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IN VIVO STUDIES OF THE TRYPTOPHAN-5-HYDROXYLASE SYSTEM

QUANTITATION OF SEROTONIN AND TRYPTAMINE USING GAS CHROMATOGRAPHY–MASS FRAGMENTOGRAPHY

H.-Ch. CURTIUS* and HELEN FARNER

Division of Clinical Chemistry, Department of Pediatrics, University of Zurich, Steinwiesstrasse 75, 8032 Zurich (Switzerland)

and

FRANÇOISE REY

Hôpital des Enfants Malades, Paris (France)

SUMMARY

An *in vivo* determination of tryptophan-5-hydroxylase (E.C. 1.14.16.4) activity is described. Subjects were loaded with deuterated L-tryptophan-d_5 (50 mg/kg body weight) and the deuterated serotonin-d_4 in urine was analysed using mass fragmentography. Four control subjects were dosed orally and two of them also intravenously with 50 mg/kg of L-tryptophan-d_5. One patient with atypical phenylketonuria (PKU) due to a tetrahydrobiopterin (BH_4) deficiency was dosed without and during BH_4 treatment. Without BH_4, the patient showed only minor formation of deuterated serotonin. After BH_4 administration (2.5 mg/kg body weight) the serotonin formation increased about four-fold but was not normalized.

Serotonin in urine and blood was analysed as the pentafluoropropionyl (PFP) derivative using gas chromatography–mass fragmentography. Deuterated serotonin was used as internal standard.

The analysis of tryptamine can be performed with the same procedure.

INTRODUCTION

The tetrahydrobiopterin (BH_4)-dependent enzyme tryptophan-5-hydroxylase is known to be the rate-limiting enzyme of the serotonin biosynthesis deriving from tryptophan. Normally about 1% of the tryptophan contained in the food intake is converted to serotonin. Tryptophan is first hydroxylated to 5-hydroxytryptophan, followed by decarboxylation to 5-hydroxytryptamine. The second enzymatic reaction is about 100 times faster. The enzyme that catalyses the conversion from 5-hydroxytryptophan to serotonin is the relatively unspecific aromatic L-amino acid decarboxylase which is also involved in the synthesis of the catecholamines.

Tryptophan-5-hydroxylase occurs mainly in the enterochromaffin cells, in the neurons and in the rodent mast cells. It is assumed that the tryptophan-5-hydroxylase in the brain is not saturated by its substrate and that the *in vivo* synthesis of serotonin

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depends to some extent on the tryptophan concentration in blood. It has not yet been ascertained whether BH_4 is the natural cofactor in the brain, but it is suspected to be a pterin.

Serotonin possesses a variety of pharmacological effects and acts as a very important neurotransmitter in the brain. In mammals about 90% of the serotonin present in the body, which in an adult human probably totals up to 10 mg, is lodged in the gastrointestinal tract, mainly in enterochromaffin cells and enterochromaffin-like cells. A few such serotonin-containing cells are also present in other tissues. Most of the remaining serotonin is present in platelets and in the brain. Several workers have reported that disorders of the tryptophan metabolism might play an important role in affective disturbances, *e.g.*, mania, endogenous depression, schizophrenia and neurological diseases. Pare *et al.*[1], Berendes *et al.*[2] and Matsuda *et al.*[3] reported for the first time a decreased serotonin blood level and a decreased 5-hydroxyindoleacetic acid excretion in phenylketonuric (PKU) patients. We have recently shown by *in vivo* investigations that the conversion of tryptophan to 5-hydroxytryptophan by the enzyme tryptophan-5-hydroxylase is inhibited by elevated phenylalanine concentrations in blood and tissues[4].

In patients with atypical PKU suffering from a BH_4 deficiency[5], we have recently shown that the serotonin excretion was reduced to only 10% compared with the values of normal controls[4]. With BH_4 substitution the serotonin excretion increased about four-fold.

For these reasons, an *in vivo* measurement of the tryptophan-5-hydroxylase activity is of great importance. An indirect *in vivo* measurement is possible by loading patients with deuterated L-tryptophan and subsequently measuring the deuterated serotonin formed in urine by using the gas chromatography–selective ion monitoring (GC–SIM) method.

