JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2012, 63, 6, 613-621
www.jpp.krakow.pl

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CONVERSION L-TRYPTOPHAN TO MELATONIN IN THE GASTROINTESTINAL TRACT: THE NEW HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD ENABLING SIMULTANEOUS DETERMINATION OF SIX METABOLITES OF L-TRYPTOPHAN BY NATIVE FLUORESCENCE AND UV-VIS DETECTION

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Melatonin is a major biosynthetic product of pineal gland exerting a potent antioxidant and the reactive oxygen metabolites scavenging activities but the mechanism of formation of this indole at extrapineal sources has not been fully elucidated. It is known that the gastrointestinal (GI)-tract plays an important role as a source of melatonin synthesis but the conversion of L-tryptophan into melatonin in the GI-tract of experimental animals and humans should be further examined. In this study, the conversion of L-tryptophan to melatonin was determined in the serum collected from rats administered intragastrically with this amino acid acting as melatonin precursor. For this purpose, a simple, sensitive and reliable method was developed for simultaneous determination of six L-tryptophan metabolites in rat serum, namely, 5-hydroxytryptamnie (5-HT), 5-hydroksytryptophan (5-HTR), kynurenin (KYN), antranilic acid (AA), indole-3-acetic acid (IAA) and melatonin that were analyzed in one chromatographic run by high-performance liquid chromatography (HPLC) with UV and native fluorimetric detection with multiple wavelengths. We used nucleosil Supelco C18 5 µm 4.6 mm × 250 nm column with the standard mobile phase consisting of solvent A (water/0.1% trifluoroacetic acid (TFA) and solvent B (methanol/0.1% TFA) in gradient elution. Fifty five rats received vehicle (saline) of L-tryptophan (50 mg/kg) or melatonin (50 mg/kg) by means of intragastric gavage and they were anesthetized and sacrificed at 0, 10, 20, 30, 60, 120 or 240 min upon L-tryptophan or melatonin administration for the venous blood withdrawal. The serum collected samples were kept on ice for the HPLC determination. The average recovery of 5-HT, 5-HRT, KYN, AA, TRP, IAA, and melatonin were 99±3%, 97±1.5%, 94±2.5%, 99±2.46, 98±1.5 and 98±2%, respectively. We conclude that 1) L-tryptophan is converted to melatonin in the GI-tract during the day when the pineal gland synthesis is inhibited, and 2) the reverse phase high performance liquid chromatography (RP-HPLC) is a new sensitive and reliable method that could be successfully applied to the study of kinetics and metabolism of L-tryptophan in GI-tract.

Key words: *melatonin, L-tryptophan, gastrointestinal tract, melatonin metabolites, kynurenine, 5-hydroxytryptamine, 5-hydroxytryptophan, antranilic acid, indoleacetic acid*

INTRODUCTION

L-tryptophan is an essential amino acid that acts as building blocks in protein biosynthesis but besides that another important function of L-tryptophan is that this amino acid serves as a precursor in the synthesis of various biologically active substances *in vivo* such as melatonin and other metabolites such as 5-HT, 5-HTR, KYN, AA and IAA (1-3). Following discovery of melatonin in the pineal gland, subsequent studies revealed that this indole is also distributed in many extra-pineal organs including retina, Harderian gland, placenta, kidneys, respiratory tract and gastrointestinal (GI)-tract (4-8). Moreover, melatonin has been localized in non-neuroendocrine cells such as mast cells, natural killer cells, eosinophils, platelets and endothelial cells. For instance, the GI-tract appears to be abundant and particularly rich source of melatonin. Previous studies revealed that total amount of melatonin in the GI-tract exceeds by about 500-1000 times the amount of this indole secreted by the pineal gland (7, 8) with highest concentration in bile, stomach, ileum and colon (9-12).

The conversion of L-tryptophan leads to synthesis of major metabolite melatonin due to rate-limiting activity of the two enzymes N-acetyltransferase (NAT) and hydroksyindolo-O-methyl-transferase (HIOMT) (*Fig. 1*). This indole can be released into blood-stream by pineal gland during the circadian rhythm with lowest plasma concentration during the day (13). The GI-tract conversion of L-tryptophan triggered attention of many investigations in the past in different organs (8) but little is known on melatonin and other metabolites and their kinetics after administration of L-tryptophan in rodents. Therefore, we

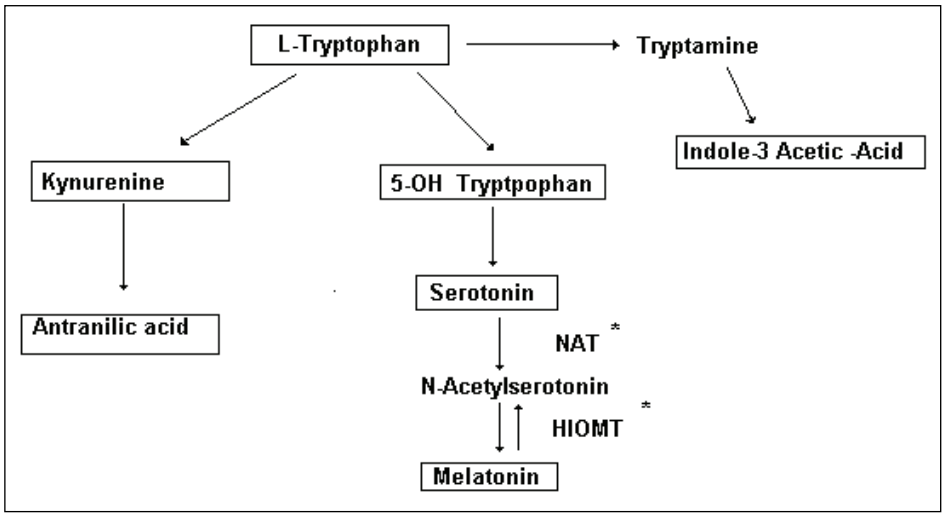

Fig. 1. Metabolic pathways of physiological conversion of L-tryptophan into serotonin and melatonin *via* activity of two rate-limiting enzymes NAT and HIOMT.

