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Evaluation of antiparkinson activity of PTUPB by measuring dopamine and its metabolites in *Drosophila melanogaster*: LC-MS/MS method development

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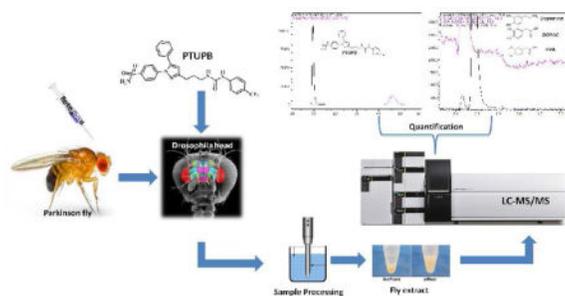
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Abstract

Soluble epoxide hydrolase (sEH) inhibition is reported to elevate endogenous epoxyeicosatrienoic acids (EET's), which are known to play an important role in neuroprotection by inhibiting oxidative stress and neuroinflammation. In the present study, PTUPB, a dual inhibitor of sEH and COX-2, has been tested for its antiparkinson activity against rotenone (ROT) induced neurodegeneration in *Drosophila* model of Parkinson's disease (PD). To determine the efficacy and brain bioavailability of PTUPB a simple, rapid and sensitive LC-MS/MS method was developed and validated for the estimation of PTUPB (Method-I), dopamine (DA) and its metabolites (Method-II) in fly head. Mass spectrometric acquisitions of analytes signals were performed in positive and negative electron spray ionization MRM mode by monitoring the daughter ions. The isocratic elution using formic acid (0.1% v/v) and acetonitrile (20:80 v/v) (for method I), and acetic acid (0.1% v/v) and methanol (for method II) on Jones C₁₈ was carried out to achieve the separation. The results of brain PTUPB, DA and its metabolites estimation shows a dose dependent increase in PTUPB concentration and a dose dependent prevention of ROT induced changes in DA and its metabolites levels ($p < 0.05$), indicating a significant neuroprotection activity of PTUPB. In the present study, we have successfully developed and validated LC-MS/MS methods to identify and quantify PTUPB, DA and its metabolites using a UFLC-ESI-QqQ mass spectrometer for the screening of neuroprotective agents in *Drosophila Melanogaster*.

Graphical abstract

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1. Introduction

Parkinson's disease (PD) is characterised by progressive loss of dopaminergic neurons in the nigrostriatal system of the brain which results in bradykinesia, rigidity, resting tremor and posture instability. The major pathological events leading to neurodegeneration includes increased ROS production resulting in oxidative stress, neuroinflammation and apoptosis [1–3]. This in turn results in the imbalance in dopamine (DA) synthesis and metabolism (Figure 1). The arachidonic acid (AA) released from membrane phospholipids by phospholipase A2 (PLA2) is metabolized to prostaglandins and thromboxane by cyclooxygenase (COX), to leukotrienes by lipoxygenase (LOX), and to epoxyeicosatrienoic acid (EETs) by cytochrome P450 (CYP450) oxidases [4, 5]. The EETs are quickly metabolized to inactive or less active metabolites by soluble epoxide hydroxylases (sEH). EETs' are reported to play a vital role in cytoprotection, due to their ability to attenuate oxidative stress, inflammation, and apoptosis [3]. It has been previously reported that progressive neuroinflammation induces the release of COX-2 which is rapidly expressed in several cell types in response to cytokines, and pro-inflammatory mediators [6]. One of the novel strategies, therefore, is simultaneous inhibition of both sEH and COX-2 enzymes. Since the pathogenesis in PD is multifactorial, involving mitochondrial dysfunction, oxidative stress and inflammation, the novel compound (s) that simultaneously target multiple degenerate pathways are required [1]. PTUPB (4-(5-phenyl-3-3-(4-trifluoromethyl-phenyl)-ureido-propyl-pyrazol-1-yl)-benzenesulfonamide), a dual inhibitor of sEH and COX-2 is expected to stabilize the EETs and thereby promote its cytoprotective actions such as anti-oxidant, anti-inflammatory, and anti-apoptosis. The simultaneous inhibition of COX-2 by PTUPB is also expected to reduce neuroinflammation mediated cell death [1,7, 8].

The flies such as *Drosophila melanogaster* have been employed in elucidating various pathological mechanisms of human neurodegenerative disorders. The *Drosophila* model has been widely studied in case of genomic, cellular and developmental knowledge and the flies display surprisingly intricate behaviours and have complicated brain and nervous systems [9]. The fly model, therefore, provides a well-characterized system that is relatively easy to manipulate but complex enough to be relevant to the development of human disease models [10, 11]. The rotenone (ROT) induced mitochondrial dysfunction, oxidative stress and inflammation in *Drosophila* is a well-accepted model for PD, which has been widely exploited in screening of potential molecules [12–14]. The global animal testing regulations that permit and control the use of non-human animals for research vary greatly around the world, but most governments aim to control the number of times individual animals may be

used; the overall numbers used; and the degree of pain that may be inflicted without using anesthetic. All the regulatory bodies in general recommend adopting the principles of 3 R's i.e., replacement, refinement and reduction [15, 16]. In this changing scenario, development of alternatives are, therefore needed. A number of computer simulations, *in vitro* techniques and other alternative models have been recommended in the place of animals. The *Drosophila* model is considered as one of the appropriate replacement models for rodents and other higher animals used in PD research. The model is inexpensive to propagate, maintain and can produce a large number of genetically homogenous progeny [17]. The present study, aims to evaluate antiparkinson's activity of PTUPB and also to develop and validated LC-MS/MS methods to identify and quantify PTUPB, DA and its metabolites in PD model of *Drosophila melanogaster* using UFLC-ESI-QqQ mass spectrometer.

