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Note

Quantitative aspects of urinary indole-3-acetic acid and 5-hydroxyindole-3-acetic acid excretion

JOHN A. HOSKINS and RODNEY J. POLLITT

Medical Research Council Unit for Metabolic Studies in Psychiatry, University Department of Psychiatry, P.O. Box 134, Middlewood Hospital, Sheffield S6 1TP (Great Britain)

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In a recent paper the thin-layer separation of a large number of indolic tryptophan metabolites from human urine was described[1]. Some of these metabolites were quantitated by *in situ* photometry. The results for indican and indole-3-acetic acid (IAA) excretion were some 10–20 times lower than previously accepted values and these discrepancies were attributed by the authors to the greater specificity of the thin-layer method. 5-Hydroxyindole-3-acetic acid (5-HIAA) excretion was also apparently much lower than revealed in previous studies, averaging only 0.1 mg per 24 h (6 subjects, 16 collections, 10 after tryptophan load). A considerable proportion of the 24-h urine specimens contained less than 50 μg of 5-HIAA.

These findings are particularly crucial to the field of biochemical psychiatry, where a large number of studies (for example ref. 2) of the excretion of IAA and 5-HIAA under various conditions have been undertaken. 5-HIAA is an end metabolite of 5-hydroxytryptamine (serotonin), a neurotransmitter, though probably about 80% of urinary 5-HIAA is of extraneuronal origin. If the currently accepted methods for measuring IAA and 5-HIAA were indeed grossly erroneous, many basic concepts of human indole metabolism would need re-examination.

We are currently studying human tryptophan metabolism using stable isotopic tracers and have available a specific and accurate method for measuring both IAA and 5-HIAA using gas chromatography–mass spectrometry (GC–MS) with internal isotopic standards. The principle of this method is quite different from that used by Byrd and coworkers[1] and from other colorimetric or fluorimetric methods. Hence it seemed an appropriate technique to resolve this conflict.

MATERIALS AND METHOD

Synthesis of indole-3-acetic acid-(methylene-$^2H_2$)

Indole-3-acetic acid (100 mg) in ^2H_2O (1.2 ml) containing 10% (w/v) of NaO^2H was heated for 3 h at 120°. Acidification with 2 *N* hydrochloric acid gave a product with 92% $^2H_2$ and 8% $^2H_1$, which was used without further purification.

Synthesis of 5-hydroxyindole-3-acetic acid-(methylene-$^2H_2$)

5-Benzyloxyindole-3-acetic acid (Sigma, St. Louis, Mo., U.S.A.) (100 mg)

dissolved in 1.7 ml ^2H_2O containing 10% NaO^2H was heated for 6 h at 120°. After acidification with 2 *N* hydrochloric acid a 2.5-ml methanolic solution of the product was debenzylated by catalytic hydrogenation for 2 h at room temperature and atmospheric pressure using palladium on asbestos (75 mg)[3]. The catalyst was removed by filtration, washed with 5 ml methanol and the combined filtrate and washings treated with 200 mg neutral alumina (Camag M·FC). Filtration and evaporation of the solvent gave 5-HIAA containing 85% $^2H_2$, 6% $^2H_1$, and 9% $^2H_0$ as an oil which crystallized on standing.

Urine analysis

Complete 24-h urine collections were obtained from 19 individuals, age range 5–50 years (av. 26 years). Strict dietary control was not observed but bananas were avoided[4].

For the estimations standardized solutions of the deuterated internal standards (0.5-ml aliquots, each containing about 25 μg of compound) were added to 10-ml aliquots of the urines. The mixture, adjusted to pH 4 with 2 *N* hydrochloric acid, was saturated with sodium chloride and then extracted with 3 × 10 ml ether. The ethereal extract after drying with sodium sulphate was evaporated under reduced pressure and the residue treated with bis-trimethylsilyltrifluoroacetamide containing 1% of chlorotrimethylsilane (1 part) and pyridine (1 part).

