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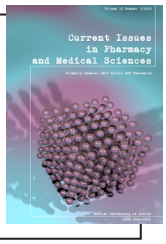

The effect of a selective estrogen receptor modulator – raloxifene – on the levels of tryptophan and kynurenic acid in the livers of rats as studied via RP-HPLC-FL

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ABSTRACT

The levels of tryptophan (TRP) and its main metabolite kynurenic acid (KYNA) were measured in rat livers treated with raloxifene – a selective estrogen receptor modulator. The research was conducted by applying high-performance liquid chromatography on a 5 µm Zorbax Eclipse XDB-C18 column. Selective fluorescence detection (FL) was performed at an excitation of 219 nm and emission of 360 nm for TRP and KYNA. The assays showed good linearity (R^2>0.95) within the tested ranges of 0.045-0.20 µg mL^{-1}, 0.025-0.32 µg mL^{-1}, respectively, for KYNA and TRP. The limits of the detection were found to be 0.1480 ng mL^{-1} for KYNA and 0.0332 ng mL^{-1} for TRP. The deproteinization of the liver homogenate samples was accomplished by 80% methanol addition combined with boiling precipitation. The average recovery values were between 94.84% and 99.54% with RSDs no more than 5.5%. The work revealed that raloxifene decreased the mean value of tryptophan, as compared with the control group, while simultaneously leaving kynurenic acid at the same level. For the first time the research suggests that, in the case of raloxifene therapy, tryptophan is not metabolized via the kynurenine pathway.

INTRODUCTION

Tryptophan (TRP) is an essential amino acid participating in protein synthesis. It may also be considered as the precursor of biologically active metabolites generated along several pathways (the kynurenine, serotonin pathways). These include, among others, neuroactive tryptamine, kynurenic acid, melatonin, immunosuppressive kynurenine, and important enzyme redox co-factors such as nicotinamide adenine dinucleotide (NAD). Recently, the potential effects of various drugs on the hepatic metabolism of TRP have received more attention. Several studies have focused on the changes in the enzyme activity in the kynurenine pathway induced by some chemicals [1,2].

It should be emphasized that only two papers are dealing with the quantification of TRP and KYNA in homogenates of liver tissue [3,4]. Badawy *et al*. [3] homogenized rat liver with ice-cold water and 12% (w/v) $HClO_4$. The supernatants were further analyzed by an HPLC using a reversed-phase Synergi fusion-RP 80 Å column and a mobile phase consisting of 73:27 (v/v) sodium dihydrogen phosphate: methanol. In this case, a run took almost 13 minutes and detection limits were 4.92 ng mL^{-1} and 1.01 ng mL^{-1} for KYNA and TRP, respectively. Recoveries of TRP and KYNA were on the average level of 96.8±5.93% and 105.7±7.3%, respectively. Chen *et al*. [4] applied polarity switching UHPLC-SRM-MS quantification to tryptophan and its main catabolites in different mouse tissues, among others, in liver homogenate samples. In this case, the liver tissues were homogenized in ice-cold methanol. After centrifugation, the supernatants were analyzed by UHPLC on a HSS T3 column by the use of the gradient elution mode. This system enabled the researchers to obtain satisfactory recoveries for studied analytes and detection limits on the level of 1.56 ng mL^{-1} and 0.78 ng mL^{-1} for TRP and KYNA, respectively.

Ours is the first study on the relationship between the rat liver levels of TRP and its major metabolite – KYNA, formed by the kynurenic pathway, following raloxifen

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administration at a dose of 2 mg/kg/m.c.s.c. We developed a new HPLC-FL procedure to quantify the aforementioned metabolites.

MATERIALS AND METHODS

Chemicals

HPLC organic solvents of reagent grade were obtained from Merck (Darmstadt, Germany). Water was deionized and purified by ULTRAPURE Millipore Direct-Q 3UV-R (Merck). Zinc acetate, sodium acetate, and sodium hydroxide were purchased from P.O.Ch (Gliwice, Poland). L-tryptophan and L-kynurenic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Chromatographic Conditions

All of the processed samples were analyzed using an Elite LaChrom HPLC Merck-Hitachi (Merck, Darmstadt, Germany) consisting of a fluorescence detector (L-2485U) and column thermostat Jetstream 2 Plus (100375, Knauer). HPLC separation was performed on a column (250 mm x 4.6 mm I.D.) packed with 5 µm Zorbax Eclipse XDB-C18 (Agilent Technologies, Santa Clara, CA, USA). The column was thermostated at 20°C±0.1. Retention data were recorded at a mobile phase flow-rate of 1.0 mL min^{-1} with online degassing using a L-7612 solvent degasser.

