Development of Ketoprofen-p-Aminobenzoic Acid Co-Crystal: Formulation, Characterization, Optimization and Evaluation

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Development of Ketoprofen-p-aminobenzoic acid Co-crystal: Formulation, characterization, optimization and evaluation

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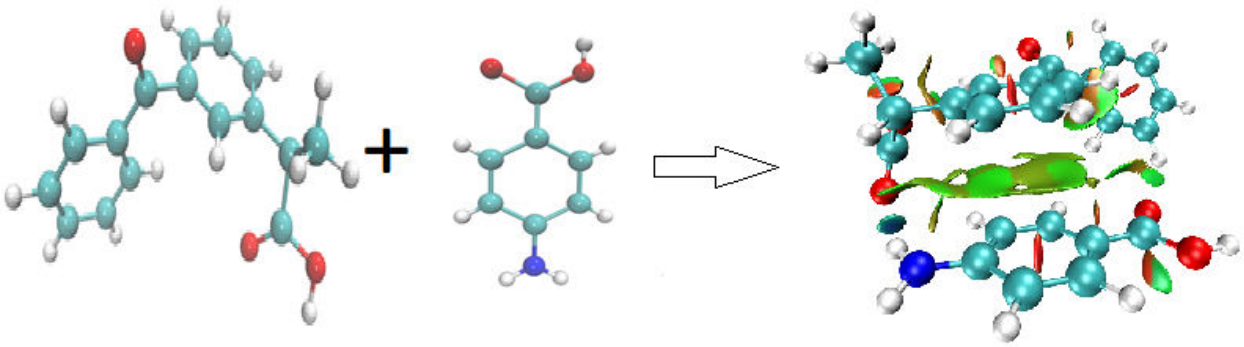
Grapical abstract



ketoprofen p-aminobenzoic acid RDG iosurface of ketoprofen-PABA cocrystal

ABSTRACT

Co-crystal is a promising class of solids that may provide options for improved properties. In the present study, ketoprofen- *p*-aminobenzoic acid (KP-PABA) co-crystal were prepared to sought enhanced solubility and dissolution rate of drug. KP-PABA co-crystal were prepared by solvent evaporation technique employing central composite experimental design, selecting independent variables as concentration of drug and PABA whereas dependent variables were assumed to be solubility and % drug release. The optimized batch as suggested by the experimental design was characterized by FTIR, DSC, XRD, SEM and NMR studies and further, evaluated for *in-vitro* and *in-vivo* anti-inflammatory and analgesic activities. The solubility and % drug release of different batches of co-crystal was found to be between 34.20-60.11 µg/ml and 68.11-93.45%, respectively. Co-crystal containing ketoprofen and PABA in molar ratio (1:1) was found to be optimized formulation batch. Physical characterization by X-ray diffraction spectra and differential scanning calorimetric studies confirms the crystallinity of prepared co-crystal. The half maximal inhibitory concentration (IC_{50}) values for *in-vitro* anti-inflammatory activity comes out to be 34.04 µM for ketoprofen and 4.373µM for optimized formulation, exhibiting almost 8-fold amplification indicating higher anti-inflammatory effect of optimized batch as compared to drug ketoprofen. The results of *in-vivo* anti-inflammatory activity carried out by rat paw edema method revealed that the optimized batch of co-crystal preparation provided a significant % inhibition in paw volume in contrast to standard drug in wistar rats. In this case, a crystalline molecular complex of drug Ketoprofen, that demonstrate poor aqueous solubility, with p-aminobenzoic acid was recognized that further set out an improvement in solubility and also in anti-inflammatory activity of the drug in wistar rats.

KEY WORDS: Ketoprofen. *p*-aminobenzoic acid. Co-crystal. Solubility enhancement. Solvent evaporation.

INTRODUCTION

Co-crystal are described as a multicomponent crystalline material possessing two or more molecules (i.e., drug and coformer) that are connected by noncovalent interactions in the same crystal lattice [1,2]. Co-crystal have the ability to improve the drug dissolution, bioavailability, hygroscopicity, solubility, processability and physical/chemical stability of the compound as compared to active pharmaceutical ingredient (API) [3, 4]. Co-crystallization is an advanced method to improve pharmaceutical solid dosage form. The preference of a method to produce co-crystal is crucial and depends on different factors for example thermal stability, alteration in solubility of coformers and API in particular solvents, tendency to form stable polymorphs or solvates and availability of a sufficient amount of the substance [5]. The selection of suitable coformer for co-crystallization is based on aqueous solubility of coformer, hydrogen bonding, non-covalent bonding, carbon chain length and molecular recognition. The difference between pKa value of API and coformer (*i.e.,* ΔpKa) also depicts the co-crystal formation capability of a coformer with a given API [6, 7]. According to Berry and Steed, salt formation occurs when ΔpKa value remains in close proximity to that of a base however co-crystal will be formed if this value exists close to acid [8]. The formation of co-crystal is formed if multiple H-bonds are formed [9, 10]. Co-crystal of various drugs with different coformers are listed as ketoconazole with *p*-amino benzoic acid and nicotinamide, telmisartan with chitosan, aceclofenac with chitosan, tadalafil with methylparaben, carbamazepine with nicotinamide, piroxicam with PEG 4000, PVP K30, acetazolamide with theophylline and piperazine and 5-Fluorouracil with gentisic acid, 3,4-dihydroxybenzoic acid and 4-aminopyridine etc. that have been synthesized employing different techniques *viz.* solvent evaporation [11], solvent change approach [12, 13], solvent drop grinding [14], solution cooling crystallization, solvent evaporation, melting and cryomilling [15], slow crystallisation [16], liquid-assisted grinding and solvent evaporation [17], solvent-assisted grinding and solution crystallization [18].