2. Experimental

2.1. Chemicals and reagents

Dopamine, DOPAC, HVA, rotenone, L-Phenylalanine (an internal standard for dopamine and its metabolites) and celecoxib (an internal standard for PTUPB) were obtained from Sigma-Aldrich (St. Louis, USA). The acetonitrile (ACN) and methanol of LC-MS grade were purchased from SD Fine chemicals (Mumbai, India). Dimethyl sulfoxide (DMSO) and activated charcoal of AR grade were obtained from SD fine chemicals (Mumbai, India). The ultra-pure water was obtained using a Milli-Q RO system (Millipore India, Bangalore, India). The molecule PTUPB was received in the form of gift from Dr. Hammock's laboratory (University of California, Davis, USA).

2.2. Preparation of stock solutions

The Celecoxib (IS-1) was dissolved in 0.1% formic acid and ACN (20:80, v/v) to obtain desired concentration. Stock solution of PTUPB was prepared by solubilising it in MeOH and further diluted with 0.1% formic acid and ACN (20:80, v/v). The standards solutions of DA, DOPAC, HVA and L-Phenylalanine (IS-2) were prepared by dissolving in ultra-pure millipore water.

2.3. Fly maintenance and treatment

2.3.1. Estimation of LD₅₀—The wild type (Oregon K) adult, male, synchronized 10-day-old flies were grown and maintained at 24±1°C with 70–80% relative humidity and were fed on a standard wheat flour-agar diet with yeast granules as the protein source. [18, 19] The synchronized flies were exposed to ROT (10–1000 µM) and PTUPB (100–1000 µM) separately for about 12 days to determine the median lethal dose, LD₅₀ on Day 6.

2.3.2. Neuroprotection study—Based on LD₅₀ results 500 µM ROT; 100 µM and 250 µM PTUPB doses were selected for the neuroprotection study. The flies were divided into 4 groups with 50 flies in each group. Group 1 and 2 received vehicle DMSO (0.25% v/v) in sucrose solution (7% v/v) and served as normal and control respectively. Group 3 and 4 received PTUPB at a concentration of 100 and 250 µM, respectively for 12 days. The vehicle and test solutions were administered as soaked filter paper discs. All treatments were started 4 days before ROT treatment. On day 5, except for the normal cohort, all groups

received ROT (500 μ M). On day 12 the flies were sacrificed by freezing at -80°C for 3 minutes. The heads were separated using a sharp cutter from the rest of body and stored at -80°C to determine amounts of DA and its metabolites.

2.3.3. Estimation of PTUPB in fly head—50 flies in each group were treated with 100 and 250 μ M of PTUPB dissolved in sucrose solution (7% v/v) via soaked filter paper discs. Then the flies were sacrificed by freezing at -80°C for 3 min at different time points of 0.02th, 4th and 7th day. The *Drosophila* heads were separated using a sharp cutter from the rest of body and stored at -80°C to determine PTUPB concentration.

2.4. Extraction of PTUPB, DA and its metabolites in fly head

Fly heads were homogenized in water with a hand homogenizer. The homogenates were centrifuged at 2500 *g* for 10 min at 4°C . The supernatant was collected and spiked with 200 ng/mL of celecoxib (IS-1). The proteins were precipitated with MeOH and centrifuged at 5000 *g* for 10 min. The 10 μ l of the clear supernatant was injected into UFLC-ESI-MS/MS mass spectrometer (Shimadzu 8030, Japan) to determine PTUPB concentration. The same procedure was used for DA and its metabolites except ACN was used as an extraction solvent and L-phenylalanine was used as an internal standard (IS-2).

2.5. Protein estimation

Protein concentrations in homogenates were determined by the Bradford's method using bovine serum albumin as a standard. The concentration of PTUPB, DA and its metabolites were expressed in terms of ng/mg proteins (Table S3) [20].

2.6. Preparation of calibration curves

The calibration curves of PTUPB (0.15–1000 ng/ml), Celecoxib (IS-1, 200 ng/ml) DA (0.3–2000 ng/ml), DOPAC (3–2000 ng/ml), HVA (25–2000 ng/ml), L-phenylalanine (IS-2, 250 ng/ml) was constructed by spiking PTUPB, DA, DOPAC and HVA along with the respective internal standards into blank. The blank was prepared by homogenizing pooled normal fly heads ($n=40$). The endogenous analytes were removed by agitating with activated charcoal (1.25g) overnight, followed by centrifugation at 2500 *g* for 10 min at 4°C and the supernatant was filtered using Whatman filter paper [21]. The proteins were precipitated using MeOH and ACN for estimation PTUPB, DA and its metabolites, respectively. After centrifugation at 5000 *g* for 10 min 10 μ l of clear supernatant of calibration standards were injected into UFLC-ESI-QqQ mass spectrometer. The data acquisition was performed by using Lab solutions software (Shimadzu, Japan) (Table-1).