Table 1. Fluorescence (FL) made by stop-flow technique and UV detection, limit of detection LOQ and limit of quantification LQS of standards in aqueous solution.

Compound	LOD pmol/ml	FL(λ ex./ λ em.,nm)*	LOQ pmol/ml	LOD nmol/ml	UV(λ, nm)	LOQ nmol/ml
5HT	3.42	300/335	6.8		Not detected	
KYN	----------	Not fluorescent	----------	0.4	365	1
5HTR	6.25	300/335	13.6		Not detected	
AA	6.85	339/419	13.7		Not detected	
L-Trp	6.06	285/365	27.4	1.25	285	3
IAA	6.85	282/349	13.7		Not detected	
MLT	1.7	295/339	3		Not detected	

* ex-excitation; em.- emission

studied the time-dependent process of conversion L-tryptophan to melatonin and other L-tryptophan metabolites. We considered that there are several different methods developed to determine conversion of L-tryptophan to its biogenic amines. The major commonly employed to determine these aspects are high performance liquid chromatography (HPLC) with UV (14-15), fluorescence detection (15, 17-19) electrochemical detection (18, 20, 21) and system HPLC coupled to mass spectrometry (22, 23). There are variety of mobile phases developed to provide satisfactory chromatograph resolution, isocratic (14-20) and gradient elution (19-21). Herein, we propose an alternative method for the determination of indole compounds in rat serum by gradient elution HPLC with UV and native fluorimetric detection in standard gradient elution without any derivatization process in one chromatographic run. Furthermore, the sample handling and processing for our method seems to be simple and reliable.

MATERIAL AND METHODS

The study was approved by the Institutional Animal Care and Use Committee of Jagiellonian University Medical College in Cracow and was conducted in accordance with the statements of Helsinki Declaration regarding handling of experimental animals.

Experimental animals

Fifty five Wistar rats of both sexes, weighing of 150-200 g were used. Animals were housed in cages under standard conditions, on commercial pellet chow, at room temperature with a 12-h light:12-h dark cycle. Rats were fasted for 24 hours prior to the start of the experiment, while drinking water was available *at libitum*. All experiments were carried out in the morning at the same time at 10 a.m. During the experiments with vehicle (saline) or tryptophan administration all rats were kept in individual Bollman cages.

Melatonin was dissolved in a drop of absolute ethanol and then in physiologic saline, while L-tryptophan was dissolved in physiologic saline containing a drop of 0.1 HCl. Melatonin or L-tryptophan was administered in a volume of 0.5 ml by intragastric (i.g.) gavage and rats were sacrificed at 0, 10, 20, 60, 120 and 240 min after each compound or vehicle being administered. After administration of L-tryptophan (50 mg/kg, i.g.) and melatonin (50 mg/kg, i.g.) rats were anesthetized with phentobarbital (60 mg/kg i.p.), weighed and their abdominal cavities were opened. The blood samples were taken from vena cava immediately centrifuged and the serum kept at -20°C until analysed.

Chemical reagents and chromatographic system

L-tryptophan (L-Trp), kynurenine (KYN), 5-hydroxytryptamine (5-HT), anthranilic acid (AA), indole-3-acetic acid sodium salt (IAA), 5-hydroxy-L-tryptophan (5-HTR), melatonin, trifluoroacetic acid (TFA) were obtained from Sigma (Schelldorf, Germany). Trichloroacetic acid (TCA), methanol and acetonitrile (ACN) gradient grade were purchased from Merck (Darmstadt, Germany). Water was deionized by passing through an EASY pure RF compact ultrapure water system.

HPLC system (Shimadzu Duisburg, Germany) consisted of two solvent delivery module LC10AT vp and DGU-14A degasser, a CTO-10 ASvp column oven, a SIL-10ADVp auto injector, SPD-M10Avp diode array detector and RF-10Axl fluorescence detector. The CLASS-VP 7.2.1. was used for data

collection and processing. The samples were separated at 20°C on a nucleosil Supelco C_{18} (4.6×250 mm i.d.) fitted with Phenomenex guard column (4×3 mm i.d.) packed with the same material, with a mobile phase A: water with 0.1% trifluoroacetic acid (TFA) and mobile phase B: methanol with 0.1% TFA. After injection the sample were eluted with 19.5% B followed with 5 min; linear gradient to 24.5% B, then 25 min; linear gradient to 49.5%, 1.5 min; linear gradient to 100% B and 18.5 min isocratic period. The column was then re-equilibrated to the initial condition for 15 min. Analyses of 40 µl of samples were performed at flow of 1.0 ml/min with UV-VIS and fluorescence detection with the multiple wavelengths change.

Sample preparation and detection of L-tryptophan metabolites

Frozen serum specimens were thawed at 37°C for 15 min and 100 µL of serum was diluted with 100 µL of potassium phosphate buffer (0.05 mol/L, pH 6.0). Proteins were precipitated with 250 µL of frozen aqueous solution of TCA (2 mol/L): acetonitrile (1:4). The capped tubes with precipitate were immediately vortexed and centrifuged for 10 min at 11,000 g. The resulting supernatants were filtrated through a PTFE 0.2 µm Supelco filter and a 65 minute procedure of separation of the analyzed compound was carried out on HPLC system.