The elution was carried out in the isocratic mode by a mobile phase consisting of 7% acetonitrile in an appropriate concentration for the acetates. The mobile phase was filtered through a Nylon 66 membrane filter (0.45 µm) Whatman (Maidstone, England) by the use of a filtration apparatus. Typical injection volumes were 20 µL corresponding to the volume of the Rheodyne injector loop. The equipment was operated using EZChrom Elite software (Merck). The purity of TRP and KYNA was estimated to be >99% by HPLC-DAD analysis – based on a peak area normalization method.

Animal Experiments

Procedures involving animals were conducted in accordance with European Community and Polish legislation on animal experimentation (Resolution No. 18/2014). The experiments were carried out using eight to ten weeks old female Wistar rats weighing 277.5±67.5 g. The retrospective case series of 16 rats were divided into two groups: group 1 (control); group 2 (treated). Control animals received orally a veterinary paste, whereas group 2 received raloxifene suspended in veterinary paste in the dose of 2 mg/kg bw/day. On the 14^{th} day of the experiment, the animals were decapitated. The liver was immediately excised and frozen in liquid nitrogen and then stored at -80°C until further analysis.

The rat livers were homogenized within 4 min. (70% of power) at the temperature of 0°C, using water (1:3), in a BANDELIN SONOPULUS HD 2070 Ms 72. Sample deproteinization was accomplished by the addition of 1 ml of 80% methanol per 1 ml of a homogenate of the rats' liver tissue. The obtained mixture was placed in an ultrasonic bath for 15 min. at 90°C. Afterwards, the mixture was centrifuged at 4,000 rpm for 30 min, filtered through a millipore filter (0.45 µm), and injected into the HPLC column. Due to the high TRP content, its quantification was accomplished after 100 times dilution of the sample by water.

Method Validation

The analytical method validation was carried out according to ICH Q2 (R1) method-validation guidelines. The following validation parameters were established: selectivity, precision, accuracy, linearity, recovery, LOD and LOQ.

Linearity, Recovery, Precision

The calibration curves for each analyte were obtained by injecting various amounts of the studied metabolites into the HPLC while using referential calibration curves for their quantification. TRP was accurately weighed and dissolved in water to produce a concentration of 25-320 ng mL^{-1}. KYNA solutions were prepared in 1.5 mM NaOH to produce a concentration of 45-200 ng mL^{-1}. Peak area of the analytes was measured for creating the calibration curve by employing least squares regression analysis and uniform weighing. Limit of quantification (LOQ) and limit of detection (LOD) values were based on 10:1 and 3:1 signal to noise ratio, respectively.

The recovery study was accomplished by adding the reference standards to the liver homogenate of rats. Spiked samples were prepared as follows: 50 µL of the standard solution of different concentrations was added to 1000 µL of the liver tissue homogenate. The quality control (QC) samples: low-, medium- and high were then prepared. All calibration standards were triplicated. Spiked samples were formulated and analyzed according to the procedure described above. The determined peak areas were substituted into the calibration curve equation and the percentage extraction yield was calculated by the ratio of [(after spiking – before spiking)/added] × 100.

The analysis was repeated six times – providing intraday precision values and six times in another day – giving intermediate precision values. Inter-assay and intra-assay variability were determined by computing the percentage relative standard deviation (%RSD).

Data Analysis

Multiple regression analysis was performed using Microsoft Excel 2010. Experimental values were expressed as means ± SD. Differences between measurements were considered as significant when $p < 0.05$ in Student's t-test. The Shapiro-Wilk's W test and the Mann-Whitney U test for detecting a normal distribution of data were performed by the use of Statistica v.12.

RESULTS AND DISCUSSION

Choice of detection and chromatographic conditions

In our study, a chromatographic system consisting of 7% acetonitrile in aqueous solution with 20 mM sodium acetate and 3 mM zinc acetate was chosen as being the most effective and selective. Because of native fluorescence, it is possible to generate a very sensitive quantification of kynurenines by means of fluorescence detection. Here, chelation

with zinc ions enhances the intense fluorescence of KYNA that occurs physiologically at a very low concentration. Accordingly, excitation and emission maxima were observed at 219 and 360 nm for TRP and KYNA. The fluorescence detection conditions ensuring the strongest fluorescence for both analytes were applied in subsequent experiments.

