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Development and Validation of a RP-HPLC Method for Simultaneous Determination of Metformin Hydrochloride, Phenol Red and Metoprolol Tartrate for Intestinal Perfusion Studies

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***SUMMARY*. A novel, fast, easy liquid chromatographic method was developed for simultaneous determination of metformin hydrochloride, phenol red, metoprolol tartrate for intestinal perfusion studies. The analysis was performed on a C_{18} column with a mobile phase composed of phosphate buffer (pH 3.0, 100 mM)-acetonitrile (30:70, v/v). Method was validated for selectivity, sensitivity, linearity, precision, accuracy, stability. All calibration curves were linear (r > 0.9986). Quantification limit was 2.5 μg/mL for metformin, 0.38 μg/mL for phenol red, 0.77 μg/mL for metoprolol. Detection limit was 0.85 μg/mL for metformin, 0.12 μg/mL for phenol red, and 0.25 μg/mL for metoprolol. Precision and accuracy values of the method fulfilled the required limits. The mean permeability values of metformin across jejunum, ileum and colon were 5.59 ± 3.09, 11.5 ± 1.26, 7.86 ± 2.65 x 10^{-5} cm/s, respectively; the corresponding values for metoprolol were 5.77 ± 3.37, 4.33 ± 0.48 and 10.5 ± 3.15x10-5 cm/s. These results demonstrate that permeability of metformin across jejunum and colon can be classified as low, whereas its permeability is high across ileum.**

RESUMEN. Se desarrolló un método nuevo, rápido y fácil de cromatografía líquida para la determinación simultánea de clorhidrato de metformina, rojo fenol y tartrato de metoprolol en estudios de perfusión intestinal. El análisis se realizó en una columna C_{18} con una fase móvil compuesta de tampón fosfato (pH 3,0, 100 mM)-acetonitrilo (30:70, v/v). El método fue validado para la selectividad, sensibilidad, linealidad, precisión, exactitud y estabilidad. Todas las curvas de calibración fueron lineales (r > 0,9986). El límite de cuantificación fue 2.5 μg/mL para la metformina, 0.38 μg/mL para el rojo fenol y 0,77 mg/mL para el metoprolol. El límite de detección fue 0,85 g/mL para la metformina, 0,12 g/mL para el rojo fenol y 0,25 mg/mL para el metoprolol. Los valores de precisión y exactitud del método cumplen los límites requeridos. Los valores de permeabilidad media de metformina a través de yeyuno, íleon y colon fueron 5,59 ± 3,09, 11,5 ± 1,26 y 7,86 ± 2.65 x 10^{-5} cm/s, respectivamente; los valores correspondientes para el metoprolol fueron 5,77 ± 3,37, 4,33 ± 0,48 y 10,5 ± 3.15 x 10^{-5} cm/s. Estos resultados demuestran que la permeabilidad de la metformina en todo el yeyuno y el colon se puede clasificar como baja, mientras que su permeabilidad es alta en todo el íleon.

INTRODUCTION

Diabetes mellitus is a major health risk in many countries, and the incidence rates are increasing [1]. Type 2 diabetes is the most common type of diabetes in the world. It is a metabolic disorder and characterized by hyperglycemia (high blood sugar) in the context of insulin resistance and relative lack of insulin [2,3]. A biguanide derivative metformin (Fig.1) is the first-line drug of choice for the treatment of type 2 diabetes. Metformin decreases hyperglycemia by suppressing hepatic glucose production [4]. Additionally, metformin increases insulin sensitivity, enhances peripheral glucose uptake, decreases insulin-induced suppression of fatty acid oxidation, and decreases absorption of glucose from the gastrointestinal tract [5]. Metformin has high solubility and low permeability, and is classified as Class 3 compound according to Biopharmaceutics Classification System [6]. It is primarily absorbed from the small intestine and it has a relatively low (50-60%) bioavailability [7].