2.7. Method validation

The developed LC-MS/MS methods (I and II) to identify and quantify PTUPB, DA and its metabolites in PD model of *Drosophila* was validated as per the USFDA Guidelines. The method of determination was validated for specificity, accuracy, precision, linearity, and stability using standard protocols [25].

3. Chromatographic conditions

Ultra force LC system coupled with tandem quadrupole mass spectrometer (Shimadzu 8030, Tokyo, Japan) equipped with electrospray ionization (ESI) interface, LC-20AD pump, SPD-M20 PDA detector, CTO-20AC column oven, CBM-20 Alite controller and SIL-20AC auto sampler. Two separate methods were developed for both PTUPB and DA (along with its metabolites). The chromatographic separations were achieved using Jones C₁₈ (50 × 4.6 mm; 3μ) column at ambient temperature.

3.1. Estimation of PTUPB (Method-I)

The positive and negative ionization modes were employed for the detection of PTUPB and Celecoxib (IS-1), respectively. The formic acid (0.1% v/v) and acetonitrile in the ratio 20:80 v/v, were used as the mobile phase at a flow rate was 0.4 ml min⁻¹. The main working parameters were set as follows; capillary voltage: 1.3 kV; heat block temperature: 350°C and desolvation line temperature: 250°C, Ultra pure nitrogen gas was used as nebulising gas (3 L/min) and drying gas (15 L/min). For collision induced dissociation (CID) experiments ultrapure argon gas (230 Kpa) was used. Multiple reaction monitoring (MRM) transitions as well as collision energy voltages applied for the estimation of PTUPB were summarized in Table. 2 and Figures 2, and 3.

3.2. Estimation of DA and its metabolites (Method-II)

Analyses of DA, DOPAC, HVA, and L-phenylalanine (IS-2) were performed with a mobile phase of acetic acid (0.1% v/v) and methanol (80:20 v/v) at a flow rate of 0.5 ml min⁻¹. The positive (DA and IS-2) and negative ionization (DOPAC, HVA) modes were employed. All other working parameters were set as per method-I (Table-2 and Figure-2, and 3).

4. Results and discussion

4.1. UFLC-MS/MS method development and validation

4.1.1. Estimation of PTUPB (method-I)—PTUPB contains sulphonamide function on its side chain as a result it shows intense protonated molecules in the positive ion mode. Whereas the IS-1 (Celecoxib) contains fluorine function on the side chain, hence it shows deprotonated molecules in the negative mode. MS acquisition of PTUPB and IS-1 were, therefore, performed in positive and negative electron spray ionization MRM mode by monitoring the reaction *m/z* at 544.20 → 382.85 (PTUPB), 380.20 → 316.30 (IS-1), respectively. It was observed that PTUPB and IS-1 loses a 4-(trifluoromethyl) aniline moiety followed by carbonyl group and SO₂ group, respectively during fragmentation process in the collision cell (Figure 2).

The ultra-fast chromatography of PTUPB and IS-1 were performed using Jones C₁₈ stationary phase. Initial chromatographic separation was performed using different aqueous (water, 0.1% acetic acid, (ammonium acetate (2–10 mM) and ammonium formate (2–10 mM) with pH (2.5–7) and 0.1% formic acid) and organic phases (methanol and acetonitrile) in different ratios. A good separation of analytes was achieved using 5mM ammonium acetate and methanol (35: 65 v/v, pH 4.5) but MS detection was poor. After repeated trials,

use of 0.1% formic acid and acetonitrile (20:80 v/v) at a flow rate of 0.4 mL/min resulted in high sensitive detection and good peak shape which was further used in MRM method optimization of PTUPB and IS-1. These analytes were separated well from endogenous matrix interferences. Retention times of PTUPB (ESI positive) and IS-1 (ESI negative) were nearly 1.70 and 1.75 min, respectively. The standard and sample (in *Drosophila*) chromatograms of PTUPB and IS-1 are shown in (Figure 3 and S1 a, b). The maximum runtime for determination of analytes was 6 min which indicates high-throughput analysis of samples in batches.

4.1.2. Estimation of DA and its metabolites (Method-II)—DA and IS-2 (L-phenylalanine) contains amino function on side chain hence it shows intense protonated molecules in the positive ion mode. DOPAC and HVA contain a carboxyl group and hence they can easily deprotonated in the negative mode. MS acquisition of analytes were, therefore performed in positive (DA and IS-2) and negative (DOPAC and HVA) electron spray ionization MRM modes, by monitoring the reaction at m/z 155.05 \rightarrow 137.05 (DA), 166.15 \rightarrow 120.10 (IS-2), 167.05 \rightarrow 123.10 (DOPAC), 181.05 \rightarrow 137.15 (HVA), respectively (Table 2 and Figure 2). It was observed that during the CID experiments, a stable product ion of m/z 137.05 was obtained for DA presumably resulted from loss of ammonia from precursor ions. Loss of carboxyl moieties from molecular ions was a characteristic fragmentation attribute for other three analytes (DOPAC, HVA and IS-2).

Liquid chromatographic separation of DA, DOPAC, HVA and IS-2 were achieved on same stationary phase as that of PTUPB. The peaks of DA and its metabolites and IS-2 were free from endogenous substance. In positive ionization mode, the retention times of DA and IS-2 were approximately were 2.91 and 2.38 min, respectively; where as in negative ionization mode, the retention times of DOPAC and HVA were nearly 2.35 and 4.22 min, respectively. The chromatograms (standard and sample) are shown in Figure 3, S1a, and S1b. The total run time was 8 min for determination of all the four analytes indicating rapid analyses of the developed method.