Several methods for the determination of serotonin in the brain[6–8] and also in urine[9] by SIM have been described. For the present purpose we adopted the extraction procedure of Yuwiler *et al.*[10] with some modifications. For the GC–SIM determination we used pentafluoropropionyl (PFP) derivatives. We added deuterated serotonin and deuterated tryptamine as internal standards prior to the analytical procedure. This method allows the determination of deuterated and non-deuterated tryptamine in the same urine or blood sample.

EXPERIMENTAL

Materials and methods

All chemicals were of the highest purity available and solvents were redistilled before use. Helicase was obtained from Industrie Biologique Française (Gennevilliers, France). Reference compounds were obtained from Fluka (Buchs, Switzerland). L-Tryptophan-2,4,5,6,7-d_5 (as loading material) was synthesized by Professor P. Hemmerich, University of Constance, G.F.R. The internal standards serotonin-α-d_2-β-d_2 and tryptamine-α-d_2 were obtained from IC Chemicals (Munich, G.F.R.).

Free serotonin. The procedure is shown schematically in Fig. 1. After the addition of 0.5 μg of tryptamine-d_2 and 2.5 μg of serotonin-d_4 as internal standards to 5 ml of urine, or 0.1 μg of tryptamine-d_2 and 0.5 μg of serotonin-d_4 to 2 ml of whole blood, the samples were mixed with 1 ml of 3% aqueous ascorbic acid solution satu-

rated with potassium chloride and disodium EDTA and with 5 ml of 2 *M* phosphate buffer (pH 10.5) saturated with potassium chloride. The amines were extracted with 20 ml of butanol-1 and the butanol phase was washed with 5 ml of 0.05 *M* ammonia solution. After the addition of 25 ml of cyclohexane and centrifugation, the amines were re-extracted into 2 ml of 0.5 *M* formic acid containing 30 mg per 100 ml of ascorbic acid and 1 mg per 100 ml of creatinine sulphate. The extraction was repeated three times with 1 ml of the formic acid solution. After evaporation of the combined extracts to dryness, the amines were converted to their PFP derivatives by heating for 1 h at 60°C in acetonitrile–pentafluoropropionic anhydride (1:1).

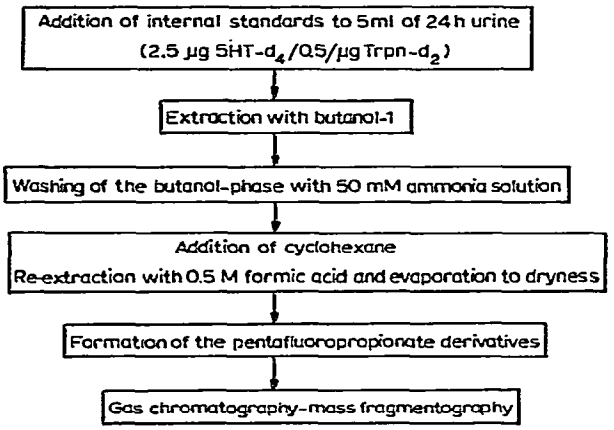

Fig. 1. Schematic diagram of the procedure for the determination of serotonin and tryptamine.

The following apparatus and conditions were used for GC–SIM: a Carlo Erba GI 450 gas chromatograph, Fractovap 2101 AC; glass column, 300 × 2 mm I.D., packed with 6% QF-1 on Chromosorb W AW DMCS (80–100 mesh); injector temperature, 250°C; helium carrier gas pre-pressure, 0.3 kg/cm^2; oven temperature, 160°C for tryptamine and 170°C for serotonin.

A Vacuum Generator mass spectrometer, Micromass 16F, with a Data System 2000, was used. The separator was a single jet, temperature 260°C, and the ion source temperature was 220°C; other parameters were 20 eV, 50 µA and SEV 2.5 kV. Serotonin was detected at *m/e* 438 (d_0), *m/e* 440 (d_2 = internal standard), *m/e* 441 (d_3) and *m/e* 442 (d_4), and tryptamine at *m/e* 289 (d_0), *m/e* 291 (d_2 = internal standard), *m/e* 293 (d_4) and *m/e* 294 (d_5).

Total serotonin. A 5-ml volume of urine was mixed with the internal standards and 1 ml of the ascorbic acid solution mentioned above. Then the pH was adjusted to 11 and 0.25 ml of saturated aqueous barium chloride solution were added. After centrifugation, the clear supernatant was acidified to pH 4.65 and 0.25 ml of 1 *M* acetate buffer (pH 4.6), 0.25 ml of chloroform and 10 mg of helicase were added. The mixture was incubated for 24 h at 25°C. Total serotonin was then extracted as described for the free serotonin.