The identification of the peaks was accomplished by use of three procedures: (a) by spiking samples with reference compounds, (b) by comparing retention time of separated compounds with standards, and (c) by recording fluorescence spectra with the stop-flow technique.

Stock solution standard curves and limit of detection

The amount of standards was accurately weighed on an analytical balance and dissolved with potassium phosphate buffer (0.05 mol/L, pH 6.0) to make a stock standard solution in target concentration of 1 mmol/L. The standard solutions were divided into 1 ml aliquots in Eppendorf tubs and stored at -20°C before use. A series of mixed standard working solutions of different concentration were prepared by combining standards and phosphate buffer (0.05 mol/L, pH 6) to achieve proper concentrations. The solutions of seven standards within a concentration range (225-3.51 nM/ml) for L-Trp, (28-0.43 nM/ml) for KYN at UV detection, (7031-13.5 pM/ml) for 5-HT, (439-13.7 pM/ml) for 5-HTR and AA, (3515-27.4 pM/ml) for IAA, and (5625-3.43 pm/ml) for melatonin and L-tryptophan in fluorescence detection, respectively. All dilutions of standards were prepared immediately before use.

Standard curves were prepared in the same way as rat serum samples by adding 100 µl of appropriate amount of standard solution to 100 µl of potassium buffer 0.05 mol/L or to the thawed frozen rat serum. The next steps of sample preparation was similar as described above.

The calibration curves were constructed in triplicate by plotting the ratio of the peak area of mixed working solution to IS (y) against standard concentration (x), respectively. The calibration curves in serum were run in triplicate by plotting ratio of the peak area of mixed working solution to IS subtract the ratio of peak area pooled serum to IS (y) against standard concentration (x), respectively.

Linearity was established by least squares linear regression analysis of the calibration curve.

Limit of detection (LOD) for all standards was performed by diluting standards solutions until signal to noise (S/N) ratio was approximately 3. The limit of quantification (LOQ) was defined as the analyte concentration resulting in the lowest measurable peak height with acceptable precision (coefficient of variation (C.V.)≤15%).

The precision of this method was examined by adding 100 µl standard working solution of seven standards and low, middle and high concentration, respectively, into pooled serum. The intra-day reproducibility was assessed of five samples in a day while inter-day reproducibility was determined by measuring the same samples on 5 consecutive days.

RESULTS

Precipitation process

To optimize the separation procedure, the precipitation procedure, analytical columns and gradient elution were examined. In the beginning according to the previous method (24) we used only TCA (2 mol/L) to precipitation process, but we have a problem with stability of the prepared samples over time. Our modification of precipitating process rely on combining two precipitants in one cold mixture of acetonitrile (ACN) and trichloroacetic acid (TCA) 4:1. It allowed for changing of the ratio of precipitant to serum volume from 1:8 to 1.25:1, and gave a maximal stability of prepared samples within 72 hours without significant decrease of detected compounds. Our observations were corresponding to the earlier observations by Polson *et al.* (25).

HPLC separation and detection conditions

To achieve the best separation of detected compounds during one chromatographic run we check several gradient methods on various analytical columns. The optimal separation was obtained on a nucleosil Supelco C_{18} (4.6×250 mm i.d.) with gradient elution described above.

The presented method of simultaneous determination of L-tryptophan and tryptophan metabolites in rat serum is based on the programmed wavelength change detection. To optimize the condition of standard detection the fluorescence spectra by a stop-flow technique without standards of KYN were recorded. These standards cannot emit native florescence.

For excitation and emission of 5-HT we have chosen the 5-HTR fluorescence detection because concentration and native fluorescence intensity of serotonin in rat serum is higher than 5-HTR. The programmed wavelength changing detection permit to determine concentrations of detected compounds with maximum limit of detection during one analysis. The fluorescence excitation end emission wavelengths were set to 300/335 nm respectively at the beginning of the run (detection 5-HT and 5-HTR). At 11.5 min later, excitation and emission wavelengths were changed to 339/419 nm (detection of AA) and at 4.5 min later, the excitation and emission wavelengths were changed to 285/365 nm (detection of L-tryptophan). At 8 minutes later, excitation and emission wavelengths were changed to 282/349 nm (detection of IAA) and after another 3.5 min excitation and emission wavelengths were changed to 295/339 nm (detection of melatonin). UV signals were monitored at 360 nm and 280 nm for detection KYN and L-tryptophan respectively. The excitation and emission of detecting standards made by the stop-flow technique in aqueous solution, limits of detection (LOD) and quantification (LOQ) as shown in *Table 1*.

Concentration of L-tryptophan in this method can be measured by florescence and UV detection, respectively. As the concentration of L-tryptophan in rat serum is high the detection in UV seems to be sufficient. The retention times of 5-HT, KYN,

Table 2. Calibration curves of L-tryptophan (L-Trp) and its metabolites including melatonin (MLT).