GC–MS was carried out as previously described[5,6] on a Perkin-Elmer 270 gas chromatograph–mass spectrometer, using both OV-101 and OV-17 stationary phases. IAA was measured by repetitive scanning of the molecular ions at *m/e* 319 and 321 for the natural compound and the isotopic standard, respectively, and the 5-HIAA was measured using the molecular ion minus $CO_2Si(CH_3)_3$ peaks at *m/e* 290 and 292. The deuterated standards were calibrated using authentic unlabelled IAA and 5-HIAA correcting for the contribution of the small amount of undeuterated material in the standard. With IAA the $^2H_0$ content was too small to detect, but with 5-HIAA it contributed the equivalent of 0.2 mg/l to the ratio readings.

RESULTS AND DISCUSSION

GC–MS has previously been used for the determination of 5-HIAA[7] and IAA[8] in cerebrospinal fluid though by methods that differed from those used here. The combination of the advantages of gas chromatographic resolution with the selective detection afforded by the mass spectrometer gives a high degree of specificity. In particular, indole carboxylic acid which Byrd and coworkers[1] regarded as a possible interfering substance in the colorimetric determination of IAA will not contribute to the values obtained by GC–MS. The internal isotopic standard compensates for losses and instrumental variability and gives a check on the gas chromatographic retention time of the natural compound to within a few seconds. On the OV-101 column, replicate analyses of the nineteen samples gave a standard deviation (S.D.) of 2.0% for the IAA and 2.3% for the 5-HIAA. The excretion of IAA ranged from 0.46–6.20 (2.55 ± 1.54 S.D.) mg per 24 h and that of 5-HIAA ranged from 1.66–5.01 (3.36 ± 0.80 S.D.) mg per 24 h. These results are for the free acids. Those samples examined also on the OV-17 column gave similar results.

The excretion of IAA in our subjects is somewhat lower than the values usually

obtained by solvent extraction and colorimetry[1]. IAA is a variable component of urine, the output being influenced by the state of the gut flora and by the pH of the urine[9]. Nevertheless, our values are roughly 10 times those obtained by Byrd *et al.*[1]. The excretion of 5-HIAA was much more consistent in spite of the varied food intake of our subjects and our values are very little lower than those obtained by previous workers using non-specific extraction methods followed by colorimetry. Brown *et al.*[10] have compared urinary 5-HIAA values obtained by extraction and colorimetry using 1-nitroso-2-naphthol with those given by chromatography on a short column and continuous monitoring of the effluent by fluorimetry using *o*-phthalaldehyde. Except in cases with drug interference, the more specific method of column chromatography gave only slightly lower results, these being in excellent agreement with ours.

The discrepancy between our results and those of most other workers on the one hand, and those of Byrd *et al.* on the other, is not easy to explain. Dietary differences and the small number of subjects studied by Byrd *et al.* may be contributing factors. Nevertheless, the general agreement between the colorimetric and fluorimetric methods and our GC–MS method, which is based on entirely different principles, is striking. At least as far as IAA and 5-HIAA are concerned, this study does not support the idea that the commonly used colorimetric methods are erroneous by a factor of 10 or more.

REFERENCES

1 D. J. Byrd, W. Kochen, D. Idzko and E. Knorr, *J. Chromatogr.*, 94 (1974) 85.
2 A. Coppen, B. W. L. Brooksbank, E. Eccleston, M. Peet and S. G. White, *Psychol. Med.*, 4 (1974) 164.
3 A. Stoll, F. Trozler, J. Peyer and A. Hofmann, *Helv. Chim. Acta*, 38 (1955) 1452.
4 J. R. Crout and A. Sjoerdsma, *New Engl. J. Med.*, 261 (1959) 23.
5 C. R. Lee and R. J. Pollitt, *Biochem. Med.*, 6 (1972) 536.
6 C. R. Lee and R. J. Pollitt, in A. Frigerio and N. Castagnioli (Editors), *Mass Spectrometry in Biochemistry and Medicine*, Raven Press, New York, 1974, p. 365.
7 L. Bertilsson, A. J. Atkinson, J. R. Althaus, A. Härfast, J.-E. Lindgren and B. Holmstedt, *Anal. Chem.*, 44 (1972) 1434.
8 L. Bertilsson and L. Palmer, *Science*, 177 (1972) 75.
9 M. D. Milne, M. A. Crawford, C. B. Girao and L. Loughridge, *Clin. Sci.*, 19 (1960) 165.
10 H. H. Brown, M. C. Rhindress and R. E. Griswold, *Clin. Chem.*, 17 (1971) 92.