Journal of Chromatography, 158 (1978) 305–312

CHROM. 11,082

SELECTIVE ION MONITORING OF TRYPTOPHAN, N-ACETYLTRYPTOPHAN AND KYNURENINE IN HUMAN SERUM

APPLICATION TO THE *IN VIVO* MEASUREMENT OF TRYPTOPHAN PYRROLASE ACTIVITY

HELEN WEGMANN, H.-Ch. CURTIUS* and U. REDWEIK

Chemistry Laboratory, University Paediatric Department, Children's Hospital, CH-8032, Zurich (Switzerland)

SUMMARY

A specific method is described for the determination of deuterated and non-deuterated N-acetyltryptophan, tryptophan and kynurenine in human serum and urine using gas chromatography–mass fragmentography. N-Acetyltryptophan was analysed as the N-trimethylsilyl methyl ester derivative; tryptophan and kynurenine were converted into their N-pentafluoropropionyl methyl esters. N-Acetyl-DL-tryptophan-d_{11}, tryptophan-d_8 and kynurenine-d_2 were used as internal standards. The coefficients of variation were found to be about 8% ($n = 9$) for tryptophan and N-acetyltryptophan and about 2.4% ($n = 9$) for kynurenine.

Using this method, an *in vivo* determination of the tryptophan pyrrolase activity [L-tryptophan oxygen 2,3-oxidoreductase (decyclizing), E.C. 1.13.11.11] is possible by loading the subjects with deuterated L-tryptophan-d_5 and subsequently measuring the deuterated L-kynurenine-d_4 formed and the residual L-tryptophan-d_5.

INTRODUCTION

Synthesis of serotonin in the brain is dependent upon the amount of tryptophan present in the brain. This amount may possibly be regulated by the level of free tryptophan in plasma[1,2]. Several workers have demonstrated that the concentration of free tryptophan in plasma decreases during depressions[3,4]. Comparative studies of the kynurenine excretion in patients with depressions and normal control subjects showed an increased excretion of the metabolites of kynurenine in patients with depressions[5,6]. This indicates that the decreased level of free tryptophan in depressed patients is due to an increased activity of the tryptophan pyrrolase (E.C. 1.13.11.11).

According to another hypothesis, schizophrenia is also the result of a disorder in the tryptophan–niacin pathway[7]. For these reasons, an *in vivo* measurement of the tryptophan pyrrolase activity is of great importance. An indirect *in vivo* measurement is possible by loading patients with deuterated L-tryptophan and subsequently

* To whom correspondence should be addressed.

measuring the deuterated kynurenine formed and residual L-tryptophan in serum using the gas chromatography–selective ion monitoring (GC–SIM) method.

A GC assay for measuring tryptophan in blood was described by Gehrke and co-workers[8,9]. The only GC method for the determination of kynurenine described to date[10–12] measures the alkaline hydrolysis product *o*-aminoacetophenone and not the original kynurenine. For the present purpose, we developed a specific method for the quantitation of deuterated and non-deuterated tryptophan and kynurenine in human serum using the SIM technique. The substances are converted into their N-pentafluoropropionyl methyl esters without any other chemical treatment.

With this procedure it is also possible to determine N-acetyltryptophan from the same blood sample as its trimethylsilyl methyl ester derivative. N-Acetyltryptophan is at present being considered as a substitute for tryptophan in dietary nutrients.

EXPERIMENTAL

Materials

All chemicals were of the highest purity available, and solvents were redistilled before use.

L-Tryptophan and N-acetyl-L-tryptophan were obtained from Fluka (Buchs, Switzerland) and DL-kynurenine from Sigma (St. Louis, Mo., U.S.A.). L-Tryptophan-d_5 (loading material) with five deuterium atoms in the indole ring and kynurenine-d_2 (with deuterium in positions 3 and 5) as internal standards were synthesized by Professor P. Hemmerich, University of Constance, Constance, G.F.R. The internal standard tryptophan-d_8 was obtained from Merck, Sharp & Dohme (Munich, G.F.R.); N-acetyl-DL-tryptophan-d_{11} was synthesized by acetylation of DL-tryptophan-d_8 with acetic anhydride-d_6 in NaOD (both obtained from Merck, Darmstadt, G.F.R.) according to the Schotten–Baumann method.

