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Simultaneous determination of hydrazinocurcumin and phenol red in samples from rat intestinal permeability studies: HPLC method development and validation

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Abstract

Hydrazinocurcumin (HZC) is a patented multiactivity compound and is a potent derivative of curcumin which is not yet explored for further development as formulation and requires the determination of biopharmaceutical suitability of the molecule. Intestinal permeability and log *P* of a compound are two vital biopharmaceutical properties by which, any "hit" molecule proceeds towards NCE (new chemical entity) and govern formulation design of bioactive molecules. In this report, a simple, precise and accurate isocratic reverse phase (RP) liquid chromatography method for simultaneous analysis of HZC and phenol red, for the application in rat *in situ* single-pass intestinal perfusion (SPIP) was developed and validated using FDA bioanalytical guidelines. RP-HPLC method was developed on C-18 column with UV detection at 332 nm for both the compounds. Isocratic run with the mobile phase consisting of organic phase (methanol:acetonitrile:: 50:20 v/v) and water in the ratio of 80:20 v/v provided a short run time of 7 min with resolution of more then two without interference of blank matrix. The working/expected concentration range for HZC and phenol red were 0.5–50 and 2–200 μg/ml. LOQs for HZC and phenol red of the method was found to be 0.167 and 0.271 μg/ml respectively. The validation parameters indicate that method was suitable for the intended purpose. Permeability, considering water flux with the help of non-permeable marker phenol red was calculated to be 0.34×10^{-4} cm/s. Along with other descriptors, log *P* (1.78) and MW (<500) of HZC makes it a potential candidate for oral formulation.

Keywords: Intestinal permeability; Hydrazinocurcumin (HZC); Partition coefficient; Method validation

1. Introduction

Traditionally, drugs are obtained from natural source or synthesized one at a time but after implementation of high throughput screening (HTS) and ultra-high throughput screening (UHTS) rapid identification of large number of biologically active compounds (i.e. "hit") has occurred. In spite of high potency approximately 40% of new chemical entities failed to be new drugs [1]. This development suffers from the serious drawback of very low peroral bioavailability on administration because of poor biopharmaceutical properties, namely solubility and permeability across gastrointestinal tract (GIT) epithelia. The range of oral absorption rate constants for most pharmaceutical molecules is ~40-fold, whereas the range for the solubility of the same compounds is over one-million-folds [2]. Solubility is easily quantifiable *in vitro* and can be manipulated by formulation strategies, however the same is not true in permeability which governs absorption of orally administered drugs. Hence, screening of the drug candidates for the permeability properties is imperative to select right candidate and to prevent the killing of the developing candidate at the end of drug development program. Intestinal drug absorption depends on two major parameters: permeability across the epithelial mucosa and gastrointestinal transit time. Under the assumption of a similar gastrointestinal transit time for different compounds, a difference in mucosal permeability should suggest a difference in potential intestinal absorption *in vivo*. Of all permeability screening methods, *in situ* intestinal perfusion study offers a simple and relevant method of permeability assessment and correlates significantly with the true absorption properties in

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human beings [3]. The single-pass intestinal perfusion (SPIP) is an *in situ* technique wherein the blood supply, innervations and clearance capabilities of the animal remain intact and provides experimental conditions simulating oral administration. This technique has lower sensitivity to pH variation because of a preserved microclimate above the epithelial cells and input of drug can be closely controlled in terms of concentration, pH, osmolality, composition, intestinal region and flow rate [4]. The concentration changes could also be affected by the water content of the intestine, calling for use of volumetric markers that has zero permeability. For this purpose phenol red is co-perfused with drug compound. It was first introduced as non-absorbable marker by Gorham [5].