Loading tests

Four control subjects, members of the Clinical Chemistry Department at the

University of Zurich, aged 20–35 years, were dosed orally with deuterated L-tryptophan-d_5 (50 mg/kg body weight) which was administered in chocolate cream after an overnight fast. Urine was collected 24 h before and 0–4, 4–12 and 12–24 h after dosing. The urine samples were acidified to pH 4 with acetic acid and kept frozen.

To two of these control subjects, L-tryptophan-d_5 (50 mg/kg body weight) was also administered intravenously after an overnight fast as a 10% solution in physiological sodium chloride solution over a time span of 30 min. The urine was collected in the same manner as after the oral dosing.

A 21-month-old patient with PKU due to a BH_4 deficiency was dosed orally with L-tryptophan-d_5 (50 mg/kg body weight) without and during BH_4 substitution (therapy) in order to evaluate the effectiveness of an *in vivo* BH_4 therapy. Urine was collected before and 0–8 and 8–24 h after dosing.

RESULTS

Figs. 2 and 3 show the mass spectra of the derivatized compounds. The most intensive fragments of the tryptamine-PFP derivatives were found at *m/e* 289 for the non-deuterated compound and at *m/e* 291 for the deuterated internal standard.

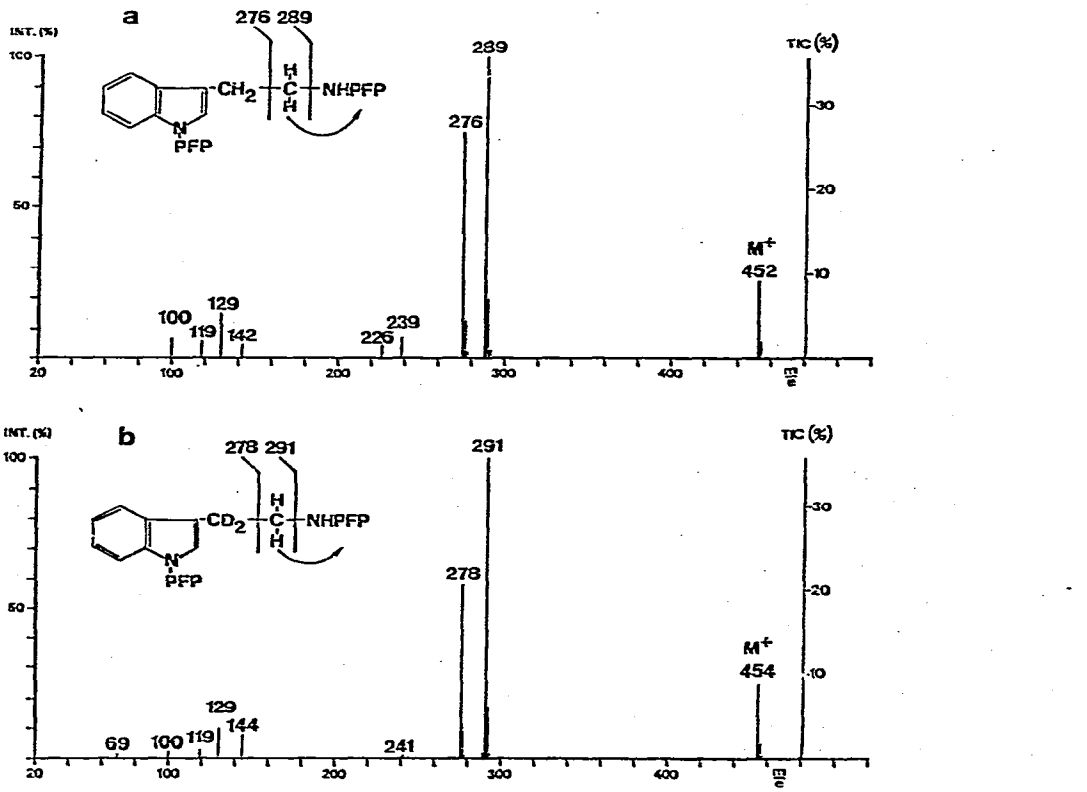

Fig. 2. Electron-impact mass spectra of pentafluoropropionyl (PFP) derivatives of (a) tryptamine-d_0 and (b) tryptamine-α-d_2 (internal standard) at 20 eV.

