

Research Article



Pharmacokinetic Studies on Chimeric Conjugate of Biphenylacetic Acid by HPLC in Wistar Rats

Suneela Dhaneshwar*, Anuradha Sutar, Latika Budhalakoti

Department of Pharmaceutical Chemistry, Bharati Vidyapeeth Deemed University, Poona College of Pharmacy, Pune, Maharashtra, India.

*Corresponding author's E-mail: suneeladhaneshwar@rediffmail.com

Accepted on: 15-05-2013; Finalized on: 31-07-2013.

ABSTRACT

We report here *in vitro* and *in vivo* stability and release studies performed on prodrug of biphenylacetic acid with L-tryptophan (BPTP) which was designed as a gastro-protective option for the latter and its bioprecursor fenbufen. Chemical stability of the prodrug was investigated in aqueous buffers in the range of acidic and weakly alkaline pH and *in vivo* release profile was recorded in blood, urine and feces of Wistar rats by HPLC. Prodrug resisted pH- dependent hydrolysis in HCl buffer (PH 1.2) but 12.5% BPA was released in phosphate buffer (pH 7.4). *In vivo* release studies in blood indicated that BPTP bypassed absorption/hydrolysis in stomach while around 21% release of BPA was furnished in small intestine during 2-6h. Release of 31.27 - 68.5 % BPA at 8h and 24h respectively indicated that the intact prodrug might have reached the colon where it was hydrolyzed by N- acyl amidases resulting in the release of BPA that might have entered into systemic circulation after absorption through colonic mucosa. 0.3 -1.5 % BPA was observed in 24h urine and 24h feces while 1.43 % intact BPTP was also seen to be present in 24h feces emphasizing its transport to colon.

Keywords: Amino acid, Fenbufen, Gastro-protective, HPLC, Prodrug, Release kinetics, Rheumatoid arthritis.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by joint swelling, joint tenderness and destruction of synovial joints, leading to severe disability and premature mortality.^{1,2} It is the most common systemic rheumatic disease, affecting approximately 1% of the adult population.³ Chronic pain associated with rheumatic diseases such as rheumatoid arthritis (RA) and osteoarthritis affects millions of people every year. Patients of RA have to depend upon nonsteroidal anti-inflammatory drugs (NSAIDs) to help reduce joint pain, stiffness and swelling throughout their lives. Gastrointestinal upset, irritancy, ulceration and hemorrhage are devastating side effects of NSAID therapy.³

Fenbufen is a bioprecursor prodrug of active metabolite biphenylacetic acid (BPA), the latter being two folds more potent than fenbufen. We have reported synthesis, characterization and pharmacological screening of gastro-protective prodrugs of BPA with L-tryptophan (BPTP) and L-glutamine (BPGU).⁴ Kinetics of degradation and release of active parent molecule from a prodrug are important considerations in prodrug design that prove the bioreversible nature of the covalent linkage between the drug and the promoiety or carrier. So the aim of present study was to investigate *in vitro* and *in vivo* stability/release characteristics of BPTP (Figure 1) in various buffers and blood/ urine/feces of Wistar rats.

MATERIALS AND METHODS

Biphenylacetic acid (BPA) was purchased from Sigma Aldrich, Germany. The HPLC system used to study release kinetics consisted of a pump (Jasco PU 2080); with sampler programmed at 20 µl capacity per injection. The

