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ORIGINAL RESEARCH

High-performance liquid chromatography method development and validation for simultaneous determination of five model compounds, antipyrine, metoprolol, ketoprofen, furosemide and phenol red, as a tool for the standardization of rat *in situ* intestinal permeability studies using timed wavelength detection

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ABSTRACT: A simple, precise, accurate and rugged reversed-phase high-performance liquid chromatography (HPLC) method has been developed and validated for the simultaneous determination of five permeability model compounds, viz. antipyrine, metoprolol, ketoprofen, furosemide and phenol red. The method was intended to standardize rat *in situ* single-pass intestinal perfusion studies to assess the intestinal permeability of drugs in the market as well as new chemical entities. Optimum resolution was achieved by gradient elution on a Symmetry Shield C-18 analytical column with the mobile phase consisting of a mixture of aqueous potassium dihydrogen orthophosphate (pH 5.5; 0.01 M) and methanol at a flow rate of 1.5 mL/min. The retention times of antipyrine, metoprolol, ketoprofen, phenol red and furosemide were about 9, 12, 13, 16 and 17 min, respectively. Data acquisition was carried out using a photo diode array detector in the wavelength range 210–600 nm. Extraction of chromatograms was carried out by timed wavelength. Data obtained in all studies indicated that the method was suitable for the intended purpose. The validated method was found to be linear and precise in the working range. Suitability of storage under various conditions and freeze/thaw impact at cold temperature were established to ensure complete sample recovery without any stability issues. Recovery very close to the spiked amounts indicated that the method was highly accurate and suitable for use on routine basis.

KEYWORDS: antipyrine; ketoprofen; metoprolol; furosemide; phenol red; intestinal permeability

INTRODUCTION

The biopharmaceutical properties, viz. solubility and permeability, are pivotal factors that are considered for entry of new chemical entities (NCEs) into the development phase. It is estimated that about 40% of the NCEs fail to reach the market due to poor biopharmaceutical properties (Prentis *et al.*, 1988). The solubility can be altered by various formulation strategies, making permeability the major rate-controlling step.

Several methods have been given in the literature to assess the permeability (Hidalgo, 2001; Le Ferrec *et al.*, 2005). The rat *in situ* single-pass intestinal perfusion study is commonly performed during drug discovery and is well accepted as a predictive tool to estimate intestinal permeability (Boisset *et al.*, 2000). Permeability values based on rat *in situ* intestinal permeability studies have been used to rank drugs as high/low permeability compounds (USFDA, 2000). In spite of gaining popularity and wide acceptance, *in situ* intestinal perfusion studies may have minor flaws/discrepancies which in turn can lead to inter-laboratory variability of permeability experimentation. The standardization of the experiment with a model drug has therefore been suggested to increase the reliability and suitability of *in situ* intestinal perfusion studies (USFDA, 2000).

A list of 20 model drugs has been reported by the USFDA which may be considered for the standardization

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Abbreviations used: NCE, new chemical entity.

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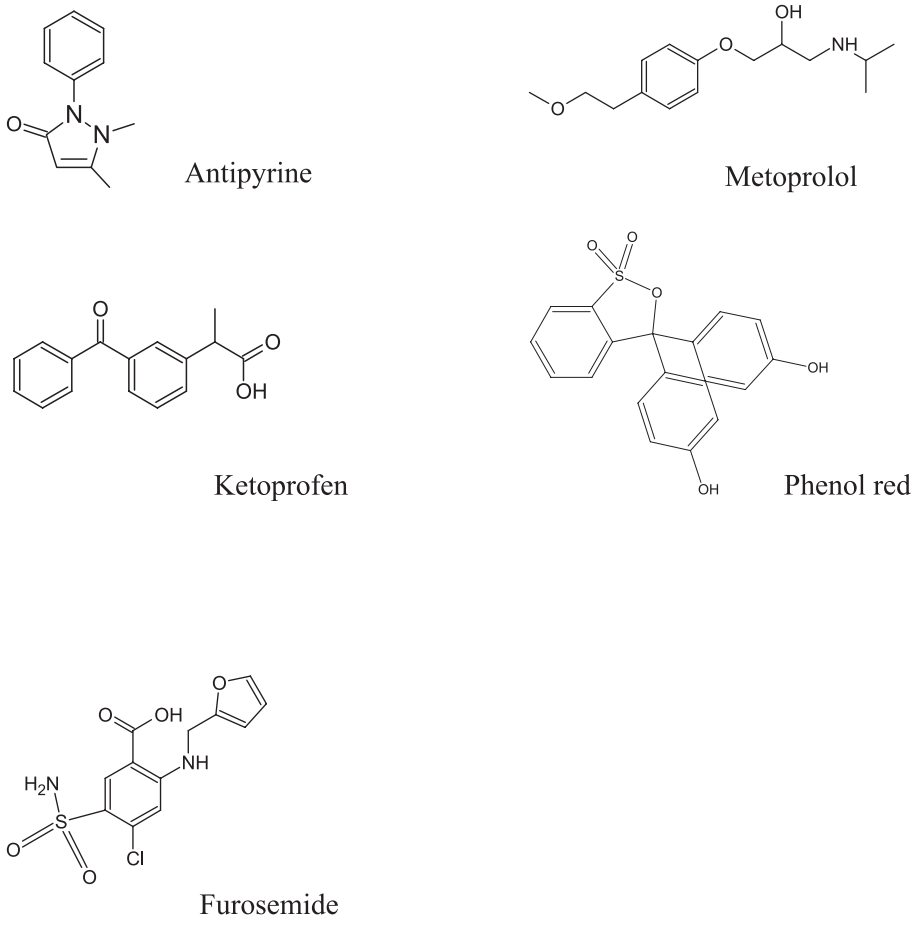