In recent decades, pharmacological studies on curcumin (*Curcuma longa*, *Zingiberaceae*) suggested the promising biological activities like anti-oxidant, anti-angiogenic, cyclooxygenase inhibitory, anti-inflammatory, anti-microbial, cancer-chemo preventive, anti-leishmanial and anti-HIV [6]. The suppression of multiple biochemical pathways may explain the antitumor effect of curcumin. Due to its low molecular weight and lack of toxicity, curcumin could be ideal candidate as a chemotherapeutic agent. However, studies have demonstrated that its use is limited *in vivo* due to low potency and poor absorption characteristics [6]. This withstanding, curcumin remains a good lead compound for the design of analogs with similar safety profile, but favorable biopharmaceutical properties. Recently we have reported synthesis of HZC along with evaluation for its antioxidant, COX-2 inhibitory and anti-inflammatory activity [7]. *In vivo* and *in vitro* angiogenesis experiments showed that HZC has 30-folds higher inhibitory activity against bovine aortic endothelial cells (BAECS) proliferation [8–10].

In light of above discussion, we report a validated HPLC method for simultaneous determination of novel analogue of curcumin, i.e. HZC and phenol red in intestinal perfusate along with partition behavior at different pH for further pharmaceutical development as a potential molecule.

2. Experimental

2.1. Chemicals

HZC (Fig. 1) was synthesized and purified using a method previously reported by us [7]. The conversion of curcumin to pyrazole derivative was ascertained by absence of C=O peak in the range of 1820–1600 cm^{-1} of FTIR (KBr) spectra. Further, a sharp peak was observed at 3319 cm^{-1} due to N–H stretching which may also be of O–H but sharpness in the peak is due to N–H stretching. Another peak at 3479 cm^{-1} was assigned to the phenolic O–H stretching. N–H bending vibration was observed at 1593 cm^{-1} which further confirmed the presence of pyrazole group. ^{1}H NMR spectrum in $CDCl_3$ (ppm): 7.16 (2H, CH_{phenol}); 6.76 (2H, d); 6.97 (2H, dd); 7.09 (2H, d); 6.95 (2H, d); 6.64 (1H, s); 3.86 (6H, s, OMe) and mass spectrum (M + 1 peak at 366.1 in APCI mode) that complied with true structure of the desired product. Its purity was further evaluated by melting point, TLC and HPLC with photodiode array detector (PDA) (purity ≥97%). Phenol red used as a low permeability marker was obtained from Merck (Mumbai, India) and used without further purification. Hydroxypropyl-β-cyclodextrin (HP-β-CD) (Pharma grade) was purchased form Wacker chemical (Germany). All other reagents were of AR grade obtained from s.d. fine chemicals (Mumbai, India) and used as received. Water purified by reverse osmosis (ELGASTAT, UK) was used for all purposes. HPLC grade solvents from J.T. Baker (USA) were used for drug analysis.

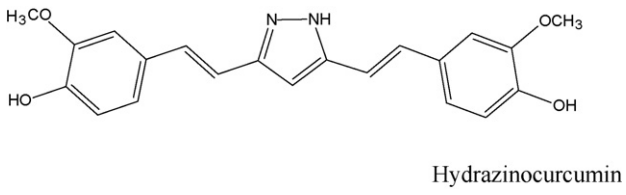

Fig. 1. Chemical structure of hydrazinocurcumin (HZC).

2.2. Animals and legal prerequisites

Male Wistar rats (300–370 g) were used for *in situ* single-pass perfusion. Anesthesia, surgical and perfusion procedure were justified in detail and were approved by the Institutional Animal Ethics Committee (IAEC, NIPER). The study complied with federal and local requirements for animal studies.

2.3. Instrumentation

A liquid chromatographic system (HPLC) (Shimadzu, Japan) with a solvent pump (LC-10AT VP), on line degasser (DGU-14AM), autoinjector (SIL-10AD VP) with temperature controller (CTO-10AS VP column oven) and UV–vis dualwavelength spectrophotometric detector (SPD-10AVP) was used. CLASS-VP software was used for data acquisition, reporting and analysis. The C-18 column used for chromatographic separation was Lichrospher-100 (particle size 5 μm, pore size 10 nm, dimension 4.6 mm × 250 mm) with guard column Lichrospher (Merck, Germany). Osmolality of perfusion solution was determined by vapor pressure osmometer (Wespor, USA), calibrated against various osmolality standards (100, 290, 1000 mOsm/kg).