Dowex 50W-X2 (H^+), 200–400 mesh, was regenerated several times with sodium hydroxide solution and hydrochloric acid and then washed before use with 1 *N* hydrochloric acid and water until neutral.

Methylation was carried out with diazomethane according to Vogel[13].

Preparation of samples

A 100-μl volume of a 10 mg per 100 ml N-acetyl-DL-tryptophan-d_{11} solution, 100 μl of a 10 mg per 100 ml tryptophan-d_8 solution and 20 μl of a 10 mg per 100 ml kynurenine-d_2 solution as internal standards were mixed with 1 ml of human serum and 1 ml of 0.9% sodium chloride solution, or with 2 ml of urine. Then 2 ml of a 4 g per 100 ml sulphosalicylic acid solution were added for deproteinization and, after centrifugation twice for 15 min at 2000 *g*, the supernatant was applied on to a Dowex 50W-X2 (H^+) column (8 × 0.65 cm). The residue was washed with 1 ml of a 0.5 g per 100 ml sulphosalicylic acid solution, centrifuged again, and the supernatant applied on to the column.

After rinsing with 7.5 ml of 10% acetic acid, N-acetyltryptophan was eluted with 13 ml of this solution. After further rinsing with 20 ml of water, 100 ml of 25 m*M* pyridine–formic acid buffer (pH 3.0) and 5 ml of 25 m*M* pyridine–formic acid buffer (pH 4.55), kynurenine and tryptophan were eluted with 41 ml of the latter buffer.

Derivatization of N-acetyltryptophan

The N-acetyltryptophan fraction was evaporated to about 150 μl under reduced pressure at 30° and mixed with 150 μl of methanol. Diazomethane in diethyl ether was added dropwise with shaking until methylation was completed. The sample was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 100 μl of acetonitrile and 30 μl of bis(trimethylsilyl)trifluoroacetamide (BSTFA). These samples were then ready for injection.

Derivatization of kynurenine and tryptophan

The fraction was evaporated to dryness (*in vacuo* with repeated addition of distilled water) at 30° in order to remove trace amounts of pyridine. The remaining water was removed azeotropically by repeated evaporation with benzene, and the residue dried under vacuum over phosphorus pentoxide for at least 1 h. The samples prepared in this manner were derivatized to their N-pentafluoropropionyl methyl esters by the addition of 100 μl of acetonitrile and 100 μl of pentafluoropropionic acid anhydride (PFPA). Immediately after this solution had been mixed it was evap-