Figure 1. Molecular structures of antipyrine, metoprolol, ketoprofen, phenol red and furosemide.

of the *in situ* intestinal perfusion experiment. Once suitability of the method has been demonstrated by the suggested model drugs, there is no need to retest these compounds in further permeability studies provided the same protocol is being used. However the ultimate results of the permeability studies for compounds being tested rely on the authenticity and integrity of the data generated by an analytical method.

Although analytical methods have been reported for the quantitative estimation of the individual standard drugs (Carr *et al.*, 1995; Xie and Zhou, 1995; Farca *et al.*, 2003; Zuo *et al.*, 2004; Lanchote *et al.*, 1997; Danhof *et al.*, 1979; El Saharty, 2003; Smith *et al.*, 1980), to the best of our knowledge, a single validated analytical method for quantitative analysis of a mixture of five standard drugs, antipyrine, metoprolol, ketoprofen, phenol red and furosemide, has not been reported in the literature.

The aim of the present study was to develop and validate a simple HPLC method for the simultaneous determination of two high-permeability compounds (antipyrine and ketoprofen), one moderate-permeability compound (metoprolol; Mistry *et al.*, 1998) and a low-permeability compound (furosemide) along with a zero-permeability marker (phenol red; Issa *et al.*, 2003; Sutton *et al.*, 2001). Structures of the given permeability standards and the zero permeability marker are presented in Fig. 1.

EXPERIMENTAL

Materials

Antipyrine, metoprolol, ketoprofen and furosemide were procured from Sigma (St. Louis, MO, USA). Phenol red and sodium dihydrogen orthophosphate were from Qualigens (Mumbai, India). Methanol (HPLC-grade, Lichrosolv) and *ortho*-phosphoric acid (GR grade, minimum purity 88%) were from Merck (Mumbai, India). Xterra RP 18, Symmetry C-8 and Symmetry C-18 were from Waters (Milford, CA, USA). Inertsil ODS 3V was procured from GL sciences Inc., Japan, the Kromasil RP-18 from Flexit Jour Laboratories, Pune, India and the Hichrom RP-18 from Hichrom Limited, Berks, UK.

Equipment

A Waters Alliance 2695 separations module appended with a 2996 PDA detection system and Empower software (version 5.0) was used for chromatography. A Thermo Orion pH meter (model 420A+), a Sartorius balance, a Branson sonicator and Eppendorf Research Pro Digital Pipettes were used during experiment.

Chromatographic conditions

A Symmetry Shield C-18 (150 mm × 4.6 mm, 5 μm) column was used for the validation studies. The column temperature was set at 35°C. The mobile phase consisted of a mixture of aqueous potassium dihydrogen orthophosphate (pH 5.5; 0.01 M) and methanol in gradient composition. A gradient of time (min)–% methanol of 0:5, 22:60, 23:60 and 23.10:5 was programmed. The mobile phase was pumped at a flow rate of 1.5 mL/min. The separation had a run time of 23.10 min and an injection delay of 3 min. The injection volume was set to 50 μL. Data acquisition was carried out using PDA detector in the wavelength range of 210–600 nm. Extraction of chromatograms was carried out by timed wavelength [time (min)–λ, 0:242; 10.5:222; 13:228; 14.5:420; 16:258].

