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INFLUENCE OF TRYPTOPHAN LOADING ON URINARY EXCRETION OF ANTHRANILIC ACID AND 3-HYDROXYANTHRANILIC ACID BY MEN AND WOMEN AS DETERMINED BY ALKALI FLAME IONIZATION GAS CHROMATOGRAPHY

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SUMMARY

A gas chromatographic method with alkali flame ionization detection is described for the determination of urinary total (free and conjugated) anthranilic acid (AA) and 3-hydroxy-anthranilic acid (HAA) as their pentafluorobenzyl esters Prior to analysis, urine was hydrolysed using hydrochloric acid in a boiling water bath The highest AA and HAA yields were obtained with 4 M hydrochloric acid and a hydrolysis time of 4 h The coefficients of variation of the between-run analyses of AA and HAA at the endogenous level were 7 2 and 5 8%, respectively The average recovery for both substances was 84% The method described has been used to study the excretion of AA and HAA in the urine of healthy males and females before and after an oral load of tryptophan Furthermore, the influence of oral contraceptives has been investigated Results indicate that for both sexes the excretion of AA in the urine was higher than that of HAA, except after tryptophan loading The excretion of AA by women was higher than by men For HAA, the results of both sexes were comparable Furthermore, for neither of the sexes was a diurnal variation of AA or HAA observed After tryptophan loading, the formation of HAA was increased by more than that of AA Results obtained for women on oral contraceptives indicate a hormonal-induced inhibition of AA formation

INTRODUCTION

Anthranilic acid (AA) and 3-hydroxyanthranilic acid (HAA) are oxidative degradation products of tryptophan, appearing in the urine [1] The excretion of tryptophan metabolites is still a subject of increasing interest [2]. This results especially from the fact that in many clinical conditions an abnormal tryptophan breakdown is observed

One of the major metabolic pathways of tryptophan is the kynurenine route,

which is influenced by a large number of factors, e.g. oestrogen-containing oral contraceptives, vitamin B_6 status and liver [3—5]. These influences are more clearly demonstrated in the amounts of tryptophan metabolites excreted in the urine after an oral load of L-tryptophan [4, 6]. Two important conversion products of kynurenine, AA and HAA [1], appear in the urine in both free and conjugated forms [7]

Several chromatographic methods have been described for the determination of urinary AA and HAA [1, 7—10] However, most of them lack specificity and/or high sensitivity, especially when endogenous excretion levels (i e. without tryptophan loading) have to be determined We have chosen gas chromatography (GC) for the analysis of the endogenous levels of AA and HAA in urine As AA and HAA are nitrogen-containing compounds, we used an alkali flame ionization detector to improve specificity.

In the literature, hardly any detailed information is given on the conditions (e.g acid concentration, duration and temperature) for optimal hydrolysis Therefore, we have concentrated on these conditions. Furthermore, the GC method developed was used to investigate the influence of sex, day and night period and the use of oral contraceptives on urinary excretion of AA and HAA by men and women before and after an oral tryptophan load.

EXPERIMENTAL

Reagents and apparatus

AA and HAA were obtained from Sigma (St Louis, MO, U.S.A). Standard solutions were prepared in methanol and appeared to be stable for at least one month if stored at −20°C.

Pentafluorobenzyl bromide (PFBB) was obtained from Pierce (Rockford, IL, U.S.A.). For derivatization, a 10% (v/v) PFBB solution in acetone was used. To remove excess PFBB reagent, the standards were cleaned on a column filled with deactivated silica gel (350 × 6 mm I D.) as described below for the clean-up of urine samples Standard solutions in distilled ethyl acetate were stable for at least one month if stored at +4°C.

Silica gel 60 (0 05—0 2 mm, 70—270 mesh) was obtained from Macherey and Nagel (Düren, F.R G). It was heated for 3 h at 250°C After cooling, 95 0 g of the silica gel were deactivated with 5 0 g of water, homogenized till lump-free and allowed to equilibrate overnight in a tightly stoppered bottle before use Basic aluminium oxide Woelm B-Super 1 (product number 04568) was obtained from Woelm Pharma (Eschwegen, F R.G.) and used for chromatographic purification of diethyl ether. A citrate—phosphate buffer solution (pH 3 0) was prepared by mixing 80 ml of 0 1 M citric acid solution with 20 ml of 0 2 M sodium monohydrogen phosphate solution Caesium carbonate was obtained from Janssen Chimica (Beerse, Belgium).