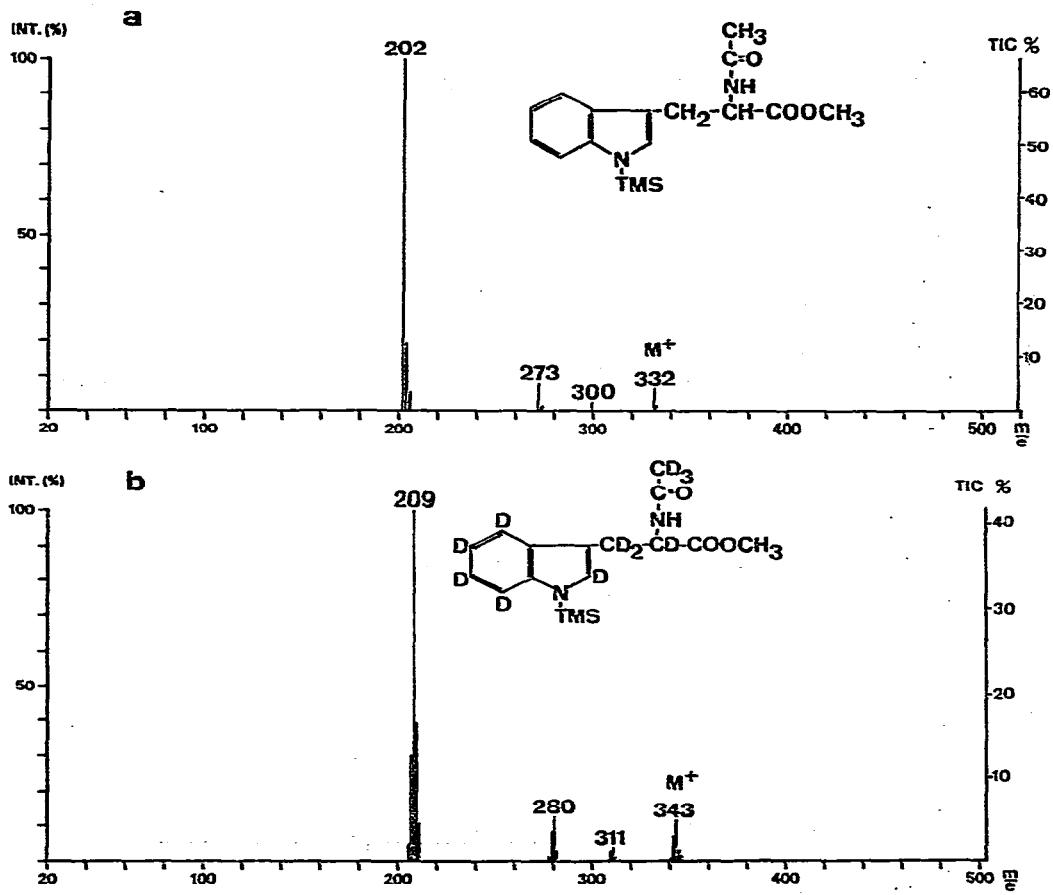

Fig. 1. EI mass spectra of trimethylsilyl methyl ester of (a) N-acetyltryptophan-d_0 and (b) N-acetyltryptophan-d_{11} (internal standard) at 20 eV.