2.4. Chromatographic conditions

Isocratic chromatographic separation accomplished using mobile phase consisting of organic phase (methanol: acetonitrile:: 50:20 v/v) and water in the ratio of 80:20 v/v respectively. The pH of water was 8.5 (it was adjusted to the value by 0.1N KOH or acetic acid if required). Mobile phase was filtered through a 0.45 μm PVDF membrane (Millipore, India) and degassed by sonication. Freshly prepared mobile phase was run at a flow rate of 0.6 ml/min, column was thermostated at 35 °C and injection volume of 10 μl was made with autoinjector. Effluent was monitored with UV detector at a wavelength of 332 nm which was selected on the basis of UV–vis scan recorded at a double-beam UV-spectrophotometer (Analytik-Jena, Germany). Though the absorption maxima of both the drugs were different, analytical wavelength of 332 nm was selected which

is λ_{max} for HZC, because phenol red is a non-absorbable marker and its concentration is not expected to be too low for accurate quantification.

2.5. Preparation of standard solutions

Primary stock solution was prepared by dissolving HZC and phenol red in methanol (HPLC grade) to produce concentration of 1 and 4 mg/ml respectively in the same solution. This was diluted 20 times to make a secondary stock solution at concentration of 50 μg/ml for HZC and 200 μg/ml for phenol red. From a secondary stock solution six standard solutions of different concentration were prepared by spiking in blank perfusion fluid. 'Blank perfusion' used here and onwards means, the perfusion fluid without drugs perfused by SPIP method as described in Section 2.10.

2.6. Method validation

2.6.1. Selectivity

For selectivity, analysis of blank samples of the biological matrix (blank perfusion) was obtained from six different animals and each was tested for interference and selectivity was ensured at LOQ level separately for both the drugs, i.e. HZC and phenol red. Moreover, purity of the HZC and phenol red peaks were determined using photodiode array (PDA) detector.

2.6.2. Accuracy and precision

Quality control samples were prepared by spiking of secondary stock solution in the blank perfusion fluid. Accuracy was determined at three different concentrations of QC samples (2.5, 15, 40 μg/ml for HZC and 10, 60, 120 μg/ml for phenol red) each in five replicates. Similarly, precision was measured using five determinations per concentration for all QC samples. Intra- and inter-assay precision was measured by determinations at a particular day and also at three consecutives days, respectively. Recovery experiments were not performed because no extraction procedure was involved in sample preparation.

2.6.3. Calibration curve

The concentration range selected was 0.5–50 and 2–200 μg/ml for HZC and phenol red respectively and was chosen on the basis of expected concentration in the study, moreover it can take care of water flux as well and other type of studies required for formulation development. Standard curve was generated with six standard solutions in five replicates and was characterized by its regression coefficient, slope, intercept, detection and quantification limits. Calibration curves thus obtained was used to quantify HZC and phenol red in permeability samples.

2.7. Stability

2.7.1. Freeze and thaw stability

Stability of both the drugs after freeze and thaw cycles was determined at all three QC concentrations in triplicate. The QC samples were frozen at −20 °C for 24 h and then thawed unassisted for next 24 h, this cycle was repeated three times before analysis.

2.7.2. Bench-top stability

Bench-top stability of the drugs in the blank matrix was determined by holding separate QC samples at above three concentrations and in triplicate at room temperature for 24 h. Samples were analyzed thereafter with the same method, and accuracy and precision was calculated.

2.7.3. Stock solution stability

Stability of the stock solution stored at −20 °C for seven days and subsequently for 6 h at room temperature was also determined. Stock solution was spiked in blank perfusion fluid as mentioned above to produce three QC concentrations in triplicate and analyzed against the QC samples prepared from fresh stock solution.