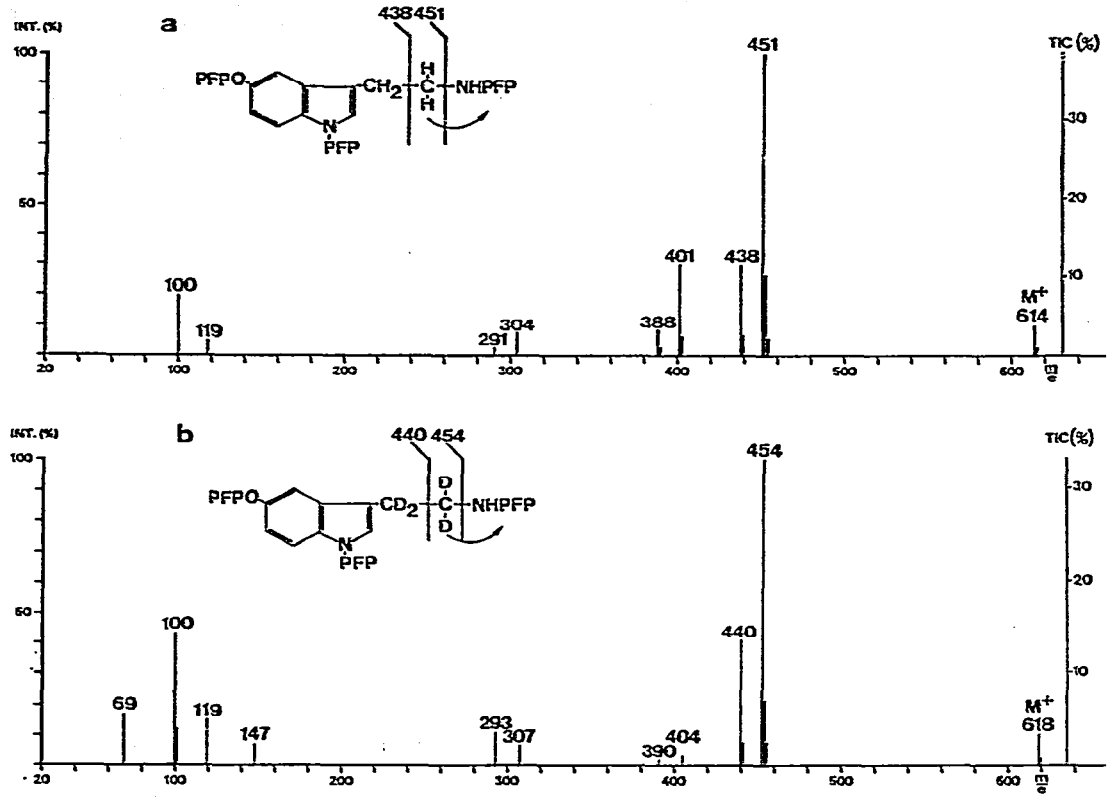

Fig. 3. Electron-impact mass spectra of pentafluoropropionyl (PFP) derivatives of (a) serotonin-d_0 and (b) serotonin-α-d_2-β-d_2 (internal standard) at 20 eV.

The most intensive fragment of the serotonin-PFP derivative is at *m/e* 451. However, for the mass detection, the ion at *m/e* 438 was chosen, because only this fragment allows the distinction between the side-chain-deuterated internal standard and the ring-deuterated serotonin-d_3 and -d_4, deriving from the dosing material tryptophan-d_5. SIM chromatograms of serotonin in urine samples from a control subject before and after dosing with tryptophan-d_5 are shown in Fig. 4. The first peak at *m/e* 438 represents the undeuterated compound serotonin-d_0 and the peak at *m/e* 440 the internal standard. The peaks at *m/e* 441 and *m/e* 442 derive from the administered tryptophan-d_5. The coefficients of variation were found to be about 9.5% ($n = 4$) for serotonin and 6.8% ($n = 6$) for tryptamine.

Blood samples showed an approximate loss of serotonin of 15% after 10 days at −20°C.

The amounts of conjugated serotonin in urine ranged from 5 to 28% of the free serotonin, with a mean of 14.2%.

After the intravenous administration of tryptophan-d_5 the urinary excretion of free serotonin-d_4 was higher than after oral dosing. In 24 h after the intravenous application, two control subjects excreted 154 and 185 μg of serotonin-d_4 compared with 60 and 99 μg, respectively, after oral dosing. Fig. 5 shows these results.