DA and its metabolites are small polar analytes and are difficult to be retained inside stationary phase.[22] To improve the retention times gradient elution and ion-pairing agents are commonly used. However, the use of non-volatile peak modifiers in mobile phase is harmful to electro spray ionization and will typically hinder mass spectrometry detection capacity.[23] Moreover, the use of gradient elution program will alter ESI conditions during the LC run which can makes quantification of analytes difficult.[24] Initial chromatographic runs were tried with water, formic acid (0.1% v/v), acetic acid (0.1% v/v), ammonium acetate and ammonium formate, acetonitrile and methanol in different combinations. However, good peak shape and high sensitive detection were obtained using acetic acid (0.1% v/v) and methanol (80:20 v/v) at a flow rate of 0.5 mL/min and these conditions were employed for the optimization of these four analytes in MRM mode (Table 2 and Figure 3).

4.2. Method validation

The chromatographic peaks of analytes showed no interference from endogenous components in retention times of the analytes indicating the specificity of the developed

methods. Recovery of analytes were determined by comparing the mean peak areas obtained from the extracted fly head homogenate samples with the peak areas obtained by the direct injection of the corresponding spiked standard solutions of analytes. Three different concentrations of analytes were measured in fly head and the percentage mean recoveries of analytes were reported (Table S1).

The precision (intra- and inter-day) and accuracy experiments in head samples were measured at three different QC levels (n=6) on the same day and on three different days, respectively. For the precision and accuracy, acceptance deviation was set within 15% of the nominal concentration except at LLOQ, which was set at 20% deviation from the nominal concentration. The results were found to be within the acceptable limit which indicates that the assay methods were accurate and precise for replicate analysis of analytes in fly head (Table S1).

All the four analytes were spiked separately into *Drosophila* head homogenate to determine the calibration curves using their respective methods. The linearity of each analyte was ascertained by plotting the response factor versus concentration of standard solution. The linearity range for PTUPB, DA, DOPAC, and HVA was found to be 0.15–1000, 0.3–2000, 3–2000, 25–2000 ng/mL respectively. The results for linear regression analysis are given in Table 1. The results show that the Pearson correlation of for four analytes in head was >0.999. The lowest limit of quantitation (LLOQ) for PTUPB, DA, DOPAC and HVA in head sample with acceptable accuracy and precision (20%), was found to be 0.15, 0.3, 2.4 and 21 ng/mL, respectively (Table 1).

The stability testing of PTUPB, DA, DOPAC and HVA in head samples were performed by the analysis of QC's at three different concentration levels (n=6) subjected to various storage conditions like freeze thaw (3 cycles at $-70 \pm 2^\circ\text{C}$), short-term (at 25°C for 6 h), long term (at $-70 \pm 2^\circ\text{C}$ for 30 days) and stock solution (at 25°C for 6 h) stability. For freeze-thaw (3 cycles) stability, the spiked head samples were frozen at -70°C for 24 h and thawed at room temperature. After completion, the samples were refrozen for 12–24 h under the same conditions, the freeze-thaw cycle was repeated more than two times, at the end of third cycle samples were analyzed and compared with the freshly prepared QC's (n = 6) in fly head. For the short term and stock solution stability study, fly head QC's were kept at 25°C for 6 h and samples were processed, analyzed and compared with the freshly prepared QCs. The long-term stability was evaluated by analyzing stored head samples. The samples were considered to be stable when the deviation from the nominal values was within $\pm 20\%$. The results indicate that the four analytes were stable under all the stability conditions. Table S2 depicts the percentage changes in the mean concentration of analytes under all tested conditions in fly heads.

4.3. Assessment of neuroprotection of PTUPB in *Drosophila Melanogaster*

Dopamine and its metabolites were measured using the developed procedure (Method II) to assess the neuroprotective activity of PTUPB. The rotenone treatment significantly reduced the DA and its metabolite levels in the control flies compared to untreated normal indicating its neurodegenerative effects (Figure 5A–B and Table 4). PTUPB showed a dose dependent neuroprotection against rotenone induced changes in the levels of DA and its metabolites

(Figure 5A–B, S1b and Table 4), indicating its potential neuroprotective benefits. In addition, we also measured the levels of PTUPB in fly head (Method I) to establish the bioavailability of PTUPB in brain. It was observed that there was a dose dependent increase in concentration of PTUPB in fly head (Figure 4, S1a and Table 3). The concentration of PTUPB in fly head from 0.02 to 7 days treatment in fly head was found to be 4.99 to 17.64 nM and 3.66 to 19.64 nM at a dose of 100 μ M and 250 μ M, respectively.

4.4. Statistical analysis

The data are expressed as the mean \pm standard deviation of mean (SEM). Statistical significance was determined by one way ANOVA followed by Bonferroni *post hoc* test to assess differences between the groups. Values were considered significant, if $p < 0.05$.

5. Conclusion

We have developed two simple LC-ESI-MS/MS methods for the *in vivo* quantification of PTUPB (Method I), DA and its metabolites (Method II) in *Drosophila* head. The procedure is rapid, reliable, reproducible and sufficiently sensitive to detect and quantify the PTUPB, DA, and DOPAC in fly head samples except for HVA. The methods enable us to gain insights into the neuroprotective action of PTUPB against ROT induced neurodegeneration in flies. The developed methods provide a direct means to measure the concentration of PTUPB, DA and its metabolites at the site of action in a small, fruit fly head samples which represents an important step forward in quantitative studies *in vivo*. The data generated, therefore were useful to gain insight into the *in vivo* distribution of the PTUPB as well as its impact on the dopaminergic nervous system in ROT induced PD model of *Drosophila melanogaster*.

