International Journal of Chemical Science
Online ISSN: 2523-2843, Print ISSN: 2523-6075
Impact Factor: RJIF 5.22
www.chemicaljournals.com
Volume 2; Issue 6; November 2018; Page No. 19-29

Development and validation of spectrophotometric method for estimation of sodium [2-[2, 6-dichlorophenyl [(4-AMINO N-ACETYL) phenyl acetate]-amino] phenyl acetate] in rat serum and its application in pharmacokinetics studies

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Abstract
A simple, selective and sensitive spectrophotometric method is described for the determination of sodium [2-[2, 6-dichlorophenyl [(4-amino n-acetyl) phenyl acetate]-amino] phenyl acetate] (DPAAPA) in rat blood sample. The assay was based on protein precipitation with mixture of methanol and ethyl acetate (1:2) then alkaline oxidation reaction with potassium ferricyanide. DPAAPA reacts with potassium ferricyanide in alkaline medium to give a yellow color after 5 min at 30 ˚C temperature having maximum absorbance at 451 nm. The reaction obeys Beer's Law from 2-12 µg mL^{-1} for DPAAPA with molar absorptivity 4.2837×10^4 L mol^{-1} cm^{-1}. The recoveries of DPAAP from spiked blood samples were 99.20-101.20 µg mL^{-1} with relative standard derivation was less than 2.0%. The analytical recovery, sensitivity and accuracy of this assay method were adequate for characterization of the pharmacokinetics of oral administration of DPAAPA to Wistar albino rats and the assay has been successfully applied to provide pharmacokinetic data.

Keywords: Sodium [2-[2, 6-dichlorophenyl [(4-amino n-acetyl) phenyl acetate]-amino] phenyl acetate], Spectrophotometry, $K_3[Fe(CN)_6]$, Pharmacokinetics

1. Introduction
Paracetamol is widely used as an analgesic [1] and antipyretic drug [2]. In text book of pharmacology, it is classified as a Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) [3, 4]. A Literature survey indicates that numbers of paracetamol derivatives have been synthesized [5-10]. The synthesized paracetamol derivatives have been shown superior pharmacological potencies than paracetamol [5-10]. Sodium [2-[2, 6-dichlorophenyl [(4-amino n-acetyl) phenyl acetate]-amino] phenyl acetate] (DPAAPA) is one of the paracetamol derivative. It was synthesized by condensation reaction between (4-amino N-acetyl) phenyl 2-chloroacetate and 4-amino phenyl sulfonamide. It shows analgesic, antipyretic, anti-inflammatory and antibacterial activities [11]. DPAAPA show superior pharmacological activities than parent paracetamol and diclofenac sodium drugs [11].
A number of analytical methods have been described for the drug determination in pure form, tablet form and biological fluids etc. like, liquid chromatography [12], titrimetry [13], capillary electrophoresis [14], chemiluminescence [15], HPLC methods [16, 17], spectrofluorimetry [18], spectrophotometric methods [19, 20]. DPAAPA is a new paracetamol derivative. It is very much essential to develop a suitable and sensitive analytical method for the estimation of DPAAPA at low levels in serum or other biological samples. No any official method is available for determination of DPAAPA. It is the first method for determination of DPAAPA in spiked blood samples and also used for evaluating pharmacokinetics parameters.
In the present study, a simple, accurate, sensitive and reproducible analytical method with better detection range and simple spectrophotometric determination of DPAAPA in spiked blood samples is developed. The method is based on alkaline oxidation of DPAAPA with potassium ferricyanide. The developed method was validated by International Conference on Harmonisation (ICH 1996) [21] and United States Pharmacopoeia (USP 2000) [22] and recoveries studies. The developed method was further used for evaluating pharmacokinetics study of DPAAPA after oral administration (100 mg kg^{-1} body weight) in Wistar albino rats. All animal experimental protocols were approved by the Institution Animal Ethical Committee.

2. Experimental
2.1 Chemicals
The synthesized compound; Sodium [2-[2, 6-dichlorophenyl [(4-amino n-acetyl) phenyl acetate]-amino] phenyl acetate] was used after recrystallization and purification. Potassium ferricyanide, Sodium hydroxide, Methanol and Ethyl acetate were purchased from Sigma-Aldrich, Mumbai, India. Further analytical grade chemicals and double distilled water were used.

2.2 Apparatus
A double beam UV-Visible spectrophotometer (Shimadzu-1700) equipped with a quartz cell of 1.0 cm path length was used. Analytical balance with minimum weigh capacity 10 mg and maximum 220g, AUW220D, Shimadzu, Japan was used. A cooling centrifuge (C-24BL, maximum rpm 20,000, minimum cooling temperature -8 ˚C, Remi Equipment Pvt. Ltd., Mumbai, India) was used for preparation of serum samples.

2.3 Preparation of Stock Solutions

The stock solutions of NaOH and $K_3[Fe(CN)_6]$ were prepared at concentration of 3.0 M and 3.0 mM in distilled water respectively and were further appropriated diluted for the preparation of working solutions. An aqueous standard stock solution-1 and a standard solution-2 of DPAAPA (1.0 mg mL^{-1}) was prepared by dissolving 100.0 mg of DPAAPA in 100.0 mL of distilled water and 100.0 mL of methanol, respectively. Working solutions were prepared by appropriate dilution of the standard solution. Spike stock solutions of 100.0 µg mL^{-1} of DPAAPA were prepared in blank serum by properly mixing of 100.0 µL of 100.0 µg mL^{-1} stock solution-2 of DPAAPA with 100.0 µL blank serum.

The appropriate amounts of possible interfering substances such as Ca^{+2}, Na^{+}, K^{+}, Mg^{+2}, Zn^{+2}, Fe^{+2}, L-Alanine, Glycine, Tyrosine, glucose, uric acid, paracetamol and L-ascorbic acid were dissolved in distilled water. After further dilution with the same solvent, the final concentrations for interference study were obtained 100.0 µg mL^{-1}. The appropriate concentrations were prepared during the study by using dilution method.

2.4 Spectrophotometric determination of DPAAPA

To an aliquot from the stock solution-1 of DPAAPA was properly mixed with 2.0 mL of 1.5 M NaOH and 2.0 mL of 1.0 mM $K_3[Fe(CN)_6]$ and finally, the volume was made up to 10 mL with distilled water. The resulting absorbance of the yellow color was measured at 451 nm at optimum conditions (30 ˚C temperature and 5 min.) employing all reagents except DPAAPA as a blank.

2.5 Optimization Studies

2.5.1 The Effect of Diluting Solvent

The effect of diluting solvent was examined using acetonitrile, ethanol, methanol, water, propanol and butanol solvents. A 10.0 µg mL^{-1} of DPAAPA solution was used to determine the effect of diluting solvent. An aliquot of DPAAPA was taken in 10 mL volumetric flask and properly mixed after addition of 2.0 mL of stock solutions of NaOH and $K_3[Fe(CN)_6]$. The finally, resulting solution was diluted up to the mark with acetonitrile, ethanol, methanol, water, propanol and butanol solvents respectively. The absorbance was measured at λmax against respective blank solvents.

2.5.2 The Effect of Reagent Concentration

The effect of NaOH and $K_3[Fe(CN)_6]$ concentration on absorbance were investigated in the range of 0.1-3.0 M and mM respectively. Aliquots of DPAAPA were taken in 10 mL volumetric flasks and 2.0 mL of various concentrations of NaOH and 2.0 mL of stock solution of $K_3[Fe(CN)_6]$ in each flask were added. The resulting solutions were diluted up to the mark with water. Similarly, a fixed amount of NaOH and various concentrations of $K_3[Fe(CN)_6]$ was studied. The maximum absorbance was noted at 451 nm λmax against the blank selected as an optimum concentration of the reagents.

