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Article

Determination of Tryptophan in Raw Materials, Rat Brain and Human Plasma by RP-HPLC Technique

Najma Sultana[1], M. Saeed Arayne[1], Moona Mehboob Khan[2,3], Darakhshan M. Saleem[4] and Agha Zeeshan Mirza[5*]

[1]United Biotechnologies, Gulistan-e-Jauhar, 75290 Karachi, Pakistan, [2]Research Institute of Pharmaceutical Sciences, Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Karachi, Karachi, Pakistan, [3]Jinnah College of Pharmacy, 22.23, Shaheed-e-millat Road, Karachi, 74000 Pakistan, [4]Department of Biochemistry, University of Karachi, Karachi, 75270 Pakistan, and [5]Department of Chemistry, University of Karachi, Karachi, 75270, Pakistan

*Author to whom correspondence should be addressed. Email: zee_amm@hotmail.com

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This paper describes tryptophan (TRP) estimation in raw human plasma and rat brain by reversed-phase high-performance liquid chromatography (RP-HPLC). Estimation was carried out on a Purospher STAR C18 column using water–acetonitrile (90:10 *v*/*v*, at pH 2.7) mixture at a rate of 1.5 mL/min as mobile phase. Eluents were monitored at 273 nm by an ultraviolet detector. The method was linear ($R^2 > 0.999$), precise (intra-day and inter-day precision <2%) in the range of 0.25–20 μg/mL. The detection and quantification limits were 0.0144 μg/mL and 0.0437 μg/mL, respectively. In human plasma, Day 1 and Day 2 precision were 0.054–2.29% and 1.66–3.7%; whereas precisions in rat brain were 1.23–2.3% and 0.677–4.2%, respectively. The method was applied to study TRP level in human smokers and in arthritic rat brain. An efficient RP-HPLC method was developed for TRP determination that worked for clinical and research purposes.

Introduction

Tryptophan (TRP) (Figure 1) is an essential amino acid (1). It performs many fundamental roles in the human body, primarily synthesis of 5-hydroxytryptamine (5-HT) (Figure 2). As the precursor of 5-HT, it is involved in mood, consciousness and sleep (1–2). It is metabolized to various other biologically active substances *in vivo* as melatonin, nicotinic acid and nicotinamide adenine dinucleotide (NAD) (3). TRP catabolism increases due to hyperactivation of indoleamine-2,3-dioxygenase (IDO) in many pathological conditions like viral infections, autoimmune disorders (such as rheumatoid arthritis) and malignant diseases. IDO is an enzyme that is produced in various cells by interferon-gamma (γ) during inflammation. The net consequence of this action is the decrease in TRP concentration in the body. This depletion in turn affects 5-HT synthesis in brain and sends the patient into depression (1–5). Therefore, TRP depletion is widely used to identify the susceptibility of patients toward depression (6).

The present method to quantitate TRP in biological samples will help to understand its role in many psychological conditions.

Previously, numerous methods have been reported to estimate TRP levels in biological fluids. Among them, in most cases, the TRP level was determined in human plasma and human serum (3, 4, 7–12) and in human cultured cell in one method (12). Kawai *et al.* (3) established a column-switching method for kynurenine and TRP measurement in plasma. In this method, two types of phosphate buffers were used as mobile phases: one without acetonitrile and one with 5% of acetonitrile. The total elution time was 35 min. Morita *et al.* (7) and Kraft *et al.* (8) determined serum and plasma TRP levels along with their metabolites, whereas Widner *et al.* (9) and Vignau *et al.* (10) measured serum TRP levels by high-performance liquid chromatography (HPLC)–fluorescence detection. Herve *et al.* (11) proposed a method for TRP, kynurenic acid (Kyn) and 3-hydroxyanthranilic acid (Kyn metabolite) in serum by HPLC–ultraviolet (UV) or fluorescence. Vaarmann *et al.* (12) determined TRP (under +900mV) and its metabolites in human plasma and cultured human cells by HPLC with electrochemical detection. They used 50 mM sodium phosphate acetate buffer, 0.42 mM octanesulphonic acid and 10% methanol as mobile phase. Maneglier *et al.* (4) determined TRP and Kyn in human plasma and a supernatant of human cultured cell by HPLC-coulometric detection using phosphate buffer and acetonitrile as mobile phase with a total run time of 14 min.