Abbreviations

PD	Parkinson disease
DA	Dopamine
DOPAC	3,4-dihydroxyphenylacetic acid
HVA	Homovanillic acid
BBB	blood brain barrier
sEH	soluble epoxide hydrolase
mEH	microsomal epoxide hydroxylases
COX-2	cyclooxygenase-2
ROS	reactive oxygen species
AA	arachidonic acid
EETs	Epoxyeicosatrienoic acids

LOX	lipoxygenase
PLA2	phospholipase A2
CYP450	cytochrome P450
ROT	rotenone
IS	internal standard
ACN	acetonitrile
CON	control
CPCSEA	Control and Supervision of Experiments on Animals
UGC	University Grants Commission
MCI	Medical Council of India

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Highlights

- LC-MS/MS methods development for dopamine, DOPAC and HVA in *Drosophila melanogaster*
- LC-MS/MS method to determine efficacy and bioavailability of PTUPB in *Drosophila*
- Evaluation of antiparkinson activity of PTUPB in Parkinson model of *Drosophila*
- *Drosophila* an alternative to animal models for screening antiparkinsons activity

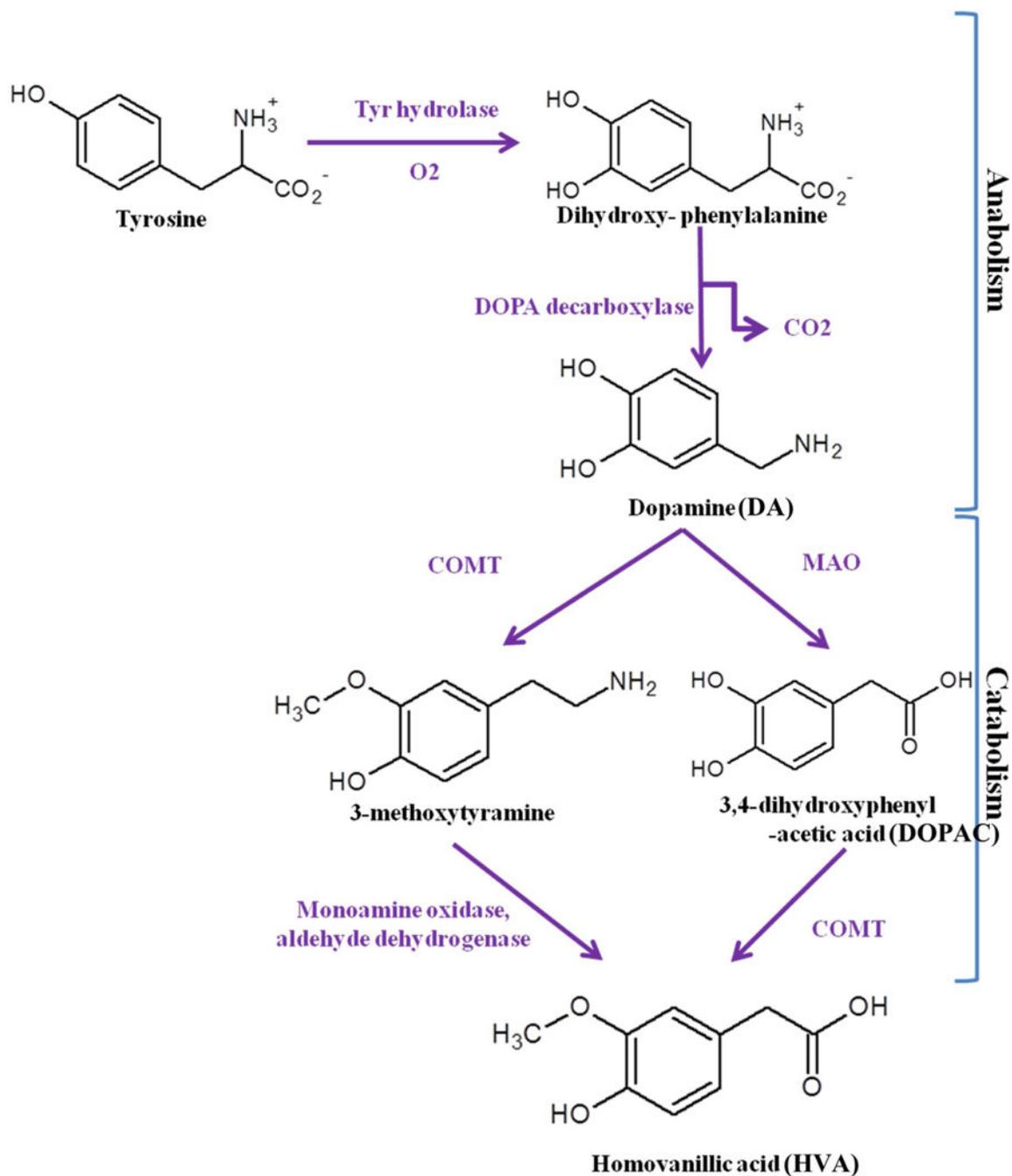


Figure 1. Synthesis and metabolism of Dopamine (Abbreviations: MAO- Monoamine oxidase, COMT-catechol-o-methyltransferase).