Preparation of mobile phase

A 1.74 g aliquot of dipotassium hydrogen orthophosphate (K_2HPO_4) was dissolved in about 950 mL of Milli-Q (MQ) water. The pH was adjusted to 5.5 with 10% orthophosphoric acid (H_3PO_4). The final volume was then made up to 1000 mL with MQ water to obtain a 0.01 M buffer solution. The aqueous K_2HPO_4 buffer solution (0.01 M; pH 5.5) and methanol were filtered separately through 0.22 μm GV filters and sonicated for about 15 min to degas before use.

Preparation of diluent

Diluent for stock solutions and test samples was prepared by mixing 6 parts of MQ water and 4 parts of HPLC-grade methanol. The mixture was sonicated for about 15 min and filtered using 0.45 μm HVLP filter.

Preparation of stock solutions

The stock solution of each model drug was prepared at 1500 μM strength. The amount required to prepare a concentration of 1500 μM of each component was transferred to individual volumetric flasks and dissolved in diluent. In the case of furosemide, a stock solution was prepared in methanol instead of the diluent. The stock solution of phenol red was prepared at concentration of 1 mg/mL in diluent.

Preparation of test mixture

The test mixture was prepared by mixing the stock solutions of individual components in equal proportions and making up to the final volume with the diluent to obtain a test concentration of 150 μM of each model drug and 50 μg/mL of phenol red.

Method validation

System suitability. The suitability of the system was established at 100% test concentration on each day before performing the actual validation. Parameters like USP tailing, number of theoretical plates, USP resolution, capacity factor and percentage RSD in area as well as in retention time (R_t) were determined.

Specificity. The specificity of the method was assessed by injecting individual components and recording their retention times and UV spectra.

Linearity. Linearity was established by the construction of a seven-point calibration curve over a range of about 5–150% (5, 10, 25, 50, 100 and 150%) of the nominal analyte concentration of 150 μM for each standard and 50 μg/mL for phenol red based on the applicability of the method.

Accuracy. The accuracy of the method was established at 80, 100 and 120% of the test concentration and calculated as percentage recovery. Tyrode's buffer was taken as the matrix for performing recovery studies. Each model drug was prepared at a concentration of 3000 μM strength individually and phenol red was prepared at a concentration of 1000 μg/mL. All the components were mixed in equal proportions to obtain the stock mixture for accuracy study. Aliquots of 0.4, 0.5 and 0.6 mL of the stock mixture were spiked in 1.6, 1.5 and 1.4 mL of intestinal blank perfusate to obtain 80, 100 and 120% of the nominal analyte concentrations, respectively. The samples were prepared in triplicate. The resultant solutions were vortex-mixed for 3 min and diluted 2-fold with the diluent. These samples were centrifuged at 10,000 rpm for 10 min. The clear supernatant was filtered through 0.22 μm PVDF filter into HPLC vials and analyzed. Another set of neat standards was prepared in diluent. The recovery was determined by comparing the peak areas of the spiked rat perfusion extracts and neat standards prepared in diluent.

Precision. Precision was established in terms of repeatability and intermediate precision. Repeatability was determined by analyzing six replicates at 100% test concentration. The %RSD in area of each component was calculated for the replicates independently as a measure of repeatability.

Intermediate precision was determined at two levels, intra-day and inter-day. Three concentration levels were chosen to cover the entire range (5, 100 and 150% of nominal analyte concentration). All the injections were carried out in triplicate. Intra-day precision data were obtained by analyzing three sets of freshly prepared standards at different time intervals on one day. The chromatographic peak areas were compared and the %RSD in the area of each component between measurements was estimated. Inter-day precision was measured as %RSD in the area of each component obtained on three consecutive days. The samples for the intermediate precision (intra-day and inter-day) were freshly prepared and were corrected for their individual weights.