Ketoprofen ($C_{16}H_{14}O_3$) belongs to BCS-II, is a propionic acid derivative and can be taken orally to treat inflammatory diseases for instance rheumatoid arthritis, osteoarthritis, musculoskeletal disorders or postoperative pain etc. [19]. The anti-inflammatory property of ketoprofen is due to inhibition of enzyme cyclooxygenase-I and II, thus curtailing the formation of precursors of

prostaglandins and thromboxanes. Several methods of improving the solubility or dissolution rate of ketoprofen have been put forward like soild dispersion [20, 21], liquisolid [22], microemulsion-based gel [23], emulgels [24], nanoparticles [25-27], solid lipid nanoparticles (SLNs) [28], prodrug with pectin [29], micro and nanocomposite with PLGA [30] etc. Although the co-crystal of ketoprofen with conformer nicotinamide [31] are known. *P*-aminobenzoic acid (PABA) as a coformer purposed in this study is a member of the vitamin B complex and is generally recognized as safe (GRAS) [32]. The ketoprofen and PABA were selected on the basis of the pKa rule. Benzoic acid has pKa value of 4.21 whereas pK_a value for ketoprofen is 3.88 and the value of ΔpKa ($pKa_{acid} - pKa_{base}$) is -1.36 and correlate to Bhogala et al., at negative values of ΔpKa, co-crystal development is predictable [33].

In the present piece of research-work, easy and consistent technique of solvent evaporation was employed for composing the ketoprofen-PABA co-crystal seeking improved dissolution of drug. The preparation of co-crystal was accomplished as per central composite experimental design (CCD) by experimental design protocol selecting 2-factors at 3 levels (Design Expert software version 11.0). DSC, FT-IR, XRD, SEM and NMR studies were utilized to characterize the optimized formulation. The optimized formulation was further evaluated for both *in-vitro* and *in-vivo* activities.

MATERIALS AND METHODS

Materials

Ketoprofen (KP) was obtained from Infinity Laboratories Pvt. Ltd (Behra, India). *p*-amino benzoic acid (PABA) was supplied by Central Drug House (P) Ltd., New Delhi. Sodium chloride, acetic acid, carrageenan, ethanol, di-sodium hydrogen orthophosphate, potassium di-hydrogen orthophosphate was procured from Hi-Media lab. Pvt. Ltd. All other chemicals & reagents used in the study were of analytical grade and used as received.

Methods

Synthesis of Ketoprofen - p-amino benzoic acid (KP-PABA) co-crystal

Ketoprofen - *p*-amino benzoic acid (KP-PABA) co-crystal was prepared by the solvent evaporation method as reported earlier [34]. Ketoprofen and PABA were used in stoichiometrically equal ratio and after carefully weighing were dissolved in10mL of acetic acid

in a sealed flask and is kept under continuous stirring on a magnetic stirrer for 2 h while maintaining the temperature at 60 ^{o}C. The clear solution so formed was then filtered through 5µm filter paper and allowed to evaporate slowly at room temperature under a fume hood.

Experimental Design

The preparation of co-crystal using ketoprofen and PABA was optimized using 2-factor, 3 level CCD. The concentration of ketoprofen (254.29-508.58 mg) (X_1) and concentration of PABA (137.14-274.28 mg) (X_2) were designated as formulation variables whereas the % drug release and solubility (µg/ml) were picked as response variables (Table I). Each independent variable was considered at three levels (i.e. -1, 0, and 1).

Solubility studies

Ketoprofen pure drug and each batch of KP-PABA co-crystal formulations containing ketoprofen equivalent to 10 mg was dispersed in 10 ml of distilled water, separately and are kept on continuous shaking for 48 h for equilibration at room temperature (25°C) to determine the solubility of ketoprofen. The obtained solution was filtered by 0.45µm millipore filter paper and the drug content was determined by taking absorbance at 260 nm using *uv-vis* spectrophotometer. The amount of drug was calculated using the calibration curve in water.

Determination of drug content

The different batches of KP-PABA co-crystal formulations containing drug equivalent to 5mg were dissolved separately in 25 ml of phosphate buffer (pH 7.4). The samples were filtered through 0.45µm milipore filters and after appropriate dilution the samples were analyzed by *uv-vis* spectrophotometer at 260nm [35].

Total Drug Content (TDC) was calculated by the following equation.

$$\text{Total drug content (\%)} = \frac{\text{Weight of drug in cocrystal}}{\text{Weight of cocrystal}} \times 100 \qquad (1)$$

***In vitro* drug release profile**

In vitro dissolution studies were performed using USP type II dissolution apparatus. Dissolution studies of pure drug (Ketoprofen) and each batch of KP-PABA co-crystal formulation containing drug equivalent to 20 mg were conducted in 900ml phosphate buffer (pH 7.4) at 37±0.5^{o}C with a

continuous stirring speed of 50 rpm. The powder was dispersed over the dissolution medium. Aliquots of sample (5ml) was withdrawn at different time intervals for 60 minutes and replaced with an equivalent amount of the dissolution medium to retain sink conditions during the experiment. Samples were filtered through 0.45µm milipore filters and the concentration of ketoprofen in the samples was determined by measuring the absorbance of the samples at a wavelength of 260 nm using the *uv–vis* spectrophotometer followed by determination of mechanism of release by fitting the release rate data in various release kinetic models.

Characterization

Fourier Transform Infrared Spectroscopy (FT-IR)

Ketoprofen, PABA and optimized batch of KP-PABA co-crystal formulation were exposed to FT-IR spectroscopy. FTIR spectroscopy was directed by a Perkin-Elmer, Spectrum, US spectrophotometer and the spectrum was documented in the wavelength region of 4000cm^{-1} to 400 cm^{-1} using KBr pellet method. The method involves dispersing of sample in KBr and compressing into disc by applying a pressure of 50 kg/cm^2 in hydraulic press.

Powder X-ray diffraction (PXRD)

The ketoprofen, PABA, and optimized batch of KP-PABA co-crystal formulation powder samples were examined using an X-ray diffractometer (Miniflex 2, Rigaku, Japan) from 0◦ to 80◦ diffraction angle (2Θ). The Miller index (d_{hkl}) signifies direction and plane in the crystal and is determined using Bragg's equation (equation 2).

$$n\lambda=2d_{hkl}Sin\theta \qquad (2)$$

Here λ and n denote the wavelength (1.5418Å) and order (n=1, first order), respectively; θ is the Bragg's angle.

Differential scanning Calorimetry (DSC)

Thermal behavior of ketoprofen, PABA and optimized batch of KP-PABA co-crystal formulation was studied using a DSC (Mettler Toledo, Switzerland), heated the samples at

temperatures within the range of 20-250°C with a scanning rate of 10°C/min in aluminum pans under nitrogen flow at a rate of 50 ml/min.