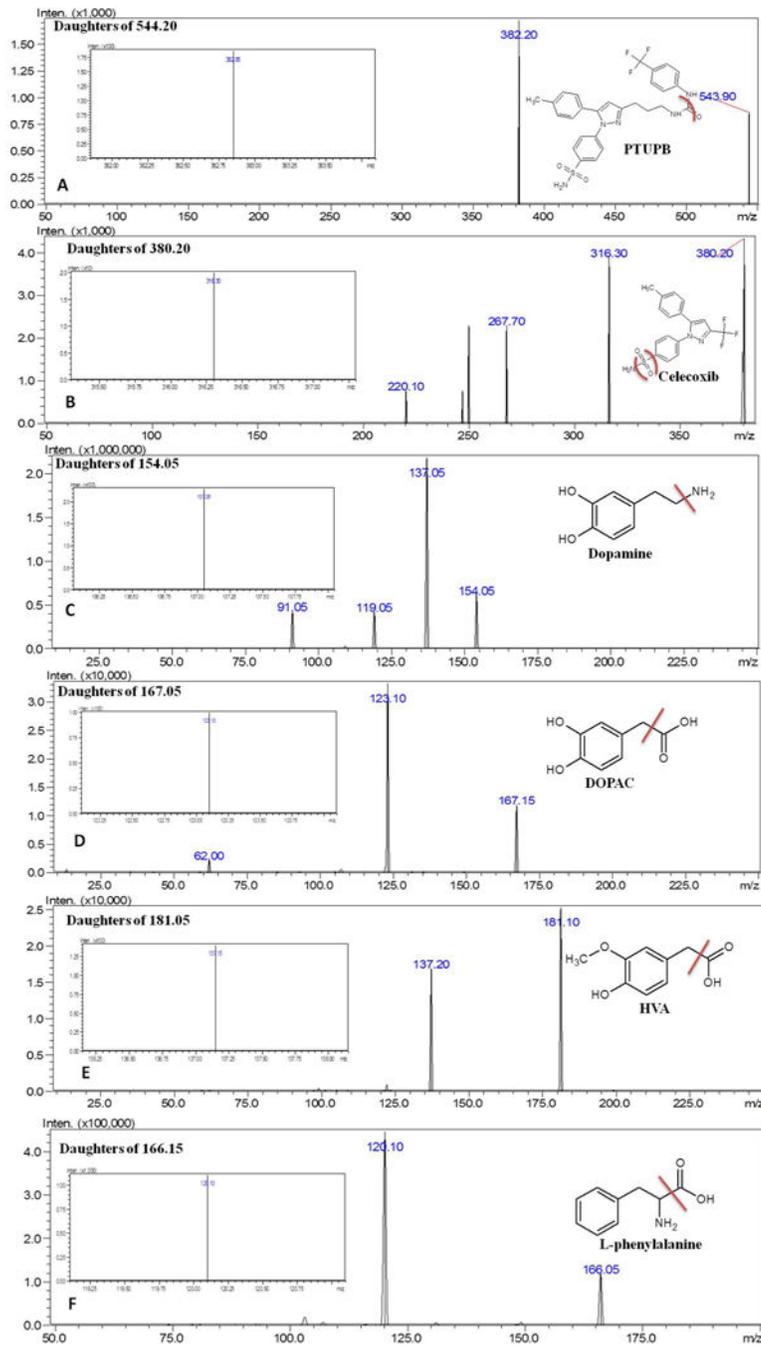


Figure 2. Product ion scan and MRM (image shown inside the box) spectra of (A) PTUPB, (B) Celecoxib (IS-1), (C) DA, (D) DOPAC, (E) HVA, (F) L-Phenylalanine (IS-2).