All other chemicals were of analytical grade and were checked for the absence of interfering impurities by means of blank determinations In the respective cases, only distilled ethyl acetate was used Evaporations were carried out at 40°C (water-bath) in a vacuum rotary evaporator.

Gas chromatography

Chromatography was performed with a Tracor 550 gas chromatograph using

a Tracor 702-N-P nitrogen detector and a Tracor 770 autosampler The instrument was equipped with a glass column (55 cm × 2 7 mm I D) packed with Utrabond 20 M (100—120 mesh), stock number 4904, obtained from Alltech (Arlington Heights, IL, U.S A) The flow-rates of the carrier and detector gases were 25 ml/min for helium, 3 ml/min for hydrogen and 120 ml/min for air

The temperatures applied were as follows injector 200°C, for the column initially 150°C for 2 min, then programmed 5°C/min up to 250°C with a final hold of 2 min, detector 250°C. A 1- or 5-mV f.s.d recorder, chart speed 5 mm/min, was used Peak heights obtained for sample extracts and standards were used for the calculation of AA and HAA concentrations.

Gas chromatography—mass spectrometry

The gas chromatograph (Varian Model 3700) was equipped with a glass CP-Sil 5 column (10 m × 0 5 mm I.D) of Chrompack (Middelburg, The Netherlands) The flow-rate of the carrier gas (helium) was 5 5 ml/min. The temperatures were as follows injector 210°C, column temperature for AA—PFB 135°C, then programmed 10°C/min to 250°C, for HAA—PFB 185°C, then programmed 10°C/min to 250°C

The gas chromatograph was coupled to a Finnigan-MAT 8200 mass spectrometer with an open split coupling The ion source of the mass spectrometer was heated at 220°C The spectra were recorded at 70 eV The emission and acceleration voltages were 1 mV and 3000 V, respectively

Urine samples

Three equal groups (*n* = 3 × 7) of apparently healthy males and females, with and without regular use of oestrogen-containing oral contraceptives and all in the age range 19—43 years (mean age 30), volunteered in the study of excretion of AA and HAA in the urine

Urine produced during a period of ca 32 h in the sequence night—day—night was collected directly in polyethylene vessels and stored in the dark during collection at a temperature not exceeding 18°C Collection of urine during the second night was done after an oral load of 2 g of L-tryptophan [6], taken shortly before bed-time This was done to see whether there are differences between men and women in that respect and whether oral contraceptives have an effect on the tryptophan metabolism [3, 11] at the level of AA and HAA The urine excreted during and immediately after the sleeping period was considered as night urine Urine excreted during the remainder of the 24-h period was defined as day urine After collection, urine was stored at − 20°C till GC analysis

The creatinine content of urine was determined by the picrate method [12].

The excretions of the tryptophan metabolites AA and HAA have been calculated as the total amounts excreted during a 24-h period and on the basis of urinary creatinine. The latter was done to minimize the influence of differences in duration of the collection periods between individuals The excretion during the 24-h period was calculated from the individual results obtained for the day and night periods The fraction of AA relative to HAA (% AA) was defined as [AA]/([AA] + [HAA]) × 100% and was calculated to compare rates of formation of AA and HAA in different groups (men and

women) and with different treatments (oral contraceptive and tryptophan loading)

Hydrolysis and extraction

In routine analysis, a 10-ml aliquot of urine was mixed in a tube with 5 ml of concentrated hydrochloric acid The closed tube was heated in a boiling water bath for 4 h After cooling in melting ice, 5.5 ml of 10 M sodium hydroxide solution and 1 ml of citrate buffer were added. The solution was adjusted to pH 3 with a 2 M sodium hydroxide solution and saturated with sodium chloride (ca 8 g). Thereafter, it was extracted with 8, 6 and 6 ml of diethyl ether, respectively The diethyl ether extracts were combined and the volume was adjusted to 20 ml with diethyl ether