In almost all methods, either buffers were used as mobile phase that were corrosive to the column, or the total elution time was very high. Measurements of TRP and its metabolites in samples by HPLC were conducted either by changing the UV wavelength and voltage according to the substances measured, or by the use of two types of detectors at a time.

The present paper describes a rapid, precise and economical method (90% water in mobile phase) for the determination and quantification of tryptophan by HPLC–UV. Although the pH of the mobile phase was adjusted through orthophosphoric acid, its percentage was below 0.1%, whereas in most of the previous HPLC methods, buffers were used as mobile phase that are highly corrosive to columns. In most methods, total elution time was usually high, in contrast to our proposed method, in which total estimation was completed in less than 7 min. This setup made our method more economical in terms of time and money, but with satisfactory precision and accuracy. This method also provided a procedure to determine TRP not only in human plasma, but also in rat brain samples, which work as an animal model in many pathological disorders related to human beings. 5-Hydroxytryptophan, which is always present in biological samples, does not interfere with the method and has been used as an internal standard. Conversely, 5-hydroxytryptophan can be quantitated by this method using TRP as an internal standard. Finally, the application of this

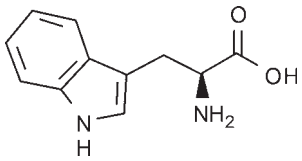

Figure 1. TRP.

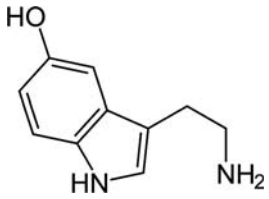

Figure 2. 5-Hydroxytryptamine.

method to study the TRP role in human plasma (smokers and non-smokers) and in the brain of rats suffering from arthritis highlighted its utilization in many clinical investigations.

Materials and Methods

Chemicals and reagents

Reference standard of TRP and 5-hydroxytryptophan were purchased from Sigma chemicals. All reagents used were of analytical grade and obtained from Merck Germany. Acetonitrile of HPLC grade (Tedia, Fairfield, OH). Freshly prepared deionized filtered water was used to prepare the mobile phase.

Chromatographic conditions

The liquid chromatographic system consisted of a Shimadzu model LC-10AT VP pump with a SPD-10AT VP, variable wavelength UV-Visible detector. The chromatographic system was integrated via a Shimadzu model CBM-102 Communication Bus Module to a Pentium IV PC loaded with Class-GC Software. Analysis was conducted on a Purospher STAR C18 (250×4.6) analytical reversed-phase column with mobile phase of water–acetonitrile (90:10 v/v) at pH 2.7 maintained by orthophosphoric acid. The samples were introduced through a rheodyne injector valve with a 20-μL sample loop. Assays were performed at ambient temperature at a flow rate of 1.5 mL/min. The eluents were monitored at 273 nm.

Standard solutions

Stock solution (100 μg/mL) of aqueous TRP was prepared and diluted into 20.0, 10.0, 7.0, 5.0, 3.0, 1.0, 0.5 and 0.25 μg/mL to prepare calibration curves. All solutions were stored at -20°C, at which TRP is stable.

Calibrating sample solution

Perchloric acid (0.4M) (13), which was used as an extraction medium, was added to 100 μg/ml TRP solution and diluted to the previously mentioned concentration, which worked as calibrating sample solution to validate the method in the presence of extraction medium.

Biological samples

Studies on humans

Thirty microliters of human plasma from a healthy male volunteer was used for this purpose. This amount was mixed with 60 μL of 0.4M perchlorate extraction medium and centrifuged at 12,000 rpm for five minutes. Then, the supernatant containing TRP was separated and stored at -20°C.

To study TRP levels in smokers and non-smokers, blood plasma was obtained from 20 healthy male volunteers. This portion of the study was conducted under the ethical committee research guidelines by the National Bioethics Committee, Pakistan. Among 20 volunteers, 10 were non-smokers (that worked as control) while 10 were smokers (test group). Samples were prepared in the same way as described previously.