Two important parameters control the oral absorption of compounds namely aqueous solu-

KEY WORDS: intestinal perfusion, metformin hydrochloride, metoprolol tartrate, phenol red

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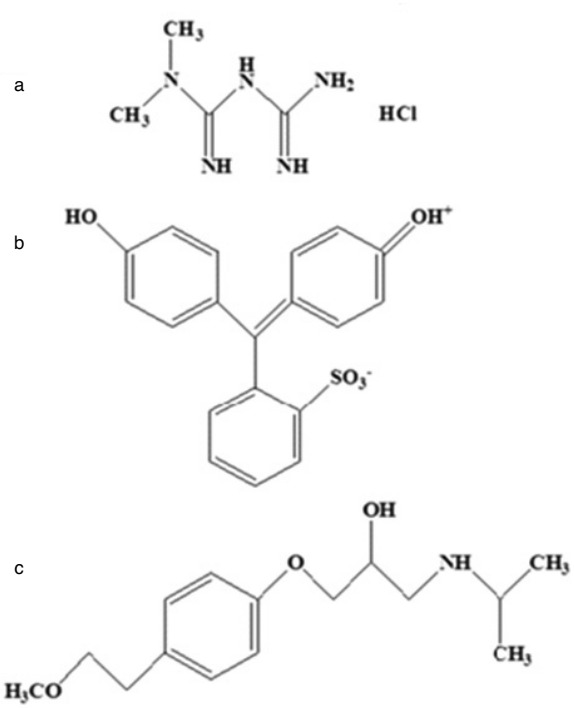

Figure 1. The chemical structures of metformin hydrochloride (**a**), phenol red (**b**) and metoprolol tartrate (**c**).

bility and intestinal permeability. Variety of methods are recommended for estimation of intestinal permeability and hence absorption, of a compound, including *in vivo* studies in humans (*e.g.,* mass balance studies, absolute bioavailability studies), intestinal perfusion studies using suitable animal models, and *in vitro* studies (*e.g., in vitro* permeation studies across a monolayer of cultured epithelial cells) [8]. Of these methods, in situ intestinal perfusion studies are simple and show similar absorption results with human studies [9]. However, for accurate estimation of permeability values, it is necessary to use a validated HPLC method for analysis of compound in the samples obtained from the intestinal perfusion studies.

Several HPLC methods are available in literature for separate determination of metformin hydrochloride [10-14], metoprolol tartrate [15,16], and phenol red [16-18] (Fig. 1). Although there is an HPLC method available for simultaneous determination of metformin hydrochloride and phenol red [17] in intestinal perfusion samples, all chromatographic conditions are different from our method, and also total analysis time is longer. To our knowledge, there is no study available in the literature for simultaneous determination of metformin hydrochloride, metoprolol tartrate and phenol red. Therefore, the primary aim of this study was to develop and validate a novel reversed-phase liquid chromatographic method for the simultaneous determination of metformin hydrochloride, metoprolol tartrate and phenol red in intestinal perfusion samples. The secondary aim of this study was to demonstrate the applicability of developed method by analyzing the samples containing all these compounds obtained from the simultaneous perfusion jejunum, ileum and colon. Metformin hydrochloride was selected as the model compound, metoprolol tartrate as a reference standard to compare the permeability coefficient of metformin hydrochloride and also as a marker for suitability of permeability method [19]. Phenol red was used as a zero permeability marker for correction of permeability coefficient for water transport [20].

MATERIALS AND METHODS

Chemicals

Metformin hydrochloride was kindly supplied by Bilim Pharmaceuticals-Turkey, and metoprolol tartrate from Novartis Pharma AG (Turkey). Phenol red was purchased from Merck (Germany). HPLC grade acetonitrile was obtained from Sigma-Aldrich. Water was purified using a Milli-Q system (Millipore).

Instrumentation and chromatographic conditions

The HPLC system used for analysis was Shimadzu LC-20 A/Prominence Alliance (Japan) equipped with a solvent degasser, LC-20 AT pump, a temperature-controlled autosampler, and was coupled to a SPD-M20 photodiode array (PDA) detector and operated with the LC solution software. Metformin hydrochloride, phenol red and metoprolol tartrate were separated using a Waters Spherisorb ODS2 C_{18} (250 × 4.6 mm, 5 µm; USA) column. The HPLC system was operated at 25 °C using a mobile phase consisted of phosphate buffer (pH 3, 100 mM)-acetonitrile (30:70, v/v). The mobile phase was filtered through a 0.45 µm membrane filter, degassed before use, and delivered to the HPLC system at a flow rate of 1 mL/min. PDA detection was performed at 232 nm. Total run time for analysis was 8 min and the injection volume was 20 µL.

