ± 1.6	64
6	2.19 ± 0 31	14
This study	1.07 ± 0.493	45

In conclusion, we have demonstrated an improved, direct assay for P2C which is simple and rapid. The procedure as described can measure P2C levels below 0.05 μg/ml. This method may be useful to monitor collagen metabolism in a variety of clinical situations.

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