2.5.3 The Effect of Reaction Temperature

The temperature was optimized using the method of steepest ascent [23]. The optimization of temperature studies for DPAAPA was carried out by using 10.0 µg mL^{-1} solution-1 of DPAAPA. According to spectrophotometric method of DPAAPA, the standard solution-1 of DPAAPA was mixed with reagents in a vortex mixer followed by incubating at various temperatures in the range of 5-80 ºC. The absorbance of the resulting solution was measured for each temperature at λmax on visible spectrophotometer. Each determination was done in triplicate.

2.5.4 The Effect of Reaction Time

The steepest ascent method was used for optimization of reaction time [23]. The optimal reaction time of DPAAPA was carried out as similar to the effect of temperature. The absorbance of the resulting solution was measured at various time intervals in the range of 0-60 min. The optimal reaction time at an optimized temperature was taken as the time corresponding to the maximal absorbance of the sample.

2.5.5 Stoichiometric Ratio Determination

The Job's method was used to check the stoichiometric reaction between synthesized compound and reagents [24]. The stoichiometric ratio was determined by using equimolar of solutions of the $K_3[Fe(CN)_6]$ and DPAAPA (1.0 mg mL^{-1} each). Seven different volumes of 0.00, 0.4, 0.66, 1.00, 1.34, 1.60 and 2.00 of $K_3[Fe(CN)_6]$ were taken in 10 mL volumetric flasks and diluted up to 2.0 mL with DPAAPA solution. Finally, the resulting solutions were diluted up to 10.0 mL with water. The absorbance was measured at 451 nm against the blank, prepared similar way except DPAAPA.

2.5.6 Determination of DPAAPA in Blood Sample [25]

Blank blood (0.5 mL) sample was collected from Adult Wistar albino rat, through retro-orbital sinus puncture [26] using 0.1 mL capillary tubes into 2 mL polyethylene centrifuge tube. A 1.0 mL ethyl acetate was previously added into 2 mL polyethylene centrifuge tube. A 0.5 mL blood was spiked with a 0.5 mL stock solution-2 of DPAAPA prepared in methanol (1.0 mg mL^{-1}). The mixture was hand shaken for about 5 minutes until the contents were properly mixed. The mixture of methanol and ethyl acetate (1:2) was found to be an ideal solvent for blood extraction because of blood proteins were not precipitated by using only methanol or ethyl acetate solvent. Then the tubes were shaken for 5 minutes on a vortex mixture and centrifuged at 2000 rpm for 5 minutes at 30 ºC temperature. The clear supernatant was obtained which did not require further clean up. The convenient volumes of the supernatants were collected in 10 mL volumetric flasks and analyzed by developed method. The final concentrations of each synthesized compound in blood supernatants were 1.25, 2.50 and 3.75 µg mL^{-1} respectively. The absorbance was measured at λmax against the blank reagent which were prepared in the same way except the addition of DPAAPA. The calibration curves were plotted for DPAAPA both with and without spiked serum sample in concentration range of 2-12 µg mL^{-1}.

2.6 Method Validation

Evaluation of the spectrophotometric method was based mainly on linearity assay, precision and accuracy. This method was developed and validated as per ICH guideline 1996 and USP 2000 [21, 22].

2.6.1 Accuracy and Precision

The accuracy and precision of the developed methods [23, 27] were determined from intra-day and inter-day analysis of spiked DPAAAP at three different concentrations levels in blood (1.25, 2.50 and 3.75 µg mL^{-1}) samples. The precision of the developed methods was described as the percentage relative standard deviation (%RSD). The accuracy was described as a percentage of measured concentration of DPAAPA. Intra-day precision and accuracy of the developed method for blood samples were evaluated by analyzing each sample three times on the same day, while inter-day precision and accuracy of the developed method for blood samples were evaluated by analyzing each sample for six consecutive days.

2.6.2 Recovery Study

The recoveries study was carried out using three different spiked concentrations of DPAAPA in blood (1.25, 2.50 and 3.75 µg mL^{-1}) samples. The recovery study was described as a percentage of measured concentration of DPAAPA.

2.6.3 Robustness

The robustness was examined by evaluating the influence of small variation in the method. In these experiments, one parameter was changed, whereas the others were kept unchanged, and it was calculated in terms of standard deviation and %RSD. The robustness of the proposed method was assessed by changes in the analytical wavelength (±1 nm) at two different concentrations (5.0 and 10.0 µg mL^{-1}) of analytes and changes in working temperatures (±2 ºC) and reaction time (±3 min) at 10.0 µg mL^{-1} concentration levels of analytes (DPAAPA).

2.6.4 Ruggedness

The ruggedness of the proposed methods was checked by using two different analysts at two different concentrations (5.0 and 10.0 µg mL^{-1}) of analytes and also examined day-to-day reproducibility in blood (2.5 µg mL^{-1}) samples for six consecutive days. Ruggedness was calculated in terms of standard deviation and % RSD.

2.6.5 Stability Study

The stability of DPAAPA in blood samples was measured for typical storage and handling conditions. At three different concentration levels of Spiked blood (1.25, 2.50 and 3.75 µg mL^{-1}) samples were stored at bench top (at room temperature for 6-24 hr) and long-term (at -20 ºC for a week) conditions. The bench-top and long-term stability were examined by analyzing the stored sample using developed assay method for DPAAPA and compared with freshly prepared intra-day blood samples.

2.6.6 Specificity and Selectivity

The specificity of the method was investigated by possible interferences caused through other potential substances on the determination of DPAAPA. Under the developed experimental conditions for DPAAPA, the effect of interfering species was evaluated by addition of various concentrations of interfering species (e.g. Ca^{+2}, Na^{+}, K^{+}, Mg^{+2}, Zn^{+2}, Fe^{+2}, L-Alanine, Glycine, Tyrosine, glucose, uric acid, paracetamol and L-ascorbic acid) to spiked blood samples containing a fixed amount of DPAAPA (10.0 µg mL^{-1}). The tolerance of the various interferences and selectivity of the proposed method was examined.

2.7 Pharmacokinetics

2.7.1 Animals Used

A crossover study with respect to the synthesized compound DPAAPA and route of administration was conducted on four rats. Adult Wistar albino rats weighing between 250–320 g were used for the pharmacokinetics studies. The animals were considered to be healthy on the basis of preliminary physical examination and maintained under similar environmental and manage mental conditions. The animals were weighed before the day of drug administration to determine the requirement of dose. They had received no medications before two weeks and during washout period. The animals were kept off feed 18 hrs before the administration of DPAAPA and have accessed to drinking water *ad libitum*. Pharmacokinetic parameters were calculated by non-compartmental analysis and according to the Trapezoidal rule [28-31].

2.7.2 Administration of DPAAPA

The animals were divided into two groups and each group consists of four rats. The dose of synthesized compound DPAAPA and paracetamol were prepared in 2.0% aqueous suspension of acacia gum. The animals of the group I was given paracetamol (500 mg kg^{-1}) served as control as well as reference standard. The animals of groups II was orally administered 100 mg kg^{-1} DPAAPA.