detector consisted of a UV/VIS (Jasco UV 2075 plus) model operated at a wavelength of 254 nm to monitor the effluent. Data was integrated using Jasco Borwin version 1.5, LC-Net II/ADC system. Thermo Scientific ODS Hypersil (HiQ Sil C₁₈HS; 250mm × 4.6mm, 5.0 µm) from MA, USA. in the reversed phase partition chromatographic condition. The system was used in an air-conditioned HPLC laboratory atmosphere (20 ± 1°C). Mobile phase used was methanol: 5% acetic acid (75: 25) at flow rate 1ml/min. Before analysis, the mobile phase was degassed using sonicator and filtered through a 0.45 membrane filter. Human plasma utilized for method development was purchased from Bharati Vidyapeeth Medical College, Dhankawadi, Pune. The animals were procured from National Toxicological Centre, Pune for the present study. The animal facilities of Department of Pharmacology at Poona College of Pharmacy are approved by Committee for the Purpose of Control and Supervision of Experimental Animals (Reg. No.100/1999/CPCSEA). The protocols for *in vivo* kinetic studies were approved by The Institutional Animal Ethics Committee (IAEC) of Poona College of Pharmacy, Pune.

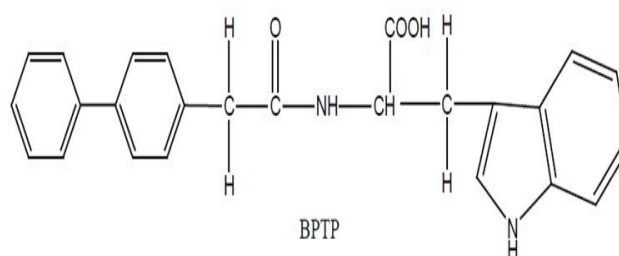


Figure 1: Prodrug of biphenylacetic acid with L-tryptophan



In Vitro Release Studies

pH-dependent activation and stability of BPTP was studied in aqueous buffers (pH 1.2 and pH 7.4) to simulate the pH of stomach and small intestine. The studies were carried out in triplicate. The release of BPA from BPTP was monitored by HPLC and the methods developed were validated as per U.S.P. XXIV edition using different parameters like accuracy, selectivity, sensitivity and reproducibility.

Construction of calibration curve of BPTP in HCl buffer (pH 1.2)

Stock solution of BPTP was prepared by dissolving 10 mg in minimum amount of methanol in a volumetric flask and finally making up the volume to 10 ml with mixture of methanol and HCl buffer (4:6) (1000 µg/ml). 1ml of this solution was transferred to 10 ml volumetric flask and volume was made up to 10 ml using HCl buffer (pH 1.2) (100 µg/ml). This was considered as the stock solution. To each 10 ml volumetric flask, 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 ml of stock solution of BPTP was added and volume was made up by HCl buffer. All prepared dilutions of 10 ml volume were mixed in a vortex mixer for 2 min, filtered through a membrane filter (0.45 µm). The filtrate (20 µL) was injected in the C₁₈ column and eluted with the mobile phase methanol: acetic acid (5%) (75: 25 v/v) at a flow rate of 1.0 ml/min and elute was monitored at wavelength of 254 nm and overlay spectra of all the components were taken by measuring the absorption with a sensitivity of AUFS 0.01. A calibration curve was constructed by plotting concentration versus the peak area in the range 10-60 µg/ml. The equation generated from the calibration curve ($y=6866x-26943$; $R^2 = 0.996$) was used to calculate the concentration of BPTP in the HCl buffer (pH 1.2).

In vitro release in HCl buffer (pH 1.2)

BPTP (10 mg) was introduced in 500 ml of HCl buffer taken in 1000 ml beaker at $37 \pm 1^\circ\text{C}$. The solution was continuously stirred and 1ml aliquot portions were withdrawn at various time intervals over a period of 3 h. The aliquots were estimated on HPLC using methanol: acetic acid (5%) (75:25) as the mobile phase at a flow rate of 1 ml/min at 254 nm with the retention time of 10.1 min for BPTP and min 8.05 for BPA. Chromatogram at 3 h is illustrated in figure 2.