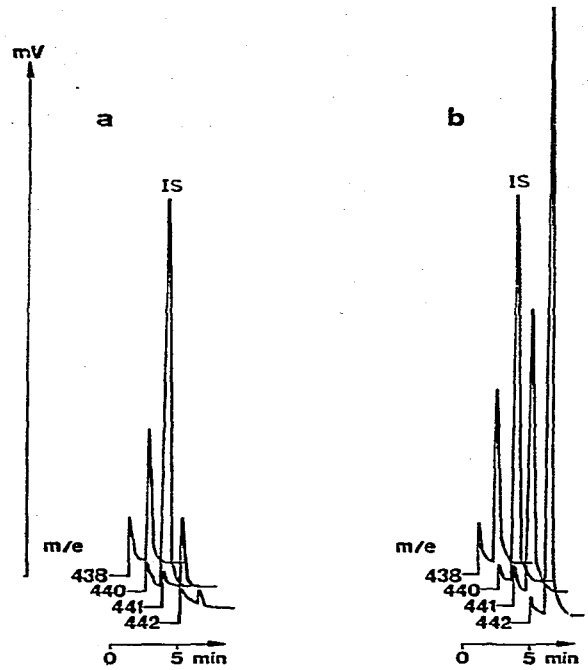

Fig. 4. Selective ion monitoring (SIM) chromatograms of serotonin in urine samples from a control subject (a) before and (b) 0–4 h after intravenous administration of L-tryptophan-d_5 (50 mg/kg body weight): *m/e* 438, serotonin-d_0; *m/e* 440, serotonin-d_2 (internal standard, IS); *m/e* 441, serotonin-d_3; and *m/e* 442, serotonin-d_4 (deriving from the dosing material).

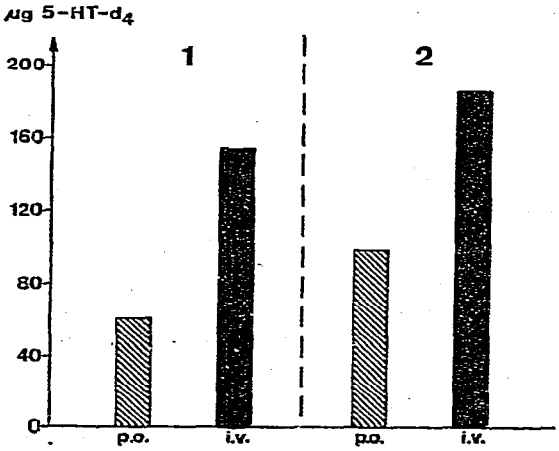

Fig. 5. Urinary excretion of serotonin-d_4 (5-HT-d_4) by two control subjects (1, 2) in 24 h after oral dosing (p.o.) with L-tryptophan-d_5 (50 mg/kg body weight) and after intravenous (i.v.) administration of L-tryptophan-d_5 (50 mg/kg body weight).

In two other control subjects, the serotonin-d_4 excreted in 24 h after oral dosing with tryptophan-d_5 (50 mg/kg body weight) amounted to 60 and 53 µg.

In Fig. 6, the urinary excretion of serotonin-d_4 by a patient with PKU due to

BH_4 deficiency is shown after oral dosing with 50 mg/kg of L-tryptophan-d_5 without and during BH_4 therapy. The values were found to be 0.19 μg of serotonin-d_4 without BH_4 therapy and 0.79 μg during BH_4 treatment.

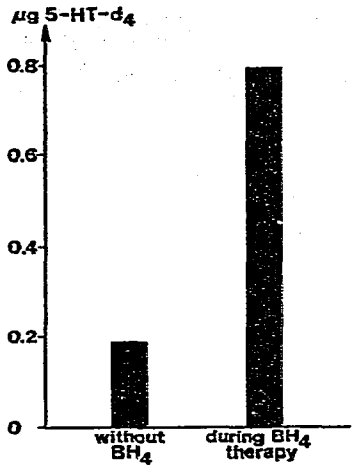

Fig. 6. Urinary excretion of serotonin-d_4 (5-HT-d_4) by a patient with PKU due to a tetrahydrobiopterin (BH_4) deficiency in 24 h after oral loading with L-tryptophan-d_5 (50 mg/kg body weight) without and during BH_4 therapy.