2.7.3 Collection of Blood Samples

Blood samples (approximately 1.0 mL) were collected in Eppendorf test tubes from the tail vein of Wistar albino rat [32]. A mixture of methanol and ethyl acetate (1:2) was previously added in an Eppendorf test tube. The DPAAPA (100 mg kg^{-1}) was administered orally to the rat and blood samples were withdrawn at the time intervals of 0.0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 10, 12, and 24 hr. Immediately, a blood sample and the mixture of methanol and ethyl acetate (1:2) was shaken for 5 minute by hand until the contents were properly mixed. Then after, the tubes were shaken for 5 minute on a vertex mixture and centrifuged at 2000 rpm for 5 minute at room temperature. Blood supernatants were separated and after necessary labeling stored at –20 °C until assayed.

2.7.4 Assay Method for Paracetamol

Blood samples of paracetamol were measured by spectrophotometrically [33]. After treatment, 0.2 mL of blood supernatant sample was mixed with 1.0 mL of 1.0 M hydrochloric acid and 2.0 mL of 1.0 mM ferric sulphate. The resulting solution was heated at 100 ºC in water bath for 10 min. Then after, adding 2.0 mL of 1.0 mM potassium ferricyanide and was diluted up to 10 mL with distilled water. The resulting samples were analyzed by spectrophotometrically at λmax 700 nm, after 24 min. A calibration curve was constructed (concentration range between 0.2-2.0 µg mL^{-1}) by spiking drug-free rat blood in duplicate with a standard solution of paracetamol (100.0 µg mL^{-1}).

The pharmacokinetic parameters were calculated as a mean

value ± standard deviation (SD) (n = 4). Statistical analysis was performed by student t-test and one way ANOVA. A value of $P < 0.05$ was considered to be statistically significant.

3. Results and Discussion

The literature survey suggested that α-carbon containing tertiary amine group was oxidized by potassium ferricyanide in alkaline medium [34, 35]. The α-carbon containing tertiary amine side chain is present in the structure of DPAAPA. Thus, the oxidation reaction of DPAAPA is possible in alkaline medium by the reaction with potassium ferricyanide. Sodium hydroxide provides an alkaline medium for the oxidation reaction of DPAAPA and also acts as a catalyst in the reaction. The mechanism of oxidation of DPAAPA involves the formation of α-carbanion and α-carbene. The pathway of oxidative product formation between DPAAPA and $K_3[Fe(CN)_6]$ is illustrated in Scheme 1.1. The yellow colored oxidative product was formed during the reaction between DPAAPA and $K_3[Fe(CN)_6]$ in basic medium (Figure 2). The absorption spectra of the yellow colored oxidative product in basic media was measured in the range of 350-800 nm against the blank solution and shows a maximum absorbance at 451 nm (Figure 3).

3.1 Optimization Studies

3.1.1 The Effect of Diluting Solvents

Different diluting solvents like, acetonitrile, methanol, ethanol, propanol, butanol, water were used in this study. Experimental results show that the maximum absorbance for the oxidative product was achieved using water as diluting solvent (Table 1).

3.1.2 The Effect of Reagent Concentration

The effect of reagent concentration for the formation of oxidative product was checked at a fixed concentration of DPAAPA (10.0 µg mL^{-1}). The various concentrations of NaOH and $K_3[Fe(CN)_6]$ were tested in the range of 0.1-3.0 M and 0.1-3.0 mM respectively. The results show that maximum response was achieved by using 1.5 M NaOH and 1.0 mM $K_3[Fe(CN)_6]$ (Figure 4). Higher and lower concentrations had no significant effect on absorbance for the formation of oxidative product.

3.1.3 The Effect of Reaction Temperature

The temperature effect on the formation of oxidative product was performed at different temperatures (10, 20, 30, 40, 50 ºC). The results are demonstrated in Figure 5. The maximum absorbance value was obtained at 30 ºC temperature. Thus, 30 ºC temperature was selected as an optimal temperature for the formation of oxidative product. The results reveal that the lower absorbance values were obtained at the below and higher temperature than optimal temperature.

3.1.4 The Effect of Reaction Time

Performing the oxidative product formation at different time intervals (0, 5, 10, 15, 20, 30 min) shows that the maximum absorbance was achieved at 5 min after mixing the DPAAPA and $K_3[Fe(CN)_6]$ in a basic medium. The results are depicted in Figure 6.

3.1.5 Stoichiometric Ratio Determination

The Job's method of continuous variations by using varied volumes of equimolar solutions (1.0 mg mL^{-1}) of DPAAPA and $K_3[Fe(CN)_6]$ was employed to find out the stoichiometry of the oxidative product. The absorbance was plotted over the mole ratio of DPAAPA:$K_3[Fe(CN)_6]$ (Figure 7). The plot reached a maximum value at a mole fraction of 1.6 which indicates an equimolar ratio 1:4 for formation of oxidative product. Therefore, four moles of $K_3[Fe(CN)_6]$ and one mole of DPAAPA took part in oxidation reaction. The proposed structure of the stoichiometric ratio of the oxidative product is presented in Figure 7.

3.2 Validation Study

Under the optimum conditions, the calibration curves correlating the absorption intensity with the corresponding concentration of DPAAPA in the range of 2-12 µg mL^{-1} was constructed (Figure 8). The calibration curves were plotted in the range of 2-12 µg mL^{-1} in spiked DPAAPA in blank serum (Figure 9). All the plots were linear with very small intercepts and good correlation coefficients. The analytical parameters are given in Table 2. The limit of the detection (LOD) and limit of quantification (LOQ) were determined using the standard formula, LOD= 3.3 × [Standard deviation/Slope] and LOQ = 10 × [Standard deviation/Slope]. Based on the three replicate measurements, the LOD and LOQ were found to be 0.32 µg mL^{-1} and 0.97 µg mL^{-1} respectively.

3.2.1 Precision and Accuracy

The inter-day and intra-day precision and accuracy were evaluated for selected concentrations of DPAAPA from spiked blood samples in one day and six consecutive days. The results are summarized in Table 3. Low values of RSD and error indicated that the developed method is the acceptable precise and accurate. The recovery of DPAAPA from spiked blood samples was found to be about 99.20-101.20%.

3.2.2 Robustness

The robustness of the proposed method was established by deliberately changing the wavelength (451±1 nm), working temperature (30±1 ºC) and reaction time (5±3 min) for the determination of DPAAPA. The 5.0 and 10.0 µg mL^{-1} concentrations of DPAAPA were used for robustness study and three times analyzed by the proposed method. %RSD were found to be less than 1.0% for different analytical parameters, indicating the robustness of the proposed method (Table 4).

3.2.3 Ruggedness

Ruggedness of the proposed method was evaluated at different concentration levels of DPAAPA (2.50, 5.0 and 10.0 µg mL^{-1}) under the different analysts in same laboratory condition and various day-to-day analysis of spiked blood samples. The values of %RSD were found to be less than 1.0% indicating the ruggedness of the proposed method (Table 5).

3.2.4 Stability Study

The stability of the DPAAPA-$K_3[Fe(CN)_6]$ oxidative product

was evaluated for bench-top and long term stability. The results are shown in Table 6. The result shows that the oxidative product was relatively stable for storage conditions. The intra-day recoveries from blood samples were comparable with bench-top and long-term stability data of blood samples and very minor changed would be observed between them.

3.2.5 Specificity and Selectivity

The Effect of Interfering Radicals

The specificity of the method was determined by measuring the absorbance of solutions containing 10.0 µg mL^{-1} of DPAAPA with various amounts of diverse species. It was observed that the interferents produce below ± 10% error. Typical results are given in Table 7.