2.8. Partition coefficient

Experimental log *P* values were determined using a modified micro shake-flask method [11]. To overcome the anticipated error in weighing of less quantity, stock solution of 1 mg/ml in methanol was used and 1 ml of this solution was evaporated under vacuum at 25 °C (Maxi dry lyo, Denmark) until constant weight. The residue was dissolved in 2 ml of 1-octanol which was presaturated with corresponding aqueous phase (phosphate buffer pH 3.0 or 7.4 or water). At last, 2 ml of aqueous phase saturated with 1-ocatanol was added and 30 piston strokes were applied to this solution in a 10 ml glass syringe. The resultant dispersion was placed in shaker water bath at 37 °C for two hrs for clear phase separation. Each phase was transferred to a 2.0 ml eppendorf tube and centrifuged at 2500 × *g* for 15 min. Ten microlitres of this solution was transferred into an eppendorf tube and mobile phase was added to make it appropriate for HPLC injection. The concentration of HZC in 1-octanol was determined by validated HPLC method (not published) and retrospective concentration in the aqueous phase was also determined. This experiment was carried out prior to the permeability experiments and the same HPLC method was not found suitable for simultaneous determination of HZC and phenol red.

2.9. Composition of perfusion solution

The perfusion solution consisted of 48 mM NaCl, 5.4 mM KCl, 2.8 mM Na_2HPO_4, 4 mM NaH_2PO_4 and 1 g/l D-glucose. The pH of buffer was adjusted to 7.4 by the 0.1N KOH solution. The final osmolality of the buffer and perfusion solution was kept in the range of 200–250 mOsm/kg as measured by vapor pressure osmometer.

Owing to low solubility, HZC was difficult to formulate into perfusion solution and thus, hydroxyl propyl-β-cyclodextrin (HPβCD) (5% w/v) was used to overcome the solubility barrier [12,13]. Lukewarm Phosphate buffer saline (pH 7.4) consisted 8 g/l NaCl, 1.38 g/l Na_2HPO_4, 0.19 g/l KH_2PO_4 was used to maintain the body temperature by soaking a pad of cotton and

placing it on exposed excised portion. Concentration of HZC and phenol red in the drug containing perfusion solution was kept 18 and 50 μg/ml, respectively. Stability of the perfusion solution for the experimental period was trailed by giving parallel conditions to the perfusion solution except passing it through the intestine and determining the change in drug concentration and osmolality.

2.10. In situ permeation studies

In situ permeation studies were performed according to the reported method [14,15]. Briefly, male Wistar rats were kept in a 12 h light–dark cycle and were fasted 12–18 h, water *ad libitum* before experiment. Rats were anesthetized by intraperitonial injection of thiopental sodium (50 mg/kg) and placed on a heated pad to keep a normal body temperature. Laparotomy was performed after onset of deep anesthesia by making a midline abdominal incision of approximately 5 cm. A 15–20 cm section of the proximal rat jejunum was located, rinsed gently with saline (37 °C) and used as perfusion assembly. Care was taken in handling the small intestine to minimize the surgery in order to maintain an intact blood supply. This segment was rinsed with phosphate buffer saline (10 ml) and perfusion solution was pumped at a flow rate of 0.2 ml/min using syringe pump (Harvard Apparatus PHD 2000 pump, MA, USA). HZC concentration was sufficient enough to avoid precipitation in the lumen during the course of study. Single-pass perfusion procedure was performed to determine the permeability. The entire excised area was covered with an absorbable cotton pad soaked in phosphate buffer saline as mentioned above. Samples were acquired from the distant part of cannulated jejunum in eppendorf tubes at 5 min interval (approximately 1 ml) for 120 min perfusion period, after 20 min of equilibration. The equilibration time prior to sampling was sufficient to washout and to reach initial steady state. Water flux was quantified with the help of concentration change of phenol red. The samples were stored (not more than a day) at −20 °C until analysis where they were thawed, filtered and directly injected into HPLC for simultaneous quantification of HZC and phenol red. The binding possibility of perfusion solution to plastic tubing was mitigated by rinsing it with 8–10 ml of perfusion solution and discarded. The complete study was performed in quadruplet.