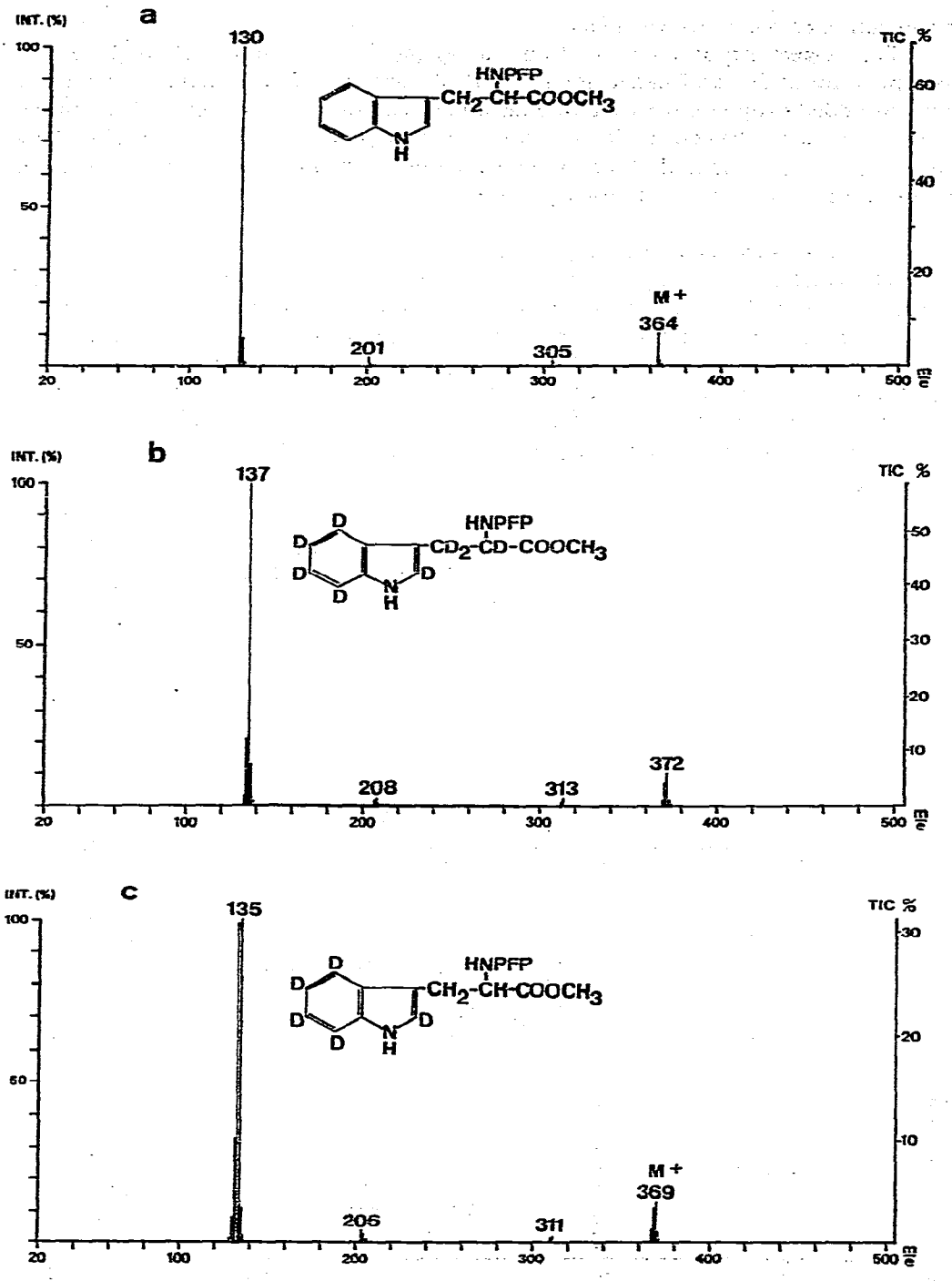

Fig. 2. EI mass spectra of N-pentafluoropropionyl methyl ester of (a) tryptophan-d_0, (b) tryptophan-d_8 (internal standard) and (c) tryptophan-d_5 at 20 eV.

orated to dryness under a stream of nitrogen. Then diazomethane in diethyl ether was added until methylation was completed. The samples were dried again and the residue was dissolved in 100 μl of acetonitrile.

GC–MS SIM

A Carlo-Erba GI 450 gas chromatograph, Fractovap 2101 AC and a Vacuum Generator mass spectrometer, Micromass 16F, with a Data System 2000 were used. The separator was a single jet, temperature 260°, and the ion source temperature was 220°, other parameters being 20 eV, 50 μA and SEV, 2.5 kV.

The GC column conditions for N-acetyltryptophan were as follows. A glass column, 300 × 2 mm, packed with 2.5% SE-30 on Chromosorb G (80–100 mesh) with an injection block temperature of 250°, helium as carrier gas, pre-pressure 0.4 kp/cm^2 and oven temperature 190°, was used.

Tryptophan and kynurenine were gas chromatographed using a glass column, 300 × 3 mm, packed with 6% QF-1 on Chromosorb W AW DMCS (80–100 mesh) with an injection block temperature of 250°, helium as carrier gas, pre-pressure 0.5 kp/cm^2 and oven temperature 185°.