Studies on animals

Female Sprague–Dawley rats, weighing 215–230 g (8–10 weeks), kept at $21 \pm 2^{\circ}$C on a 12-h light/dark cycle with free access to standard laboratory rat food pellets and water for one week before use, were used in this study under the ethical guidelines of International Association for the Study of Pain in Conscious Animals. Their brain samples were collected and homogenized with the help of an electric homogenizer in the presence of 5 mL 0.4M perchlorate extraction medium per brain. Homogenates were then allowed to stand for 10–15 min for the precipitation of proteins. The supernatant was decanted in a separate Eppendorf tube and centrifuged at 12,000 rpm for 5 min. Finally, the supernatant containing TRP was separated and stored at -20°C until analysis.

To study brain TRP levels in nonarthritic (which work as control) and adjuvant induced arthritic (AIA) rats, the adopted procedure was the same as described previously (14). In brief, arthritis was induced in an adjuvant treated group by intradermal injection of 0.1 mL suspension of 1 mg of fresh Lyophilized Mycobacterium tuberculosis H37Ra (MT H37Ra; DIFCO Laboratories, Detroit, MI) in liquid paraffin oil into the tail base, using a sterile hypodermic needle. Paw edema (in both right and left) in both groups was used as an indicator to evaluate arthritic severity. When arthritis was fully developed at Day 22 in AIA group, rats of both groups were chopped, their brain samples were collected and TRP in these samples were extracted in the same way as described previously.

In all solutions of standard, calibrating samples and biological samples, 5-hydroxytryptophan was added as an internal standard.

Validation study

ICH guidelines (15) were followed for method validation. Parameters such as system suitability, selectivity, specificity, linearity, accuracy (% recovery), precision (robustness, ruggedness), sensitivity and limits of detection and quantification were evaluated in calibrating the solution. To evaluate precision and accuracy in the human plasma and rat brain samples,

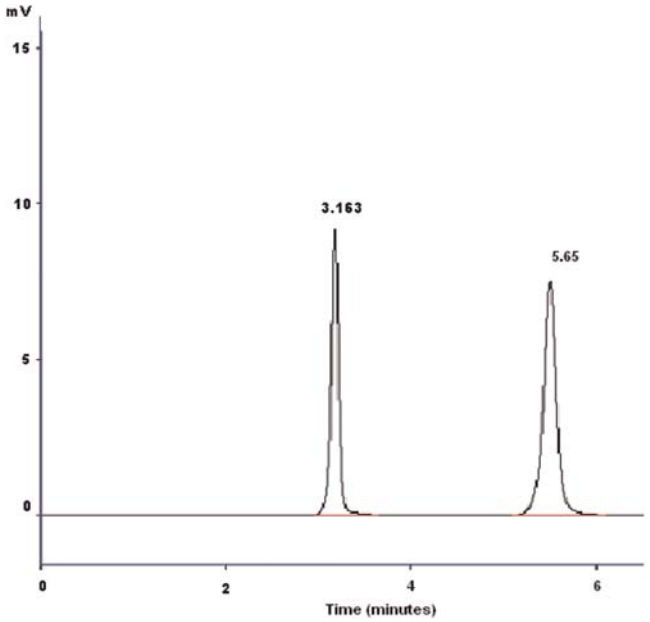

Figure 3. Separation of 5-hydroxytryptophan (3.163 min) and TRP (5.65 min) in standard solution.