It should be emphasized that the addition of 250 mmol L^{-1} of zinc acetate to the eluent system has been previously proposed by Hervé [5]. Considering the fact, that such a high concentration of zinc acetate may hamper HPLC analysis, in this study, we applied 3 mM of this additive in the eluent system to avoid practical problems.

Elution was monitored by fluorescence (FL) detector for the identification and quantification of the analytes. Figure 1 presents a typical chromatogram obtained for the patterns.

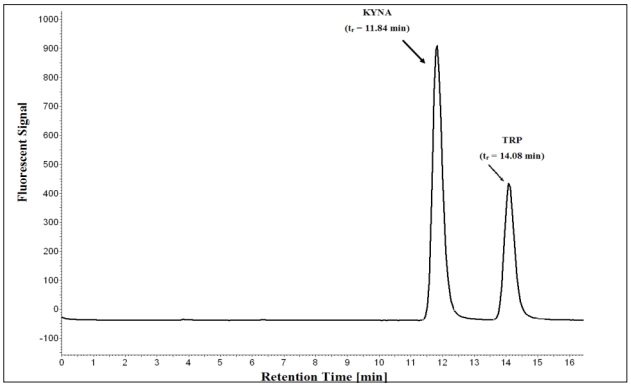

Figure 1. HPLC chromatograms obtained for standard mixture containing TRP, and KYNA at the concentration of 0.5 and 0.15 ng/ml, respectively. The analysis was performed using a Zorbax Eclipse XDB-C18 column and 7% acetonitrile containing 20 mM sodium acetate and 3 mM zinc acetate as the mobile phase. Peak identity: (TRP) tryptophan, (KYNA) kynurenic acid. The injection volume was 20 µl. Fluorescence detection was performed at excitation 219 nm and emission 360 nm, respectively

The approximate retention times were 11.84 min for KYNA and 14.08 min for TRP. The eluent system composed of 7% ACN in water containing zinc acetate and sodium acetate allowed a complete resolution (α = 1.25) of the analyzed compounds in an analysis time lower than 15 minutes with sufficient efficiency expressed in the theoretical plates number (USP) (N = 60 900 m^{-1} for TRP and 57 320 m^{-1} for KYNA, respectively) and peak symmetry (A_s = 1.17 for both).

The limits of the detection under fluorescence detection were found to be 0.1480 and 0.0332 ng mL^{-1} for KYNA and TRP, respectively – taking into account 3:1 signal to noise ratio. Comparing the obtained results with that already published, the detection limit for TRP (0.033 µg L^{-1}) is lower than the 24.91 µg L^{-1} obtained by Ma *et al*. via the use of HPLC with ultraviolet detection and Hypersil C-18 column [6]. A different study dealt with the quantification of TRP and KYNA on Restek C18 Aqueous column [7]. Here, although the retention time is about two times shorter, the resolution of KYNA and TRP appears to be worse. The above paper declares about 0.3 minutes of difference between KYNA and TRP retention time, whereas our method ensures 2.24 min. of this difference.

Recovery Study

The calibration curves of peak area *vs*. concentration were obtained by fitting the data using linear regression analysis. Acceptable linearity (R^2 >0.95) within the test range was found for the examined compounds. Collected results of linearity, LOD and LOQ are presented in Table 1.

Table 1. The linear regression parameters obtained for the calibration curves of KYNA and TRP

Analyte	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	Linear range of the calibration curve (µg mL^{-1})	Regression equation parameters (y = ax + b)		
				a ± s_a	b ± SD	r
KYNA	0.1480	0.4941	0.045-0.200	7572935± 351021	95971± 47964	0.9894
TRP	0.0332	0.1107	0.025-0.320	51936859± 1874850	1569872± 297450	0.9948

Abbreviations: LOD - the detection limit, LOQ - the quantification limit

The accuracy of the method was established by the standard addition method. The blank liver tissue homogenate samples (Fig. 2) were spiked with the analyte standard solution at three concentration levels representing low, middle and high analyte contents. The mean recovery values determined from three different concentrations were above 90%, and the obtained precision results were satisfactory. RSD% for repeatability was always lower than 5%, and for intermediate precision – lower than 8.5%. The results are summarized in Table 2.