Scanning electron microscopy (SEM)

The surface morphology and shape of optimized batch of KP-PABA co-crystal formulation was examined using SEM (JSM-6100 scanning microscopy, Japan). The sample (optimized batch) after coated with gold was mounted on aluminium stub containing double adhesive carbon tape. The photographs were observed at acceleration voltage of 10 kV.

Nuclear Magnetic Resonance (NMR) Spectroscopy

The NMR spectra of ketoprofen, PABA and optimized batch of KP-PABA co-crystal formulation after dissolving in DMSO-d_6 were examined using Bruker Avance AV 400 NMR spectrometer (Bruker, Karlsruhe, Germany) to get solution ^{13}C NMR data at a temperature of 293 K using Tetramethylsilane (TMS) as an internal standard [36]. Data was interpreted using Mnova program (Mestrelab Research, Santiago de Compostela, Spain).

Stability studies

The optimized batch of co-crystal was kept for the accelerated stability studies according to ICH guidelines (40 ± 2 ^{o}C and 75 ± 5% RH) for a period of 6 months in a stability chamber. The samples were placed in hermetically sealed vials containing rubber plugs and aluminum bung. The stored co-crystal were taken out after 6 months and evaluated for the drug content (according to the method described in earlier section of drug content, *n=3*) and for any physical changes [13].

Biological evaluation of KP-PABA co-crystal

***In-vitro* anti-inflammatory activity**

Egg albumin denaturation method was employed to evaluate *in-vitro* anti-inflammatory activity of drug ketoprofen and optimized batch of KP-PABA co-crystal [37]. The mixture contained of pure fresh hen's egg albumin (0.2ml), Phosphate buffer saline, pH 7.4 (2.8 ml)) and 2ml of different concentrations of KP-PABA co-crystal formulation (containing ketoprofen equivalent to 10 mg) (125, 250, 500, 750 & 1000 µg/ml in Dimethyl sulfoxide as solvent). After incubation (Caltan, NSW, India) for 15 min at a temp of 37±2 ^{o}C, the temperature of mixture was raised to

70°C. After cooling at room temperature, the absorbance measured at λ_{max} of 260nm. Similar procedure is followed for the model drug ketoprofen containing same concentrations as for the formulation, as a reference or control [38].

The % inhibition of protein denaturation was calculated as per equation 3

$$\% \text{ Inhibition} = \frac{Abs.of\ control - Abs.\ of\ sample}{Abs.of\ control} \text{ X } 100 \qquad (3)$$

The half maximal inhibitory concentration (IC_{50}) values of ketoprofen and optimized formulation was measured by nonlinear regression analysis using Graph Pad Prism 5.0 for Windows.

***In-vivo* carrageenan-induced anti-inflammatory activity**

The protocol with registration no. CPCSEA Reg. no-IAEC/2020/10-18 was approved for animal study by the Animal Ethical Committee, Guru Jambheshwar University of Science and Technology, Hisar, India. Wistar rats (180-210 g) of either sex were divided into three groups comprising of six animals each. Group I (Control treated) with carrageenan was kept as control, Group II (standard Drug) was treated with drug-ketoprofen (10 mg/kg) and Group III (Test compound) was treated with KP-PABA co-crystal (equivalent to ketoprofen 10 mg/kg) body weight administrated orally. 0.1 ml of 1% suspension of carrageenan in normal saline, was administered as subplantar injection in the left hind paw of albino wistar rats, after 1 h after of oral administration of the test materials. The paw volume was measured using vernier caliper at 1, 2, 3, 4, 5 and 6 h after the carrageenan injection [39, 40]. The % inhibition in paw volume was calculated by using equation 4,

$$\% \ inibition = \frac{Vc - Vt}{Vc} \times 100 \qquad (4)$$

Where V_c and V_t is the inflammatory increase in paw volume control group and the inflammatory increase in paw volume in test group respectively [41].

Analgesic activity (Tail flick method)

Analgesic activity was measured by tail flick method using a radiant type analgesiometer. In tail flick method pain was produced by placing the tip of the tail on the heat source. Mice (25-30g) of either sex were divided into three groups (test, standard and control) containing six animals in each group. The tail flick reaction time for each animal was recorded six times before administering the drug and the mean was used as predrug reaction time. A dose of the standard drug (Ketoprofen) and test compound (optimized batch of KP-PABA co-crystal) containing

ketoprofen equivalent to 5 mg/kg of body weight in 0.9% w/v sterile saline was administered to animals. The test and standard group received drug orally while the control group was catered with vehicle only. After administration of the drug, the tail flick reaction time was measured at different time interval as 0, 1, 2, 3, 4 and 5 h [42].

% Analgesic activity (PAA) was calculated by using the equation 5

$$PAA = (T2 - T1)/T1 * 100 \quad (5)$$

Where, T_1 and T_2 are reaction time in seconds before and after treatment with drug, respectively. Data was analyzed by one-way ANOVA method and further followed by Tukey's post-hoc test, statistically and is denoted as P value.

RESULTS

The preparation of co-crystal using ketoprofen and PABA was optimized using 2-factor, 3 level CCD. The concentration of ketoprofen (X_1) and concentration of PABA (X_2) were designated as formulation variables whereas the % drug release and solubility (µg/ml) were specified as response variables Formulation parameters and responses for experimental design for different batches of KP-PABA co-crystal as exhibited in Table 1. The TDC of different batches of KP-PABA co-crystal was observed between 97.01 to 98.92 %. No physical changes were observed during stability studies and even after six months.

Solubility studies

The outcome of solubility (Y_1) and *in-vitro* drug release profile (Y_2) of the KP-PABA co-crystal, formulated according to the experimental design expert protocol is exhibited in Table I. The responses generated were fitted into numerous polynomial models.

Table I. Formulation parameters and responses for experimental design.