Preparation of standard solutions

All stock and working solutions used in the calibration and validation studies were prepared

in the perfusate obtained from simultaneously perfused single pass rat intestinal perfusion preparation.

Standard stock solutions of metformin hydrochloride (1000 µg/mL) and metoprolol tartrate (400 µg/mL) were prepared in perfusion solution. These primary stock solutions were then diluted with the perfusion solution to obtain secondary standard stock solutions. Because of the limited solubility of phenol red, its standard stock solution (200 µg/mL) was prepared in a mixture of methanol (40%) and perfusate (60%), and then diluted with the perfusate to obtain secondary standard stock solution. All these solutions were kept at 4 °C. Working standard solutions for the calibration curves were prepared from the secondary stock solution diluted with the perfusate within the concentration range of 2.5-200 µg/mL for metformin hydrochloride, 1-60 µg/mL for metoprolol tartrate, and 1-30 µg/mL for phenol red.

Method validation

The developed HPLC method was validated as to selectivity, linearity, sensitivity (limit of detection and lower limit of quantitation), accuracy- precision (intra-day and inter-day), and stability, according to the ICH Guidelines [21].

Selectivity

The selectivity of the analytical method was assessed by injecting drug free perfusate which was collected from jejunum, ileum and colon during the perfusion studies, into the HPLC system.

Linearity

The linearity of the method was evaluated by spiking seven different concentrations within the concentration range of 2.5-200 µg/mL for metformin hydrochloride, 1-60 µg/mL for metoprolol tartrate, and 1-30 µg/mL for phenol red, (six different series were prepared). Six calibration curves were carried out for each compound. Calibration curves for metformin hydrochloride and reference compounds (metoprolol tartrate and phenol red) were constructed by plotting the peak areas of each compound against the corresponding nominal concentrations. Linearity of the method was demonstrated by the calibration equation which is characterized by determination coefficient, slope and intercept.

Sensitivity

The sensitivity of the analytical method was evaluated by determining the limit of detection (LOD) and lower limit of quantitation (LLOQ). LOD is defined as the lowest amount of analyte in a sample which can be detected, and LLOQ as the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The signal to noise ratios of 3:1 and 10:1 were taken as LOD and LLOQ, respectively.

Precision and accuracy

Precision of the method was determined by analyzing drug-free perfusate spiked with model and reference compounds. The intra- and inter-day precision studies were carried out for assessment of the assay precision. Three different concentrations of compounds (10, 25, and 200 µg/mL for metformin hydrochloride; 5, 30, and 60 µg/mL for metoprolol tartrate; 2.5, 12.5, and 30 µg/mL for phenol red) within calibration range were analyzed six consecutive days (inter-day) and six times within the same day (intra-day) to determine the precision of the method. The relative standard deviations (RSD) of intra- and inter-day studies were calculated for assessment of precision of the method. The inter- and intra-day accuracies of the method was determined at three test concentrations and calculated as percentage Bias.

Stability

At the end of the perfusion experiments, perfusion samples were stored at -20 °C until analysis. Therefore, stability studies were conducted only for frozen samples. Stability of perfusion samples containing all compounds (metformin hydrochloride, metoprolol tartrate, phenol red) obtained from simultaneous perfusion of jejunum, ileum and colon, were performed 24 h, one week, and one month after storing at -20 °C. All samples were thawed at room temperature and then analyzed by HPLC. The results were compared with initial concentrations.