3. Results and discussion

HZC, pyrazole analogue of curcumin (Fig. 1), is hydrophobic weak base having pK_a of 5.8 (unpublished data) and solubility of 4–5 μg/ml. Reverse phase chromatography is recommended for separation of hydrophobic compounds however ionic compounds varies in retention time with ionization which in turn depends on the pH of mobile phase. Thus it is recommended for reproducibility that pH of the mobile phase for ionic samples should always be more than ±1 pK_a so that compound either remains almost completely in ionized or unionized form [16]. In this case, ultrapurified water (pH 8.5) was used as pK_a of phenol red is of 7.4.

3.1. Chromatography and specificity

RP-HPLC method was validated for drug specificity (selectivity as per FDA-CDER guideline), linearity, accuracy, precision and stability mainly as per validation guideline of FDA-CDER for bioanalytical method and ICH (Q2B) [17]. Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. The influence of matrix was negligible in samples obtained from all six animals, further the small peaks appearing with blank (blank perfusate) have RT other then the peak of interest. Selectivity for both the compounds was good as can be observed from the value of accuracy and precision in Table 1 at LOQ level in the matrix obtained from six different sources (animals). Specificity of the method was further evaluated by strata of two tests that is separation of compounds and compatibility (chemical interaction). The former was assessed by well-separated peaks of two compounds in HPLC as shown in Fig. 2 with the RT of 2.8 ± 0.2 and 5.1 ± 0.2 min for phenol red and HZC respectively. For compatibility, TLC was used. R_f

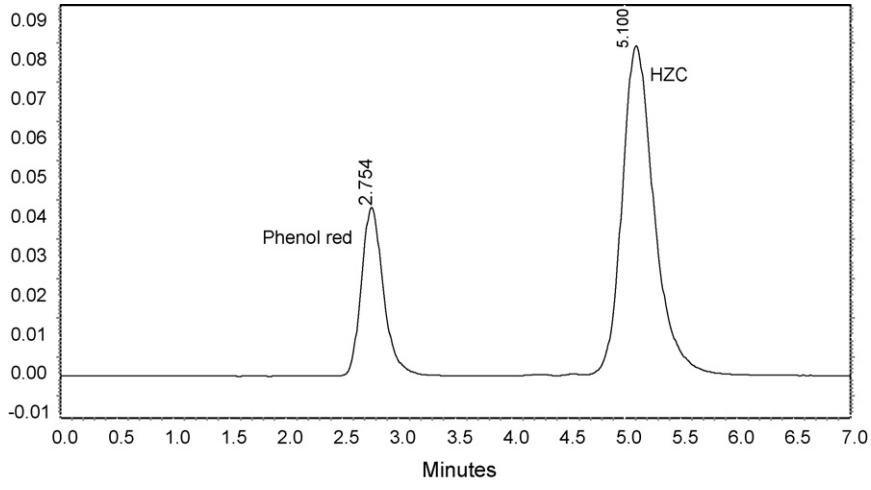

Fig. 2. Representative chromatogram showing well-separated (resolution 2.6) peaks of phenol red and HZC in real samples.

Table 1
HPLC method validation parameters for determination of HZC and Phenol red at 332 nm

Parameter		Hydrazinocurcumin			Phenol red	
Regression equation						
Range (μg/ml)		0.5–50			2–200	
Slope (±S.D.)		157711.89 (±8617.69)			10691.95 (±729.74)	
Intercept (±S.D.)		109874.22 (±1376.74)			70094.87 (±1526.66)	
Validation	Concentration (μg/ml)	Accuracy (%)	%R.S.D. (CV)	Concentration (μg/ml)	Accuracy (%)	%R.S.D. (CV)
LOQ	0.167	102.41	1.38	0.271	97.63	1.04
Precision						
Intra-assay	2.5	95.77	6.99	10	101.12	3.48
	15	98.97	4.81	60	99.41	4.22
	40	98.69	4.19	120	99.25	3.98
Inter-assay	2.5	97.45	2.45	10	97.27	5.60
	15	99.35	2.05	60	98.10	1.92
	40	102.28	3.04	120	100.55	1.44
Stability						
Freeze–thaw	2.5	102.18	1.04	10	98.09	4.52
	15	96.28	8.01	60	104.74	8.02
	40	94.06	1.44	120	104.31	4.52
Bench-top	2.5	99.14	4.61	10	93.42	7.13
	15	97.52	4.82	60	95.54	2.48
	40	104.24	2.99	120	102.11	3.90
Stock solution	2.5	94.57	1.84	10	105.96	0.94
	15	97.42	8.93	60	95.91	8.03
	40	102.44	4.86	120	93.03	1.75