Derivatization and chromatographic clean-up

A 5-ml aliquot of the diethyl ether extract was evaporated to dryness in a 50-ml round-bottomed flask. The residue was dissolved in 8 ml of acetone and mixed with 0.5 ml of 10% (v/v) PFBB solution, 50 mg of caesium carbonate and some pumice stones. The mixture was boiled under reflux (water bath at 90°C) for 15 min After cooling, 10 ml of 2,2,4-trimethylpentane were added and the solution was concentrated to ca 2 ml Thereafter, another 10 ml of 2,2,4-trimethylpentane were added and the solution was concentrated to ca 2 ml

Deactivated silica gel (1 g deactivated with 5% water) was slowly poured into a column that was filled with 10 ml of *n*-hexane. The hexane was drained until the level reached the top of the silica gel. The 2,2,4-trimethylpentane extract was transferred quantitatively to the column using 5 0 ml of 1% ethyl acetate in *n*-hexane. The column was eluted with 50 ml of 1% ethyl acetate in *n*-hexane. The first 5 ml were discarded and the remaining 45 ml were concentrated to 1.0 ml using a gentle stream of dry nitrogen

Statistical analysis

Analytical results have been evaluated statistically using the Genstat program (Rothamsted Experimental Station, U K) incorporated in a VAX 11/750 computer (Digital Equipment) Analysis of variance (split-plot) was used for the determination of statistically significant differences between means of groups For evaluation of the influence of tryptophan loading, levels of significance were determined after natural logarithmic transformation of the individual results of GC analysis

RESULTS AND DISCUSSION

Extraction

Using plasma, Douglas and Philopoulous [13] observed a critical pH (4 75) of the sample at which AA and HAA extraction was optimal We found the highest percentage (> 95%) of extraction from hydrolysed urine using purified diethyl ether and a pH range of 2—4 Sodium sulphate, which is generally used for drying ether extracts, gave poor results (60%)

Hydrolysis of conjugates

To optimize the hydrolysis conditions, samples were incubated at 100°C

mg AA/l urine
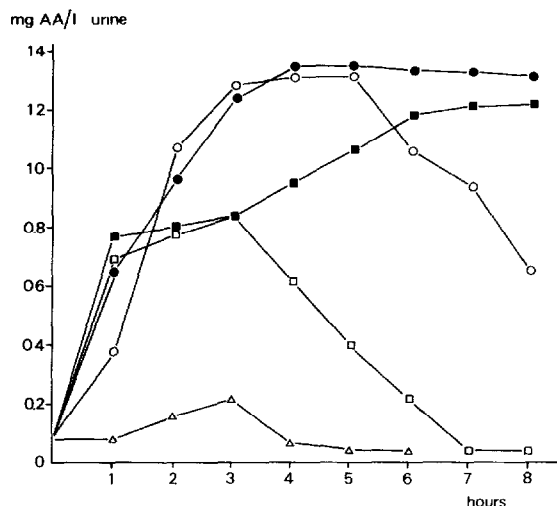
Fig 1 Time dependence of the acid hydrolysis of urinary anthranilic acid (AA) conjugates The acid concentrations applied were 0 1 M (△), 0 5 M (□), 2 0 M (■), 4 0 M (●) and 6 0 M (○) hydrochloric acid

mg HAA/l urine
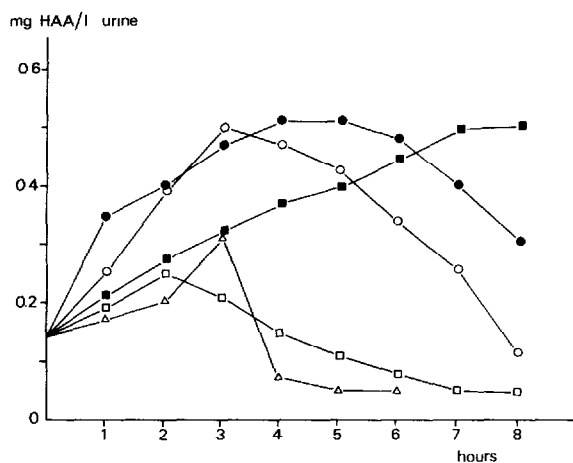
Fig 2 Time dependence of the acid hydrolysis of urinary 3-hydroxyanthranilic acid (HAA) conjugates The acid concentrations applied were 0 1 M (△), 0 5 M (□), 2 0 M (■), 4 0 M (●) and 6 0 M (○) hydrochloric acid

for 1—8 h at different hydrochloric acid concentrations The results are shown in Figs. 1 and 2