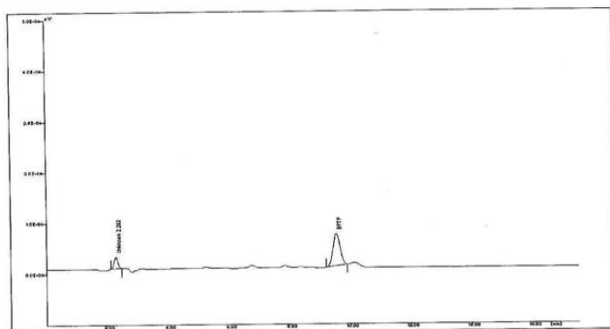


Figure 2: Chromatogram showing stability of BPTP at 3h in HCl buffer (pH 1.2)

Construction of calibration curve of BPTP in phosphate buffer (pH 7.4)

Same procedure as described for calibration in HCl buffer was followed by replacing HCl buffer (pH 1.2) with phosphate buffer (pH 7.4). Calibration curve of BPTP was constructed in the range 10-60 µg/ml in phosphate buffer.

In vitro release in phosphate buffer (pH 7.4)

Same procedure as described above was followed. HCl buffer was replaced by phosphate buffer and release of BPA was studied over a period of 8 h. The kinetics of hydrolysis was monitored by decrease in prodrug concentration and increase of free drug concentration with time. The concentration was calculated from the equation generated from the calibration curve of BPTP in phosphate buffer ($y=6686x-17092$; $R^2=0.998$). The order of reaction and half lives ($t_{1/2}$) were calculated. The equation, $K= (2.303/t) \log (a/a-x)$, where K represents hydrolysis constant, t is the time in min, a is the initial concentration of prodrug, x is the amount of prodrug hydrolyzed and (a-x) is the amount of the prodrug remaining; was used to calculate the rate of hydrolysis. A representative chromatogram showing release of BPA from BPTP at 8 h is illustrated in Figure 3.

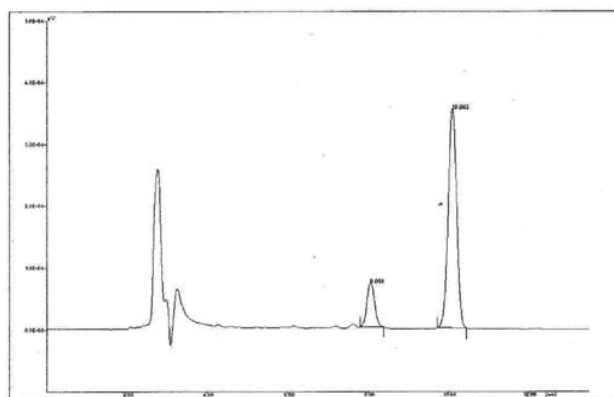


Figure 3: Chromatogram showing release of BPA from BPTP at 8 h in phosphate buffer (pH 7.4)

In Vivo Kinetic Studies

In vivo release kinetics of BPTP was studied in plasma, urine and feces of albino Wistar rats.

Construction of calibration curve of BPA in human plasma

Human plasma was utilized for method development and plotting calibration curves of BPA and BPTP. Stock solution of BPA was prepared by dissolving 10 mg of BPA in minimum amount of methanol (2-3 ml) and then making up the volume to 10 ml with methanol (1000 µg/ml). 0.1 ml of this solution was transferred to 10 ml volumetric flask and volume was made up with methanol (10 µg/ml). To each eppendorf tube (1ml capacity), 0.1 ml plasma and 0.1, 0.2, 0.3, 0.4 and 0.5 ml of the solution of drug (10 µg/ml) and an appropriate amount of methanol was added to make the final volume of 1 ml. All prepared

dilutions of 1 ml volume were mixed in a vortex mixer for 2 min, centrifuged at 5000 rpm for 5 min and filtered through a membrane filter (0.45 µm). The filtrate (20 µl) was injected on the C₁₈ column and eluted with the mobile phase methanol: acetic acid (5%) (80: 20 v/v) at a flow rate of 1 ml/min and elute was monitored at wavelength of 254 nm and overlay spectra of all the components were taken by measuring the absorption with a sensitivity of AUFS 0.01. A calibration curve was constructed by plotting concentration versus the peak area in the range 1-5 µg/ml. The equation generated from the calibration curve ($y=90651x- 6094$; $R^2= 0.995$) was used to calculate the concentration of BPA in the plasma.