Batch	Conc. Of Ketoprofen (mg) (X_1)	Conc. Of PABA (mg) (X_2)	Solubility (µg/ml) (Y_1)	% *In-vitro* drug release in 60 min. (Y_2)	Total drug content (%)
1	508.58	137.14	45.02±0.18	79.47±0.33	97.78±0.13
2	381.43	205.71	53.42±0.3	85.12±0.36	98.42±0.04

3	381.43	274.28	58.14±0.2	85.69±0.22	97.12±0.08
4	381.43	205.71	53.12±0.21	82.94±0.41	97.94±0.06
5	381.43	205.71	55.19±0.19	79.80±0.34	97.91±0.08
6	381.43	137.14	42.32±0.22	69.74±0.8	97.70±0.03
7	508.58	274.28	60.11±0.26	93.45±0.15	98.92±0.02
8	381.43	205.71	52.23±0.27	83.87±0.09	98.06±0.04
9	508.58	205.71	56.63±0.33	85.86±0.096	98.14±0.06
10	254.29	205.71	49.71±0.8	75.90±0.05	97.91±0.03
11	254.29	137.14	34.20±0.9	68.11±0.73	97.78±0.01
12	254.29	274.28	50.16±0.6	83.77±0.72	97.01±0.03
13	381.43	205.71	52.58±0.13	88.11±0.77	98.90±0.06
Ketoprofen			14.94±0.3	22.79±0.56	

All values are expressed as mean ±S.D., n=3.

The responses, solubility (Y_1) and *in-vitro* drug release (Y_2) were fitted best into the quadratic response surface model with none transformation of the data. In different batches of KP-PABA co-crystal solubility values range from 34.20 - 60.11µg/ml and % *in-vitro* drug release range for 68.11 to 93.45%. This upheave in solubility and thus dissolution may be due to formation of soluble complex between ketoprofen and PABA. The solubility of KP−PABA co-crystal (60.11 µg/ml) was higher than that of the ketoprofen (14.94 µg/ml). The reason for this furtherance in solubility is the intermolecular hydrogen bonding between the ketoprofen and PABA and it is surmised that this linkage may result in hydration of co-crystal and thereby, high solubility of the drug is observed from co-crystal.

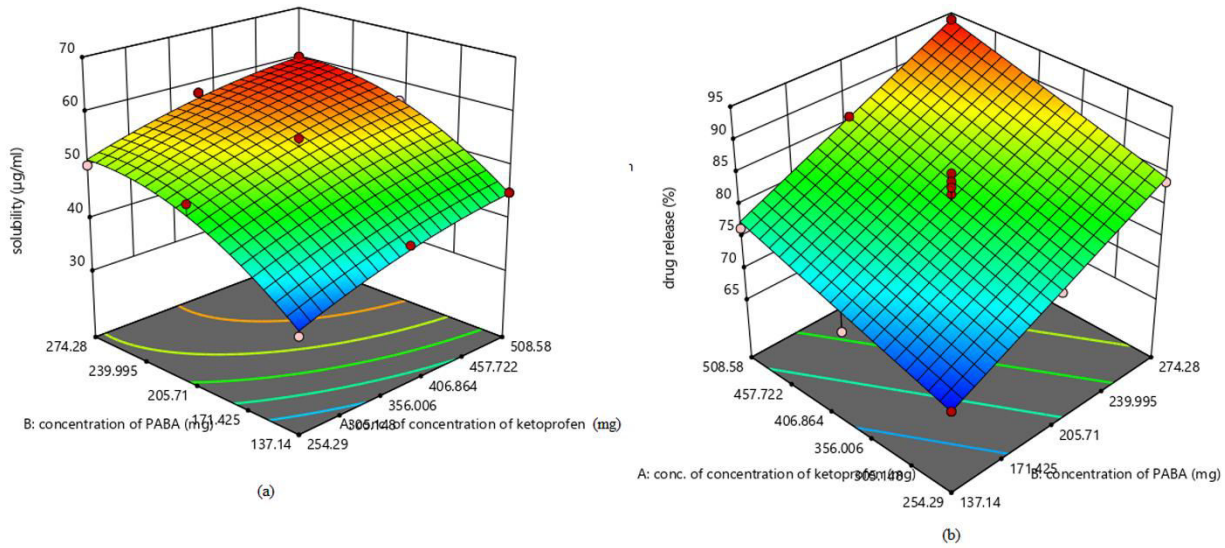


Figure 1. (a, b) Response surface plots displaying effect of concentration of ketoprofen (KP) & PABA on solubility (Y_1) and in-vitro drug release (Y_2).

The polynomial models for the responses solubility (Y_1) and *in-vitro* drug release (Y_2) can also be expressed by the equation (6) and (7), respectively. The synergistic and antagonist effect is explained by the positive and negative coefficient value for specific independent variables in the polynomial equation (6) and (7), respectively. The equations generated revealed that both factors independently exerted a significant effect on the solubility and *in-vitro* drug release. The response surface plot [Figure 1 (a, b)] revealed that solubility and *in-vitro* drug release vary in curvilinear fashion with an increase in the amount of each factor. However, the effect of the concentration of PABA (X_2) seems to be more pronounced as compared with that of concentration of ketoprofen (X_1).

$$Y_1 = 53.68+4.61X_1+7.81X_2-0.275X_1X_2-1.45X_1^2-4.39X_2^2 \quad (6)$$

$$Y_2=42.218+0.037X_1+0.119X_2-0.317X_1 X_2 -3.51X_1^2 -3.31X_2^2 \quad (7)$$

The denouement of ANOVA test on the response surface quadratic model are summarized in Table II demonstrated that model was found significant with lack of fit as non-significant. A good correlation among experimental and predicted responses was specified with a good value of R^2 (>0.9). The adequate precision measuring signal to noise ratio (greater than 4) is desirable. In this design, the ratio of 25.49 (solubility) and 23.05 (drug release) illustrated an adequate signal that let the model to navigate design space. Figure 1 (a&b) display the combined effect of

concentration of ketoprofen & PABA on solubility and drug release. It can be interpreted from the plots that independent and dependent variables exists in a curvilinear correspondence. It is also inferred from the plot that higher level of ketoprofen & PABA results in increase in solubility of formulation.

Table II. Model summary statistics

Model						Lack of Fit	
Response factors (Y)	**F-value**	**Prob.>F**	**R^2**	**Adequate Precision**	**C.V (%)**	**F-value**	**Prob.>F**
Y_1	20.58	<0.0001	0.975	25.49	2.81	2.31	0.218
Y_2	49.19	<0.0001	0.907	23.05	2.88	1.56	0.34

Optimization

The optimization equation 6 and 7, relating the response and independent factors, was fabricated based on a quadratic model. To the responses *i.e.* solubility and *in-vitro* drug release the desirability function was applied with constraints to obtain the higher magnitude of both. In this manner the formulation, containing PABA (274.28 mg) as coformer and drug (508.58 mg) with addition of acetic acid (2%) solution at 60^{o}C, established the maximum desirability, was organized and evaluated.