The highest AA and HAA yields were obtained with 4 M hydrochloric acid after a hydrolysis time of 4— 5 h The curve for 6 M hydrochloric acid showed about the same maximum after 3—4 h but the yields decreased rather sharply after ca 4 h. The same optimum was obtained with 2 M hydrochloric acid after 8 h. At these three hydrochloric acid concentrations, decomposition of AA and HAA seems to occur after 5 h of incubation. The 0 5 and 0 1 M hydrochloric acid concentrations gave poor results Under these conditions, the pH of the hydrolysate increased to 6 This indicates that no acid was left for hydrolysis
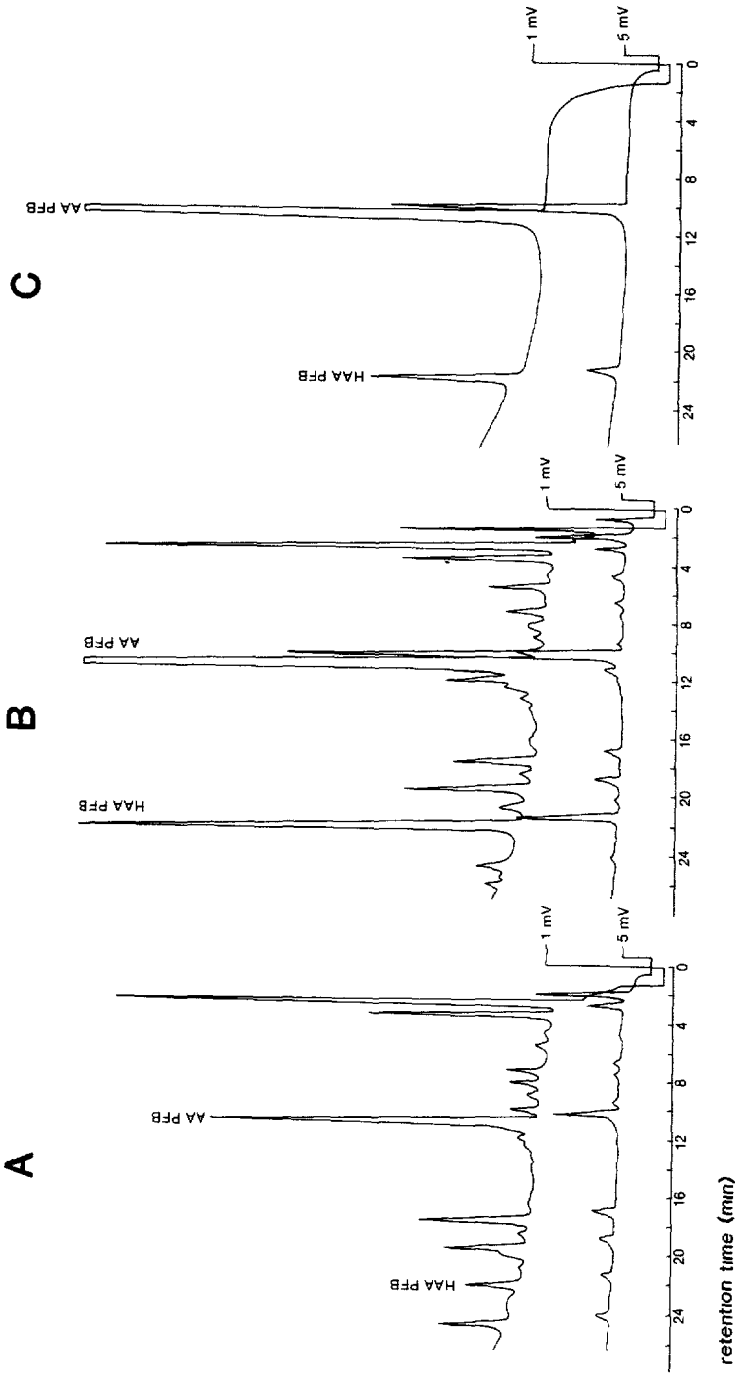
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Fig 3 Chromatograms obtained with a gas chromatography column packed with Ultrabond 20 M (100—120 mesh) (A) Night urine (5 μl), containing 1 1 mg/l anthranilic acid (AA) + 0 63 mg/l 3-hydroxyanthranilic acid (HAA), (B) night urine (5 μl) after L-tryptophan loading, containing 5 7 mg/l AA + 5 4 mg/l HAA. (C) standard, containing 20 ng AA + 8 ng HAA, 1 mg of AA or HAA is equivalent to 7 3 μmol of AA or 6.5 μmol of HAA, respectively

and that the compounds investigated may be unstable at higher pH and at high temperature