3.3 Pharmacokinetics study

The pharmacokinetics study of synthesized compound DPAAPA was evaluated by non-compartment animal model and trapezoidal rule [28, 30]. The synthesized compound DPAAPA and paracetamol [33] in blood samples were analyzed by a validated spectrophotometric method. The concentration of DPAAPA and paracetamol in blood samples are calculated using regression equation and calibration curve (Figure 1.9-2.0). The regression equation of DPAAPA and paracetamol in serum was Y = 0.0909X - 0.0568 (r = 0.9997) and Y = 0.5071b + 0.0133 (r = 0.9985) respectively.

The pharmacokinetics parameters evaluating for blood sample are summarized in Table 8. The mean concentration profile of DPAAPA and paracetamol are elucidated in Figure 10. The concentration-time profiles of DPAAPA and paracetamol clearly indicates decline in serum concentrations at different sampling time. The area under the curve AUC_{0-t} and $AUC_{0-\infty}$ values are calculated from the curve of concentration of the drug versus time (Figure 11). The concentration (Cp last) of DPAAPA and paracetamol at time t and constant (K' = slope) are obtained from the semi-log curve of concentration of the drug versus time t (Figure 12). The area under the first moment curve $AUMC_{0-\infty}$ values are obtained from the curve of concentration of drug and time vs. time t (Figure 13). From the semi-log curve of concentration of drug and time (Cp*T last) vs. time is used to obtain the concentration of DPAAPA and paracetamol at time t (Figure 14). The value of the AUC is indicative of the extent of drug absorption. DPAAPA shows 88.75 ± 9.39 µg hr mL^{-1} AUC values as compared to standard paracetamol (20.51 ± 1.79 µg hr mL^{-1}) that indicates DPAAPA shows significantly more absorption than paracetamol. Further, Tmax indicates the rate of absorption of the drug. DPAAPA was found to be higher rate of absorption, because of higher Tmax (2.0 hr) values than paracetamol (1 hr). Moreover, Cmax value, indicative of the intensity of therapeutic and toxic response, was found to be higher in DPAAPA (Cmax = 10.25 ± 0.44 µg mL^{-1}) as compared to paracetamol (Cmax = 5.18 ± 0.57 µg mL^{-1}). The mean residence time (MRT) is indicative of average total time drug molecules of giving dose remain in the body. The MRT were found to be 11.32 ± 1.86 hrs for DPAAPA that were significantly higher than the paracetamol (3.95 ± 0.16 hrs). The elimination rate constant (Kel) of DPAAPA (0.09 ± 0.01 hr^{-1}) showed significantly differ than paracetamol (0.25±0.01 hr^{-1}). The results of the elimination rate constant (Kel) indicate that DPAAPA is removed from the body of rat with lower rate than paracetamol. The half life study expose that the time required for the concentration of drug to reach half of its original value. The elimination half life ($t_{1/2}$) of DPAAPA (7.84 ± 1.29 hr) was found to be significantly higher than paracetamol (2.74 ± 0.11 hr). The apparent volume distribution (Vss/F) of DPAAPA (2.63 ± 0.73 L kg^{-1}) was found to be significantly lower than paracetamol (38.30 ± 2.13 L kg^{-1}). The apparent volume distribution study indicates that the synthesized compound DPAAPA is less distributed in rat body as compared to paracetamol. The oral clearance (CL/F) of DPAAPA was found to be 0.33 ± 0.04 L hr^{-1} kg^{-1}. The oral clearance (CL/F) values were significantly lower in DPAAPA as compared to the paracetamol (7.43±0.89 L hr^{-1} kg^{-1}). The results of oral clearance indicated that the synthesized compound DPAAPA is slowly clear from the plasma as compared to the paracetamol. The relative bioavailability in terms of $AUC_{0-\infty}$ of DPAAPA and was found to be 4.33. The results suggest that DPAAPA has lower bioavailability as compared to the previous reported bioavailability of paracetamol (60-90%) [36].

4. Figure

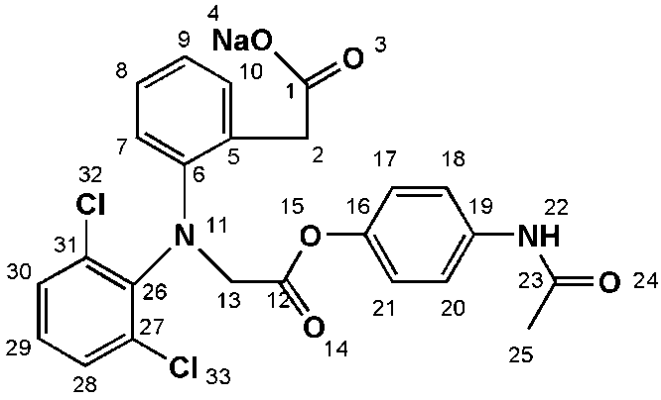
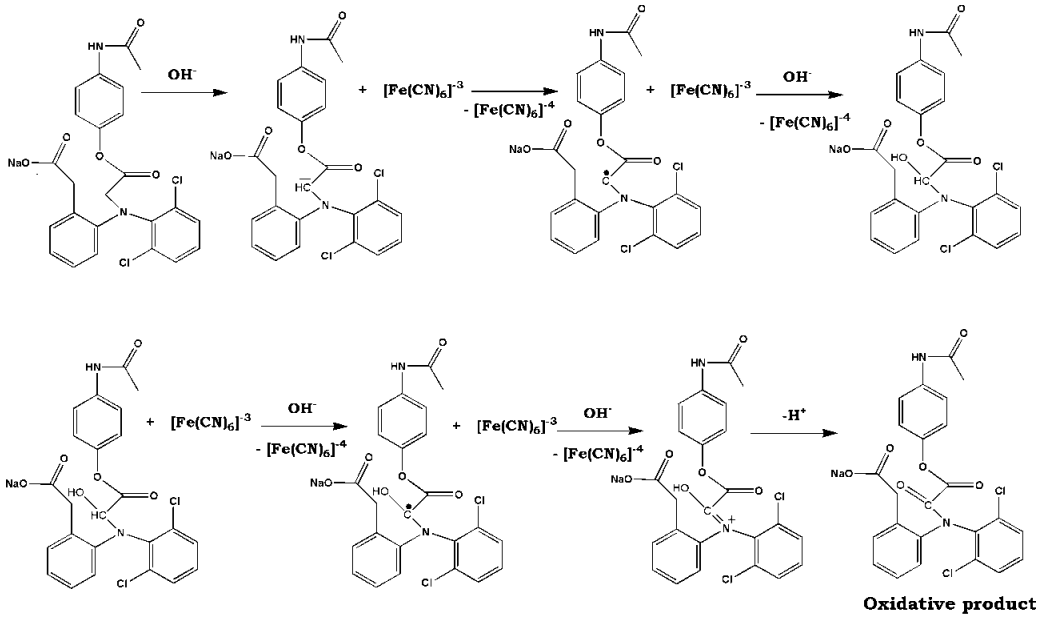


Fig 1: Chemical structure of Sodium [2-[2, 6-dichlorophenyl [(4-amino n-acetyl) phenyl acetate]-amino] phenyl acetate] (DPAAPA).



Scheme 1.1: Formation of oxidative product between DPAAPA and $K_3[Fe(CN)_6]$.