values of 0.75 and 0.65 for HZC and phenol red invalidated the chemical interaction.

Resolution dictate the separation of two adjacent peaks and a value of >1.5 represent complete separation [16]. Resolution between the two peaks was determined by equation $R = 2(t_h - t_p)/(W_h + W_p)$, where t is the retention time and W is base width for the peaks, subscript h and p represent HZC and phenol red. The value obtained for resolution is 2.6, when combined with short run time of 7 min, makes the method adequate for rapid and accurate measurement of concentrations.

3.2. Linearity

The six-point calibration curve for the HZC and phenol red was prepared in the range of 0.5–50 and 2.0–200 μg/ml, respectively. These concentration ranges were selected on the basis of drug concentration anticipated while analyzing the samples. The concentration–peak area relationships were described by simple regression analysis and analytical procedure was in a given range to obtain the test results which were directly proportional to the concentration (amount) of analyte in the defined range for the samples, i.e. correlation coefficient of $r^2 = 0.999$ for the plot of concentration versus response (peak area). Standard curve was constructed and regression parameters, range, slope and intercept were determined as shown in Table 1.

3.3. Accuracy and precision

Accuracy and precision are the closeness between experimental and true value, and closeness amongst experimental values with multiple monitoring of same sample, respectively. Both are the prerequisite for determining the concentration of any unknown sample concentration. Three concentration level quality control (QC) samples ($n = 5$) were used to measure accuracy and precision of the method. The percent recovery with both the drugs, which is an indication of accuracy, did not deviate more than 4% from true value at all three concentration with %R.S.D. of <6.99% for HZC and <5.60% for phenol red. All the values are within the limits prescribed by FDA-CDER guidelines for bioanalytical method validation. %R.S.D. of inter-day and intra-day of slopes for each standard curve obtained within day (intra-assay) and between three consecutive days (inter-assay) proved intermediate precision of inter-day and intra-day validations (Table 1). In conclusion, method was judged to be accurate and precise for intended purpose and minor variation like time and day did not affected the analysis.

3.4. Limits of detection (LOD) and quantitation (LOQ)

LOD and LOQ decide about the sensitivity of the method. LOD is the lowest concentration of the analyte detected by the method; LOQ is the minimum concentration which can be quantified with accuracy and precision. Three methods have been described in ICH guideline for determining LOD and LOQ, based on visual evaluation, signal to noise ratio and on S.D. of response and slope of standard curve. The third method was used for calculating both the numerals. LOD was calculated by equation $3.3\sigma/S$ while for LOQ equation $10\sigma/S$ was used, where σ is S.D. of response from blank and S is the slope of standard curve [17]. LOQ were further confirmed by analyzing

the blank perfusate spiked with both the compounds at the same concentration as described in specificity and selectivity procedures. Determinations at LOQ were found accurate (102.41%) and precise (%R.S.D. 1.38) for HZC and for phenol red corresponding values were 97.63% and 1.04. All the values complied with the specified limits.

3.5. Stability

Perfusion samples were found to be stable in various storage conditions used in the experimental setup. Freeze–thaw, storage at room temperature of samples and stock solution did not affected the stability of the samples thus the method provide quite flexibility for analysis of samples. The accuracy and %R.S.D. values for all stability samples are given in Table 1 and all of them are under the required limits.