DISCUSSION

For the purification, the procedure of Yuwiler *et al.*[10] was used with some modifications. The extraction of serotonin was performed with butanol-1 but the butanol-1 layer was then washed with a 50 m*M* ammonia solution for the extraction of inorganic salts. After the addition of cyclohexane, the amine was not extracted with 0.1 *N* hydrochloric acid in order to avoid losses of deuterium. For this extraction we used 0.5 *N* formic acid containing ascorbic acid to stabilize serotonin. The PFP derivatives were found to be the most suitable for the assay of serotonin and tryptamine.

The commercially available internal standard serotonin-d_4 is deuterated in the side-chain. In contrast, serotonin-d_3 and -d_4, derived from the dosing material, are ring-deuterated. Measuring the indolyl fragment at *m/e* 438 for serotonin-d_0 and *m/e* 440, *m/e* 441 and *m/e* 442 for the deuterated compounds, it is possible to distinguish between the ring- and the side-chain-deuterated serotonin compounds. The dosing material tryptophan-d_5 contained tryptophan-d_4 too, so we had to measure two masses for the compounds deriving from the dosing material. For calculation, these amounts were added.

The proposed procedure can be applied to urine and blood determinations. It was concluded from our results that samples for the determination of serotonin in blood cannot be stored indefinitely. In contrast to other workers[11], we only found a small amount of conjugated serotonin in urine compared with the free serotonin. For this reason, we decided to determine the free serotonin only. Tryptamine can also be easily analysed by the same procedure using the ions at *m/e* 289, *m/e* 291 (internal

standard), *m/e* 293 and *m/e* 294 (deriving from the dosing material) for the mass detection.

A scheme for the conversion of tryptophan-d_5 to serotonin-d_4 is shown in Fig. 7. A number of diseases are supposed to correlate with defects in the serotonin biosynthesis, *e.g.*, endogenous depression and PKU. The *in vivo* determination of tryptophan-5-hydroxylase described could enable us to investigate the assumed defects and to start a specific treatment of the patients, *e.g.*, with tryptophan, 5-hydroxytryptophan or the presumed cofactor BH_4.

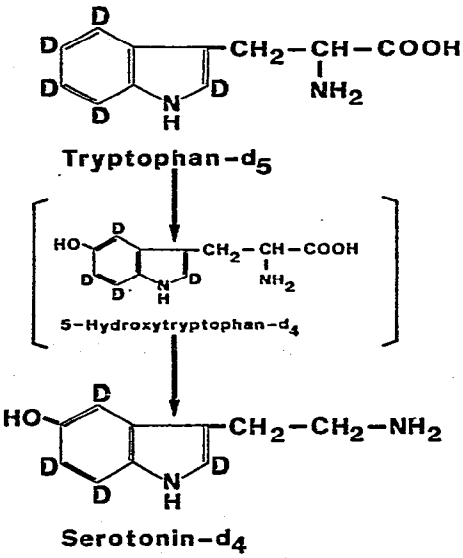

Fig. 7. Scheme of the conversion of tryptophan-d_5 to serotonin-d_4.

The measurement of deuterated serotonin reflects the natural conversion of deuterated tryptophan in the patients. An *in vitro* assay of the enzyme tryptophan-5-hydroxylase is not yet possible because the specific biopsy material is not readily available. One must also consider the fact that urinary serotonin excretion reflects mostly peripheral metabolism and is therefore not representative of the metabolism in the brain. It is a reasonable assumption that a defect in the brain metabolism is also reflected in the peripheric enzymatic steps.

The excretion of deuterated serotonin was higher after an intravenous application of deuterated tryptophan compared with the excretion after an oral administration. Nevertheless, the more convenient oral application is to be favoured. In our experience, the analysis of deuterated serotonin in urine after dosing with tryptophan-d_5 has given more significant results than blood analysis.

In a patient with PKU due to BH_4 deficiency, the *in vivo* determination of the activity of tryptophan-5-hydroxylase without BH_4 treatment showed only minor formation of deuterated serotonin. After BH_4 administration to the BH_4-deficient patient, the deuterated serotonin formation increased about four-fold, but never reached normal values. It might be possible that the administration of higher doses of BH_4 could lead to normalization of the serotonin values. This example clearly de-

monstrates the usefulness of the *in vivo* determination of an enzyme activity by dosing with a deuterated precursor and the analysis of the deuterated product by SIM.

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