Perfusion studies

The study protocol for the intestinal perfusion studies was approved by the local ethics committee of Hacettepe University. Before the perfusion experiment, male Sprague Dawley rats weighing 250 to 350 g were fasted overnight. Anesthesia was induced by intraperitoneal administration of ketamine (100 mg/kg) and xylazine (10 mg/kg) and additional doses were administered when necessary. Under anesthesia, abdomen was opened by a 3-4 cm midline longitudinal incision to expose intestinal segments (jejunum, ileum and colon). Intestinal segments were isolated and cannulated at both ends with plastic tubings. Each segment was first rinsed with saline to remove the debris until the per-

fusate run clear. Thereafter, the segments were perfused (0.2 mL/min) with perfusion medium containing MES buffer (10 mM), NaCl (135 mM), KCl (5 mM) and phenol red (0.1 mg/mL) [19] at a flow rate of 0.2 mL/min, perfusate was collected from each segment for use in calibration and validation studies, and stored at -20 °C until use.

For permeability studies, metformin hydrochloride (200 µg/mL) and reference compound (metoprolol tartrate, 100 µg/mL) were added into the perfusion solution. Then, intestinal segments (jejunum, ileum and colon) were perfused simultaneously at a flow rate of 0.2 mL/min for a total period of 60 min. Samples from each segment were collected separately every 5 min into the test tubes. When the experiment was completed, the animal was euthanized by exsanguination and length of jejunum, ileum and colon were measured again. The concentration of metformin hydrochloride in the perfusion medium was selected based on literature [17]. The concentration of metoprolol tartrate in the perfusion medium was selected based on the oral dose of drug products. All perfusion samples were diluted with the mobile phase, and then concentrations of metformin hydrochloride, metoprolol tartrate and phenol red were determined using the developed HPLC method. To monitor the performance of a bioanalytical method and to assess the integrity and validity of the results of perfusion samples, quality control samples within the linearity range (2.5-200 µg/mL for metformin hydrochloride, 1-60 µg/mL for metoprolol tartrate, and 1-30 µg/mL for phenol red) were prepared and analyzed in an individual run.

For calculation of effective permeability values (P_{eff}), the measured C_{out}/C_{in} ratio was corrected for water transport using Eq. [**1**].

$$\frac{C'_{out}}{C'_{in}} = \frac{C_{out}}{C_{in}} x \frac{C_{inphenolred}}{C_{outphenolred}} \qquad [1]$$

where $C_{in\ phenol\ red}$ is the inlet phenol red concentration, and $C_{out\ phenol\ red}$ is the outlet phenol red concentration [19,22].

The effective permeability (P_{eff}) values of the drug across the rat gut wall were calculated using Eq. [**2**].

$$P_{eff}(cm/s) = \frac{-Q \ln(\frac{C'_{out}}{C'_{in}})}{2\pi RL} \qquad [2]$$

where Q is the flow rate of perfusion solution (mL/s); C'_{out} / C'_{in} is the corrected concentration ratio of outlet to inlet concentration. R is the radius of the perfused intestinal segment (for jejunum and ileum R = 0.2 cm, for colon R = 0.25); L is the length of the intestinal segment (cm) [19,22].

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

An HPLC method was developed for simultaneous determination of metformin hydrochloride, phenol red and metoprolol tartrate in samples obtained from intestinal perfusion studies. Various mobile phase compositions (*e.g.* phosphate buffer pH 3.0: acetonitrile; phosphate buffer pH 2.5: acetonitrile; phosphate buffer pH 3.0: methanol) were used in order to obtain the best chromatographic separation of metformin hydrochloride, phenol red and metoprolol tartrate in perfusion samples. When phosphate buffer pH 3.0: methanol (30:70) was used as the mobile phase, the retention times of all three compounds were very similar without complete separation. On the other hand, replacement of methanol with acetonitrile in the mobile phase resulted in complete separation of the peaks and decrease in column back pressure. Because of simplicity, stable baseline, and unvarying response factor, isocratic elution using acetonitrile was used for analysis of all these compounds. As the retention time was reduced with an enhancement of flow rate from 0.5 to 1 mL/min [23], flow rate was set at 1 mL/min for the analysis. Waters Spherisorb ODS2 C18 (250 × 4.6 mm, 5 µm; USA) column is widely used for separation of compounds, therefore, we used the same column for analysis of metformin, phenol red and metoprolol in our study [24-26]. Furthermore, different wavelengths were used to obtain best peaks for separation of compounds. The results revealed that the peak area increased with decreasing the wavelength. Therefore, 232 nm was selected for analysis because not only the best peaks were obtained at this wavelength but also lower concentrations of compounds can be analyzed by the developed method. Although various injection volumes (10-50 µL) were tried, considering the sample volume for analysis and also the peak areas obtained, 20 µL was chosen. Based on all these results, the mixture of phosphate buffer (pH 3.0, 100 mM)-acetonitrile (30:70, v/v) as the mobile phase, 1 mL/min flow rate and 20 µL injection volume at 232 nm was selected as the optimum conditions. Using the optimum conditions, the analytical method de-