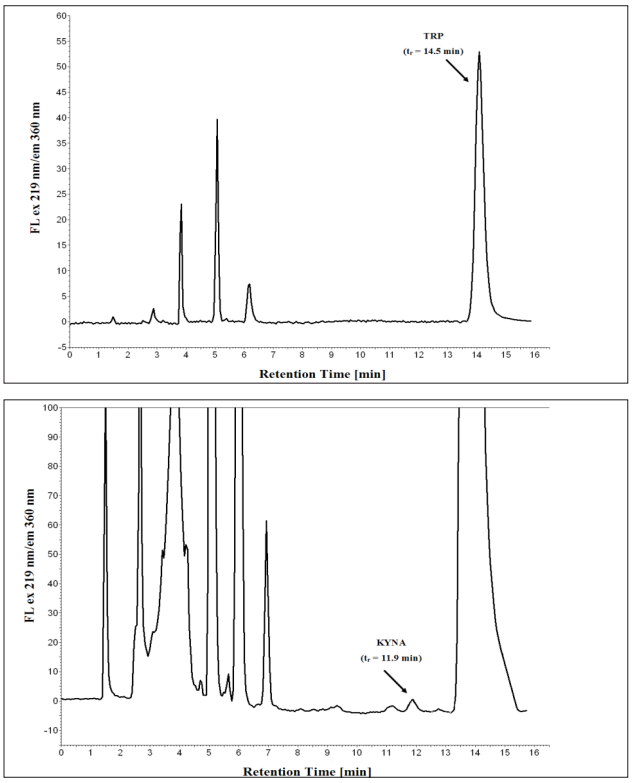

Figure 2. HPLC chromatograms obtained for: A – liver sample, B – liver sample diluted 100 times. The analysis was performed using the Zorbax Eclipse XDB-C18 column and 7% acetonitrile containing 20 mM sodium acetate and 3 mM zinc acetate as the mobile phase. Peak identity: (TRP) tryptophan, (KYNA) kynurenic acid. The injection volume was 20 µl. Fluorescence detection was performed at excitation 219 nm and emission 360 nm, respectively

Table 2. The recoveries of TRP and KYNA from the blank homogenate liver samples with the RSD% values of intra- and inter-day precision

Compound	Added (ng/mL)	Intra-day			Inter-day		
		Found (ng/mL) (mean ± SD)	RSD (%)	Recovery (%)	Found (ng/mL) (mean ± SD)	RSD (%)	Recovery (%)
Tryptophan	0	50.98± 1.42	2.79	-	33.62± 0.52	1.55	-
	25	75.74± 0.49	1.98	99.03	55.78± 1.87	8.43	88.65
	100	146.21± 4.86	5.01	95.23	124.64± 2.40	2.64	91.02
	320	369.51± 8.76	2.75	99.54	341.11± 10.22	3.32	96.09
Kynurenic acid	0	105.06± 2.53	5.18	-	66.09± 3.54	5.35	
	125	227.80± 3.62	2.95	98.19	187.77± 4.02	3.30	97.34
	175	276.40± 2.78	1.62	97.91	237.00± 3.51	2.05	97.67
	225	318.45± 7.17	3.36	94.84	282.64± 6.70	3.09	96.24

Stability

The stock solutions were assayed in triplicate at three different temperatures: 50, 70, 90°C, and after different heating times: 10 min., 20 and 30 min. The results (Tab. 3) demonstrate that both analytes were stable up to 30 min. of heating at 90°C. The total recovery of each substance after stress testing was not lower than 95%.

The stability of the samples was also investigated by way of different solvents being used for sample dilution, such as water, methanol and acetonitrile. In each case, the final concentration level obtained after sample dilution achieved concentration equals 0.12 μg/mL. The studied analytes remained stable in water, while the stability was reduced after the sample was diluted by acetonitrile (Tab. 4).

Table 3. Stability of studied analytes TRP and KYNA stock solutions at concentration of 0.12 μg/mL while heated at different temperatures

Time of heating [min]	50°C		70°C		90°C	
	Measured concentration [μg/mL]	Recovery %	Measured concentration [μg/mL]	Recovery %	Measured concentration [μg/mL]	Recovery %
TRP						
10 min	0.1276	106.3	0.1168	97.3	0.1236	103.0
20 min	0.1267	105.6	0.1178	98.2	0.1298	108.1
30 min	0.1197	99.8	0.1304	108.7	0.1293	107.8
KYNA						
10 min	0.1216	101.3	0.1169	97.4	0.1192	99.3
20 min	0.1191	99.3	0.1198	99.8	0.1167	97.3
30 min	0.1163	96.9	0.1176	98.0	0.1188	99.0