Construction of calibration curve of BPTP in human plasma

Stock solution of BPTP was prepared by dissolving 10 mg of BPTP in methanol (2-3 ml) and then making up the volume to 10 ml with methanol (1000 µg/ml). 0.1 ml of this solution was transferred to 10 ml volumetric flask and volume was made up with methanol (10 µg/ml). 1 ml (1000 µg/ml) of this solution was transferred to 10 ml volumetric flask and volume was made up with methanol (100 µg/ml). To each eppendorf tube (1ml capacity), 0.1 ml plasma and 0.2, 0.4, 0.6 and 0.8 ml of the solution of drug (10 µg/ml) were added whereas 0.1 ml (100 µg/ml) was added and an appropriate amount of methanol was added to both the series to make the final volume of 1 ml. Same procedure as described in 4.3.2 was followed. A calibration curve was constructed by plotting concentration versus the peak area in the range 2-10 µg/ml. The equation generated from the calibration curve ($y= 48612x + 33100$; $R^2=0.997$) was used to calculate the concentration of BPTP in the plasma.

In vivo release kinetics of BPTP in rat plasma

A male albino Wistar rat was starved for 24h prior to use and had free access to water. Blood (1ml) was withdrawn by retro-orbital puncture of rat and the reading was considered as zero min reading. Then an oral dose of BPTP (22.4 mg/kg) equimolar to fenbufen, was administered and rat was placed in a metabolic cage in order to collect urine and faeces. Blood samples were collected by retro-orbital puncture from both the eyes of the rat in EDTA coated tubes at an interval of 30 min for the first 1 h. Then subsequently blood was collected on 2h, 4h, 6h, 8h, and 24h. Then EDTA coated tubes were centrifuged at 5,000 rpm at 0-5 °C for 10 min. Supernatant solution of centrifuged blood (0.1 ml) was added to eppendorf tube (1ml capacity) and 0.9 ml methanol was added to it for immediate plasma protein precipitation. All the solutions were then vortexed for 2 min and again centrifuged at 5000 rpm for 10 min at 0-5 °C in order to precipitate other impurities or solid matter present in the biological samples. Then these samples were analyzed by HPLC, using same procedure as mentioned in above using the mobile phase methanol: acetic acid (5%) (80: 20 v/v). The retention time of BPA and BPTP were 7.8 and 10.5 min respectively. The concentration of released BPA and

BPTP were determined from their respective calibration curves in human plasma. A representative chromatogram showing release of BPA from BPTP at 24 h is illustrated in Figure 4.

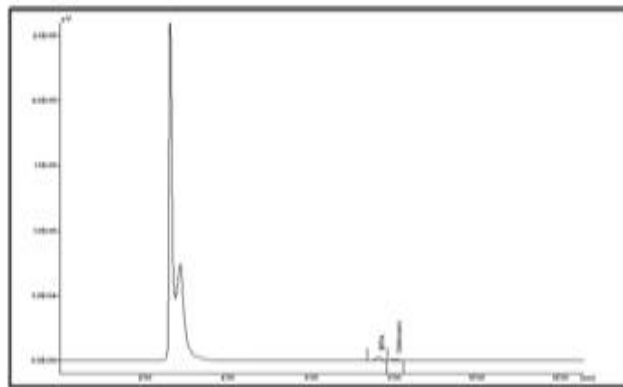


Figure 4: Chromatogram showing release of BPA from BPTP at 24 h in rat blood

Release studies in rat urine and feces

Three rats were kept in metabolic cages and their 24 h urine and feces were collected separately. Same procedure was followed as for release in rat plasma. For calculation of concentration of released BPA in urine and feces, calibration curves of in HCl and phosphate buffers were used respectively. The chromatograms of released BPA in rat urine and feces are shown in Figures 5 and 6 respectively.

