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A Newly Developed and Fully Validated HPLC Method for Simultenous Determination of in-situ Single Pass Intestinal Permeability of Domperidone, Metoprolol Tartarate and Phenol Red

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ABSTRACT: One of the most popular methods for figuring out a drug's intestinal permeability is the intestinal perfusion (SPIP) approach. Metoprolol as tartrate and phenol red are frequently utilized as reference compounds in perfusion tests to assess the permeability coefficient of the study's focus component, Domperidone maleate. Our study's goal was to create and validate a reversed-phase liquid chromatographic technique for the simultaneous detection of Domperidone maleate, metoprolol, and phenol red for use in intestinal perfusion studies. The analysis was performed on a C18 column 150 × 1.5 mm, 5.0 µm using a mobile phase consisting of methanol: 20 mM potassium dihydrogen phosphate buffer 43:57, *v/v*; pH 3.1. The procedure was validated using the FDA's standards for stability, linearity, precision, and accuracy. All calibration curves were linear $r^2 > 0.99$. The system suitability parameters of the method are the required limits all analytes. The in situ intestinal permeability experiments can easily be standardized with the help of this newly created and proven approach.

KEYWORDS: Domperidone; HPLC; validation; Metoprolol tartarate; Phenol Red.

1.INTRODUCTION

Rat intestinal perfusion studies have emerged as the most dependable and affordable non-human FDA-approved technique for permeability evaluation and BCS classification. A high correlation (r^2 = 0.8–0.95) between human and rat small intestinal permeability has been reported for drugs with both transporter-mediated absorption and passive diffusion mechanisms, but their levels may differ between species regardless of the type and expression of transporters [1, 2]. Rat perfusion studies add the advantage of being useful for assessing segment-dependent permeability throughout the entire small intestine, a factor that has hitherto been unmeasurable in humans. Recently, it was demonstrated that despite their major differences, two common rat perfusion techniques—(a) single-pass intestinal perfusion (SPIP) and (b) closed-loop Doluisio (CLD)—performed similarly [3]. The Doluisio approach normally analyzes absorption throughout the entire small intestine, whereas the SPIP model concentrates on a 10 cm piece of the intestine (often the jejunum) [4, 5]. Either method can be used to measure segment-dependent permeability with the proper adjustments. The drug solution tested in the Doluisio method is delivered into the intestinal lumen and remains within the segment throughout the experiment, in contrast to the SPIP technique, which pumps the drug solution through the intestinal segment at a constant flow rate, allowing only one drug molecule to pass through it. Different permeability estimates may result from such variations in trial design, particularly for medications with poor permeability [6].

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Domperidone (DMP), 5-chloro-1{1-[3-(2,3-dihydro-2-oxo-1H-benzimidazol-1-yl)propyl]-4-piperidinyl1,3-dihydro-2H- benzimidazole-2-one is a selective dopaminergic D2 receptor antagonist. DMP is an important antiemetic that accelerates the intestinal transit process peripherally and at the same time makes gastric emptying fast and easy. It is a potent peripheral dopaminergic antagonist widely used in the treatment of gastrointestinal disorders such as gastroesophageal reflux, dyspepsia, nausea and vomiting [7]. In addition, it reduces esophageal sphincter pressure by strengthening gastric peristalsis and allows food to pass through the digestive system faster. The drug is taken by oral administration and is rapidly absorbed in the stomach and upper gastrointestinal tract. Its transport is active, and the absolute bioavailability is around 12.7-17.6% due to first pass elimination. It cannot cross the blood brain barrier and so has fewer side effects [8]. However, using it has been linked to a number of unpleasant side effects, including hyperprolactinemia, rash, itching, hives, diarrhea, abdominal cramps, and dry mouth [9].

A review of the literature reveals that analysis of DMP was accomplished with using separation techniques such as high-performance liquid chromatography (HPLC) [10, 11], capillary electrophoresis[12], ultra-violet (UV) spectrometry [13], and LC-MS/MS [14-16] was also used. Among these, HPLC is the dominant technique because it allows quantitative determination in many different matrices.