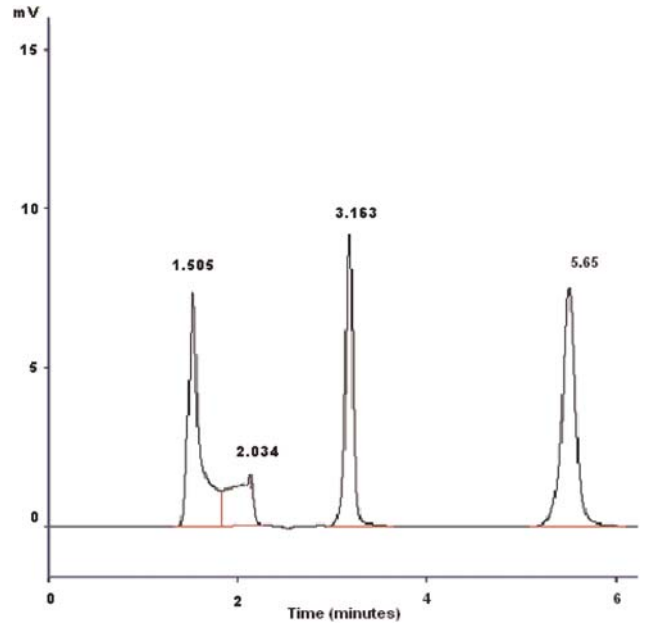

Figure 4. Separation of extraction medium peak (1.5 min), 5-hydroxytryptophan (3.163 min) and TRP (5.65 min).

initially these samples were injected directly into the system to observe their responses against naturally present TRP, then spiked with the known concentration of TRP.

Statistical analysis

Data was expressed as mean ± standard deviation (SD). To evaluate inter-group mean differences, the student's *t*-test was

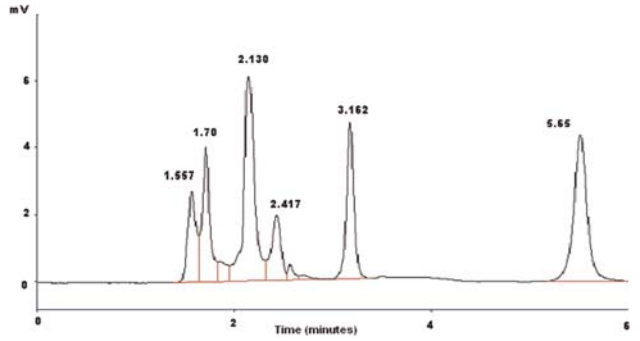

Figure 5. Separation of 5-Hydroxytryptophan (3.162 min) and TRP (5.66 min) in human plasma.

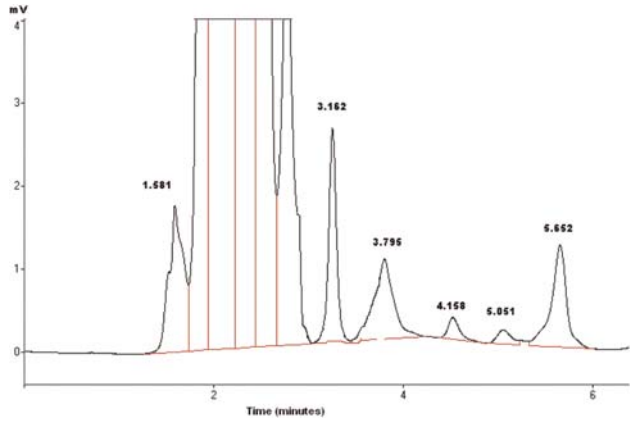

Figure 6. Separation of 5-Hydroxytryptophan (3.162 min) and TRP (5.652 min) in rat brain.

performed using statistical package for social sciences software (SPSS, Inc.) taking significance level $p < 0.05$.

Results and Discussion

Optimization of the chromatographic conditions

RP-HPLC is a high-ranking tool for drug analysis; its advantages of short retention time, method reliability, sensitivity and drug specificity substantiate the use of HPLC for various groups of drugs. Therefore, the primary objective of this study was to develop a simple, hasty and effective liquid chromatographic method to determine TRP in raw human plasma and rat brain samples. Initially, a C18 Discovery column (125 cm × 4.6 mm, 5-μm particle) was used for determination of TRP. Due to poor resolution (especially in the rat brain sample) and high retention time (more than 20 min), this column was not selected for this work. A Hiber, RT 250-4.6 Purospher Star RP-18 end-capped (5 μm) column produced good symmetrical peaks with high resolution and short retention time.