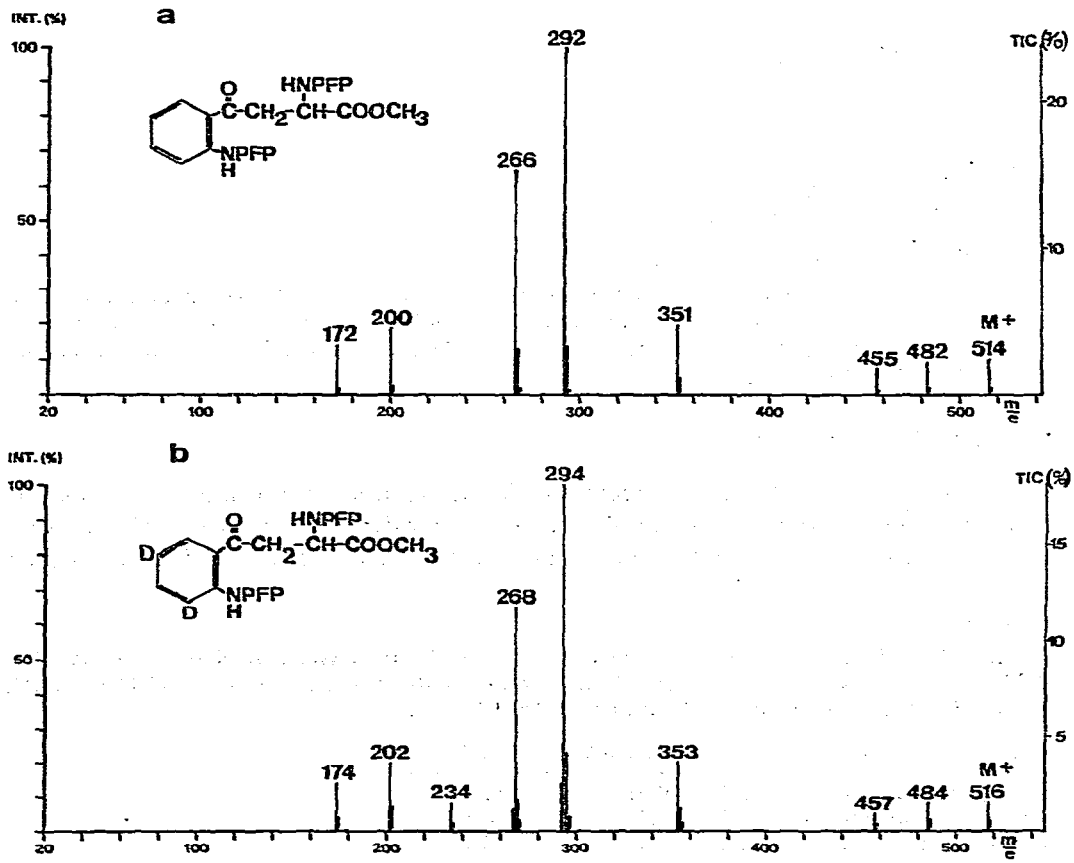

Fig. 3. EI mass spectra of N-pentafluoropropionyl methyl ester of (a) kynurenine-d_0 and (b) kynurenine-d_2 (internal standard) at 20 eV.

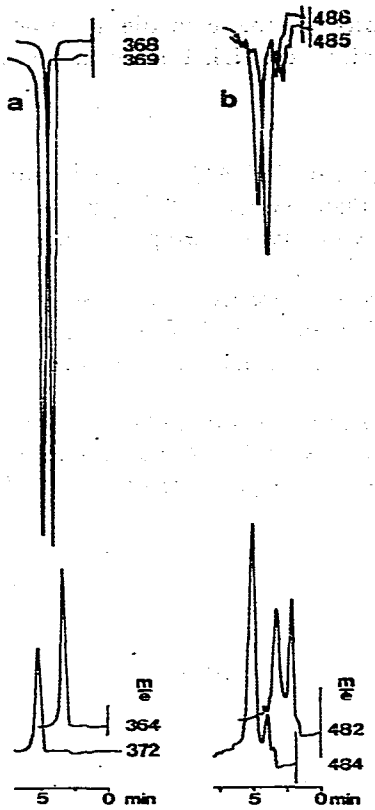

Fig. 4. SIM chromatogram of (a) tryptophan and (b) kynurenine. *m/e* 364, tryptophan-d_0; *m/e* 372, tryptophan-d_5 (internal standard); *m/e* 368 and 369, loading material; *m/e* 482, kynurenine-d_0; *m/e* 484, kynurenine-d_2 (internal standard); *m/e* 485 and 486, kynurenine formed from the loading material by the tryptophan pyrrolase.