In this investigation, DMP, PR, and MPT were all simultaneously determined using a newly designed and validated reversed-phase liquid chromatographic method in intestinal perfusion samples in order to determine segmental permeability and absorption characteristics of DMP. In this research, the permeability coefficient of DMP was compared using MPT as a reference standard. For the purpose of correcting the permeability coefficient for water transport, PR was utilized as a zero-permeability marker. The technique was approved in accordance with ICH guideline Q2 (R1) [17]. The approach was designed with the intention of being easily adaptable to clinical applications in labs conducting SPIP research**.**

2. RESULTS AND DISCUSSION

2.1. Method optimization

In the presented study, various mobile phase characteristics, such as type and ratio of organic solvent, buffer pH and concentration, and components ratio have been examined to provide a good chromatographic separation with proper system suitability. Initially, methanol and acetonitrile were tested for the organic components of the mobile phase. When the acetonitrile mobile phase component was selected, an efficient separation of the peaks could not be achieved. Metoprolol tartrate, which has a high polarity, was not separated from the solvent peak. However, when methanol, the organic component of the mobile phase, is selected, all the peaks are separated from each other according to the system suitability criteria. After a series of tries, methanol: phosphate buffer (20 mM (47:53, *v/v*) mixture was found as suitable mobile phase. pKa values of MPT, PR and DMP were 9.5, 8.0 and 7.9, respectively. The buffer type and pH was selected from this perspective, and pH was adjusted to 3.1. Under the mentioned condition, all analytes were non-ionized, and molecules had a strong hydrophobic interaction with the column's functional group (C18) according to their hydrophobicity properties. The $\log K_{ow}$ values of the MPT, PR and DMP are 1.8, 3.0 and 3.9, respectively [18-20].

In the method optimization process, all optional parameters such as column temperature, flow, organic phase type and percentage were tested in detail and the method with the most appropriate system compatibility data was selected. The most difficult part of the process was its separation from the MPT solvent front peak. The organic phase was therefore chosen as methanol. However, at low percentages (such as 20%), the solvent was well separated from the peak, while the PR column eluted too late. At high methanol percentage (such as 50%), MTP was eluted without adsorption on the column. As a result, the most suitable percentage was therefore chosen as 43% methanol. In addition, 1.2 mL/min was decided on the plan that would not deform the column at the flow rate but could provide rapid elution. In addition, studies were carried out at 30 °C column oven temperature, which gives low noise and smoother chromatograms. The system suitability data of the chromatogram obtained under optimized conditions are as in Table 1. Also, the paper is presented in the chromatogram obtained by analysis of 100% standard solution under optimized conditions in Figure 1. In the literature, there are few HPLC methods in which PR [19] or both [21, 22] as markers for SPIP studies. The optimized method appears to be more successful when evaluated in terms of chromatographic separation of previous MPT and PR analytes [21, 22]. Since both PR and MPT are highly polar, the organic components of the mobile phase, methanol and C18 in the stationary phase, and phosphate buffer were preferred. However, there are studies where acetonirile is added in small percentages according to the polarity of the drug molecule whose permeability is desired to be measured [19]. In terms of analysis

time, it is obvious that it is long. However, since the analytes have quite different physicochemical properties, they interact differently in the stationary phase.

Table 1. The system-suitability data for MPT, DMP and PR (%100).

Parameter	Observed value			Acceptance criteria
	MPT	DMP	PR	
Retention time (min)	2.84	5.22	9.28	-
Standard deviation (%) of retention time	0.54	0.02	0.54	RSD ≤1%
Precision for peak area	0.62	0.53	0.16	RSD ≤ 1%
Injection precision for retention time (min)	0.02	0.01	0.04	RSD ≤ 1%
Capacity Factor (k')	0.91	2.51	5.24	k' ≥ 2
Resolution (R_s)	9.02	9.01	9.95	>2
Tailing Factor (T)	1.4	1.15	1.16	≤2
USP Width[a]	0.21	0.31	0.50	≤1
HETP	5032133	3509967	1666757	-

[a] Calculated according to USP.