The optimization of independent variables was done with constraints of maximum solubility and maximum % release. The different set of solutions are provided by the optimization tool in design expert software. The parameters suggested by the design were concentration of ketoprofen (508.58 mg) & concentration of PABA (274.28 mg) that provide co-crystal with solubility of 60 µg/ml (predicted value 60.051 µg/ml) and % release 93.45% (predicted value 93.94%). The closer concordance between observed and predicted values discerned high predictive ability of the model. Figure 2 displays the *in vitro* release profile of ketoprofen as pure drug and the optimized batch of formulation.

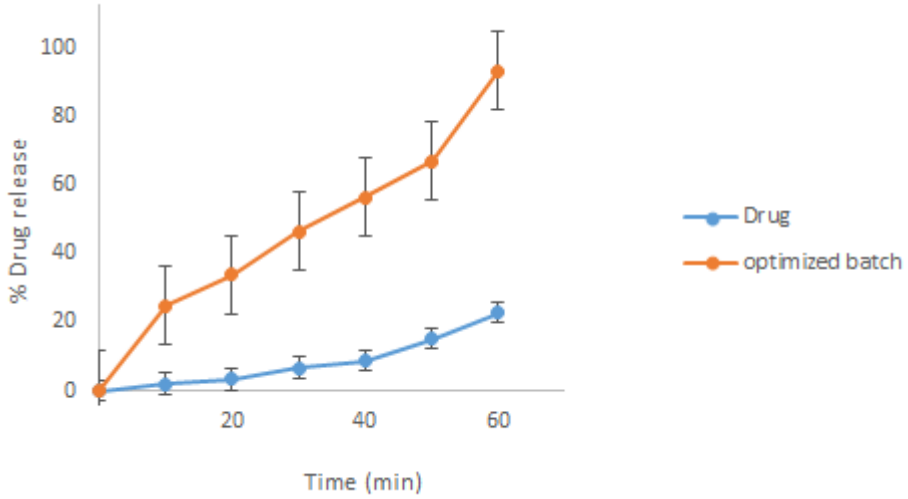


Figure 2. *In-vitro* release profile of pure drug (Ketoprofen) and KP-PABA co-crystal (optimized batch).

The release profile of co-crystal is compared with equivalent concentration of pure drug solution to study release rate kinetics. The release of ketoprofen from co-crystal formulation and drug solution was put into various kinetic models to estimate the kinetics and mechanism of release (Table III). The release rate data for the formulation was found to be fitted best into Zero order model of release kinetics (R^2 = 0.951). Further, the value of 'n' >1.0 (n=1.4) the release exponent of Korsemeyer -Peppas equation indicated that the release of ketoprofen from co-crystal occurs by super-case-2 transport *i.e.* the release occurred by relaxation or erosion of polymer after swelling in biological fluid.

Table III. Modeling and release kinetics of ketoprofen from co-crystal.

Formulation	Zero order R^2	First order R^2	Higuchi R^2	KorsmeyerPeppas R^2	n
KP solution	0.915	0.850	0.949	0.808	1.48
Co-crystal	0.921	0.813	0.932	0.913	1.52

FTIR Spectroscopy

FTIR is an analytical method used to study the changes in the position caused by the vibration modes of the functional groups. The FTIR spectra revealed the shift in characteristic peaks of

drug and coformer due to co-crystal formation involving H bonding between the corresponding functional groups. The spectra of ketoprofen figure 3 (a) showed characteristic absorption band at 2979.27 cm^{-1} due to –CH stretching. The peak appearing at 1697.76 cm^{-1} can be ascribed to -C=O stretching of acid while peak appearing at 1655.77 cm^{-1} is due to -C=O stretching of ketone. The absorption bands at 1598.67 cm^{-1} (-C=C= stretching), 1442.21 cm^{-1} (-C=O stretching of aromatic ring), 1420.59 cm^{-1} (-C-H deformation of CH_3 asymmetrical) and 1370.04 cm^{-1} (-C-H deformation of CH_3 symmetrical) also appeared. The spectra of PABA presented the characteristic absorption band of –NH stretch appearing at 3461.24 cm^{-1}, peak at 3362.37 cm^{-1} is due to –OH stretch, peak at 1681.89 is attributed to -C=C aromatic stretching while peak at 1422cm^{-1} may be ascribed to –C-C aromatic stretching. In FTIR spectra of KP-PABA co-crystal the peak due to -C=O stretching of –COOH group of ketoprofen shifted from 1697.76 cm^{-1} to 1664.16 cm^{-1}. There was shift in –NH stretch from 3362.37 cm^{-1} (PABA) to 3360.95 cm^{-1} (KP-PABA co-crystal). These observations suggested formation of hydrogen bond between amino group of PABA and –COOH group of ketoprofen in KP-PABA co-crystal.