Storage stability studies. The stability of samples on the bench top during the study, and frozen and thawed prior to processing was evaluated with the aim of addressing possible

unanticipated issues necessitating storage or holding of the samples after the experiment prior to sample processing. The test mixture was divided into four sets; one set was injected for analysis as zero-hour samples and held in the carousel in the sample compartment. This was re-injected after 24 h. A second set was held on the bench top in experimental conditions for 24 h and re-injected. The third was subjected to freezing and thawing and analyzed after 24 h. All the studies were carried out at three concentration levels (5, 100 and 150% of the test concentration). The results were analyzed as percentage recovery against the samples injected at zero hour.

RESULTS AND DISCUSSION

Method development and optimization

The method was developed systematically by exploring and optimizing each of the HPLC parameters like pH of the buffer solution, the type of the organic solvent in the mobile phase, composition of the mobile phase, the type of the bonded stationary phase, i.e. C8 and C18, temperature of the column and the wavelength of detection for each compound.

The chromatographic parameters, like optimum capacity factor (k') for each component, selectivity (α), tailing factor (T_f), number of theoretical plates (N), maximum sensitivity of each compound and resolution between each critical pair of compounds, were taken into consideration while optimizing the method. The method was optimized by changing one parameter at a time while keeping the other parameters constant.

The most challenging part of the method development was the selection of a buffer with optimum pH that would not interfere with the pK_as of any of the standards or the marker, since the standard drugs fall over almost the entire range of pH generally used in reversed-phase chromatography. The reported pK_as of the standard drugs are furosemide (3.5), metoprolol (9.2), phenol red (7.8), antipyrine (1.44) and ketoprofen (4.2). Buffers with different pH were tried, namely pH 3.0 (10 mM KH_2PO_4 buffer), pH 5.5 (10 mM K_2HPO_4 buffer), pH 6.0 (10 mM K_2HPO_4 buffer) and pH 9.0 (10 mM NH_4HCO_3 buffer). It was found that at least one of the peaks was either splitting or tailing at pH 3, 6 and 9. The best peak shapes were obtained with 10 mM K_2HPO_4 buffer pH adjusted to 5.5 with dilute orthophosphoric acid as it did not appear to interfere with the pK_a of any of the standard drugs.

The column with C-8 bonded stationary phase displayed an alternative selectivity to C-18 bonded stationary phase columns, but the metoprolol peak was found to split [Fig. 2(A)]. Different brands of C-18 bonded stationary phase columns were evaluated for efficiency, selectivity and peak symmetry (Table 1). It was found that the peaks were relatively tailing in the Inertsil ODS column. Moreover, the resolution

Table 1. Method optimization on different columns

		Components				
Columns used	Parameter	Antipyrine	Metoprolol	Phenol red	Ketoprofen	Furosemide
X-Terra RP 18	k	4.59	6.20	7.96	8.22	9.59
	T_f	0.98	2.07	1.02	1.22	0.97
	α	—	1.35	1.28	1.03	1.16
	R_s	—	6.36	8.49	1.57	7.71
Kromasil	k	10.03	12.30	16.76	13.33	18.91
	T_f	1.50	1.66	1.20	1.55	1.18
	α	—	1.22	1.26	1.08	1.13
	R_s	—	7.13	1.30	3.37	10
Inertsil ODS 3	k	7.17	7.58	9.29	10.50	11.92
	T_f	—	2.05	1.26	1.36	1.20
	α	—	1.06	1.23	1.13	1.14
	R_s	—	1.36	5.76	3.96	1.29
Inertsil C-8	k	14.86	14.45	20.54	12.00	18.11
	T_f	1.08	0.53	1.04	1.02	1.08
	α	1.03	1.20	1.13	—	1.22
	R_s	2.50	1.46	1.04	—	1.48
Symmetry Shield RP-18	k	7.93	10.36	12.84	15.62	17.53
	T_f	1.30	1.89	1.49	1.30	1.14
	α	—	1.30	1.24	1.22	1.12
	R_s	—	8.52	8.25	11.62	7.73
Hichrom RP-18	k	9.56	1.18	1.27	1.63	1.81
	T_f	1.7	2.34	1.70	1.28	1.28
	α	—	1.23	1.07	1.28	1.11
	R_s	—	7.77	3.28	1.46	8.61