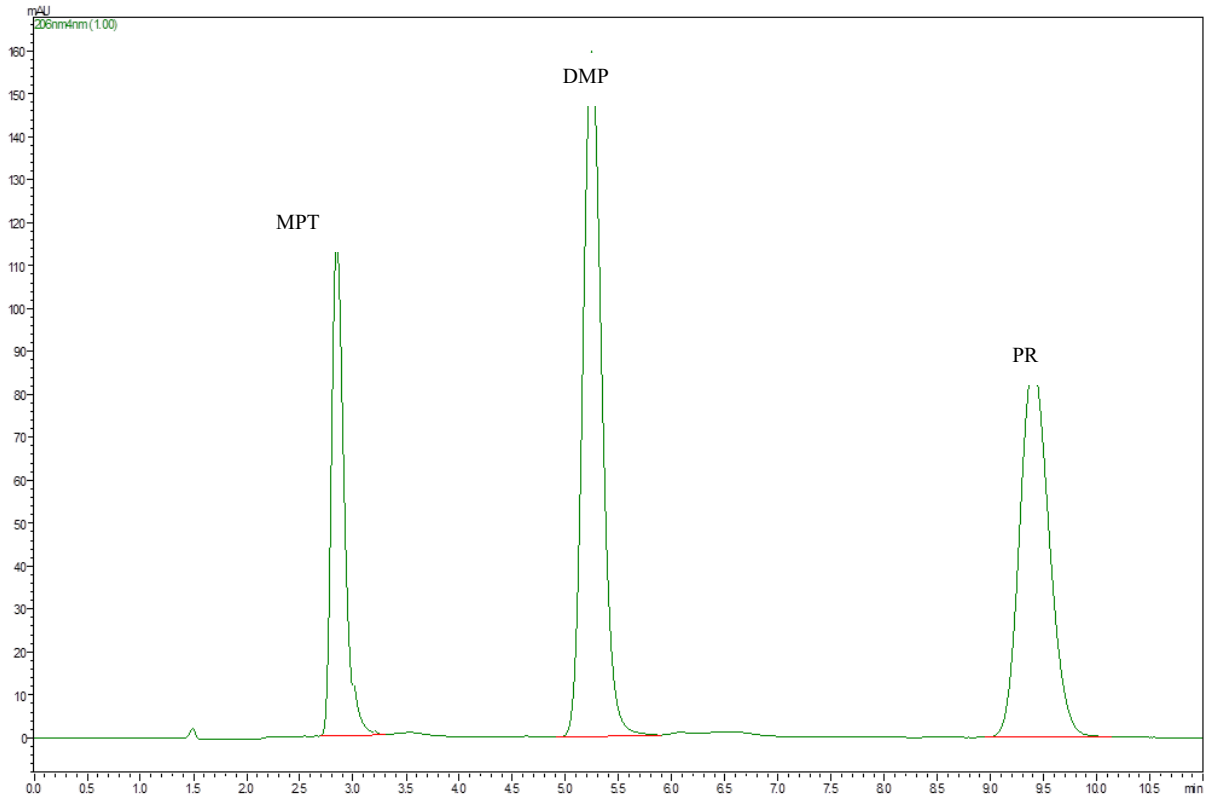

Figure 1. Chromatogram of MPT (200.7 µg/mL), DMP (50.0 µg/mL) and PR (106.2 µg/mL) under optimized conditions (%100)

2.2. Method validation

By calculating the highest areas in linearity studies, the detector signals obtained against the analyte concentration were calculated. The calibration plot was created by plotting the solutions at various concentrations against the relevant peak areas in the mass or PDA detector. It is given in Table 2 that method applicability parameters such as linearity and precision are in the appropriate range for both detector types. The specificity and selectivity of the method has been proven to eliminate possible disruptive effects. For this purpose, in the presence of degradation products, the improvement of the method and the purity of the peak were determined for each developed method, and it was demonstrated once more that the target compound alone was responsible for the current peaks. According to calculations, MTP, DMP, and PR had the greatest purity levels at 0.999119, 0.999847, and 0.9993041, respectively.