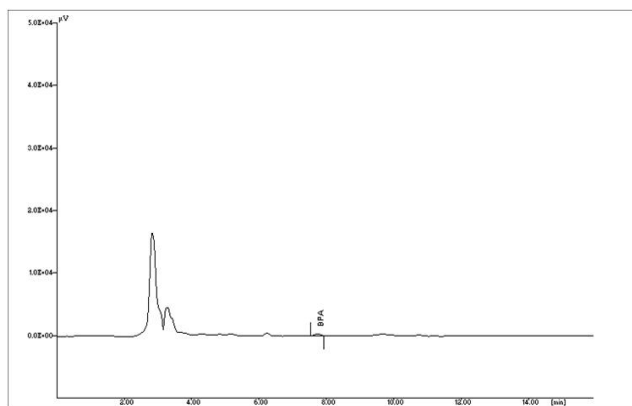


Figure 5: HPLC chromatogram for BPA in rat urine at 24 h

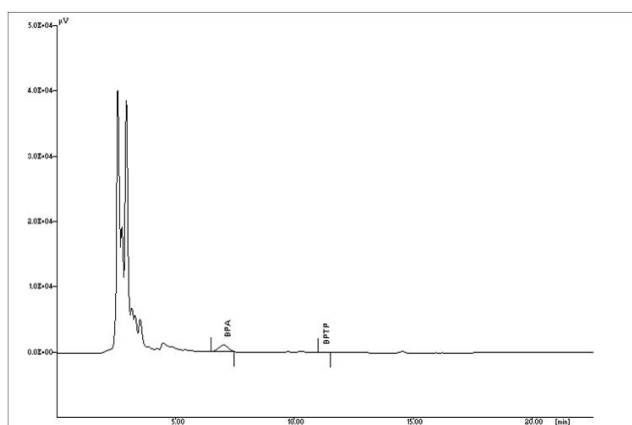


Figure 6: HPLC chromatogram for BPA and BPTP in rat feces at 24 h

RESULTS AND DISCUSSION

BPTP was synthesized with a goal of improving the bioavailability of BPA and minimizing BPA's local irritant effect. Its stability was studied in HCl buffer (pH1.2) and phosphate buffer (pH 7.4) simulating the environment in stomach and small intestine respectively. BPTP was found to be stable in HCl buffer (pH 1.2) till 3h (Figure 2). The course of hydrolysis kinetics for BPTP in phosphate buffer is shown in figure 7 where 12.45 % BPA was released at the end of 8 h. The hydrolysis followed first order kinetics ($t_{1/2}$: 2272.13 min^{-1}).

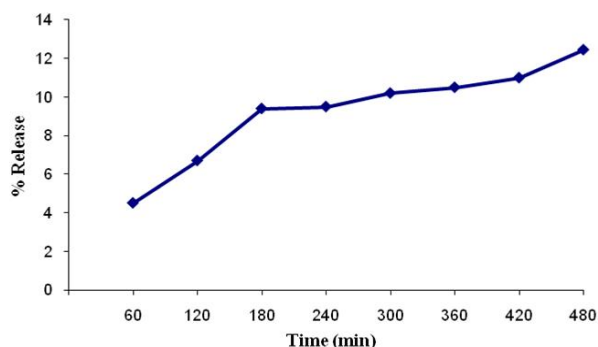


Figure 7: Percent release of BPA from BPTP in phosphate buffer (pH7.4)

First order kinetics; $t_{1/2}$: 2272.13 min; K : $4.258 \times 10^{-4} \pm 0.000245 \text{ sec}^{-1}$

In vivo release kinetics was investigated in blood, feces and urine by employing albino Wistar rats as test animals. The release kinetics of BPTP in rat plasma is shown in figure 8.