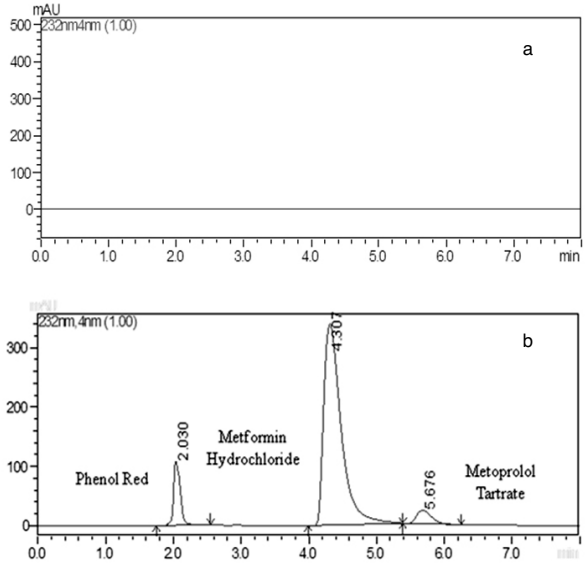

Figure 2. HPLC chromatograms of blank perfusion solution (**a**), phenol red, metformin hydrochloride, and metoprolol tartrate (**b**) at 232 nm. In b), numbers above the peaks represent the retention times.

veloped was capable of a good separation and short run time for all compounds simultaneously (Fig.2). The retention times for phenol red, metformin hydrochloride and metoprolol tartrate using optimum conditions were 2.030, 4.307, and 5.676 min, respectively, a with void volume at 1.1 min.

System suitability test parameters were checked to ensure that the system is working correctly during the analysis. The important parameter which is the time at which buffer peaks appear to, was 2 min in the analysis. For an optimum separation, capacity factor should be in the range of $0.5 < k' < 10$. Capacity factor (k) values were 2.05, 1.17, and 1.87 for phenol red, metformin, and metoprolol, respectively, indicating that these values were within the acceptable limits. For the column used in this study, the theoretical plate numbers were 1864, 2138, and 1567 for metformin hydrochloride, metoprolol tartrate, and phenol red, respectively. The calculated tailing factors of 1.66, 1.25, and 1.5 were obtained for metformin, metoprolol, and phenol red peaks, respectively which were in the acceptable range of $T \leq 2$. Collectively, all these results suggest the suitability of the system used for the analysis of the model (metformin) and reference compounds (metoprolol and phenol red) [27].

Method validation

Selectivity

Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample [28]. Following injection of blank perfusate into the HPLC column, there was no interfering peak with the retention times of phenol red (2.030 min), metformin hydrochloride (4.307 min) and metoprolol tartrate (5.676 min, Fig. 2). Based on the results, the proposed method was considered to be selective.

Linearity

Linearity of an analytical method is its ability (within a given range) to obtain results directly proportional to the concentration (amount) of analyte in the sample. Calibration curves for metformin hydrochloride and reference compounds (metoprolol tartrate and phenol red) were constructed by plotting the peak areas of each compound against the corresponding nominal concentrations. Linear regression analysis of the calibration curves showed linearity between peak areas and corresponding nominal concentrations of metformin hydrochloride, metoprolol tartrate and phenol red. Linearity data for all compounds were summarized in Table 1. All perfusion samples were diluted with the mobile phase before the HPLC analysis. Resulting con-