3.6. Partition and distribution coefficient

The properties of 1-octanol were thought to resemble those of lipid bilayer membranes. It had therefore been suggested that partition of the drug into 1-octanol simulates, to a certain extent, their ability to passively diffuse across biological membranes. The 1-octanol–water partition coefficient was 1.78 ± 0.09. The $\log D$ (thermodynamic partition coefficient) values between 1-octanol and buffer at pH 3.0 and 7.4 were 1.61 and 1.72 as shown in Fig. 3. S.D. with $\log D$ is relatively higher than log P because of different ionization with buffer. In many cases, correlations between 1-octanol–water distribution coefficients and biological permeation have been reasonably good; however, some studies also report poor or nonexistent correlations thus no definite relationship had been established [18]. However, in combination with other descriptors *in vitro* permeability using cultured monolayer cells, 1-octanol–water partition coefficients had resulted in good correlations.

3.7. Permeability calculation

Age-dependency of the rat intestinal permeability was also considered but as reported earlier altered behavior can be expected only for very young and very old rats and no influence of age on the jejunal permeability in the rat within the age interval of 5–30 weeks has been found and the animals used in the study also falls in the same range [19]. Phenol red was

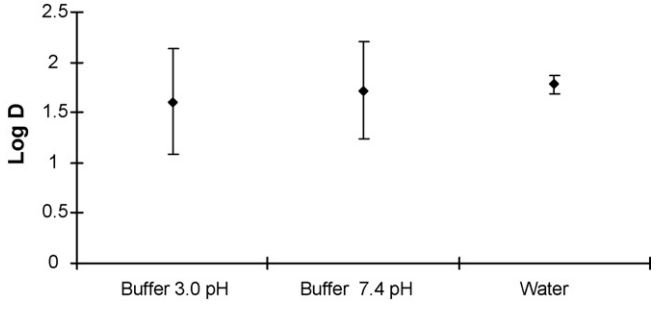

Fig. 3. Partition coefficient of HZC between 1-octanol and buffer at pH 3.0, 7.4 and water.

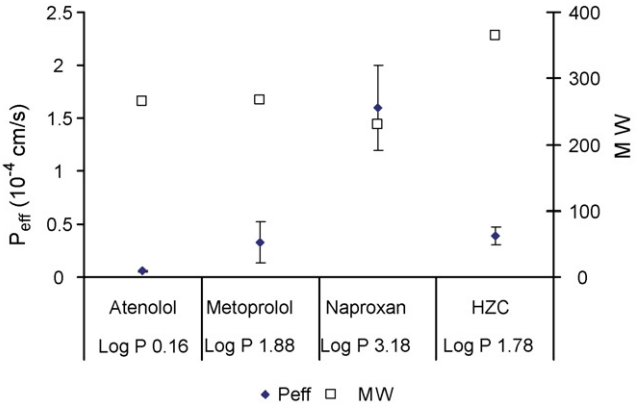

Fig. 4. Comparison of reported effective permeability (P_{eff}) of atenolol, metoprolol, naproxen with that of experimentally calculated HZC [reported P_{eff} and log P taken from Refs. [4,18]].

used as fluid marker and net water flux was calculated with the help of change in its concentration. A negative net water flux (NFW) indicates loss of fluid from the mucosal side (lumen) to the serosal side (blood). A positive net water flux indicates secretion of fluid into the segment [15]. Further, permeability of co-perused phenol red (non absorbable permeability marker) keeps a check on the intra- and inter-individual variability and presence of steady state.