	Concentration range	Aqueous solution	R^2	Pullet rat serum	R^2	LOQ rat serum
5-HT	13.5-7031 pmol/ml	Y=0.1129x -0.5901	0.9997	Y=0.1098x -0.5779	0.9998	13.7 pmol/ml
KYN	0.43 – 28 nmol/ml	Y=1.9519x -0.1063	0.9997	Y=2.0534x -1.0841	0.9997	1.5 nmol/ml
5-HTR	13.7-439 pmol/ml	Y=0.4537x -0.0665	0.9998	Y=0.4657x -1.1548	0.9991	18 pmol/ml
AA	13.7-439 pmol/ml	Y=0.1403x -0.8461	0.9995	Y=0.1436x -0.3489	0.9996	15 pmol/ml
L-Trp *	3.5- 225 nmol/ml	Y=3.087x +0.2959	0.9999	Y=3.1361x +0.3863	0.9998	3.5 nmol/ml
IAA	27.4-3515 pmol/ml	Y=0.5368x+16.788	0.9995	Y=0.5171x +7.672	0.9992	28 pmol/ml
MLT	7031.2-56250 pmol/ml	Y=0.1408x -81.607	0.9998	Y=0.1323x +11.448	0.9993	3.4 pmol/ml
	109.8-7031.2 pmol/ml	Y=1.186x -26.484	0.9999	Y=1.1145x +63.158	0.9997	3.4 pmol/ml
	3.4-109.8 pmol/ml	Y=1.0828x +1.201	0.9997	Y=1.0751x +0.2509	0.9996	3.4 pmol/ml

*- UV detection

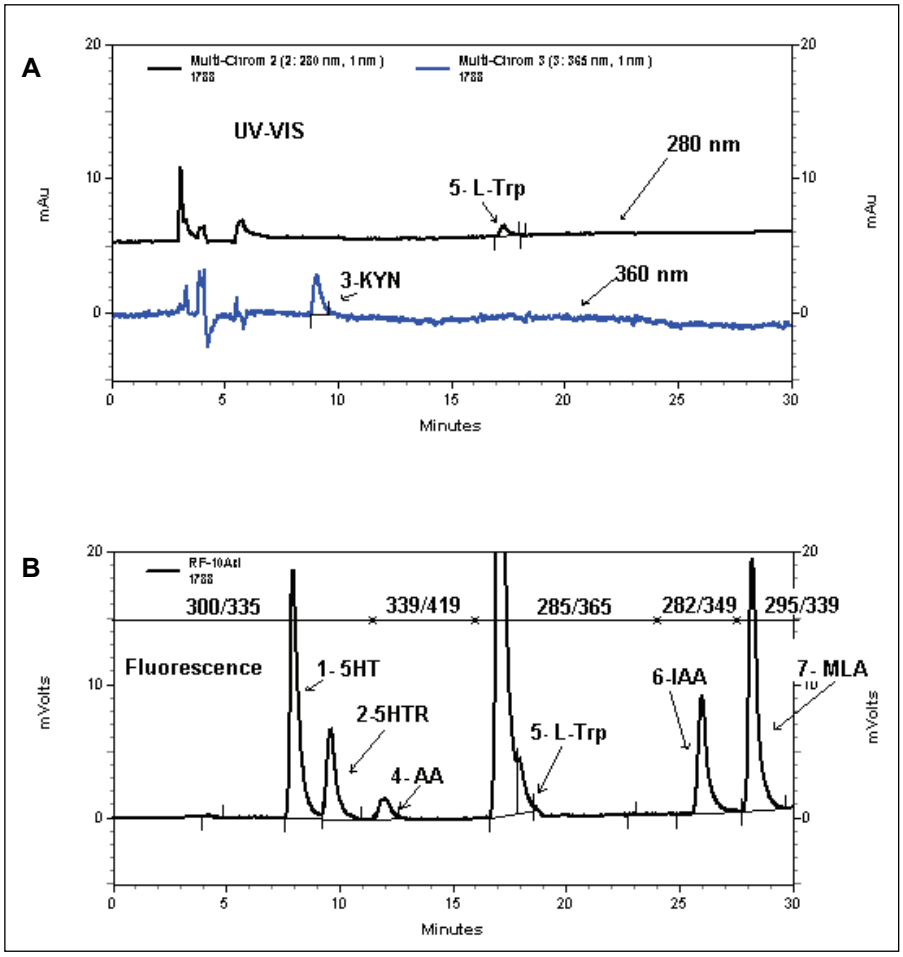

Fig. 2. Chromatographic analysis of an aqueous standard solution recorded (A) at UV-VIS and (B) by fluorescence. Chromatograms show particular metabolites of L-tryptophan (L-Trp) and melatonin. Peaks 1-5-HT, 2-5-HTR, 3-KYN, 4-AA, 5-L-Trp, 6-IAA, 7-melatonin (MLA).

5-HTR, AA, L-Trp, IAA and melatonin were 8.27, 8.15, 9.97, 11.84, 17.73, 26.68 and 28.5 min, respectively.

Fig. 2. illustrates the HPLC elution profile of seven standards in aqueous solution obtained from the gradient elution combined with the multiple wavelength change detection. Typical of analysis of rat serum is shown in *Fig. 3*.

Linearity

In this method the linearity of L-tryptophan (L-Trp) and its metabolites were investigated in water (buffer pH=6.005mM) and pooled rat serum. We mixed the buffer standard solutions with three repeats to yield combined solutions containing a concentration of the range; 3.5-225 nm/ml for L-Trp, 0.43-28 nm/ml for KYN, 13.5-7031 pm/ml for 5-HT, 13.7-439 pm/ml for 5-HTR and AA, 27.4-3515 pm/ml for IAA. Linearity of detecting melatonin was measured in three concentration ranges, low (3.4-109.8 pM/ml), medium (109-7031.2 pM/ml) and high (7031.2-56250). To detect the high concentration of melatonin in a single chromatographic run we had to changed the standards parameters of RF-10AXL fluorescence detector from Medium ×4 GAIN to Low ×16 GAIN. The linear equations for determined compounds in aqueous solution, pooled rat serum and the limit of quantification LOQ at rat serum were shown in *Table 3*.