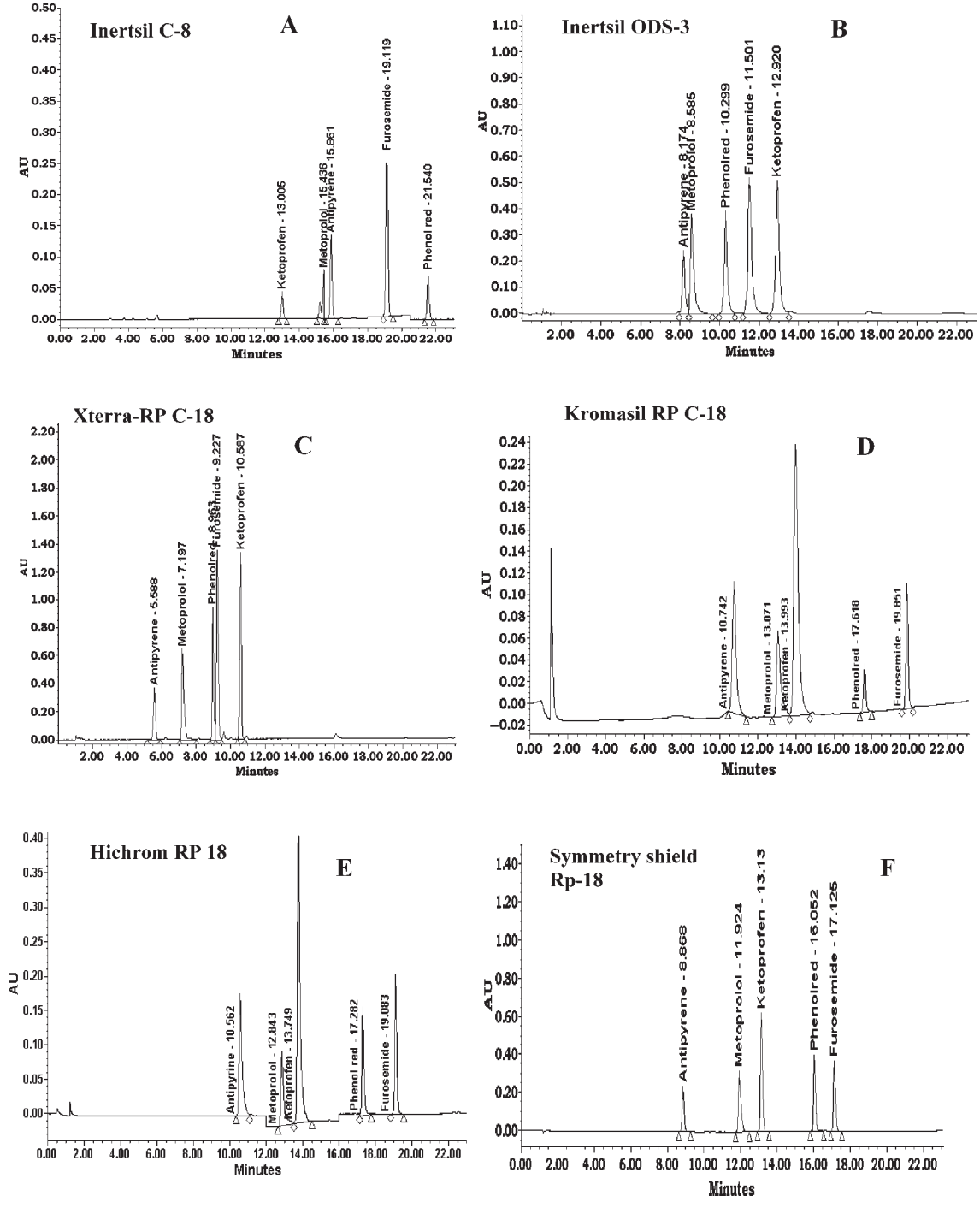

Figure 2. Representative chromatograms obtained during method development in various columns.

between antipyrine and metoprolol was found to be poor in the Inertsil ODS column [Fig. 2(B)]. The use of the Xterra column (Waters), which has hybrid particle technology, gave sharp peaks of high efficiency, but baseline resolution between phenol red and ketoprofen could not be achieved. The separation also had problems in reproducibility [Fig. 2(C)]. The Kromasil RPC-18 [Fig. 2(D)] and Hichrom RP18 [Fig. 2(E)] exhibited broader peaks with high tailing factors. The best separation with high efficiency, satisfactory peak symmetry, good resolution and reproducibility was achieved on the Symmetry Shield RP 18 column [Waters; Fig. 2(F)].