In solvent selection, an isocratic mood was applied for elution instead of gradient to avoid re-equilibration (16). Initially, methanol was used as an important component of the mobile phase, but it failed to estimate TRP in biological samples, so instead of methanol, acetonitrile was used, which had the capability to estimate and quantify TRP levels even in human plasma and rat brain samples. Different ratios of water and acetonitrile (10:90, 80:20,

Table I
System Suitability and Robustness Parameters*

Analytes	Retention time (T_R) (min)	Capacity factors (K)	Theoretical plates (N)	Tailing factor (T)	Resolution (R)	Separation factor (α)
pH: 2.7 ± 0.05						
5-Hydroxytryptophan	3.16 ± 0.21	0.29 ± 0.05	7,788 ± 54	0.97 ± 0.015	0.97 ± 0.08	1.69 ± 0.18
Tryptophan	5.65 ± 0.17	0.75 ± 0.12	7,783 ± 32	0.94 ± 0.07	0.974 ± 0.067	1.73 ± 0.27
Flow rate: 1.5 ± 0.2 mL/min						
5-Hydroxytryptophan	3.17 ± 0.11	0.291 ± 0.06	7,792 ± 79	0.97 ± 0.09	0.97 ± 0.11	1.68 ± 0.13
Tryptophan	5.64 ± 0.13	0.748 ± 0.081	7,785 ± 38	0.94 ± 0.15	0.974 ± 0.15	1.73 ± 0.24
Acetonitrile percentage: (10 ± 2)%						
5-Hydroxytryptophan	3.16 ± 0.07	0.29 ± 0.051	7,787 ± 29	0.97 ± 0.09	0.97 ± 0.12	1.69 ± 0.21
Tryptophan	5.5 ± 0.082	0.75 ± 0.11	7,779 ± 56	0.94 ± 0.14	0.974 ± 0.08	1.73 ± 0.13

*All data presented as mean ± SD ($n = 6$).

Table II
Precision and recovery for TRP determination along with extraction medium

TRP concentration added (μg/mL)	0.25	0.5	1	3	5	7	10	20
Day 1								
Concentration found	0.259	0.493	1.02	2.96	5.01	6.94	9.77	20.2
% Recovery	98.8	98.6	102.2	98.83	100.1	99.11	97.71	100.1
% RSD	0.834	0.901	0.308	0.018	0.056	0.037	0.427	0.384
Day 2								
Concentration found	0.257	0.514	1.01	2.89	4.94	6.88	9.69	19.78
% Recovery	98.2	102.9	100.9	96.56	98.75	97.14	96.87	97.84
% RSD	0.948	1.31	0.139	0.095	0.044	0.145	0.145	0.271
Day 3								
Concentration found	0.249	0.491	0.96	2.91	4.86	7.03	9.83	19.88
% Recovery	98.8	98.6	96.42	97.13	97.43	100.5	98.35	99.4
% RSD	0.958	0.85	1.06	0.58	0.19	0.276	1.12	0.243

90:10) solutions were tried at many pHs (3.4, 2.9, 2.7) by varying flow rates (0.8, 1.5, 2.0 mL/min), but the best results were obtained with a mobile phase of water–acetonitrile (90:10 *v*/*v*) adjusted at pH 2.7 flowing at 1.5 mL/min (Figures 3–6).

Peak identification

Under the optimized conditions, the peaks of TRP and 5-hydroxytryptophan were identified by comparing the standard chromatograph of TRP and 5-hydroxytryptophan with the chromatograph obtained from calibrating solutions and biological samples (Figures 3–6). Under the previously described chromatographic conditions, 5-hydroxytryptophan and TRP were eluted with retention times of 3.162 and 5.65 min, respectively.

System suitability

Six replicates of TRP (20 μg/mL) standard solution were injected on each day of method validation and appraisal was made by analyzing repeatability, relative retention (5.5 min), column efficiency (number of theoretical plates: 7783), capacity factor (0.75) and separating factor (1.73) to check system suitability. Data were summarized in Table I.

Linearity

Linearity and regression analysis were performed using Microsoft Excel 2003 software. The method showed good linearity in standard and calibrating sample solutions at seven concentrations over the range of 0.25–20 μg/mL for three consecutive days which gave a correlation coefficient (r^2) of 0.9998. The mean linear regression equation of the standard curve was $y = 5819.6.0x - 850.7$ for TRP in standard solution, and $y = 4329.7.0x - 430.9$ in calibrating solution. The lowest amount of analyte that the system can detect (limit of detection, or LOD) and the lowest amount of analyte that a system can quantify (limit of quantification, or LOQ) were determined on the basis of SDs of area under the curve and slope of the curve at low concentration levels.