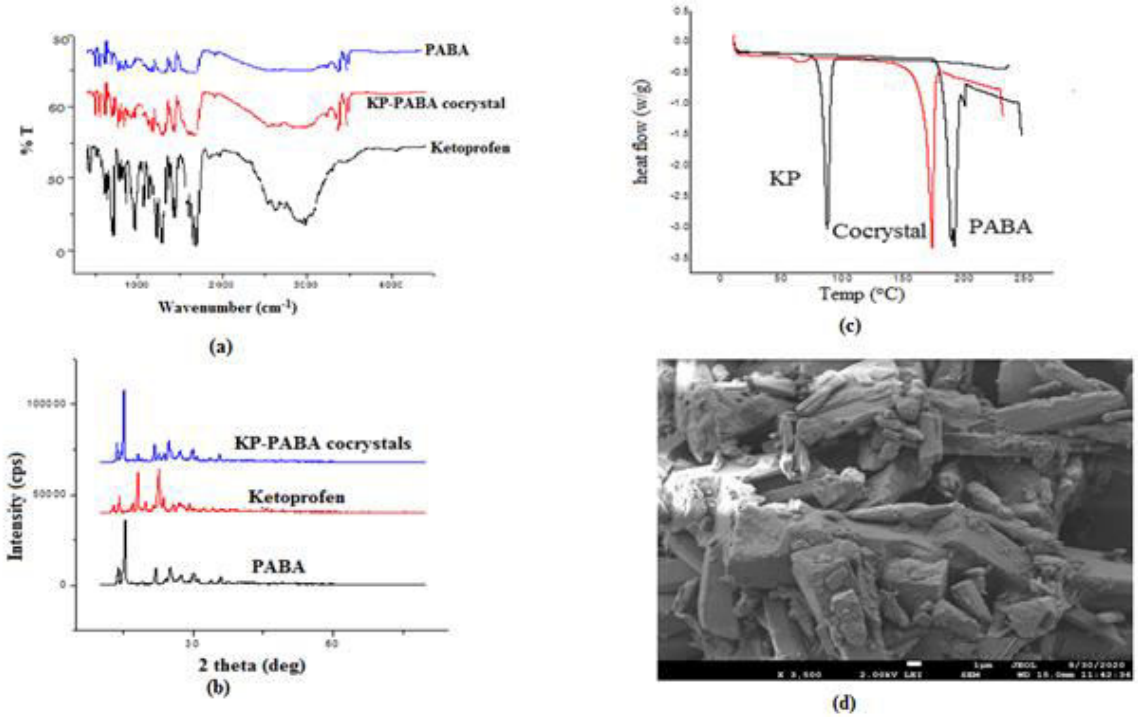


Figure 3. (a) FTIR, (b) XRD, (c) DSC spectra of ketoprofen, PABA and optimized batch of KP-PABA co-crystal, (d) SEM image of optimized batch of KP-PABA co-crystal.

Powder X-ray diffraction (PXRD)

The X-ray diffraction spectra of ketoprofen, PABA and optimized batch of KP-PABA co-crystal is illustrated in figure 3 (b). In diffraction spectra of ketoprofen the peaks (and Miller indices) appeared at 2θ of 12.74 (100), 18.52 (200), 22.85 (211), 24.00 (221), 26.32 (222), 28.920 (300) whereas, in XRD spectra of PABA the peaks (and Miller indices) appeared 15.33(121), 22.67 (211), 24.88 (221), 27.18 (222) and 30.65 (310) that manifested crystalline structure of ketoprofen and PABA respectively. The major diffraction peaks at 2θ, 13.32 (100), 15.90 (121), 18.8 (200), 21.69 (210), 25.45 (221) and 27.19 (222) were also observed is PXRD spectra of co-crystal that portrayed crystalline nature of formulation this indicating that resultant product is also crystalline in nature [43]. Moreover, some characteristic peaks of ketoprofen at 12.74, 22.85, and 28.92 disappeared while some peaks at 24.00 and 26.32 shifted to 25.45 and 27.19, respectively. Besides this, few peaks of PABA at 22.67 and 30.65 disappeared and few peaks at 15.33, 24.88, and 27.18 shifted to 15.90, 25.45, and 27.19, respectively. These changes in the PXRD data of respective co-crystal from ketoprofen and coformer are suggestive of the formation of new forms.

Differential scanning calorimetry (DSC)

DSC thermogram (Figure 3 (c)) of ketoprofen displayed a sharp single peak at 94.5^{o}C that corresponds to its melting point. The peak at 187.8ºC attributed to melting point of PABA. However, in the DSC curve of co-crystal a single endothermic peak appearing at 180^{o}C suggested the presence of a new phase (co-crystal) different from the constitutional components. The modification in the melting point is due to the alteration in the crystal lattice of the drug in the presence of coformer, indicating that certain modification has occurred.

Scanning electron microscopy (SEM)

The SEM image of the co-crystal formulation was depicted in figure 3 (d). The co-crystal showed good crystalline characteristics. This crystalline characteristic of co-crystal was reinforced by the XRD data, as discussed earlier. The pores over the surface of the co-crystal may brace the imbibition of the solvent/biological fluids, consequently, increasing the solubility and bioavaibility of ketoprofen as anticpated.

NMR Spectroscopy

Nuclear magnetic resonance spectroscopy is used to characterize the co-crystal by studying the chemical environment of their nuclei and hydrogen bonding and it also offers valuable information regarding interactions. In the NMR spectra of KP-PABA co-crystal, the carbonyl carbon of ketoprofen corresponding to 196.46 ppm and140.13 ppm has shifted to 196.12 ppm and 142.23 ppm, respectively. A deviation in the carbonyl carbon of carboxylic group in PABA shifted from 173.84 ppm and 152.13 ppm to 175.52 ppm and 153.60 ppm, respectively (Figure 4). This suggests an interaction between amino group of PABA and –COOH group of ketoprofen in KP-PABA co-crystal.