The standard drugs were monitored at different wavelengths using PDA detector, with a timed wavelength program to achieve maximum sensitivity for each drug. Similar resolution was observed when experiments were performed with either acetonitrile or

Table 2. System suitability

Component	R_t (n = 6; mean)	%RSD	Area (n = 6; mean)	%RSD	k'	R	α	T
Antipyrine	8.88 ± 0.30	3.39	2918047.67 ± 15621.75	0.53	7.88 ± 0.30	—	—	1.27 ± 0.39
Metoprolol	11.79 ± 0.46	3.95	5776985.67 ± 219909.19	3.81	10.79 ± 0.47	9.01 ± 1.66	1.37 ± 0.04	2.11 ± 0.27
Ketoprofen	13.56 ± 0.12	0.86	9307584.83 ± 318197.49	3.42	12.56 ± 0.12	5.30 ± 1.34	1.17 ± 0.05	1.55 ± 0.24
Phenol red	16.45 ± 0.06	0.38	3941345.33 ± 1485314.24	37.69	15.45 ± 0.05	11.71 ± 0.86	1.23 ± 0.01	1.33 ± 0.10
Furosemide	17.81 ± 0.35	1.98	4896872.33 ± 91450.03	1.87	16.81 ± 0.35	5.52 ± 1.27	1.62 ± 1.18	1.35 ± 0.08

Table 3. Linearity

Component	Wavelength (λ)	Range (%)	Equation	r^2
Antipyrine	240.9	5–150	$y = 30528x + 20707$	0.9975
Metoprolol	222.0	5–150	$y = 59582x + 87896$	0.9964
Ketoprofen	258.0	5–150	$y = 92960x + 54209$	0.9954
Phenol red	420.6	5–150	$y = 54389x + 33582$	0.9984
Furosemide	228.0	5–150	$y = 51546x + 49724$	0.9981

methanol, but the organic solvent of choice was methanol, considering its low cost. The effect of column temperature was studied by performing the separation at different temperatures. It was found that the resolution increased at elevated temperatures. The column temperature was optimized to 35°C as this gave a fair compromise between resolution and column life. In order to strike a balance between adequate resolution of all peaks and total run time, both gradient changes and flow rate options were tested. A flow rate of 1.5 mL/min was set for accelerating the separation process without affecting baseline separation.

Method validation

The system suitability was performed each day prior to performing the validation of each parameter. It was ensured that each of the system suitability criteria were achieved that were set for individual components (Table 2).

Specificity

The retention times were found to be about 9, 12, 13, 16 and 17 min for antipyrine, metoprolol, ketoprofen, phenol red and furosemide, respectively. No interference was observed in the retention time window of each component. Specificity was also confirmed by ensuring the spectral purity of each component with the aid of a PDA detector (Fig. 3).

Linearity

Linearity of detection in terms of response vs the analyte concentration was established in the range 5–150% of the nominal test concentration. The linearity of the method was observed in the expected concentration range, demonstrating suitability for the analysis. The goodness of the fit (r^2) for each component was found to be greater than 0.995 (Table 3).

Accuracy

The percentage recovery ranged from 95.1 to 98.5% for different components when the accuracy was performed at 80% of the test concentration. At 100% test concentration the percentage recovery ranged from 100.5 to 104.1%. The percentage recovery at 120% of the nominal concentration ranged between 96.4 and 99.9% (Table 4).

Table 4. Accuracy

	Accuracy at 80% of nominal concentration			Accuracy at 100% of nominal concentration			Accuracy at 120% of nominal concentration		
Component	Amount spiked (μg/mL)	Amount recovered (μg/mL)	Percentage recovery	Amount spiked (μg/mL)	Amount recovered (μg/mL)	Percentage recovery	Amount spiked (μg/mL)	Amount recovered (μg/mL)	Percentage recovery
Antipyrine	226.96	215.84	95.10	283.70	286.54	101.00	340.44	329.82	96.88
Metoprolol	820.64	806.52	98.28	1025.80	1031.04	100.51	1230.96	1196.64	96.40
Ketoprofen	305.44	300.88	98.51	381.80	393.78	103.14	458.16	453.06	98.89
Phenol red	398.40	391.82	98.35	498.00	518.42	104.1	597.60	595.92	99.72
Furosemide	401.36	389.52	97.05	501.70	515.74	102.80	602.04	589.16	97.86