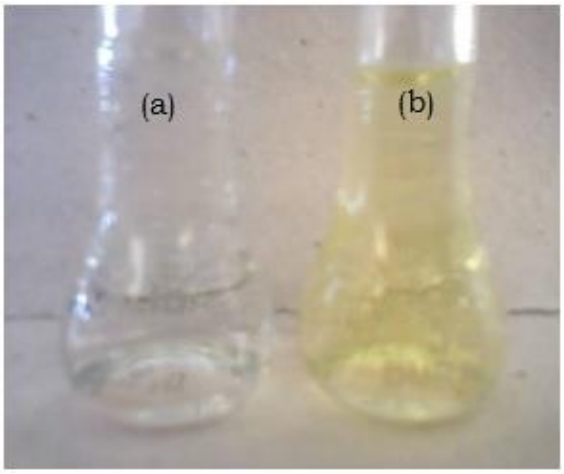


Fig 2: Oxidative product formed yellow color: (a) Colorless solution as a blank and (b) Yellow colored of oxidative product of DPAAPA with $K_3[Fe(CN)_6]$.

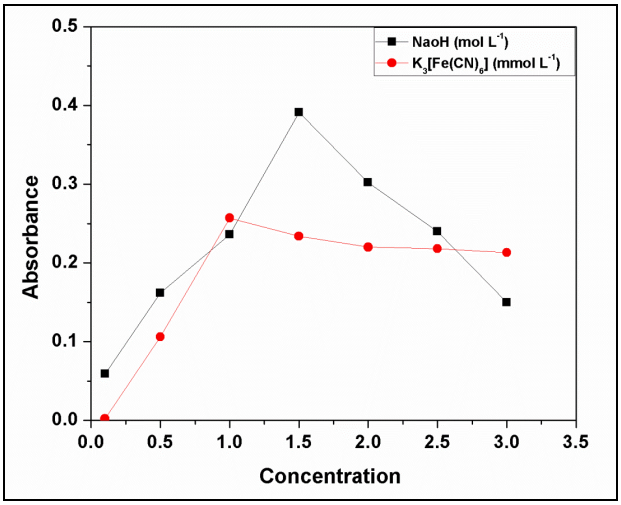


Fig 4: Effect of reagent concentrations on formation of oxidative product of DPAAPA.

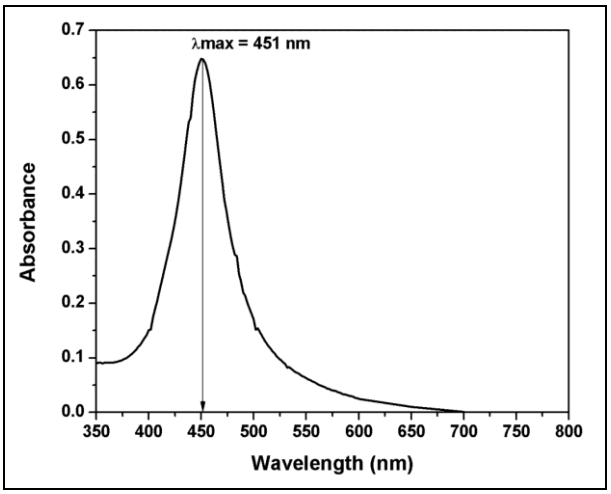


Fig 3: Absorption spectrum of the yellow colored oxidative product between DPAAPA and $K_3[Fe(CN)_6]$.

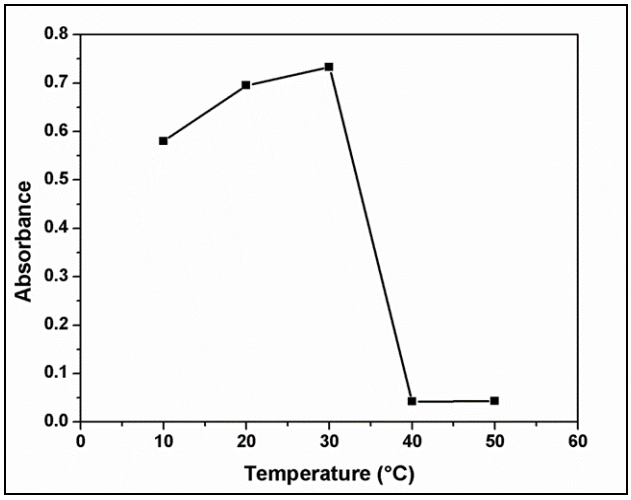


Fig 5: Optimization of reaction temperature of formation of oxidative product of DPAAPA.

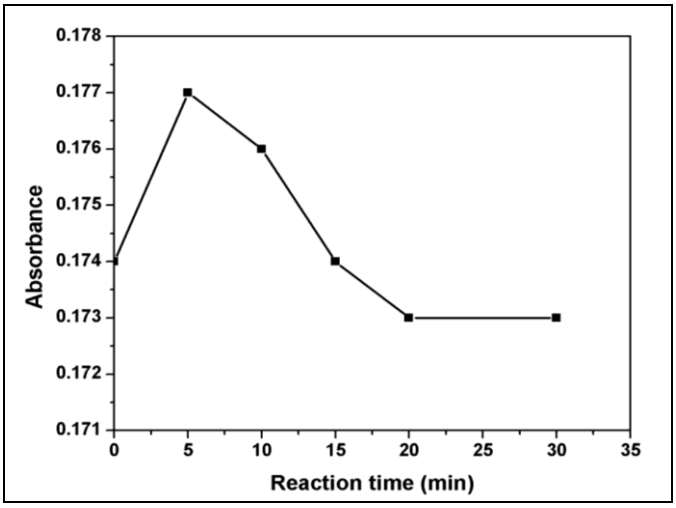


Fig 6: Optimization of reaction time for formation of oxidative product of DPAAPA.

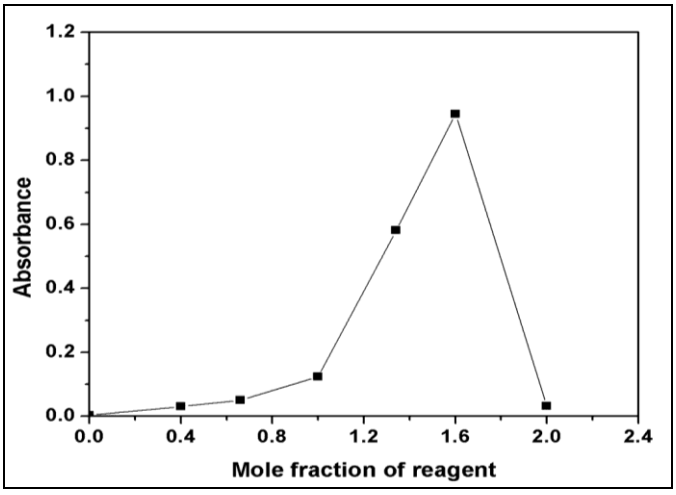


Fig 7: Stoichiometric ratio determination for the formation of oxidative product of DPAAPA.