LOD and LOQ were calculated using Equations 1 and 2, respectively:

$$\mathrm{LOD} = 3.3\sigma/\mathrm{S} \quad (1)$$

$$\mathrm{LOQ} = 3.3\sigma/\mathrm{S} \quad (2)$$

where σ is the SD of the response, and S is the slope of the calibration curve.

The LOD of TRP is standard and the calibrating solution was 0.014 and 0.012 μg/mL, while the LOQ was 0.043 and 0.0368 μg/mL, respectively, indicating that this method has the potential to estimate TRP, even in nanograms.

Precision and accuracy

Both precision and accuracy were determined by analyzing independently prepared solutions of TRP at different concentration levels. For intra-day precision (repeatability) and inter-day precision (reproducibility), analysis was performed that was expressed as the coefficient of variation (relative standard

Table III
Precision and accuracy for TRP determination in biological samples along with extraction medium ($n = 6$)

TRP concentration added (μg/mL)	0	1	3	5	7
Day 1*					
Mean ± SD	5.38 ± 0.09	6.435 ± 0.03	8.34 ± 0.22	10.26 ± 0.23	12.3 ± 0.28
% Recovery	—	105.5	98.66	97.7	98.85
% RSD	1.84	0.54	2.7	2.27	2.29
Day 2*					
Mean ± SD	5.54 ± 0.09	6.51 ± 0.12	8.48 ± 0.14	10.94 ± 0.21	12.6 ± 0.5
% Recovery		97.5	98	108	100.5
% RSD	1.78	1.84	1.66	1.87	3.7
Day 1†					
Mean ± SD	0.17 ± 0.002	1.2 ± 0.028	3.1 ± 0.056	5.17 ± 0.07	7.11 ± 0.106
% Recovery		102.75	97.58	99.95	99.17
% RSD	1.23	2.3	1.82	1.36	1.49
Day 2†					
Mean ± SD	0.174 ± 0.003	1.21 ± 0.014	3.2 ± 0.13	5.16 ± 0.19	7.3 ± 0.049
% Recovery		103.6	100.3	99.8	101.8
% RSD	1.6	1.2	4.2	3.7	0.677

*Human plasma.
†Rat brain.

Table IV
RSD During Robustness Analysis

($n = 6$)	TRP (μg/mL)								
Changes	20			10			5		
pH: 2.7 ± 0.05	2.65	2.7	2.75	2.65	2.7	2.75	2.65	2.7	2.75
RSD (%)	0.66	0.384	0.58	0.59	0.427	0.64	0.23	0.056	0.17
Flow rate: 1.5 ± 0.2 mL/min	1.45	1.5	1.55	1.45	1.5	1.55	1.45	1.5	1.55
RSD (%)	0.095	0.384	0.487	0.83	0.427	0.28	0.59	0.056	0.96
Acetonitrile (10 ± 0.2)%	89.8	90	90.2	89.8	90	90.2	89.8	90	90.2
RSD (%)	0.43	0.384	0.11	0.074	0.427	0.88	0.22	0.056	0.73

deviation, or RSD = standard deviation / mean × 100). The RSD is useful for comparing uncertainty between different measurements of varying absolute magnitude. For this purpose, six injections (n = 6) of different concentrations of TRP in the linear range were analyzed on the same day (intra-day precision) and two consecutive days (inter-day precision). The accuracy of the method was determined as percentage recovery of known amounts of TRP in calibrating solution and biological samples. Table II showed satisfactory precision and accuracy results in the calibrating solution for all three days. Table III also shows satisfactory results, because the precision (%RSD) values were in the range of 1.84–2.29% and 1.78–3.7% in human plasma and 1.23–2.3% and 0.67–4.2% in rat brain sample for Day 1 and Day 2, respectively. Accuracy values in human plasma were in the range of 97.7–105.5% and 97.5–108% and 97.58–102.75% and 99.8–103.6% in rat brain for Day 1 and Day 2, respectively. These results indicated the reliability of the method to determine TRP concentration in human plasma and rat brain samples.