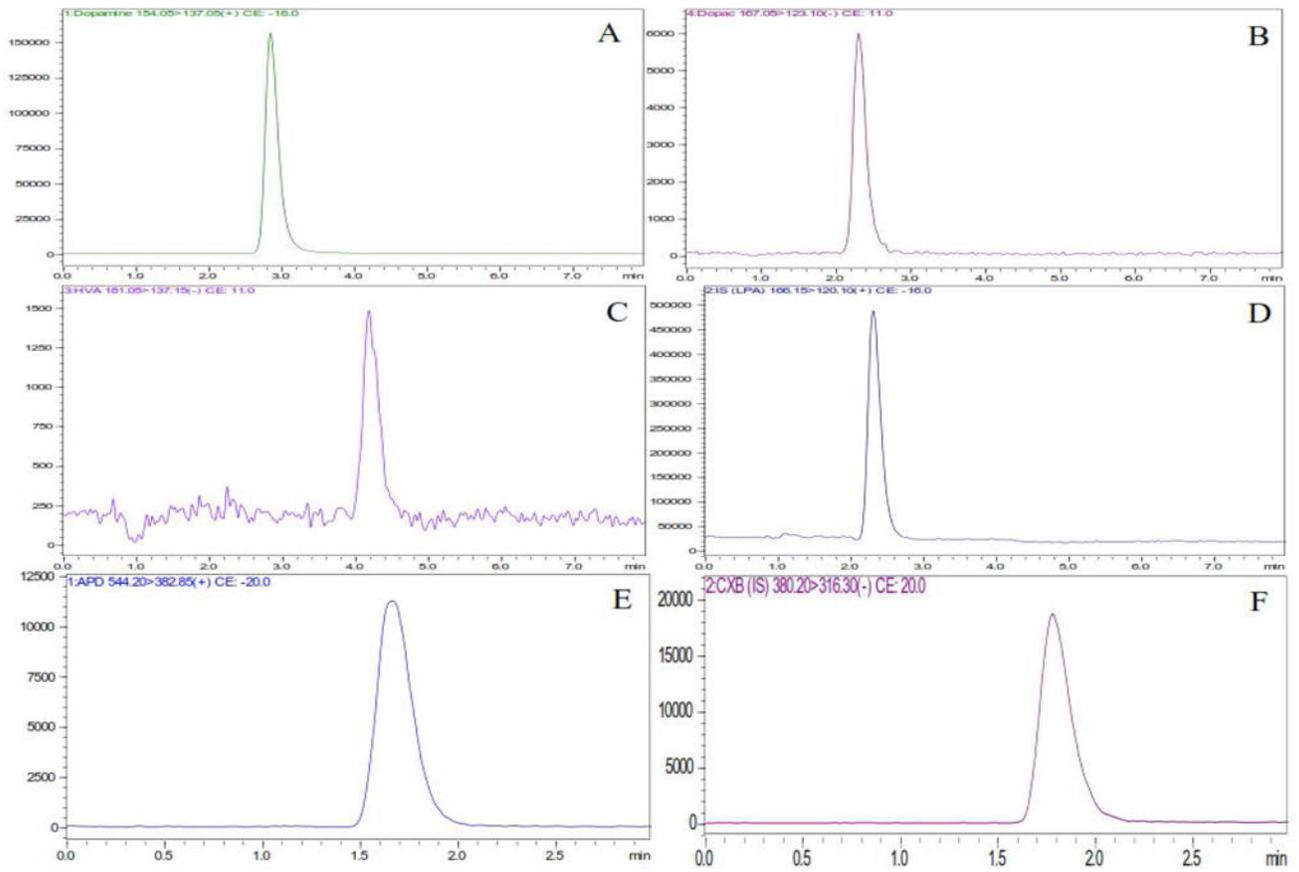


Figure 3. Standard LC-ESI-MS/MS chromatograms of (A) DA, (B) DOPAC, (C) HVA, (D) L-phenylalanine (IS-2), (E) PTUPB, and (F) Celecoxib (IS-1).

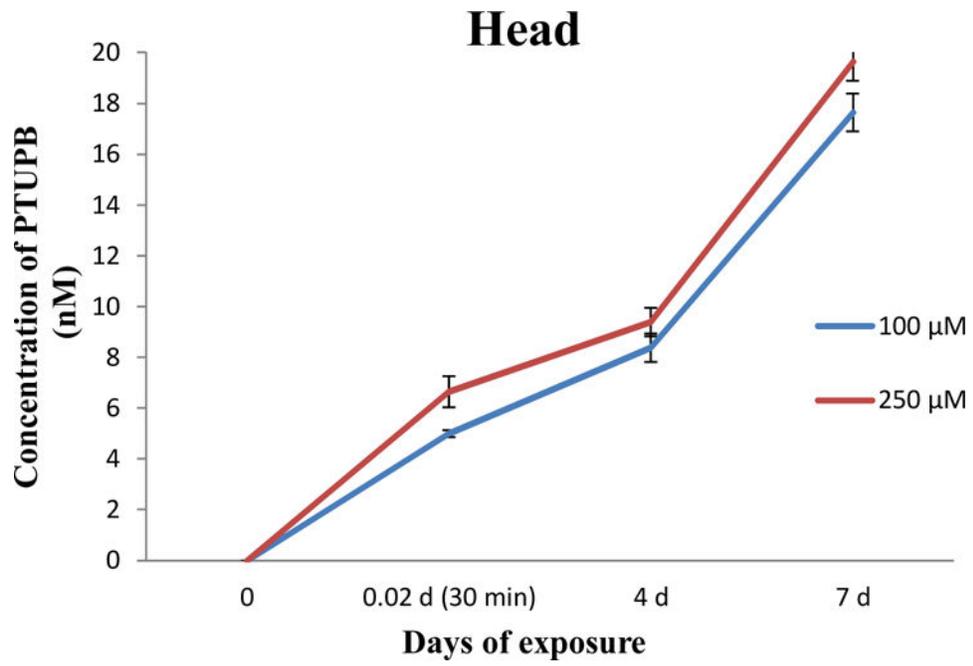


Figure 4. Correlation analysis of *in vivo* concentration of PTUPB in drosophila head corresponding to various oral doses of PTUPB (100 and 250 μM) at different time intervals. The data represented as mean ± SD, n=3.