Parameter	Metformin Hydrochloride	Phenol Red	Metoprolol Tartrate
Regression equation *	*y = 100290x + 135474*	*y = 38119x + 10290*	*y =11065x + 80167*
Standard error of intercept	3595.06	4568.2	1715.63
Standard error of slope	49.21	402.8	88.32
Determination coefficient (r^2)	0.9986	0.9994	0.9995
Linearity range (µg/mL)	2.5–200	1-30	1-60
Number of points	8	8	8
LLOQ (µg/mL)	2.5	0.38	0.77
LOD(µg/mL)	0.85	0.12	0.25

Table 1. The linearity data of the developed method (n = 6). * Linear regression analysis with a regression equation of $y = ax + b$ in which x is the concentration in µg/mL of compounds and y is the peak area.

centrations were within the linearity range for all compounds.

Sensitivity

The sensitivity of the developed method was assessed by determining the Limit of Detection (LOD) and Lower Limit of Quantification (LLOQ) values for metformin hydrochloride, phenol red and metoprolol tartrate. The LOD values were 0.85 µg/mL for metformin hydrochloride, 0.25 µg/mL for metoprolol tartrate and 0.12 µg/mL for phenol red with the signal-to-noise ratio of 3:1. The LLOQ values were 2.5 µg/mL for metformin hydrochloride, 0.77 µg/mL for metoprolol tartrate and 0.38 µg/mL for phenol red with the signal-to-noise ratio of 10:1 (Table 1). All these results show that this analytical method is sensitive enough for intestinal perfusion studies of selected test (metformin hydrochloride) and reference compounds (metoprolol tartrate and phenol red).

Precision and accuracy

Precision represents degree of proximity between successive measurements under the same analytical conditions. It is associated with random distribution of errors, actual value is irrelevant. On the other hand, accuracy is the measure of how close the experimental value is to the true value. The mean value should be within 20% of the actual value except at LLOQ, where it should not deviate by more than 25% [28]. Our results demonstrated that all bias (%) values estimated for accuracy fulfilled this requirement.

The intra-assay (intra-day) and between-assay (inter-day) precision and accuracy results determined for test and reference compounds at three different concentrations (low, medium and high concentrations) were summarized in Table 2. The RSD and bias (%) values estimated for the intra-day and inter-day precision and accuracy of the assay were within the acceptable limits (< 20%; Table 2; 28) indicating that the precision and accuracy of the proposed HPLC method were satisfactory.

Stability

The stability results were evaluated by comparing peak area ratios of freshly prepared spiked samples and perfusion samples stored at -20 °C for 24 h, one week, and one month. The difference between nominal and measured concentrations of all compounds was less than 2 % one month after storage indicating that test and reference compounds are stable for at least one month (Table 3).

Compounds	Added (µg/mL)	Intra-day			Inter-day		
		Found [a] (µg/mL)	Precision [b] RSD (%)	Accuracy [c] (bias %)	Found [a] (µg/mL)	Precision [b] RSD (%)	Accuracy [c] (bias %)
Metformin Hydrochloride	10	8.45 ± 0.04	0.1	-15.49	10.37 ± 0.22	0.55	3.72
	25	24.4 ± 0.15	0.37	-2.36	24.75 ± 0.23	0.58	-0.99
	200	199.13 ± 0.08	0.21	-0.43	199.29 ± 2.93	7.18	-0.35
Phenol Red	2.5	2.28 ± 0.01	0.04	-8.56	2.43 ± 0.05	0.13	-2.54
	12.5	12.7 ± 0.1	0.25	1.66	12.68 ± 0.04	0.11	1.51
	30	30.11 ± 0.3	0.75	0.38	30 ± 0.41	1.02	0.02
Metoprolol Tartrate	5	5.08 ± 0.09	0.23	1.6	5.09 ± 0.05	0.14	1.8
	30	29.74 ± 0.11	0.28	-0.86	29.65 ± 0.2	0.5	-1.16
	60	60.63 ± 0.62	1.52	1.05	60.34 ± 0.26	0.64	0.58

Table 2. Intra-day and inter-day accuracy and precision data of metformin hydrochloride, phenol red and metoprolol tartrate (n = 6). [a] Mean ± Standard Error, [b] RSD, Relative Standard Deviation, [c] Bias % = [(Found-Added)/Added] × 100.