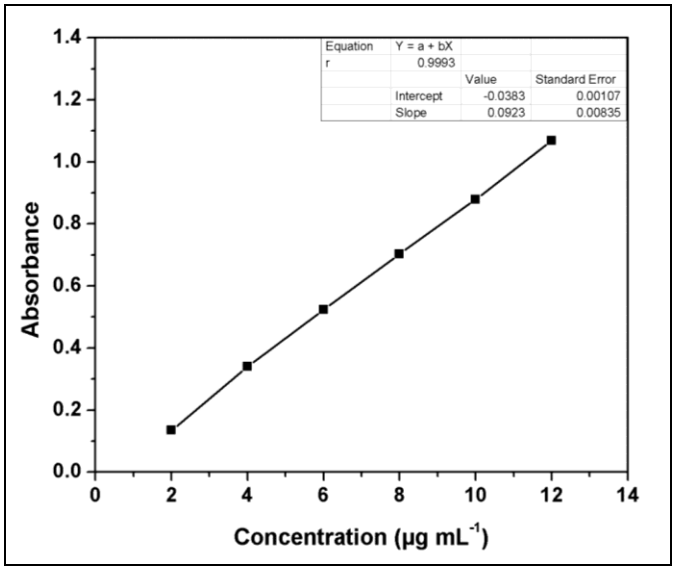


Fig 8: The calibration curve of DPAAPA.

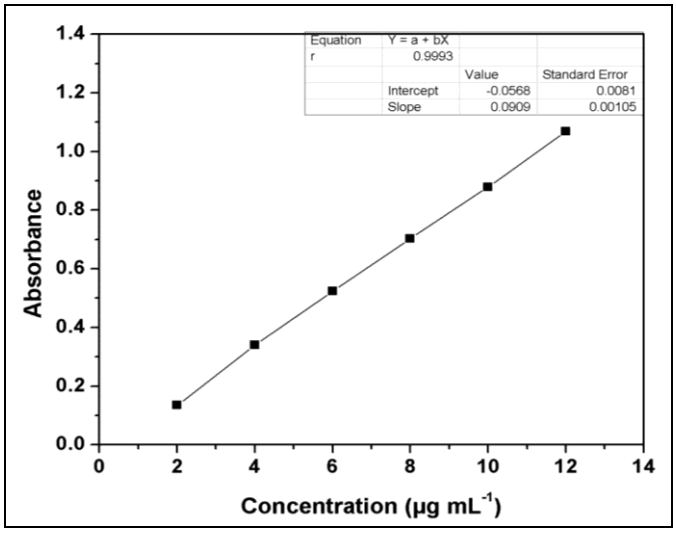


Fig 9: The calibration curve of DPAAPA in serum sample.

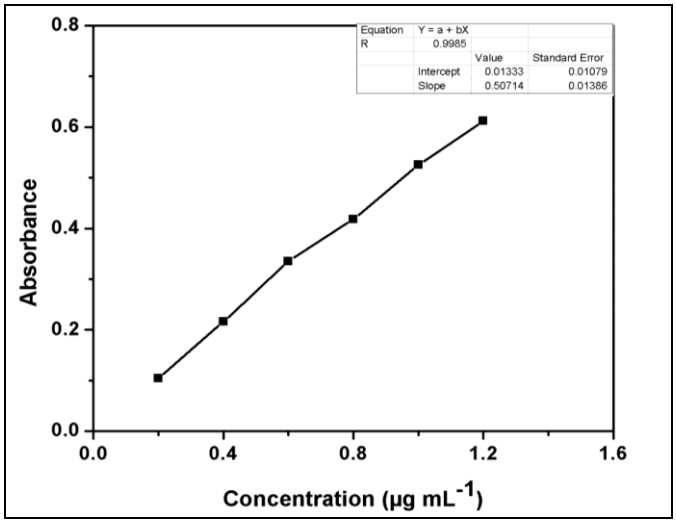


Fig 10: Calibration curve of paracetamol in serum.

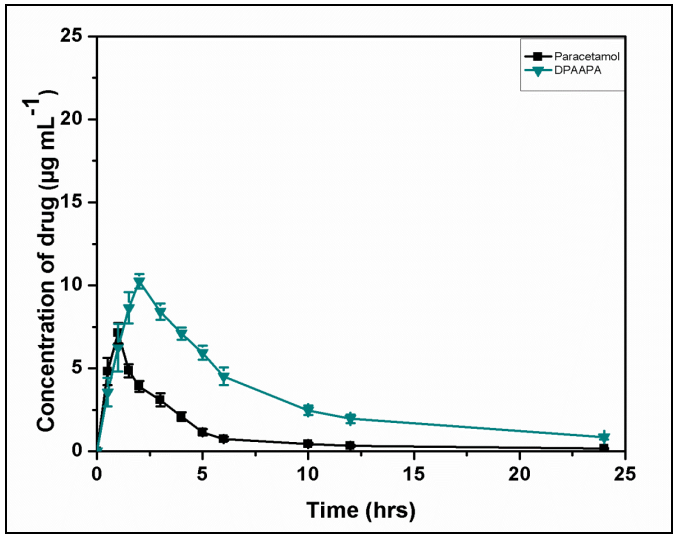


Fig 11: Mean drug concentration vs. time curves in rat after oral administration of 100 and 500 mg kg^{-1} of DPAAPA and paracetamol.

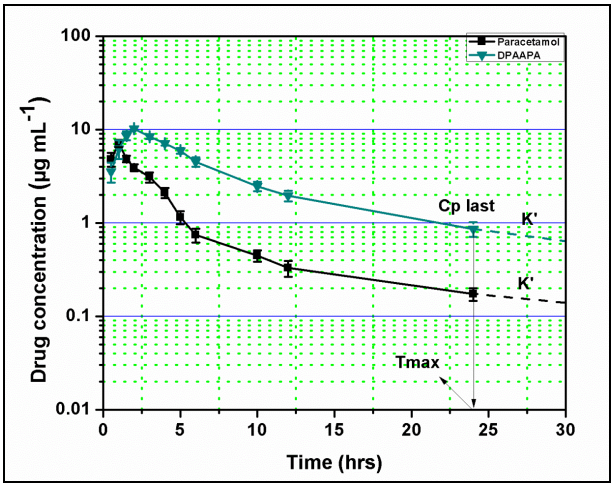


Fig 12: Semi-log curves of mean drug concentration vs. time after oral administration of 100 and 500 mg kg^{-1} of DPAAPA and paracetamol in rat.

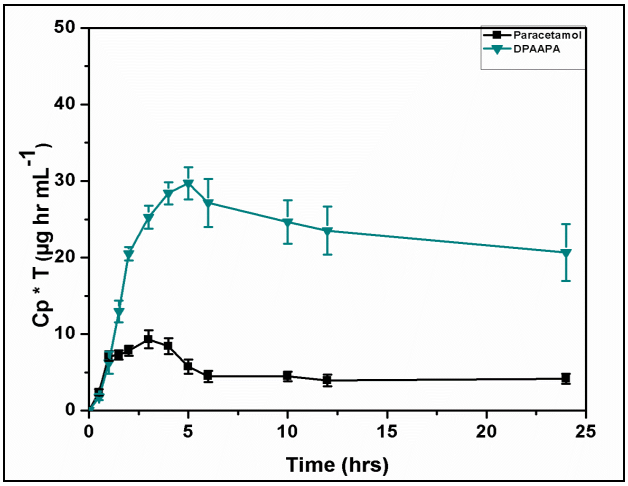


Fig 13: The area under the first moment curves after oral administration of 100 and 500 mg kg^{-1} of DPAAPA and paracetamol in rat.