RESULTS AND DISCUSSION

Figs. 1–3 show the mass spectra of the derivatized compounds. The most intensive fragment of the tryptophan PFP methyl ester derivative is at *m/e* 130 (indolyl fragment). However, for the mass detection, the molecular ion at *m/e* 364 was chosen

TABLE I

STANDARD DEVIATION (S.D.) AND COEFFICIENT OF VARIATION (C.V.) FOR THE DETERMINATION OF DEUTERATED AND NON-DEUTERATED TRYPTOPHAN, KYNURENINE AND N-ACETYLTRYPTOPHAN ($n = 9$)

Compound analysed	$\bar{x} \pm$ *S.D. (nmole/ml)*	*C.V. (%)*
N-Acetyltryptophan-d_0	3.2 ± 0.35	11.6
N-Acetyltryptophan-d_5	13.7 ± 0.6	4.5
Tryptophan-d_0	177.2 ± 16.1	9.1
Tryptophan-d_5	268.7 ± 18.8	7.0
Kynurenine-d_0	12.8 ± 0.3	2.4

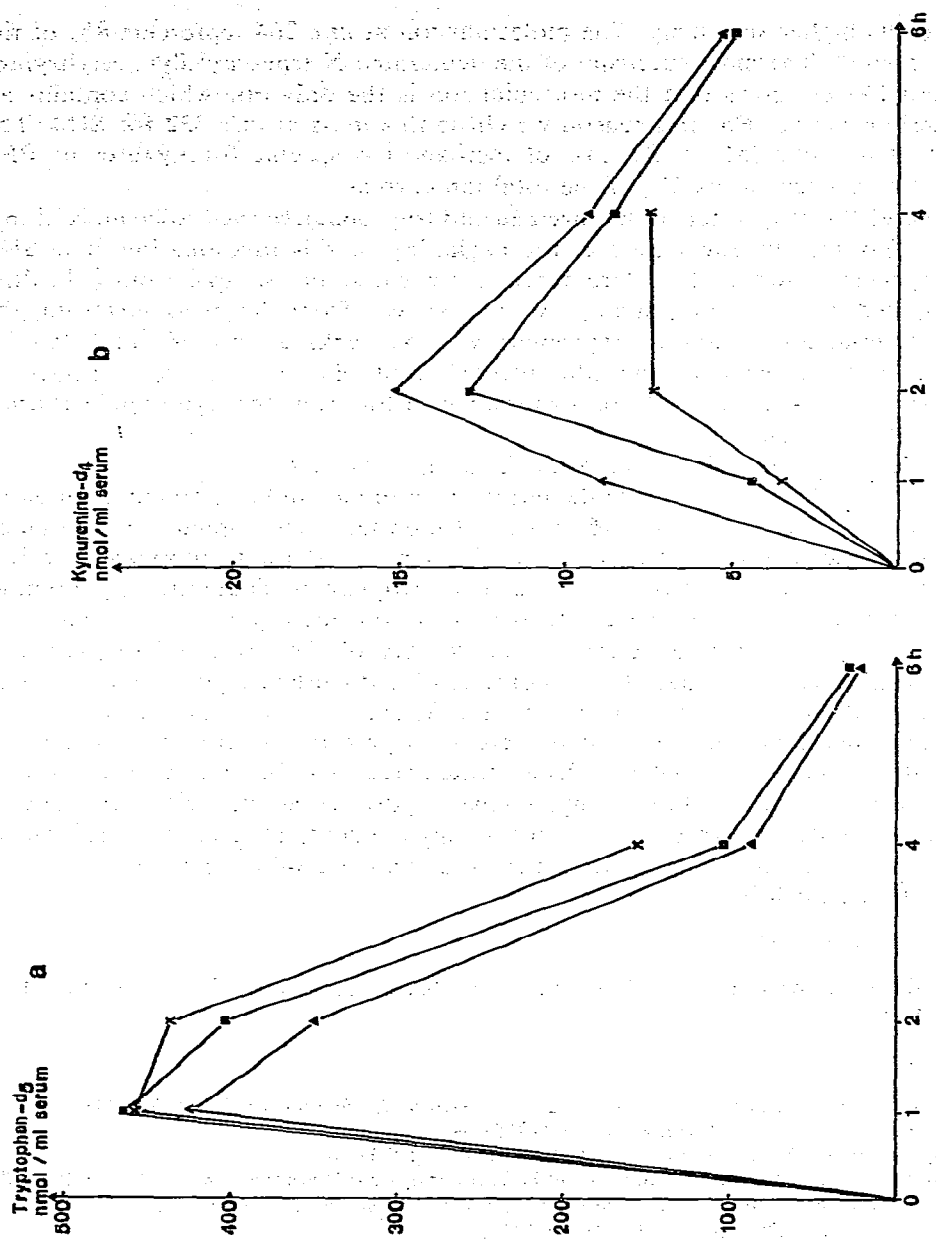

Fig. 5. Serum levels of (a) deuterated tryptophan and (b) deuterated kynurenine in three healthy persons after loading with deuterated L-tryptophan.

because of its higher specificity. The molecular ion at *m/e* 364 represents 8% of the total ion current. The mass spectrum of the deuterated N-trimethylsilyl acetyltryptophan methyl ester shows that the molecular ion is the only one which contains all the deuterium atoms. For this reason we chose this mass at *m/e* 332 for SIM. The fragment at *m/e* 482 ($M^+ - 32$, loss of methanol) is specific for kynurenine PFP methyl ester and represents 2% of the total ion current.

The PFP methyl esters of kynurenine and tryptophan proved to be more stable than the TFA methyl esters used at the beginning of this investigation. The SIM chromatograms shown in Fig. 4 represent a typical serum sample from a healthy subject loaded with L-tryptophan-d_5. As can be seen