Precision and recovery

The intra-day and inter-day precision data were evaluated at low, middle and high concentration. The within-day coefficients of variation (CVs) were less 5%, and the between a day the CVs were less then 6% per run (*Table 4*). Recovery for detecting compounds in different added concentration of standards and relatively standard deviation (RDS) were given in *Table 4*. The mean recovery and RDS was in the range 87.8-114% and less then 6%, respectively. After considering our recovery tests

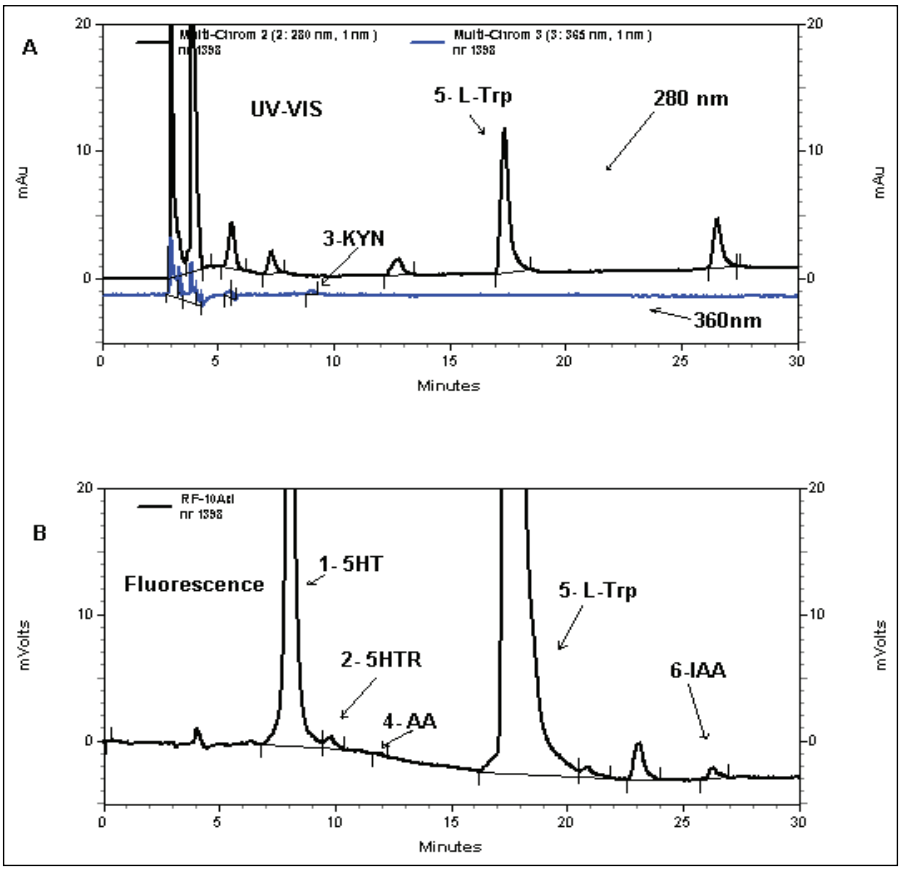

Fig. 3. Chromatographic analysis of typical rat serum recorded (*A*) at UV-VIS and (*B*) by fluorescence detection. Chromatograms show particular metabolites of L-tryptophan (L-Trp). Peaks 1-5-HT, 2-5-HTR, 3-KYN, 4-AA, 5-L-Trp, 6-IAA.

Table 3. The intra-day and inter-day precision of standards in serum (n=5).

Compound	Intra –day		Inter –day	
	x±S.D.	CV(%)	x±S.D.	CV(%)
5-HT (pmol/ml)	1757.8± 67.32	3.83	1757.4± 105.09	5.98
	438.72±21.36	4.87	438.3±12.63	2.88
	56.4±0.69	1.22	55.4±2.71	4.89
KYN (nmol/ml)	14,06±0.384	2.73	14.25±0.64	4.49
	7.03±0.275	3.92	7.12±0.19	2.70
	3,51±0.17	4.83	3.47±0.16	4.55
5-HTR (pmol/ml)	217.98±4.56	2.09	217.56±13.45	6.18
	108.49±4.71	4.34	107.92±2.69	2.49
	53.75±2.12	3.94	54.1±2.04	3.77
AA (pmol/ml)	429.9± 19.82	4.61	428.7±27.01	6.3
	219.7±5.45	2.48	218.75±8.93	4.08
	108.45±1.89	1.75	107.9±3.35	3.10
L-Trp (nmol/ml)	225.3±3.02	1.34	224.9±4.88	2.17
	112.2 ±2.56	2.28	111.7±4.75	4.26
	28.4 ± 1,01	3.55	27.4±0.83	3.05
IAA (pmol/ml)	877.8± 11.32	1.29	879.2±37.45	4.26
	439.5±10.94	2.49	438.7±5.74	1.31
	109.8± 1.96	1.78	108.5±4.26	3.93
MLT (pmol/ml)	878.6± 13.179	1.5	877.5± 25,97	2.96
	437.5 ± 12.81	2.93	46.8±2.04	4.37
	55.34 ± 1.28	2.33	54.23±2.94	5.42

(RDS) we decided not to use internal standards commonly preferred in multiple step method to monitor the precipitation process. This procedure was the similar to that described by Raikhlin *et al.* (6).