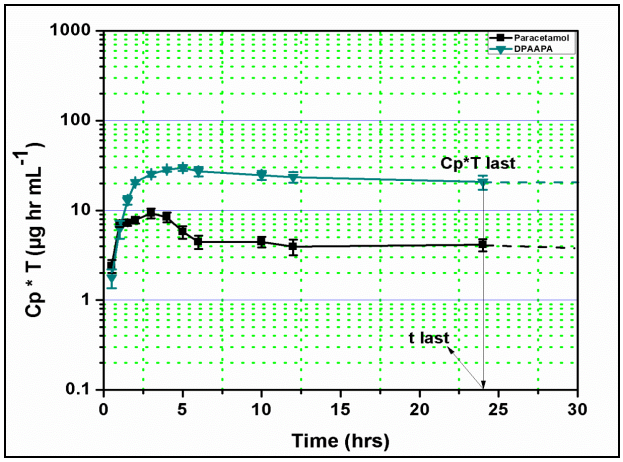


Fig 14: The area under the first moment semi-log curves after oral administration of 100 and 500 mg kg^{-1} of DPAAPA and paracetamol in rat.

5. Tables

Table 1: The effect of diluting solvents.

Sr. No	Solvents	Absorbance
1.	Acetonitrile	0.566
2.	Methanol	0.236
3.	Ethanol	0.151
4.	Propanol	0.208
5.	Butanol	0.604
6.	Water	0.740

Table 2: Selected analytical parameters obtained with optimization experiments.

Parameters	Results
Λmax	451 nm
Linear response (µg mL^{-1})	2-12
Sandell's sensitivity (µg cm^{-2})	1.495×10^{-4}
Regression equation	Y= 0.0923b–0.0382
Slope	0.0923
Intercept	-0.0382
Correlation coefficient	0.9993
Molar absorptivity (L mol^{-1} cm^{-1})	4.2837×10^{4}
LOD (µg mL^{-1})	0.32
LOQ (µg mL^{-1})	0.97
95% confidence limit	Y = 0.0923b±0.0083-0.0382±0.0010

Table3: Results of Intra-day and inter-day precision and accuracy of the method.

Spiked drug concentration of DPAAPA in blood sample (µg mL^{-1})	Intra-day			Inter-day		
	Mean found concentration (µg mL^{-1})	Accuracy %	Precision (%RSD)	Mean found concentration (µg mL^{-1})	Accuracy %	Precision (%RSD)
1.25	1.24±0.01	99.20±1.43	1.44	1.24±0.04	99.20±1.32	1.33
2.50	2.53±0.02	101.20±0.75	0.74	2.49±0.01	99.60±0.56	0.56
3.75	3.77±0.01	100.53±1.69	1.68	3.79±0.03	101.07±0.81	0.80

Table 4: Robustness of the proposed method.

Concentration (µg mL^{-1})	Parameters	Absorbance	SD (n=3)	%RSD
	Wavelength (nm)			
5	450	0.450 ±0.08	0.002	0.44
	451	0.454±0.05		
	452	0.452±0.06		
10	450	0.878±0.02	0.001	0.11
	451	0.879±0.08		
	452	0.880±0.06		
10	Temperature (˚C)			
	28	0.731±0.01	0.002	0.27
	30	0.733±0.07		
	32	0.729±0.04		
10	Reaction time (min)			
	2	0.174±0.06	0.002	0.87
	5	0.177±0.10		
	8	0.176±0.09		

Where, n = Mean value of three determinations

Table 5: Ruggedness of the proposed method.

Concentration (µg mL^{-1})	Analyst	Absorbance	SD (n=3)	%RSD
5.0	I	0.454±0.07	0.0028	0.63
	II	0.450±0.09		
10.0	I	0.879±0.05	0.0014	0.16
	II	0.881±0.07		
2.5	Blood (m=6)	0.17	0.56	0.56

Where, n = Mean value of three determinations, m= six consecutive days

Table 6: Stability of DPAAPA in blood samples (n=3).

	Spiked drug concentration (µg mL^{-1})	Intra-day Accuracy %	Bench-top Stability	Long-term Stability
	1.25	99.20±1.43	98.13±3.27	96.80±1.23
Blood	2.50	101.20±0.75	99.54±1.74	97.33±2.12
	3.75	100.53±1.69	101.61±0.92	99.17±1.48

Table 7: Effect of common interferents on the determination of 10.0 µg mL^{-1} DPAAPA.

Coexisting substance	Ratio of Coexisting substance to DPAAPA	% relative error
Ca^{+2} (Cl^-)	1:75	5.1
Na^+ (Cl^-)	1:150	2.4
K^+ (Cl^-)	1:75	0.9
Mg^{+2} (Cl^-)	1:0.4	3.4
Zn^{+2} (Cl^-)	1:0.4	0.1
Fe^{+2} (Cl^-)	1:0.4	2.9
L-Alanine	1:75	2.3
Glycine	1:25	1.4
Tyrosine	1:25	2.7
Uric acid	1:0.025	-0.7
Paracetamol	1:1	2.7
L-ascorbic acid	1:1	3.1

Table 8: The pharmacokinetics parameters (Mean ± SD) of DPAAPA and paracetamol (blood sample)

Pharmacokinetic parameter	Paracetamol	DPAAPA
Tmax (hr)	1.0±0.00*	2.0±0.00*
Cmax (µg mL^{-1})	5.18±0.57*	10.25±0.44*
MRT (hr)	3.95±0.16*	11.32±1.86*
Kel (hr^{-1})	0.25±0.01*	0.09±0.01*
$t_{1/2}$ (hr)	2.74±0.11*	7.84±1.29*
$AUC_{(0-\infty)}$ (µg hr mL^{-1})	20.51±1.79*	88.75±9.39*
$AUMC_{(0-\infty)}$ (µg hr^2 mL^{-1})	80.97±6.72*	1009.87±237.03*
CL/F (L hr^{-1} kg^{-1})	7.43±0.89*	0.33±0.04*
Vss/F (L kg^{-1})	38.30±2.13*	2.63±0.73*
F	-	4.33

The observations are mean ± SEM (n=4). Significance relative to control values: (*) for P<0.5 (ANOVA followed by Student's t-test).

6. Conclusion

The proposed spectrophotometric method is suitable for the determination of DPAAPA in blood samples. The chemical system developed for DPAAPA could be used as a very sensitive and selective determination of DPAAPA. It is a simple and precise procedure which requires inexpensive reagents and not need of highly sophisticated instruments like, HPLC and LC. The proposed method can be used for rapid and reliable routine analysis and preclinical study of DPAAPA.

7. Acknowledgement

The authors are thankful to Vidyabharti Trust College of Pharmacy, Umrakh, Bardoli, Gujarat, India for providing experimental and other facilities.