from the mass spectrum, the loading material also contains tryptophan-d_4. The peaks at *m/e* 368 and 369 for tryptophan are derived from the administered tryptophan-d_5 and -d_4, respectively. The peaks at *m/e* 485 and 486 for kynurenine result from the kynurenine formed by the loading material.

A statistical analysis of the results is given in Table I.

In Fig. 5, the serum levels of deuterated tryptophan and deuterated kynurenine of three healthy persons, after loading with deuterated L-tryptophan, are presented in relation to the time of blood collection. The activity of the tryptophan pyrrolase can be expressed by the ratio of deuterated tryptophan to deuterated kynurenine, or its logarithm. In the future we intend to load various psychiatric patients with deuterated L-tryptophan and determine the activity of their tryptophan pyrrolase. This will enable us to evaluate the hypothesis of a disturbed tryptophan pyrrolase activity in psychoses. As shown by Young and Sourkes[16], in rats an increase in liver pyrrolase activity did indeed result in an increased tryptophan degradation. An *in vivo* determination of enzyme activities with deuterated precursors has been applied successfully for the determination of the phenylalanine 4-hydroxylase activity[14]. Using loading tests with deuterated D-tryptophan and measuring the deuterated kynurenine formed, it will be possible to assay the activity of the indolamine 2,3-dioxygenase, the enzyme described by Hayaishi[15].

ACKNOWLEDGEMENT

This work was supported by the Swiss National Science Foundation, Grant No. 3.784.76.

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