Rat serum application

The described method was used to determine L-tryptophan and its metabolites during kinetic experiments in Wistar rats. The

study consisted of two series of experiments (A, B). In series A, the effects of time-dependent supplementation of melatonin was assessed, and series B which involved the effects of conversion L-tryptophan to its metabolites and melatonin at particular time after this amino acid supplementation. Each experimental group consisted of 8 rats. Typical chromatogram summarizing results of these experiments is shown in *Fig 4*.

Table 4. Recoveries of standards in serum (n =5).

Compound	Added	Recovery (%)	RDS (%)
5-HT (pmol/ml)	219.7	109.4 ± 1.5	1.37
	109.8	91.7 ± 2.52	2.74
	27	114.0 ± 3.51	3.08
KYN (nmol/ml)	14	102.2 ±3.84	3.75
	3,5	102.7 ± 4.63	4.51
	1,7	87.8 ± 3.97	4.52
5-HTR (pmol/ml)	219.7	102.6±2.56	2.49
	109	96.9 ±2.88	2.97
	27	109.7 ±5.08	4.63
AA (pmol/ml)	430	103.1 ±2.51	2.43
	109.8	96.9 ±1.49	1.54
	54	109.3 ±3.85	3.52
L-Trp (nmol/ml)	109	97.8 ± 2.54	2.59
	56	98.4 ± 4.46	4.53
	14	110.7 ± 3.27	2.95
IAA (pmol/ml)	219.7	96.4 ±1.62	1.68
	109.8	98.5 ±2.51	2.54
	54	102.4 ±5.24	5.12
MLT (pmol/ml)	56	97.2 ± 2.53	2.31
	27	96.7 ± 3.41`	3.53
	6.8	90.3 ± 4.09	4.53

The following study groups were employed: (1) control (0.5 ml of vehicle saline i.g.), (2) melatonin (50 mg /kg i.g.), dissolved in 0.5 ml of saline and given to rats at different time and sacrificed at 0, 10, 20, 60, 120 or 240 min, 3) L-tryptophan (50 mg /kg i.p.), given to rats at different times and sacrificed at 0, 10, 20, 60, 120 or 240 min.

After L-tryptophan, application, the metabolites 5-HT and 5-HTR exhibited an increase to reach their maximal serum levels up to 6 and 2 fold, respectively, at 20 min. It further showed a decline to the basic levels and once again increased slowly to the levels up to 4 and 0.6 fold. KYN, AA rose slowly and massively to reach the levels (up to 6.5, 4.5 fold) at about 60 min application and remained at the elevated level at 120 min but declined not rapidly to about 3- and 1.5-fold, respectively, at 240 min. L-tryptophan and IAA increased slowly to reach the levels up to 3.5 and 2.5 fold, respectively, at 60 min and declined gently to 2-fold and to the baseline, respectively, at 240 min.

The application of melatonin caused minor change of AA, 5-HTR. KYN and 5-HT rose slowly to reach the serum levels up to 4.5 and 3-fold, respectively, at 20 min after application and declined rapidly below the basic concentration at 60 min and 5-HRT rised gently once again to reach the serum concentration levels up to 2- and 6-fold, respectively. IAA concentration rised slowly and massively up to 3.5-fold at 120 min and then declined rapidly to the basic level.

The serum concentration of L-tryptophan metabolites after application of exogenous melatonin and L-tryptophan is presented in *Fig. 5*.

In all study groups of control rats, melatonin was not detected in the serum. A single application of melatonin at the dose of 50 mg/kg i.g. to rats produced a several thousand rise in serum melatonin levels, and this reached the highest value of 52±6.24 nM/ml at 10 min after application. The concentration of exogenous melatonin after 240 min showed a gradual decline to the value of 21.8 pM/ml (*Fig. 6*). After single i.g. application of