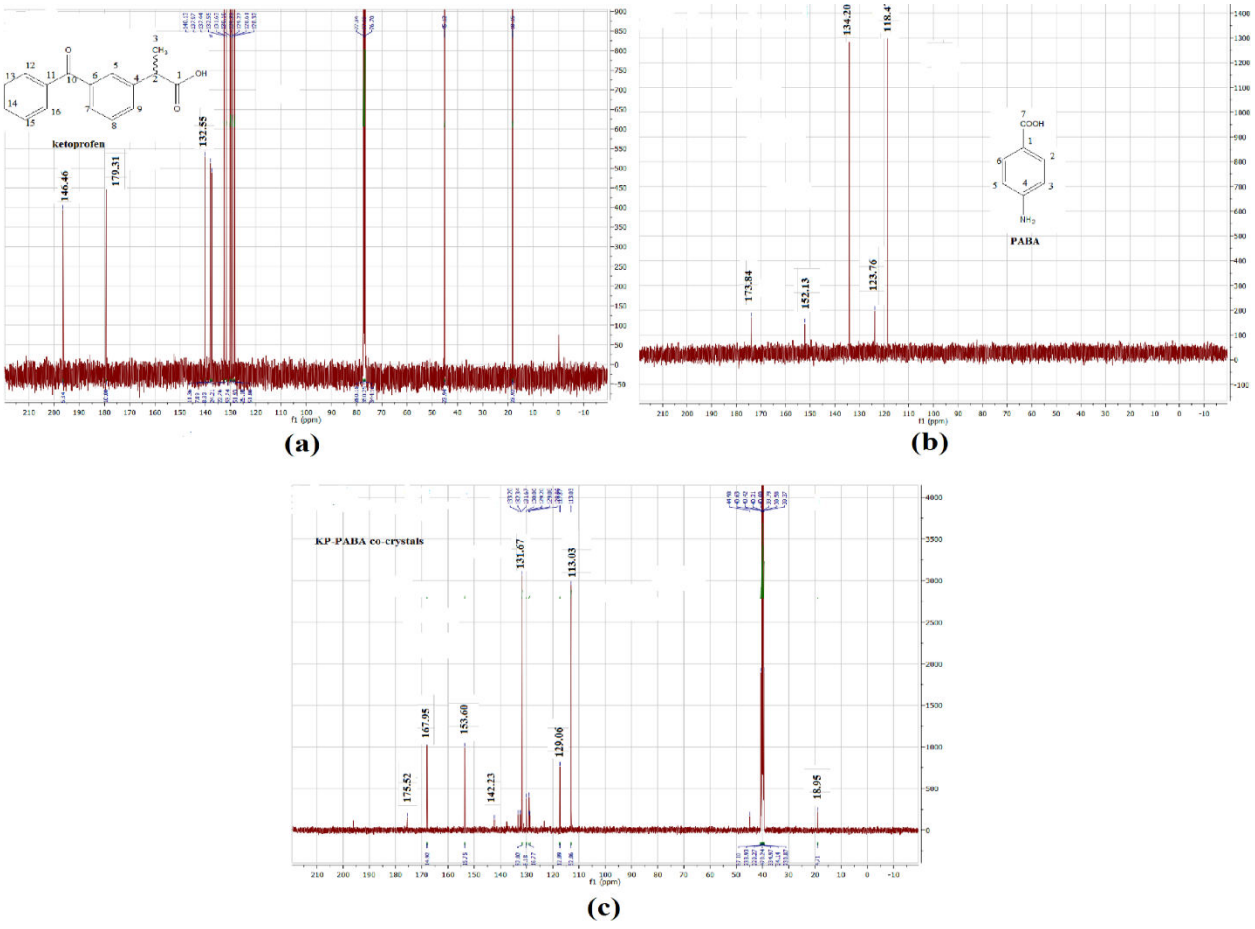


Figure 4. NMR spectroscopy of (a) ketoprofen, (b) PABA and (c) KP-PABA co-crystal.

Non-covalent interactions (NCI) analysis

Co-crystal involve no covalent modification of its constituents and different chemical constituents interact through non covalent interactions [44]. In order to identify the type of interactions that may be present in the cocrystal of ketoprofen and PABA, the NCI analysis was carried out. The chemical structures of ketoprofen and PABA were retrieved from pubchem database [https://pubchem.ncbi.nlm.nih.gov]. The two structures were combined and the system was minimized with molecular mechanics force field. This minimized system was further minimized using new and more accurate PM7 method by taking value of g_{norm} as 0.001 in MOPAC [45]. Different properties of the final minimized cocrystal were calculated by DFT using 6-31G* basis set in B3LYP method of Firefly [46]. The interactions were determined using Multiwfn [47] and VMD [48].

Reduced density gradient (RDG) calculations show that one hydrogen bond is formed between carbonyl oxygen of the ketoprofen and hydrogen atom of amino group of PABA. Aromatic rings of both the molecules stacked against each other through pi-pi stacking interactions. Carbonyl oxygen atom of PABA made weak interactions with hydrogen atoms of terminal phenyl ring of ketoprofen. These interactions are shown in figure 5 (a) below:

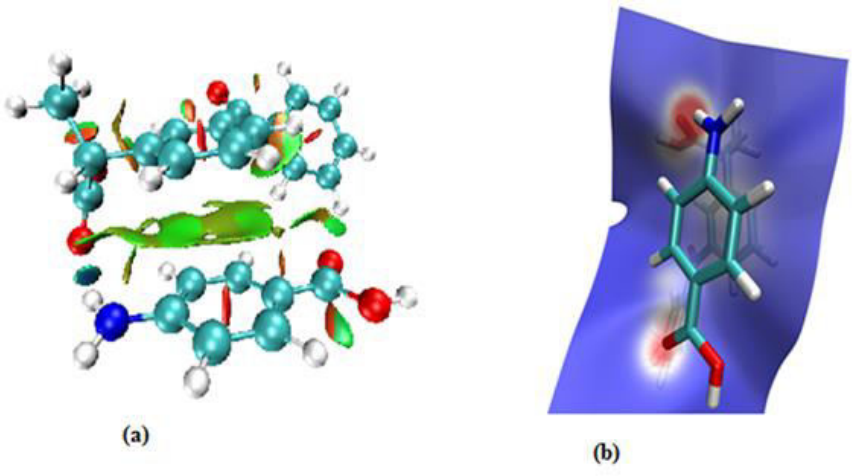


Figure 5. (a) RDG iosurface of ketoprofen-PABA cocrystal. Blue color indicates hydrogen bond and green and brown colors indicate weak Van der Waals interactions, (b) Hirshfeld surface mapped by electron density with promolecular approximation of ketopofen-PABA co-crystal.

Further, Hirshfeld surface mapped by electron density with promolecular approximation analysis was also performed to study the hydrogen bond and Van der waals interactions. Figure 5 (b) shown here clearly indicate the region of high electron density in red color between PABA amino and ketoprofen carbonyl groups. Another one is present between PABA carbonyl and terminal phenyl protons of ketoprofen whereas white region between two aromatic rings depicts lesser electron density means weaker interactions blue color indicates electron free region.

Biological evaluation of KP-PABA co-crystal

In-vitro anti-inflammatory activity

The compared plot of % inhibition of protein denaturation at different concentration of optimized formulation and pure drug is shown in figure 6 (a). The plot displayed that as the concentration of drug is increased, the % inhibition is also got increased whereas the effect is more prominent at lower concentration range of drug. Nonlinear regression analysis was used to calculate the half maximal inhibitory concentration (IC_{50}) values of ketoprofen and optimized formulation. The IC_{50} values were 34.04 µM for ketoprofen and 4.373µM for optimized formulation [Figure 6 (b)], there is almost 8 fold improvement in IC_{50} value. Hence, it is concluded that co-crystal formulation is more active in producing anti-inflammatory response than the drug ketoprofen.