Derivatization procedure and mass spectrometric indentification

Several derivatization techniques for AA, HAA and other carboxylic acids have been described in the literature Boucek et al. [8] and Rose and Toseland [9] reported methylation using methanol and ethereal diazomethane, while Grundy et al [14] and Mauthner [15] applied dimethyl sulphate. Silylation techniques with bis(trimethylsilyl)acetamide have been reported for AA [13, 16] and HAA [17]. Trifluoroacetic anhydride [18] or pentafluoro-propionic anhydride and BCl$_3$—methanol [19, 20] can be used for acetylation. However, in our hands, none of these techniques gave satisfactory results low reproducibility and/or double peaks were obtained in GC analysis at the endogenous levels of AA and HAA

As AA and HAA are nitrogen-containing compounds, we used an alkali flame ionization detector. As derivatization reagent we applied PFBB, which has successfully been used for phenols and some carboxylic acids [21, 22] The reaction time needed for optimum yields of the PFBB derivatives of AA and HAA was only ca 15 min. In GC analysis, a single reproducible peak was obtained for both compounds

The identity of the derivatives was verified by combined gas chromatography—mass spectrometry Mass spectra showed that the ions at m/e 298/317 and at m/e 314/332 are characteristic of AA-PFB and HAA-PFB derivatives, respectively. Mass fragmentograms of the PFB derivatives of AA and HAA from urine samples corresponded with those obtained for derivatized AA and HAA standards.

Gas chromatographic determination

Fig. 3 shows typical chromatograms of a night urine obtained before and after an oral load of 2 g of L-tryptophan The detection limit under the conditions described was ca 0 05 mg/l urine for AA and ca 0 1 mg/l urine for HAA This indicates that our method is suitable for AA and HAA analysis in normal urine samples at the endogenous level The results obtained were confirmed on other GC columns i.e. 3% OV-210 and 3% OV-1 both on Gas Chrom (80–100 mesh) However, the Ultrabond 20 M column gave the best separation of AA from impurity peaks in the chromatogram

TABLE I

BETWEEN-ASSAY PRECISION AND RECOVERY OF THE ANALYSIS OF URINARY ANTHRANILIC ACID AND 3-HYDROXYANTHRANILIC ACID

Acid	Precision (mean ± S E M *, n = 11) (mg/l of urine)	C V ** (%)	Recovery (mean ± S E M , n = 9) (%)	C V (%)
AA	0 58 ± 0 04	7 2	84 0 ± 5 1	6 1
HAA	0 36 ± 0 02	5 8	84 0 ± 4 0	4 7

*S.E M = Standard error of the mean
**C V = Coefficient of variation

Precision and recoveries

In order to determine the precision of the method, a pooled urine sample was analysed on eleven different days In nine series, recovery experiments were carried out using urine to which about five times the endogenous amounts of AA and HAA had been added prior to hydrolysis. Only freshly prepared standards in methanol were used The results are given in Table I.

It was found that AA and HAA in methanol and in urine samples stored at −20°C were stable for at least one month. Standard solutions of the derivatives could be stored at +4°C for at least one month without deterioration.

APPLICATION OF THE METHOD

The method described here for the analysis of urinary AA and HAA has been applied in the investigation of the influence of tryptophan loading on the excretion of these tryptophan metabolites in the urine of men and women (with and without use of oral contraceptives) Results are described below.