Table 4. Stability of investigated analytes after dilution by different solvents

Ratio of sample to diluent	Measured concentration [μg/mL]	Recovery %	Measured concentration [μg/mL]	Recovery %	Measured concentration [μg/mL]	Recovery %
TRP						
	H_2O		MeOH		ACN	
1:100	0.1231	102.6	0.0533	44.4	0.004	3.0
KYNA						
	H_2O		MeOH		ACN	
1:100	0.1223	101.9	0.0904	75.3	0.0115	9.6

Analysis of liver samples

As the liver is a complex matrix containing proteins and enzymes, there are several steps in sample preparation. The attempt to apply freezing precipitation at -20°C appeared to have failed. After defrosting, and centrifugation of the samples at 4,000 rpm for 60 min., the recovery was very low – amounting around 10%. This unsatisfactory value could be explained by enzyme activation, as the liver is a rich source of proteases.

We decided to apply a combined method to remove proteins and to deactivate enzymes. The releasing of tryptophan from protein binding via denaturation with 80% methanol in combination with boiling precipitation ensured a sufficient recovery level (>90%).

The 16 liver tissue homogenates (8 control and 8 treated with raloxifene) were analyzed for kynurenic acid and tryptophan concentrations. Here, the prepared rat liver samples were measured directly for kynurenic acid content, while quantification of TRP required a one hundred times dilution step. The results are collected in Table 5.

Table 5. Tryptophan and kynurenic acid levels in control group and group treated with a selective estrogen receptor modulator-raloxifen

The kind of series		Tissue weights [g] ± S.D. (0.0001)	Kynurenic acid		Tryptophan	
			Measured conc. (μg mL^{-1})	pmol per 1 g of tissue weights	Measured conc. (μg mL^{-1})	pmol per 1 g of tissue weights
Control group (n = 8)	1	0.9200	0.0460	674.5	0.1382	187648.1
	2	0.7850	0.1023	1344.1	0.0868	105613.3
	3	0.8281	0.1620	1810.2	0.0587	164305.5
	4	0.7821	0.1791	1816.6	0.1150	108040.3
	5	0.7472	0.0661	1123.3	0.1797	282856.5
	6	0.6960	0.0508	849.5	0.0682	105511.3
	7	0.8380	0.0498	849.5	0.0837	132182.3
	8	0.9911	0.1653	2781.9	0.1451	226116.8
The mean		-	-	1406.2	-	164034.3
Raloxifen treated group (n = 8)	1	0.5920	0.1135	2180.1	0.0299	53204.91
	2	0.7924	0.1199	2236.6	0.0583	100805.7
	3	1.0731	0.1205	2256.6	0.0675	117152.4
	4	0.8953	0.0778	1379.8	0.0733	120412.5
	5	0.8752	0.0821	1314.5	0.0437	64908.6
	6	0.7421	0.0731	1253.7	0.0474	75283.4
	7	0.8480	0.0622	1126.1	0.0674	112995.8
	8	0.6932	0.0827	1515.0	0.0664	112675.3
The mean		-	-	1657.8	-	94679.8

The data of the tryptophan liver content measured for the treated and control groups have a distribution that does not differ significantly from the normal. However, the concentration of tryptophan in the liver tissue was about 2-fold lower in the raloxifene group in comparison to the control.

The Shapiro-Wilk test in contrast shows a distribution close to the normal distribution either for the control group (p = 0.18) or treated (p = 0.11). The p-value = 0.01 for the t- test (assuming unequal variances: p-value = 0.029 for the F test), allows us to conclude that there are statistically significant differences in the arithmetic mean between the control group and the treated group in terms of the tryptophan content. In the case of kynurenic acid, the distribution of data did not follow Gaussian distribution for the treated group (p = 0.044), and the Shapiro-Wilk test shows a distribution close to the normal distribution (p = 0.26) only for the control group. In addition, the Mann-Whitney U test value of p = 0.27, allows concluding that there is no statistically significant difference in median values between the untreated and treated groups in terms of the kynurenic acid concentration.

It has been confirmed by other researchers that the ratios between tryptophan and its metabolites may be useful to estimate the activity of enzymes or to choose a possible metabolic pathway [10]. It should be emphasized that the data of the TRP/KYNA ratios exhibited normal distribution in Shapiro-Wilk's test for the treated (p = 0.92) and control group (p = 0.087). The value of p = 0.022 for the t-test (assuming unequal variances: p-value = 0.047 for the F test), allows to conclude that there are statistically significant differences in the arithmetic means measured for the TRP/KYNA ratio for the control group compared to the one treated (Fig. 3).