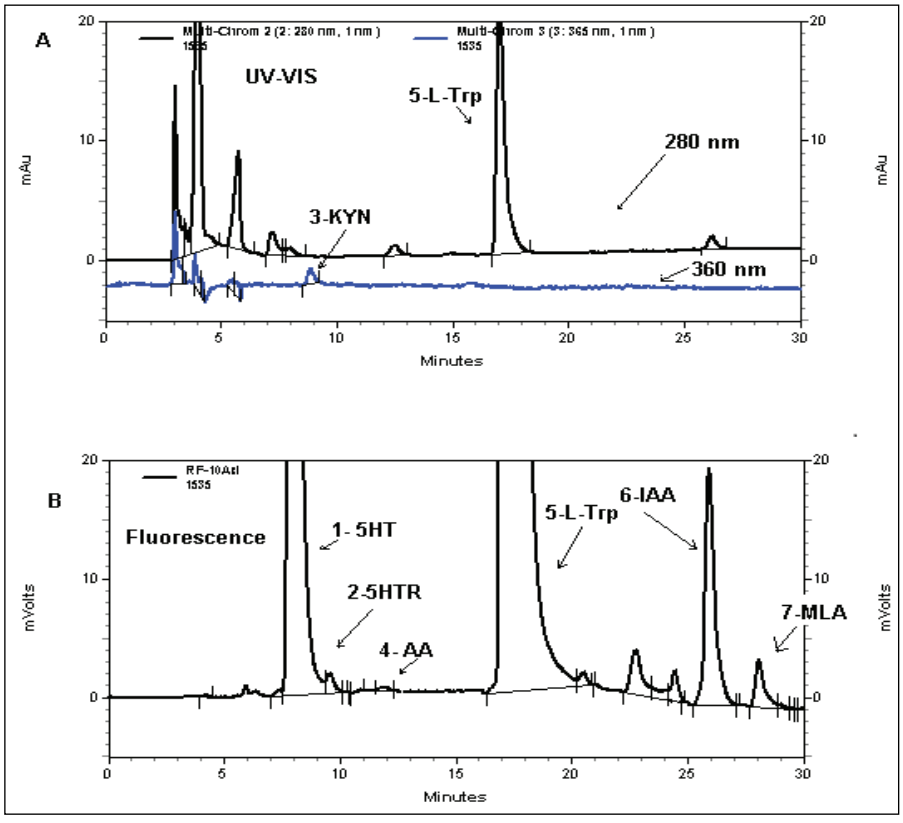

Fig. 4. Chromatographic analysis of rat serum supplemented L-tryptophan detection. Peaks: 1-5TH; 2-5HTR; 3-KYN; 4-AA; 5-L-Trp; 6-IAA; 7-MLA.

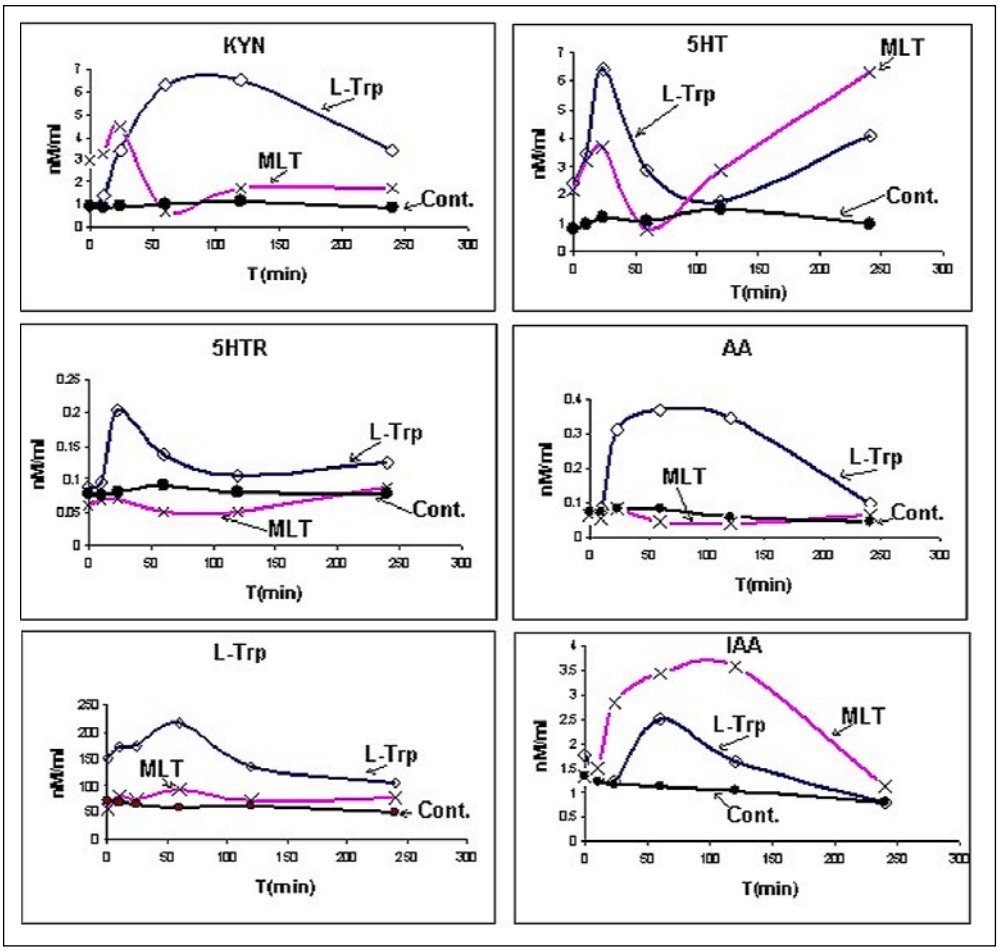

Fig. 5. Serum concentration of L-tryptophan and various L-Trp metabolites determined throughout the period of 240 min after i.g. application of 50 mg/kg of melatonin (MLT) (-x-) or 50 mg/kg of L-tryptophan. (-◇-) and of vehicle (-●-).

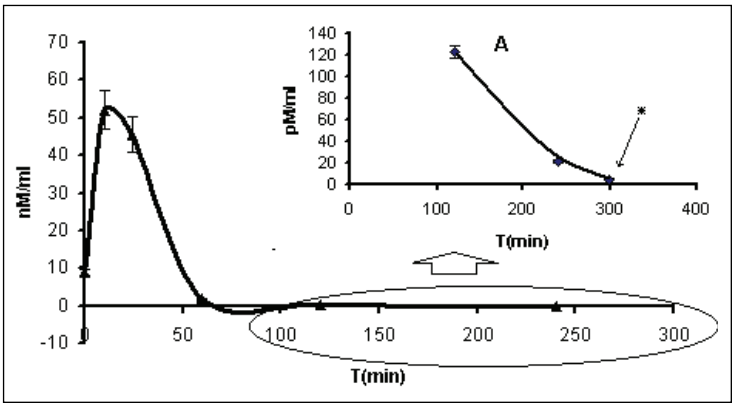

Fig. 6. Time-dependent plasma level of melatonin concentration after application of melatonin (50 mg/kg, i.g.). * - extrapolated at point 300 min.

L-tryptophan at 50 mg/kg the increment of serum melatonin levels rose up the values of about 100±5 pM/ml at 10 min, and after 240 min from the start of administration it declined to the value of about 3.86 pM/ml (*Fig. 7*).