8. References

1. Moller P, Sindet-Pedersen S, Petersen C, Juhl G, Dillenschneider A, Skoglund L. Onset of acetaminophen analgesia: comparison of oral and intravenous routes after third molar surgery. Br. J Anaesthesia. 2005; 94(5):642-648.
2. Flower RJ, Vane JR. Inhibition of prostaglan¬din synthetase in brain explains the antipyretic activity of paracetamol (4-acetamidophenol). Nature. 1972; 240(5381):410-411.
3. Brunton LL, Chabner BA, Knollmann BC. Goodman & Gilman: The Pharmacological Basis of Therapeutics. 12th ed. New York: Mac Graw-Hill. 2011; pp. 982-983.
4. Rang HP, Dale MM, Ritter JM. Flower. Pharmacology. 6th ed. London: Churchill Livingstone; 2007; pp. 226-229.
5. Van de Straat R, Bijloo GJ, Vermeulen NP. Paracetamol, 3-monoalkyl- and 3,5-dialkyl-substituted derivatives. Antioxidant activity and relationship between lipid peroxidation and cytotoxicity. Biochem. Pharmacol. 1988; 37(18):3473-3476.
6. Silva, Joseane R, Queiroz, Luana MD, Queiroz, Auriekson N, Souza, *et al.* A Theoretical Study of Paracetamol Acyl-Ether Derivatives. Computational and Theoretical Nanosci. 2011; 8(4):670-675.
7. Iakovou K, Varvaresou A, Bruni G, Massarelli P, Vavayannis A, Kazanis M. Anti -inflammatory and Analgesic Activities of Two Dicyclic Analogues of Acetaminophen and Its Regioisomer 3-Hydroxy acetanilide. Pharm. Chem. J. 2004; 38(5):239-242.
8. Al-Swayeh OA, Futter LE, Clifford RH, Moore PK. Nitro paracetamol exhibits anti-inflammatory and anti-nociceptive activity. Br. J Pharmacol. 2000; 130(7):1453-1456.
9. Ozkay Demir U, Ozkay Y, Can OD. Synthesis and analgesic effects of 2-(2-carboxy phenyl sulfanyl)-N-(4-substitutedphenyl)acetamide derivatives. Med. Chem. Res. 2011; 20(2):152-157.
10. Profire L, Sunel V, Lupascu D, Baican MC, Bibire N, Vasile C. New Theophylline Derivatives with Potential Pharmacological Activity. Farmacia. 2010; 58(2):170-176.
11. Bhavna Shah, Pravin Patil, Hirva Shah. Chemical Modification of Paracetamol and Their antimicrobial and pharmacological evaluation. Int. J Pharm. Res. Bio-Sci. 2014; 3(2):12-31.
12. Sirajuddin, Khaskheli AR, Shah A, Bhanger MI, Niaz A, Mahesar S. Simple spectrophotometric assay of paracetamol in tablets and urine samples. Spectrochimica Acta Part A. 2007; 68(3):747-751.
13. Alkharfy KM, Frye RF. High-performance liquid chromatographic assay for acetaminophen glucuronide in human liver microsomes. J. Chromatogr B: Biomed. Sci. Appl. 2001; 753(2):303-308.
14. Burgot G, Auffret F, Burgot JL. Determination of acetaminophen by thermometric titrimetry. Anal. Chimica. Acta. 1997; 343(1):125-128.
15. Ruiz TP, Lozano CM, Tomás V, Galera R. Migration behavior and separation of acetaminophen and p-aminophenol in capillary zone electrophoresis: Analysis of drugs based on acetaminophen. J Pharm. Biomed. Anal. 2005; 38(1):87-93.
16. Ruengsitagoon W, Liawruangrath S, Townshend A. Flow injection chemiluminescence determination of paracetamol. Talanta. 2006; 69(4):976-983.

17. Ameer B, Greenblatt JD, Divoll M, Abernethy RD, Shargel L. High Performance liquid chromatographic determination of acetaminophen in plasma: single-dose pharmacokinetic studies. J Chromatogr. B: Biomed. Sci. Appl. 1981; 226(1):224-230.
18. Al-Obaidy SS, Po ALW, McKiernan PJ, Glasgow JFT, Millership J. Assay of paracetamol and its metabolites in urine, plasma and saliva of children with chronic liver disease. J Pharm. Biomed. Anal. 1995; 13(8):1033-1039.
19. Vilchez JL, Blanc R, Avidad R, Navalon A. Spectrofluorimetric determination of paracetamol in pharmaceuticals and biological fluids. J Pharm. Biomed. Anal. 1995; 13(9):1119-1125.
20. Shihana F, Dissanayake D, Dargan P, Dawson A. A modified low-cost colorimetric method for paracetamol measurement in plasma. Clin. Toxicol. 2010; 48(1):42-46.
21. ICH Q2B, Validation of Analytical Procedures: Methodology, adopted in 1996, Geneva Q2B, in 2005 incorporated in Q2(R1).
22. United States Pharmacopoeia USP 24/NF 19, US Pharmacopoeial Convention, Inc., USA, 2000, pp. 214-219.
23. Anonymous. Guide for Industry, Bioanalytical Method Validation, U. S. Department of Health and Human Services Food and Drug Administration (FDA), 2001.
24. Bosque-Sendra JM, Almansa-Lopez E, Garcia-Campana AM, Caudros-Rodriguez L. Data analysis in the determination of stoichiometries and stability constants of complexes. Anal. Sci. 2003; 19(10):1431-1439.
25. West JC. Rapid HPLC Analysis of Paracetamol (Acetaminophen) in Blood and Postmortem Viscera. J Anal. Toxicol. 1981; 5(3):118-121.
26. Lhote O, Decoodt A, Moulin, Dufour A. In vivo approach to determine the route of optimal drug absorption in rats. Eur. J. Drug. Met. And Pharmacok. Proceedings of the 4th ed., European Congress of Biopharmaceutics and Pharmakinetics. 1991, pp. 43-46.
27. Causon R. Validation of Chromatographic Methods in Biomedical Analysis Viewpoint and Discussion. J Chromat. B: Biomed. Sci. Appl. 1997; 689(1):175-180.
28. Gibaldi M, Perrier D. Pharmacokinetics. 2nd ed., New York, Marcel-Dekker, 1982, pp. 121-123.
29. Ashik Ullah Md, Mohammad AKA, Sultana R, Eva RK, Mahbub Latif AHM, Hasant A. Pharmacokinetic Study of Amoxicillin Capsule in Healthy angladeshi Subjects using Urinary Excretion Data. Dhaka Univ. J Pharm. Sci. 2009; 8(1):53-59.
30. PHAR 7633 Basic Pharmacokinetics, Chapter 20, Non Compartmental Analysis. 2012, pp. 1-10.
31. Sova P, Mistr A, Kroutil A, Semerád M, Chlubnová H, Hrusková V, Chládková J, Chládek J. A comparative study of pharmacokinetics, urinary excretion and tissue distribution of platinum in rats following a single dose oral administration of two platinum (IV) complexes LA-12(OC-6-43)-bis(acetato)(1-adamantylamine)amminedic -hloroplatinum (IV) and satraplatin (OC-6-43)-bis(acetato) amminedichloro (cyclohexylamine) platinum (IV). Cancer Chemotherapy and Pharmacology. 2011; 67(6):1247-1256.
32. Fajdetic A, Vinter A, Paljetak HC, Padovan J, Jakopovic IP, Kapic S, *et al.* Synthesis, activity and pharmacokinetics of novel antibacterial 15-membered ring macrolones. Eur. J Med. Chem. 2011; 46(8):3388-3397.
33. Issa MM, Nejem RM, EL-Abadla NS, AL-Kholy M, Saleh AA. Novel atomic absorption spectrophometric and rapid spectrophotometric methods for the quantitative of paracetamol in saliva: application to pharmacokinetic studies. Indian J Pharm. Sci. 2008; 70(3):344-350.
34. Abbas K, Marji D. Oxidation of Triethylamine by Ferricyanide Ions in the Presence of Sodium Hydroxide and Potassium Hydroxide. Z. Naturforsch. 2005; 60a:667-671.
35. Al-Subu MM, Amer AA, Jondi WJ, Hanoun M, Musmar MJ. Oxidation of some Alicyclic Amines by Potassium Hexacyanoferrate (III) in Alkaline Medium: A Kinetic and Mechanistic Study. An-Najah Univ. J Res. 2001; 15(1):29-39.
36. Oscier CD, Milner QJ. Peri-operative use of paracetamol. Anaesthesia. 2009; 64(1):65-72.