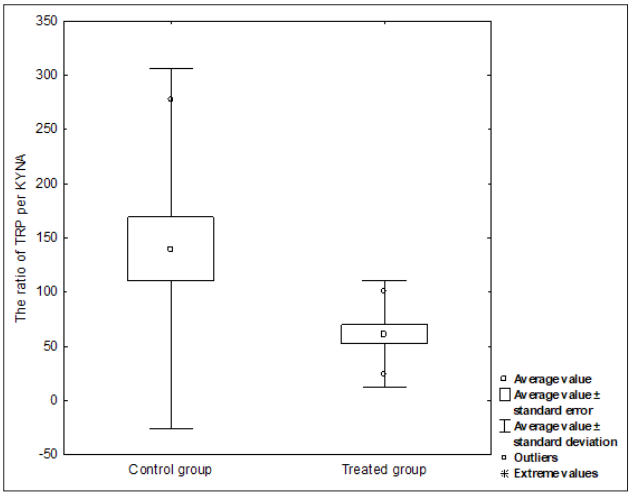

Figure 3. The ratios of TRP to KYNA in the liver tissues for the control group and group treated with raloxifene

Basing on the above observations, it should be concluded that treatment with raloxifene influences the activity of enzymes taking part in TRP degradation. Furthermore, no statistically significant differences between KYNA levels in the homogenate of the liver of rats treated with raloxifene, in comparison to control series, may suggest that part of TRP can be catabolized through minor pathways – serotonergic pathway or bacteria degradation.

DISCUSSION

Tryptophan transformation in the so-called kynurenine pathway is of growing interest as a potential therapeutic target. One of the most interesting issues is the effect of sex hormones on tryptophan metabolism. Recent studies have shown that estradiol, one of the female sex hormones, by binding to the estrogen receptor (ER) located in the cell membrane of neurons, can affect the expression of enzymes involved in the pathway from tryptophan to serotonin (tryptophan hydroxylase-2, monoamine oxidase A and B), and expression of the 1A serotonin receptor [8]. Regulation of this pathway may be a potential therapeutic target for the treatment of depression in postmenopausal women.

Experiments carried out at the beginning of the 2000s showed that administration of raloxifene, a selective estrogen receptor modulator (SERM), to patients with osteoporosis, may affect tryptophan metabolism in the so-called kynurenine pathway [9].

The results of the current experiment revealed that raloxifen administered to female Wistar rats of childbearing age at a dose of 2 mg/kg/b p.o. reduces the concentration of l-tryptophan compared to the control group, while the concentration of KYNA in the control and test group remained at the same level.

Raloxifen has a very high affinity for the estrogen receptor alpha (ERα) and less for the estrogen receptor β (ERβ). Both receptors are present in the hepatocyte membrane [10]. To receptors located in the membrane of liver cells, raloxifen acts as an agonist [11]. The reduced tryptophan concentration in the study group compared to the control group, without changing the KYNA concentration, allows the hypothesis that raloxifen affects the expression or activity of tryptophan 2,3-dioxygenase (TDO) enzyme that catalyzes the first stage of tryptophan transformation in the kynurenine pathway [12].

The obtained results suggest that as a result of the binding of raloxifene to the ERα receptor, the tryptophan transformation process increases. It could be expected that the concentration of other metabolites of the kynurenine pathway (3-hydrocynurenine, xanthurine acid, anthranilic acid) have also been increased. However, to confirm this last conclusion, additional quantitative testing of tryptophan metabolites should be performed.

CONCLUSIONS

A sensitive HPLC method with FL detection has been developed for the quantification of TRP and KYNA in the liver tissue of rats treated with raloxifene. Due to appropriate sample preparation, the proposed strategy provides suitable sample purification and gives extraction yields higher than 90% with intra-day precision ≤ 5.5%. The proposed sample pretreatment ensured satisfactory validation parameters (recovery, precision, accuracy).

The obtained levels of TRP in the control group positions itself in an utterly different range in contrast to the treated group. Performed quantifications testify to the fact that the amount of TRP decreases, whereas the concentration of KYNA remains at an almost constant level in the homogenate of the liver of rats treated with raloxifene, in comparison to the control series. This suggests that TRP catabolism is shifted from the kynurenine pathway toward minor pathways – serotonergic pathway or bacteria degradation.

The changes of TRP and KYNA levels are also reflected in the relative ratios of these metabolites in both the treated and control group. The results are of great significance for the understanding of TRP degradation induced by second-generation selective estrogen receptor modulators.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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