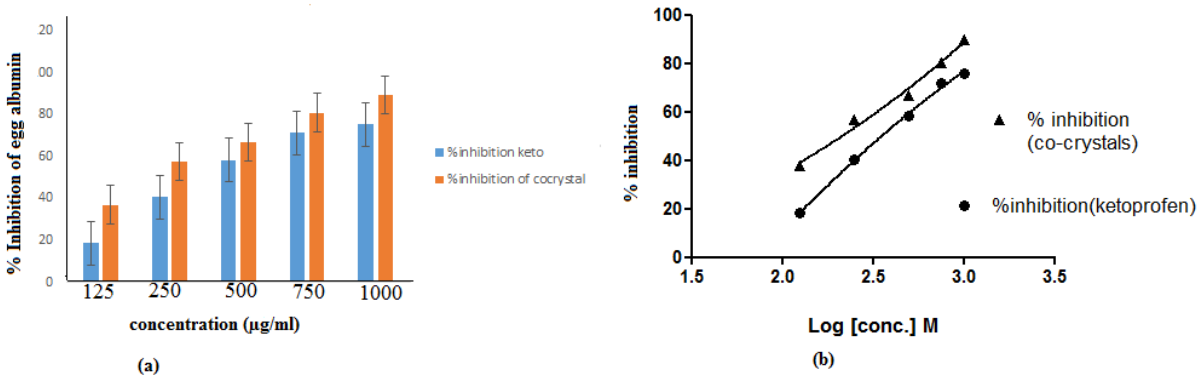


Figure 6. (a) Percent inhibition of ketoprofen and optimized formulation, (b) IC_{50} values calculated using GraphPad Prism 5.0 for Windows.

In-vivo anti-inflammatory activity

Carrageenan induced rat paw edema method was appertained to compare oral efficacy of ketoprofen and optimized batch of KP-PABA co-crystal formulation. The improvement in

activity of ketoprofen and co-crystal formulation was comparatively assesed by the increase in paw volume of control groups. The paw edema volume estimates (before and after drug administration) and % inhibition of edema at different time interval was persuaded and displayed in Table IV. The co-crystal and ketoprofen unveiled inhibition of paw edema as 66.14±0.04% and 58.16±0.03% at the end of 6 h, respectively thus demonstrating quick onset of action by co-crystal in contrast with the pure drug ketoprofen.

Statical Analysis:- Data was compared by ANOVA followed by Tukey's test. The p value is <0.0005 is considered as significant.

Table IV. Effect of ketoprofen and ketoprofen-PABA formulation on the paw edema induced by carrageenan in Wistar rats.

Time (min)	**Paw volume (mm)**			**Inibition (%)**	
	Control	**Pure drug**	**Co-crystal**	**Pure drug**	**Co-crystal**
60	4.22±0.01	4.06±0.02*	4.01±0.01*	3.73±0.03	4.77±0.05#
120	4.55±0.12	3.83±0.08*	3.71±0.14*	15.78±0.01	18.48±0.04#
180	4.90±0.101	3.44±0.04*	3.17±0.07*	30.61±0.03	35.45±0.22#
240	5.37±0.189	3.21±0.03*	3.02±0.03*	40.29±0.10	43.78±0.04#
300	6.02±0.16	3.048±0.042*	2.863±0.61*	49.31±0.18	59.64±0.17#
360	6.44±0.03	2.671±0.01*	2.138±0.03*	58.16±0.03	66.14±0.04#

*All values are expressed as mean ± S.D., n=6; *Significant ($p<0.05$) compared to control; #Significant ($p<0.05$) compared to pure drug (ketoprofen).*

Analgesic activity

The results of the % analgesic activity (PAA) of test, reference and control group are shown in Table 5. The PAA (equation 5) was comparatively evaluated for KP-PABA co-crystal and pure drug based on its potential to suppress pain. KP-PABA co-crystal showed significant effect in enhancing the pain thershold to a certain extent when compared to that of drug (ketoprofen), thus, stipulating that an improvement in solubility further tweaked the pharmacological response.

Table 5. % Analgesic effect of ketoprofen and KP-PABA by tail flick method in mice.

Treatment	PAA					
	0h	**1h**	**2h**	**3h**	**4h**	**5h**
Standard (ketoprofen)	0.03±0.020	31.30±0.71*	35.18±0.12*	42.36±0.17*	50.93±0.14*	71.96±0.17
Test (KP-PABA co-crystal)	0.02±0.011	35.51±0.15*	48.63±0.61*	55.84±0.14#	67.82±0.16#	78.92±0.32
Control (vehicle)	0.01±0.012	0.91±0.19	1.32±0.02	0.83±0.10	1.1±0.20	0.021±0.03

*All values are expressed as mean ± S.D., n=6.*Significant ($p<0.05$) compared to control.#Significant ($p<0.05$) compared to pure drug (ketoprofen).*

CONCLUSION

Ketoprofen-p-aminobenzoic acid co-crystals were prepared by solvent evaporation technique employing central composite experimental design. The optimized batch containing ketoprofen and PABA in molar ratio (1:1) after characterization by FT-IR, XRD, DSC, SEM and NMR studies was further evaluated for *in-vitro* and *in-vivo* anti-inflammatory and analgesic activities. The solubility and % drug release of different batches of co-crystal was found to be between 34.20-60.11 µg/ml and 68.11-93.45%, respectively. The results of *in-vivo* anti-inflammatory activity by rat paw edema method provided a significant % inhibition in paw volume by co-crystal formulation as compared to standard drug in wistar rats whereas *in-vivo* analgesic activity further showed that pain thershold has significantly enhanced. The half maximal inhibitory concentration (IC_{50}) values for *in-vitro* anti-inflammatory activity exhibited almost 8-fold amplification in activity indicating higher anti-inflammatory effect of optimized co-crystal batch as compared to pure drug ketoprofen. On that account, it can be concluded that co-crystallization proves to be a promissing technique for enhancing the solubility characteristics of BCS -II drugs.

CONFLICT OF INTEREST

The authors report no conflicts of interest.

Statement of Animal Rights

The procedure followed for animal handling is in accordance with the protocol as approved for animal study (CPCSEA Reg. no-IAEC/2020/10-18) by the Institutional Animal Ethical Committee, Guru Jambheshwar University of Science and Technology, Hisar, India.

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