Table 2. Statistical data for the linearity of PR, MPT and DMP

	Parameter	PR	MPT	DMP
Intra-day (n=24)	**Linearity range (µg/mL)**	21.4-212.4	40.1-401.3	10.0-100.0
	Slope	15557.9	4922.1	39681.9
	Intercept	19970.9	-1135.0	15558.5
	Regression coefficient	0.9999	0.9998	0.9998
	SE of Slope	47.6	29.6	193.5
	SE of Intercept	6355.3	7462.5	12151.7
	ANOVA	F(2.21)=0.999 p>0.05	F(2.21)=0.999 p>0.05	F(2.21)=0.999 p>0.05
Inter-day (n=24, k=3)	**Slope**	4974,2	4466807.2	0.00102
	Intercept	-3805,0	-39511.0	-0.0574
	Regression coefficient	0.9994	0.9915	0.99799
	SE of Slope	45.7	1492.3	0.0721
	SE of Intercept	11522.7	93717.9	0.000002
	ANOVA	F(2.21)=0.999 p>0.05	F(2.21)=0.999 p>0.05	F(2.51)=0.0.999 p>0.05
	LOD (µg/mL)	1.6	3.1	0.8
	LOQ (µg/mL)	2.1	4.0	1.1

After calculating the linearity and precision data of the method optimized for simultaneous SPIP analysis of MTP, DMP and PR, accuracy studies were performed. For this purpose, solutions were prepared and analyzed for each of the three analytes corresponding to the low (80%), medium (100%), and high (120%) levels of the calibration curve. Solutions of MTP, DMP and PR were prepared in a Golytely solution passed the animal intestine and diluting the required proportions with water to 80%, 100% and 120%. The obtained chromatogram for %100 percentage of recovery solution was given Figure 2 and statistical results calculated as Table 3. Analyzing standard 100% solutions after storage under various circumstances allowed researchers to look into the stability of analytes solutions. The 95% confidence level (CI) results were given in Table 4.

Robustness was investigated via analyzing analytes solutions prepared to introduce small changes in the organic component ratio in the mobile phase, mobile phase pH, flow rate, column temperature, and detector wavelength. After three repeats under final instrumental conditions, the differences in the parameters (such as retention time and tailing factor) were assessed. The results demonstrate the robustness of the procedure (Table 5). Compared to the HPLC methods developed for different drug molecules whose intestinal permeability is desired to be measured in the literature, the study has the widest range of validation parameters, and all the parameters recommended in the ICH (Q2)R1 guideline [17] were calculated and presented for each analyte component [19, 21, 22].