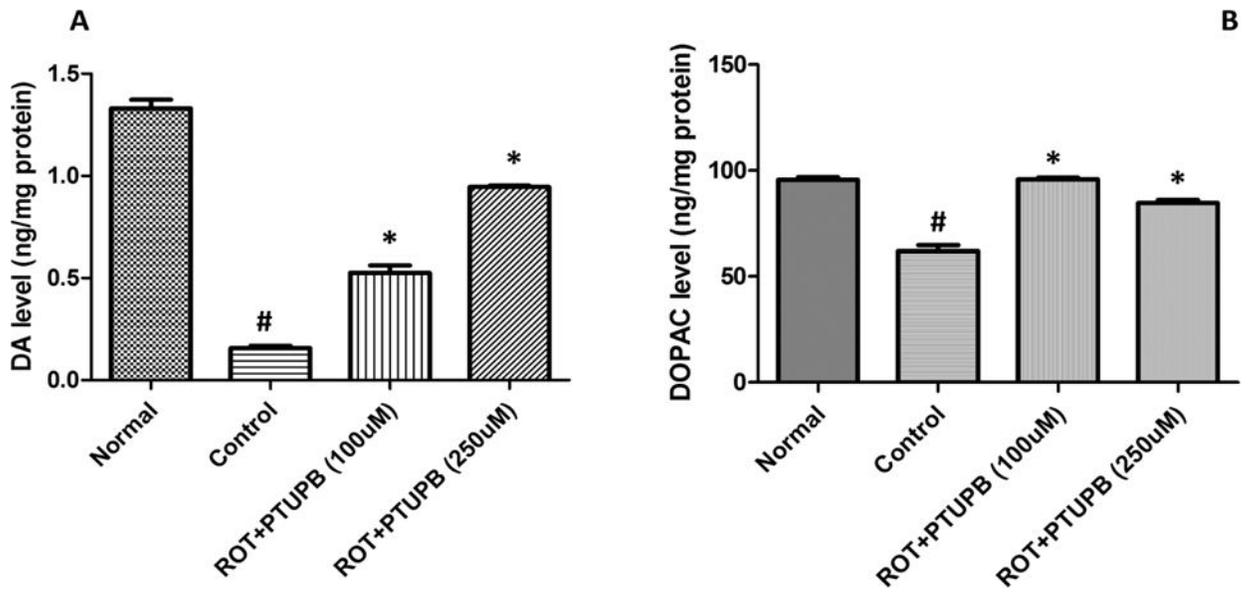


Figure 5. (A–B): Effect of PTUPB on *in vivo* concentration of DA and its metabolites in ROT (500µM) induced toxicity model of *drosophila*, corresponding to various oral doses of PTUPB (100 and 250 µM) administration; Figure A: Concentration of DA in fly head; Figure B: Concentration of DOPAC in fly head. The data represented as mean \pm SD of three independent experiments. # $p < 0.05$ versus control (CON) group and * $p < 0.05$ versus ROT treated.

Calibration data of PTUPB, DA and its metabolites by LC-MS/MS

Table 1

Analyte	Internal standard (ng/ml)	Equations	Drosophila head (ng/ml)	Linear range (ng/ml)	Correlation coefficient (R ²)	LOD (ng/ml)	LOQ (ng/ml)
PTUPB	Celecoxib (200 ng/ml)	$y = 2451.1x + 28150$		0.15 – 1000	0.999	0.05	0.15
Dopamine	L-phenylethylamine (250 ng/ml)	$y = 0.0096x - 0.0366$		0.3 – 2000	0.999	0.1	0.3
DOPAC		$y = 0.00036x - 0.00345$		3 – 2000	0.999	0.8	2.4
HVA		$y = 5E-05x - 0.0008$		25 – 2000	0.999	7	21

MS-MS conditions for multiple reaction monitoring for PTUPB, DA and its metabolites

Table 2

Analyte	Molecular weight (g/mol)	Ion mode	Precursor ion (m/z)	Product ion (m/z)	Dwell time (msec)	Q1 Pre Bias (V)	Q3 Pre Bias (V)	Collision energy (eV)	Retention time (min)
PTUPB	543.16	Positive	544.20	382.85	300	-16	-18	-20	1.70
Celecoxib	381.373	Negative	380.20	316.30	100	18	20	20	1.75
Dopamine	189.639	Positive	154.05	137.05	100	-12	-14	-16	2.91
DOPAC	168.148	Negative	167.05	123.10	100	18	17	11	2.35
HVA	182.17	Negative	181.05	137.15	100	20	11	11	4.22
L-phenylalanine	165.192	Positive	166.15	120.10	100	-24	-24	-16	2.38

Table 3

The concentration of PTUPB in *Drosophila* head upon treated with 100 μ M and 250 μ M.

SL no	Grouping	PTUPB (ng/fly)
1	100 μ M (0.02 days)	4.99 \pm 0.13
2	100 μ M (4 days)	8.38 \pm 0.56
3	100 μ M (7 days)	17.64 \pm 0.75
4	250 μ M (0.02 days)	6.65 \pm 0.62
5	250 μ M (4 days)	9.38 \pm 0.56
6	250 μ M (7 days)	19.64 \pm 0.75

Data expressed as Mean \pm SD ng/mg protein, n=3.

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Table 4

The concentration of Dopamine and its metabolites in drosophila head.

SL. No	Grouping	Dopamine (ng/mg protein)	DOPAC (ng/mg protein)	Ratio of dopamine and DOPAC (ng/mg protein)	HVA (ng/mg protein)
1	Normal	1.36±0.043	96.51±1.35	0.014	
2	Control	0.15± 0.012 [#]	64.00±2.75 [#]	0.003	
3	Rotenone + PTUPB (100 µM)	0.55±0.036*	96.48±0.76*	0.005	Not detected
4	Rotenone + PTUPB (250 µM)	0.95±0.007*	85.69±1.48*	0.011	

Data expressed as Mean ± SD ng/mg protein, n=3.