Inlet and outlet samples were not processed except filtration and analyzed for the HZC and phenol red. The difference of the concentration of Phenol red was attributed to the flux of water and corresponding concentration correction of HZC was carried out. Permeabilities were calculated using equations (1) and (2), after correcting the outlet concentration for water flux [14,20,21].

$$P_{\text{eff}} = \frac{Q[(C_{\text{in}}/C_{\text{out (corrected)}}) - 1]}{2\pi r l} \quad (1)$$

$$\text{NWF} = \frac{(1 - [\text{Ph.red}_{(\text{out})}/\text{Ph.red}_{(\text{in})}])}{l} Q_{\text{in}} \quad (2)$$

where Q is the flow rate, C_{in} and C_{out} the respective inlet and outlet concentration, r the radius of intestine (0.21 cm) and l is length of intestine measured after completion of perfusion. Values are indicated in Fig. 4 as mean ± S.D. The term NWF corresponds to the net water flux and were included in the final calculation as water across the intestinal barrier most likely occurs transcellularly instead of paracellulary at isotonic conditions [22].

Converse to the suggestion by US-FDA guidelines for BCS based biowaivers on solid orals [23], the permeability experiment does not employ the low and high permeability internal standards to prove suitability of the method because there are many reports evoking the aptness of *in situ* intestinal perfusion method in Wistar rat [12,21]. Moreover, phenol red permeability can assess any changes to the normal intestinal permeability.

Fig. 5 shows concentration of HZC leaving the intestinal segment at different time intervals. According to biopharmaceutics classification system (BCS), the human jejunal P_{eff} values for high-permeable drugs are considered to be within

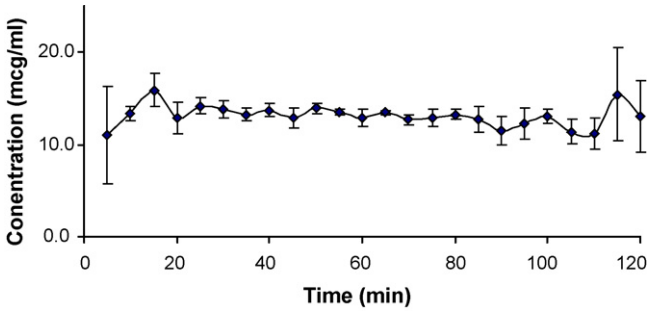

Fig. 5. HZC Concentration leaving the intestinal segment in different time intervals.

1–10 × 10^{-4} cm/s. Moreover, compounds with an average P_{eff} of approximately <0.3 × 10^{-4} cm/s in human intestine are considered to be low permeable compounds. The corresponding estimates as reported by Zakeri-Milani et al. [21] in rats for high and low-permeability are >0.3 × 10^{-4} and <0.2 × 10^{-4} cm/s, respectively. The permeability coefficient of HZC as calculated with the above formula was found to be 0.34 × 10^{-4} ± 0.08 (S.D.) cm/s, thus classified as high permeable drug. This is also supported by the data represented in Fig. 4, of permeability, log *P* along with the molecular weight (MW) of HZC together with some standards of low and high permeability suggested by US-FDA [23]. The MW of all the drugs is below 500. Thus, it is not a limitation for absorption according to Lipinski's rule of 5. Further, atenolol (log *P* = 0.16) is a low permeable class drug and naproxen with log *P* ∼ 3.18 is a highly permeable drug with permeability coefficient of 1.6 × 10^{-4} cm/s. However, the case of HZC resembles with metoprolol standard which is classified as highly permeable drug with log *P* of 0.33 and permeability constant of 0.33 × 10^{-4} cm/s.

4. Conclusion

A simple, rapid, precise, accurate and reproducible HPLC method using C18 column with UV detection was developed and validated for simultaneous determination of HZC in rat *in situ* single-pass intestinal perfusion studies. The validated method is suitable for the intended scientific work and was successfully applied to the rat SPIP studies of HZC which was found to highly permeable.

5. Application of the method

The measurement of intestinal permeability may help the development of new chemical entity (NCE) HZC. Along with the partition coefficient values, the validated HPLC method for permeability for this semisynthetic derivative of Curcumin (*C. longa*, *Zingiberaceae*) can give way to formulation development of a potent anti-oxidant, anti-angiogenic, anti-inflammatory agent (cyclooxigenase inhibitory). Since human *in vivo* studies are difficult and time consuming, this prediction is of considerable interest at an early stage of drug development.

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