DISCUSSION

In order to determine six of L-tryptophan metabolites in rat serum we combined two methods, namely, UV detection with multiple fluorimetric wavelengths changing detection. Herein, we presented efficient methods for the detection of L-tryptophan metabolites. Only melatonin detection is not enough for observation of standard concentration during the circadian rhythm in rat serum. For this purpose, many more sensitive methods were described based on isocratic HPLC elution conducted with fluorescence detection (26-28), MS detection (29) and radioimmunoassay detection (RIA) (30). Nevertheless, the use of these methods permit only of sensitive detection of one or two compounds with extraction procedure to avoid other disturbing indoles and decrease of chromatographic noise. It seems mandatory when having a large variety of melatonin concentration, the process of kinetics is preferred to use the method with average limits of detection and maximum detected compounds in one chromatographic run.

It is interest, that in order to avoid the interferences of melatonin released into blood-stream by pineal gland we decided to conduct all animal experiments during day to inhibit pineal synthesis of this indole.

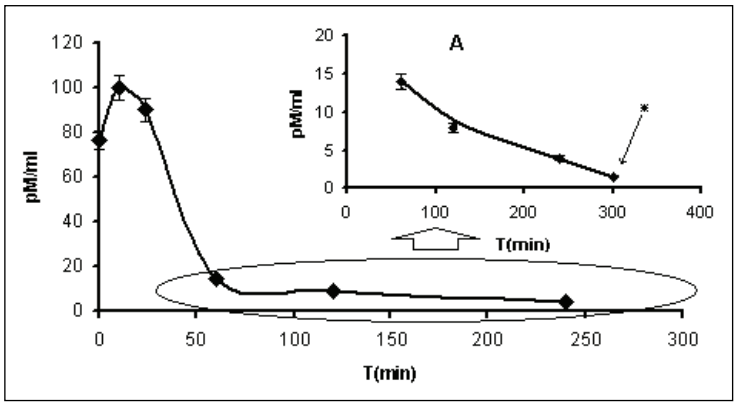

Fig. 7. Time-dependent plasma level of melatonin concentration after application of L-tryptophan (50 mg/kg, i.g.). * - extrapolated at 300 min.

Considering all our attempts to measure of serum melatonin levels, our results are similar but the concentration is higher than the values measured by others in different experiments in rodents. For example, Huether *et al.* (31) showed that plasma melatonin level during infusion of about 10 μg of melatonin/ml caused an increase in plasma melatonin levels to the value of 10.630±900 pg/ml (45.76±3.87 pM/ml) after 30 min followed by a steady-state. Jaworek *et al.* (32) showed that single application of melatonin (50 mg/kg i.p.) to rats with caerulein-induced pancreatitis (CIP) produced a several-fold rise in plasma melatonin levels, achieving value of 1030±80 pg/mL (4.43±0.34 pM/ml) at 300 min following this indole administration. Interestingly, L-tryptophan suplementation (50 mg/kg i.p.) of CIP rats resulted in a increment of plasma melatonin level to value 200±20 pg/ml (0.86±0.08 pM/ml) at 300 min of L-tryptophan administration. We compared our values on time depending sequence of events with those of Jaworek *et al.* (32) which in our study are presented in *Figs 6A* and *7A*.

Our results are corroborative with previous studies on the melatonin binding in GI-tract. For instance, Konturek *et al.* (33) showed that after application of I^{125}-melatonin tracer the maximal level of melatonin binding sites in GIT-tract was observed after 15 min and after 30 min upon the tracer administration being somewhat declined.

When compared the concentration of detected L-tryptophan metabolites with melatonin the metabolism of melatonin seems to be under control of negative feedback mechanism (28). The process is activating about 10 min after application melatonin and L-tryptophan. In the case of conversion L-tryptophan to its metabolites the process is activated when either concentration of melatonin and 5-HTR have reached 100 pM/ml of rat serum. After that time the concentration of melatonin gradually declained whereas the concentration of 5-HTR and 5-HT had increased to reach the maximum within 20 min and it declined in next 100 min.

For the other L-tryptophan metabolites KIN, IAA and AA, the process of conversion started at 20 min and the maximum concentration of these metabolites have been achieved at about 100 min after amino acid application.

In summary, we provided evidence that endogenous melatonin derived from the conversion of L-tryptophan to melatonin could be considered as a main producer of this indole in the GI-tract for instance taking place after food intake (33, 34). The GI-tract as a potential source of melatonin can influence not only the circulating melatonin level, but also the generation of this hormone by the entero-endocrine (EE) cells in the gut (35). This endogenous melatonin or that administered exogenously has been shown to provide the gastroprotective effects against acute and chronic gastric mucosal injury in animal models (33) and to accelerate the healing of *H. pylori*-infected gastroduodenal ulcers in humans, an effect, in part, involving leptin (36).

The developed reverse phase high performance liquid chromatography (RP- HPLC) method is sensitive and reliable. It has been successfully applied to the study of kinetics and metabolism of L-tryptophan in rat serum, with acceptable sensitivity. After a simple modification this method can be combined with the MS detectors for complex sample analysis. Our detailed analysis revealed that a better sensitivity of detected compounds can be achieved by changing the standard RF flow cell detectors to semi-micro cells.

Acknowledgements: This work is supported by the state grant K/ZDS/002308 from Jagiellonian University Medical College to Dr. I. Brzozowska.

Conflict of interests: None declared.

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Received: August 24, 2012
Accepted: December 20, 2012

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