Compound	% Remained		
	24 h	One week	One Month
Metformin Hydrochloride	99.67 ± 0.62	100.85 ± 1.50	99.22 ± 0.45
Phenol Red	98.14 ± 0.86	104.76 ± 1.37	98.52 ± 0.43
Metoprolol Tartrate	98.68 ± 0.29	112.28 ± 4.27	101.52 ± 2.21

Table 3. The stability of metformin hydrochloride, phenol red and metoprolol tartrate in perfusion samples (mean ± SD; n = 3).

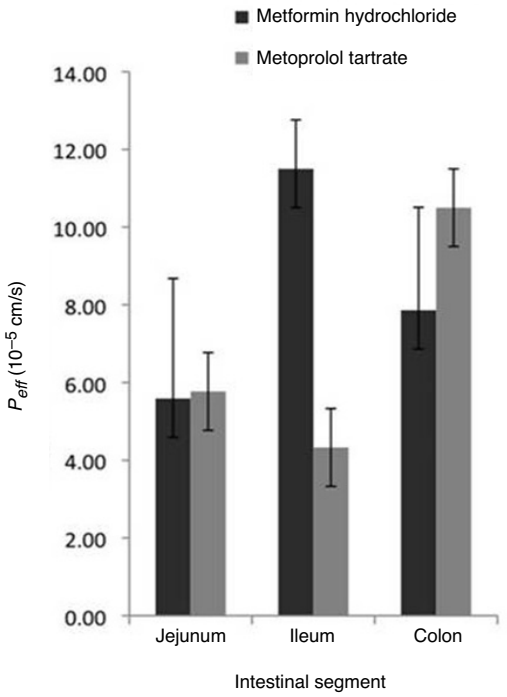

Figure 3. P_{eff} values of metformin hydrochloride and metoprolol tartrate obtained from different intestinal segments (mean ± SD; n = 6).

Perfusion studies

In perfusion studies, metoprolol tartrate was selected as a marker for the integrity of the experiment, and as a reference standard for the permeability class boundary, and phenol red as a non-absorbable marker. The permeability (P_{eff}) values calculated for metformin hydrochloride and metoprolol tartrate across jejunum, ileum and colon were depicted in Fig. 3. The permeability values estimated for metformin hydrochloride increased in the order of ileum > colon > jejunum, whereas estimated intestinal permeability values for metoprolol tartrate were in the order of colon > jejunum > ileum (Fig. 3). Comparison of the P_{eff} values indicated that metformin had higher permeability than metoprolol in all intestinal segments except ileum. These results clearly indicates that permeability of metformin hydrochloride across jejunum and colon is low (*i.e.* P_{eff}-metformin < P_{eff}-metoprolol), whereas its permeability is high across ileum (*i.e.* P_{eff}-metformin > P_{eff}-metoprolol). On the other hand, Song et al. reported that effective permeability values of metformin in the duodenum at 50 µg/mL was significantly higher than those of jejunum and ileum at the same concentration (17). For the same intestinal segments (jejunum and ileum), the permeability values we estimated for metformin hydrochloride at 200 µg/mL ($5.59 \pm 3.09 \times 10^{-5}$ cm/s for jejunum, and $11.5 \pm 1.26 \times 10^{-5}$ cm/s for ileum) were higher than those reported by Song *et al.* [17] for a lower concentration (50 µg/mL; $3.26 \pm 0.73 \times 10^{-5}$ cm/s for jejunum, and $2.96 \pm 0.36 \times 10^{-5}$ cm/s for ileum). This difference could be attributed to the differences in metformin concentrations used, and also experimental conditions *(i.e.* different perfusion mediums).

CONCLUSIONS

A new HPLC method was developed and validated for the simultaneous determination of metformin hydrochloride, metoprolol tartrate and phenol red for application in single pass intestinal perfusion studies. The developed method was successfully applied for the determination of all these compounds in samples obtained from *in situ* intestinal perfusion studies. Based on the results, permeability of metformin hydrochloride across jejunum and colon was found to be low, whereas its permeability was high across ileum.

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