Urinary excretion of AA and HAA during a 24-h period

The observed 24-h excretion of AA, HAA and creatinine in the urine by the three groups investigated is given in Table II As is regularly observed, the 24-h urinary excretion of creatinine by men was significantly higher ($p = 0.003$) than that by women With respect to AA and HAA, no significant differences were found between men and women not on oral contraceptives However, the 24 h urinary excretion of AA by women on oral contraceptives was significantly lower ($p = 0.029$). In contrast to this result, oral contraceptives appeared not to have an effect on the 24-h excretion of HAA

TABLE II

EXCRETION OF ANTHRANILIC ACID AND 3-HYDROXYANTHRANILIC ACID IN THE URINE OF MEN AND WOMEN

Each group consisted of seven volunteers

	Mean excretion per 24 h		
	AA (μmol)	HAA (μmol)	Creatinine (mmol)
Men	10 6	7 6	16 6
Women not on OCs*	10 0	5 5	11 2
Women on OCs	7 6	8 1	11 9
S E M **	2 0	3 6	2 7

*OCs = Oral contraceptives
**S E M = Standard error of the mean, values obtained in analysis of variance (18 degrees of freedom).

Diurnal variation of AA and HAA excretions

The creatinine-based excretions of AA and HAA during the day and night periods for men and women are given in Table III. In analysis of variance for both sexes, there was no significant diurnal variation for AA or for HAA.

TABLE III

MEAN CREATININE-BASED EXCRETION OF ANTHRANILIC ACID AND 3-HYDROXY-ANTHRANILIC ACID IN THE URINE OF MEN AND WOMEN

Period	Mean excretion (μmol/mmol of creatinine)					
	AA			HAA		
	Men	Women not on OCs*	Women using OCs	Men	Women not on OCs	Women using OCs
Night	0.70	0 86	0 67	0 48	0 41	0 64
Day	0 64	0 94	0 64	0 46	0 53	0 67
Night**	1 99	2 93	1 16	2 42	3 32	3 71

*OCs = Oral contraceptives
**Period of urine collection after tryptophan loading

TABLE IV

MEAN VALUES OF THE FRACTION OF ANTHRANILIC ACID IN THE URINE EXCRETED DURING VARIOUS PERIODS BY MEN AND WOMEN

Period	Fraction of AA* (%)		
	Men	Women not on OCs**	Women using OCs
Night	60	68	51
Day	60	64	52
Night***	45	47	26

*The fraction of AA has been calculated as $[AA]/([AA] + [HAA]) \times 100$
**OCs = Oral contraceptives
***Period of urine collection after tryptophan loading

The fractions of AA (percentage AA) excreted in the urine during the various periods are given in Table IV. For both sexes, no significant diurnal variation was found in analysis of variance However, the fraction of AA in women not on oral contraceptives was found to be significantly higher (p = 0.002) than that of women on oral contraceptives, but not than that of men.

Influence of tryptophan loading
The observed effects of an oral load of 2 g of L-tryptophan on the creatinine-based excretions of AA and HAA with night urine are given in Tables III and IV As was to be expected, significant increases ($p < 0$ 001) were observed for all groups For women on oral contraceptives the increase in AA after tryptophan loading was significantly smaller ($p < 0.001$) than that of women not on oral contraceptives These changes were such that the fraction of AA (percentage AA) was significantly decreased ($p < 0$ 001) as a result of tryptophan loading (Table IV) For women on oral contraceptives, the decrease in the fraction of AA after tryptophan loading was significantly larger than that of women not on oral contraceptives (p = 0.016). For men, this decrease was significantly smaller (p = 0.025) than that for women.

Comparison of AA and HAA excretion levels

With the exception of women on oral contraceptives, the excretion of AA with the 24-h urine was found to be significantly higher ($p < 0.001$) than that of HAA (Table II). This was also observed for the creatinine-based excretion of AA ($p < 0.001$) versus that of HAA for day urine as well as for night urine (Table III). These differences are also clearly demonstrated by the values calculated for the fraction of AA as given in Table IV. However, after tryptophan loading, the increase in the excretion of HAA was significantly larger ($p < 0.001$) than that of AA, resulting in fairly comparable excretions of AA and HAA for men, as well as for women not on oral contraceptives (Table III)

Our data indicate that no diurnal variation was present for the excretion of AA and HAA in the urine of men and women. The 24-h excretions by both sexes were comparable, while that of AA was higher than that of HAA. However, the use of oestrogen-containing oral contraceptives resulted in a reduced formation of AA and probably, thereby, in an increased HAA synthesis. This phenomenon, which has also been reported for some other tryptophan metabolites [12, 23], probably results from an inhibition of kynureninase by oestrogens [3], kynureninase being a key-enzyme in the oxidative degradation of tryptophan

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