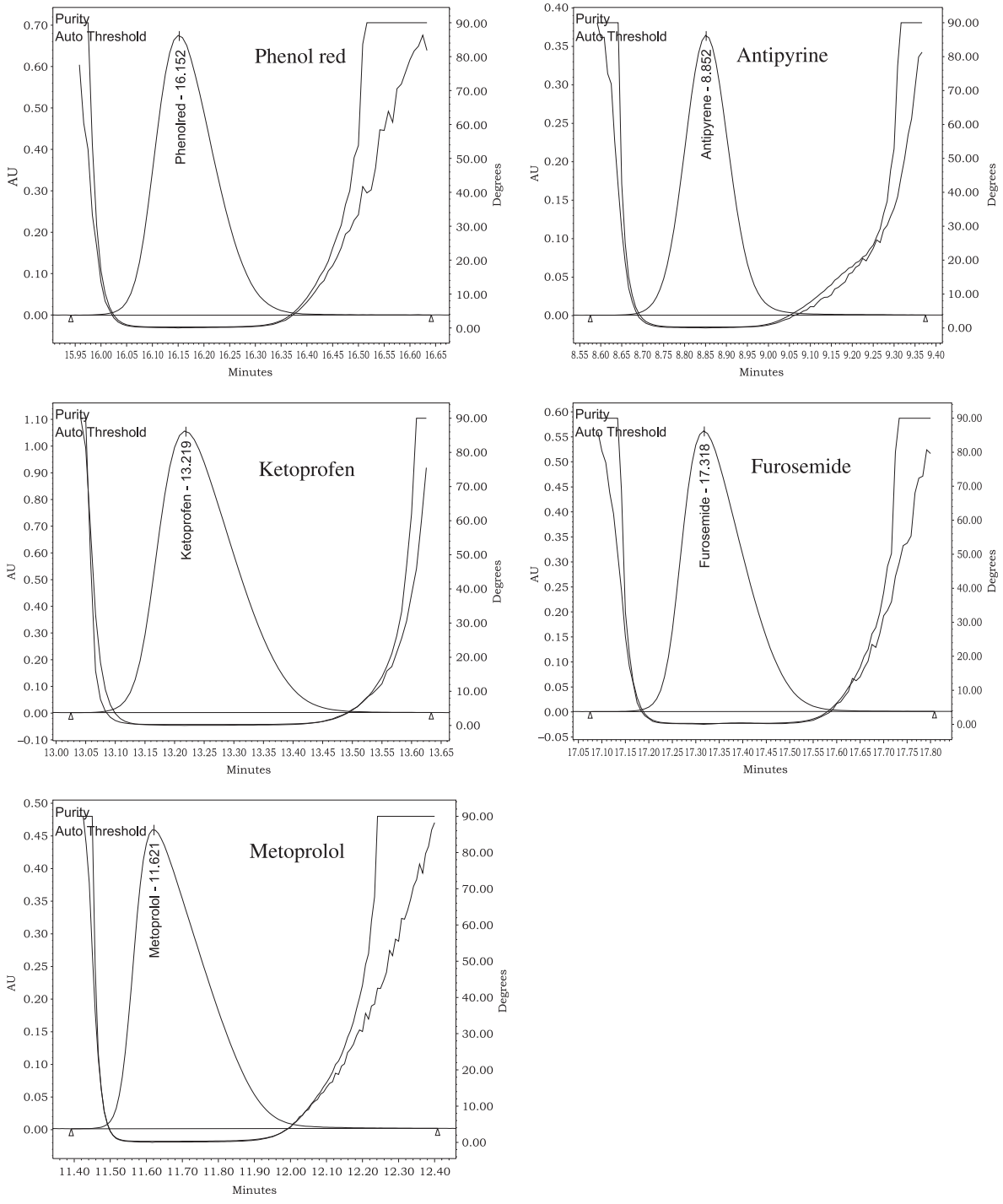

Figure 3. Purity plots obtained by Empower software for antipyrine, metoprolol, ketoprofen, phenol red and furosemide.

Precision

The repeatability was determined by analyzing six replicates at 100% test concentration. The %RSD in area was found to be less than 0.34% for each of the individual components (Table 5). The %RSD of area for each component in the studies of intra-day and inter-day precision were found to be less than 0.78% and less than 0.34%, respectively.