Sensitivity and selectivity

The method is selective and sensitive for TRP. The chromatograms (Figures 3–6) showed other peaks due to extraction medium and other biologics present in human plasma and rat brain, but these peaks did not interfere with the peaks of interest; i.e., TRP and 5- hydroxytryptophan, neither in the calibrating solutions nor in the biological samples.

Ruggedness and robustness

The ruggedness of the method was demonstrated by comparing the assay performance of two analysts on separate lots of solutions. Each analyst prepared samples in duplicate and used separate reagents and mobile phase solutions. No marked change was observed in the accuracy and precision of the method during ruggedness determination. Robustness of the method was checked by making small deliberate changes in the mobile phase (±2%), pH (±0.05%) and flow rate (±0.2%). These variations had a minor effect in operating conditions but did not show any notable deviation in results from acceptable limits, as indicated in Table I. In addition to this, to observe the effect of these deliberate changes on precision, the RSDs of TRP in calibrating solution were calculated at three different concentrations under the previously discussed (pH, flow rate and acetonitrile) changes. This gave satisfactory precision results, the data of which are summarized in Table IV.

Solution stability

The stability was evaluated of standard and sample solutions. These standard and sample solutions of TRP were kept at

Table V
Application of the Proposed Method

TRP (μg/mL) levels in human plasma of smokers and non-smokers ($n = 6$)

Smokers	Mean ± SD	p-value
	4.14 ± 1.19	$p = 0.01$
Non-smokers	5.38 ± 0.09	
Brain TRP (μg/mL) levels in non-arthritic and AIA rats ($n = 6$)		
Non-arthritic rats	Mean ± SD	p-value
	0.17 ± 0.0021	$p = 0.0035$
AIA rats	0.1117 ± 0.0025	

−20°C for one week and showed no significant variations in this time span when evaluated against fresh standard solutions.

TRP levels in human plasma of smokers and non-smokers

In this portion of the study, we applied the proposed HPLC method to compare human plasma TRP levels among male smokers and non-smokers. Table V shows the significant ($p < 0.05$) decrease in plasma TRP levels in smokers (4.14 ± 1.19) when compared with non-smokers (5.38 ± 0.09). Therefore, this result showed consistency with the pervious results. Previous studies have showed that smoking produces acute TRP depletion (17–18). In addition to this, smoking also inhibits the conversion of TRP to 5-HT (19–20). Therefore people who are addicted to smoking also suffer from depression. (19, 21–24). Therefore, by our proposed method, one can easily determine plasma TRP levels with satisfactory results.

Brain TRP levels in saline treated and AIA rats

We also applied our HPLC method to determine brain TRP levels in non-arthritic (that worked as control) and AIA rats that represented a rheumatoid arthritic model (14). Table V shows the highly significant difference ($p < 0.005$) in brain TRP concentration among non-arthritic (0.173 ± 0.0021 μg/g) and AIA rats (0.1117 ± 0.0025 μg/g). This result showed that TRP level decreased in the brains in arthritic rats, which may be one the reason for depression. This result was consistent with the previous studies, which in rheumatoid arthritis patients, showed the existence of neurological disorders such as depression and anxiety. This disorder is also associated with hyperactivity of the immune system during inflammation (24). In rheumatiod arthritis, TRP catabolism increases due to 2,3 dioxygenase enzyme, released from interferon-gamma cell during inflammation (25). Therefore, in rheumatoid arthritis patient, it may affect tryptophan transportation into the brain via common carrier system located on blood brain barrier (26). Because TRP is the precursor of 5-HT, it produces depletion in brain serotonin levels, which results in severe depression and anxiety (27–31), and our method application also supported the answer that TRP availability decreased during inflammation in AIA rats.

Conclusion

A rapid, precise, accurate, low cost and short time-consuming method for the qualitative and quantitative analysis of TRP in raw material, calibrating solutions and biological samples (human plasma and rat brain) was successfully developed. The application of our proposed method can be an important clinical tool to estimate TRP levels as low as 143.74 ng/mLin biological samples of many pathological conditions; it can also be used to study the effects of many drugs that are used in the treatment of this disorder in an easy, accurate and economical way.

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