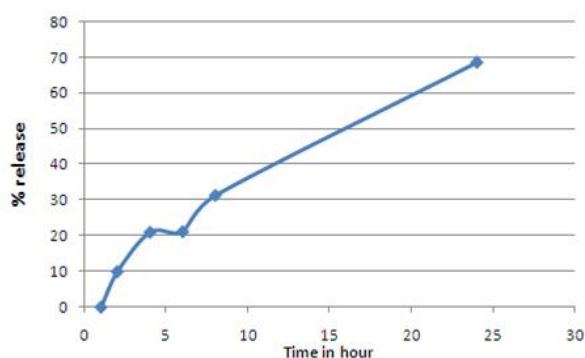


Figure 8: Percent release of BPA from BPTP in rat plasma

First order kinetics; K : $3.19 \times 10^{-5} \pm 0.0000255 \text{ sec}^{-1}$; $t_{1/2}$: 2167.5 min^{-1}

After oral administration, no traces of BPA and BPTP were observed in blood till 60 min which indicates that neither BPTP was hydrolyzed nor absorbed in stomach. First appearance of BPA was observed in blood at 120 min (9.9 %) without any traces of BPTP which indicates that

hydrolysis of BPTP might have started in small intestine. The rate of hydrolysis of BPTP was constant from 4 to 6 h corresponding to 20.95 – 21.13 % release of BPA. Release of 31.27 % and 68.5 % BPA at 8h and 24h respectively indicates that the intact prodrug might have reached the colon where it might have been hydrolyzed by N- acyl amidase resulting in the release of BPA that might have entered into systemic circulation after absorption through colonic mucosa. The hydrolysis followed first order kinetics (Figure 8) with a half life of 2167.5 min^{-1} .

0.3- 1.5 % BPA was observed in urine and feces at 24h, indicating its renal and intestinal excretion while 1.43 % intact BPTP was also present in 24 h feces emphasizing its transport to colon. Approximately 70 % of the administered dose of BPTP was recovered as BPA in blood. The results of *in vivo* studies indicate that conjugation of BPA's carboxyl group with L-tryptophan has enhanced its bioavailability.

CONCLUSION

BPTP was basically designed as a promising gastro-protective alternative to BPA and fenbufen. Stability of BPTP in acidic buffer supported its high gastro-protective effect observed in Rainsford's cold stress model in rats.⁴ Longer duration of analgesia and prolonged anti-inflammatory activity reported by us for BPTP⁴ could be justified on the basis of delayed release of BPA from BPTP as observed during *in vitro* kinetic studies performed in phosphate buffer as well as *in vivo* release kinetics observed rat blood. These findings emphasize the importance and significance of pharmacokinetic studies and their co-relationship with pharmacological activities of prodrugs.

Acknowledgements: Authors wish to thank The All India Council for Technical Education (AICTE) New Delhi, for financial support to carry out this project.

REFERENCES

1. Scott DL, Symons DP, Colton BL, Popper AJ, Long-term outcome of treating rheumatoid arthritis: results after 20 years, *Lancet*, 1, 1987, 1108–1111.
2. Mitchell DM, Spitz PW, Young DY, Bloch DA, McShane DJ, Fries JF, Survival prognosis, and causes of death in rheumatoid arthritis, *Arthritis Rheumatoid*, 29,1986, 706–714.
3. Wolfe F, The natural history of rheumatoid arthritis, *Journal of Rheumatology Supplement* 44, 1996, 13–22.
4. Dhaneshwar SS, Sutar A, Kadam P, Reducing ulcerogenic potential of biphenyl acetic acid: Synthesis and pharmacological evaluation of amino acid conjugates, *Journal of Saudi Chemical Society*, 2012, article in press, DOI:<http://dx.doi.org/10.1016/j.jscs.2012.10.008>

Source of Support: Nil, Conflict of Interest: None.