Storage stability

The storage stability was determined in terms of percentage recovery. The percentage recovery was found to be in the range 98.70–102.35% for each component when stored on the bench-top for 24 h. After two cycles of freezing and thawing, the percentage recovery was found to be between 96.26 and 104.65%. In the case of storage of the samples in sample

Table 5. Precision

Parameter	Concentration level (%)	Antipyrine (area)		Metoprolol (area)		Ketoprofen (area)		Phenol red (area)		Furosemide (area)	
		Mean ± SD	%RSD	Mean ± SD	%RSD	Mean ± SD	%RSD	Mean ± SD	%RSD	Mean ± SD	%RSD
Repeatability	100	3173303 ± 2829.94	0.09	6041535 ± 2789.29	0.05	9313472 ± 12026.69	0.13	5414349 ± 17694.55	0.33	5248834 ± 4623.71	0.09
Intra-day	5	147086.80 ± 740.16	0.50	269790.10 ± 2936.45	1.09	417280.70 ± 2693.38	0.65	287749.10 ± 671.71	0.23	255759.40 ± 451.68	0.18
	100	2860933.00 ± 6981.57	0.24	5577580.00 ± 14355.08	0.26	9137087.0 ± 23766.17	0.26	5418559.00 ± 14467.22	0.27	4883740.00 ± 7543.99	0.15
	150	3330672.00 ± 25703.19	0.77	6483458.00 ± 49122.62	0.76	10689738.0 ± 76384.9	0.71	6291715 ± 6340.37	0.10	5699857.00 ± 35599.66	0.62
Inter-day	5	141616.66 ± 329.03	0.23	3012963.66 ± 876.79	0.29	451473.96 ± 1280.86	0.28	262054.51 ± 796.65	0.30	250707.10 ± 835.83	0.33
	100	2832333.20 ± 6580.58	0.23	6025873.11 ± 17535	0.29	9029479.19 ± 25617.19	0.28	5241090.14 ± 15933.04	0.30	5014141.99 ± 16716.55	0.33
	150	4248499.79 ± 9870.88	0.23	9034523.76 ± 28585.68	0.32	13544218.79 ± 38425.79	0.28	7861635.22 ± 23899.57	0.30	25074.82 ± 752121.99	0.33

compartment for 24 h, the percentage recovery was found ranging from 98.41 to 102.43% (Table 6).

CONCLUSIONS

A simple, precise and accurate HPLC method using a C18 column was developed and validated for simultaneous quantitative determination of antipyrine, metoprolol, ketoprofen, furosemide and phenol red for the standardization of rat *in situ* single-pass intestinal perfusion studies. The validation study was carried out to prove that the new analytical method meets the following characteristics: selectivity, linearity, precision, accuracy and sensitivity. This method was found to be suitable for the determination of antipyrine, metoprolol, ketoprofen and furosemide within the range 7.5–225 μM and in the range 10–300 μg/mL for phenol red. This newly developed and validated method can be readily used on a routine basis for the standardization of *in situ* intestinal permeability experiments.

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Table 6. Storage stability

Component	Concentration (%)	Initial	Bench top		Within injector		Freeze/thaw	
		Amount injected (μg/mL)	Amount recovered (μg/mL)	Percentage recovery	Amount recovered (μg/mL)	Percentage recovery	Amount recovered (μg/mL)	Percentage recovery
Antipyrine	5	14.36	14.34	99.86	14.40	100.28	14.42	100.42
	100	287.20	287.52	100.11	287.64	100.15	287.15	99.98
	150	430.80	430.85	100.01	430.61	99.96	432.65	100.43
Metoprolol	5	51.50	51.82	100.62	52.75	102.43	54.41	104.65
	100	1030.00	1021.52	99.18	1053.12	102.24	1004.02	97.48
	150	1545.00	1556.62	100.75	1568.55	101.52	1542.17	99.82
Ketoprofen	5	19.66	19.67	100.05	20.01	101.78	19.56	99.49
	100	393.20	396.24	100.77	391.62	99.60	392.90	99.92
	150	589.80	597.91	101.38	592.66	100.48	583.64	98.96
Phenol red	5	24.62	24.15	98.09	24.23	98.42	23.70	96.26
	100	492.40	501.32	101.81	492.58	100.04	488.62	99.23
	150	738.60	743.88	100.71	738.63	100.00	734.00	99.38
Furosemide	5	25.54	26.14	102.35	25.51	99.88	26.08	102.11
	100	510.80	504.15	98.70	506.40	99.14	509.77	99.80
	150	766.20	776.39	101.33	777.78	101.51	768.61	100.31

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