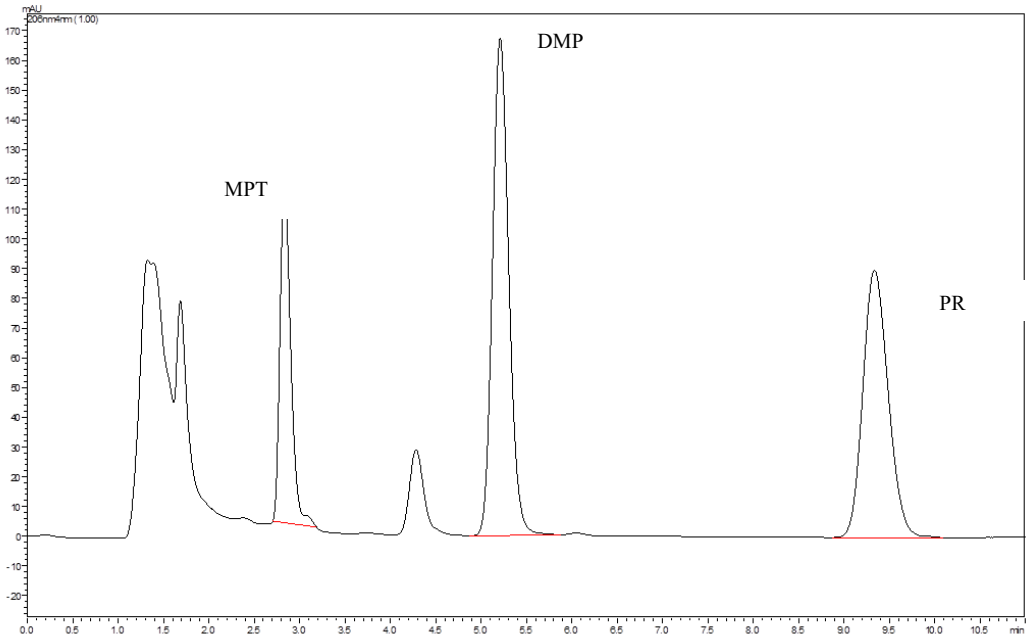

Figure 2. The chromatograms of %100 recovery solution

Table 3. Analysis of recovery studies using statistics *(n = 3)*

				Precision		Accuracy
Analyte	**Added (µg/mL)**	**Found (µg/mL)±CI***	**SD**	**RSD (%)**	**Recovery (%)**	**Error (%)**
	160.5	159.3±0.9	0.8	0.5	199.2	-0.8
MPT	200.7	199.1±0.1	0.1	0.1	99.2	-0.8
	240.8	235.1±0.9	0.8	0.3	97.6	-2.4
	40.0	39.3±0.1	0.1	0.3	98.2	-1.8
DMP	50.0	49.2±0.1	0.1	0.1	98.5	-1.5
	60.0	58.4±0.1	0.1	0.2	97.3	-2.7
	85.0	82.5±0.1	0.1	0.1	97.2	-2.9
PR	106.2	105.6±0.1	0.1	0.1	99.5	-0.5
	127.4	126.4±0.5	0.5	0.4	99.2	-0.9

**95% confidence level*

Table 4. Stability data *(n = 3)*

Compound	**Short- term stability (24 h. room temperature)**			**Short-term stability (48 h. room temperature)**		**Long-term stability (3 weeks, -20°C)**		**Freeze-thaw stability (3 cycles)**	
	Added concentration (µg/mL)	**Found (Mean±CI*)**	**Difference (%)**	**Found (Mean±CI*)**	**Difference (%)**	**Found (Mean±CI*)**	**Difference (%)**	**Found (Mean±CI*)**	**Difference (%)**
MPT	200.7	200.6±0.7	99.9	204.1±0.6	101.7	202.5±0.2	100.7	202.7±0.8	101.0
DMP	50.0	50.4±0.3	101.0	49.7±0.1	99.4	49.5±0.7	98.9	49.9±0.5	99.8
PR	106.2	106.1±0.2	99.9	105.7±0.7	99.6	105.6±0.2	99.4	105.6±0.5	99.5

2.3. Perfusion studies

There is single-pass or circulating versions of the perfusion solution for the permeability of drugs. The study was carried out in the ileum segment of the rat intestine in a 10 cm long. Samples were collected by flowing the perfusion solution from the fixed area for 60 minutes. In the current study, a single-pass perfusion technique was used to examine the permeability of DMP, an antiemetic, as a model drug. The reason for choosing this technique was that the surgical method is easy, and it is a technique close to in vivo conditions. In this technique, female rats were used. Golytely solution, which is an isosmotic solution at neutral pH, was used as the perfusion solution. MT, FK added to the perfusion solution were added according to the literature. The amount of collagen was added according to the maximum value found in the liquid solutions used in oral administration. MPT 400 µg/mL, DMP 100 µg/mL and PR 200 µg/mL were used in the perfusion solution. The mean, standard deviation, and coefficient of variation of DMP with MPT, the reference material used during the experiment, were given. MPT in BCS class I was used to compare the permeability of the model drug used. In vivo bioavailability studies are based on BCS criteria. According to the system, drugs are divided into 4 groups. According to BCS, the active substance of the orally administered drug should have high solubility and high permeability, and the drug should have a high dissolution rate. If the drug product dissolves more than 85% in the first 30 minutes in the specified environments, it is defined as rapidly dissolving. The analytes of DMP have high solubility during the experiment.

The intestinal membrane integrity was measured using PR, a zero-permeability marker. This marker is widely used in different models of permeability studies [23]. Net water flux (NWF) values indicated loss of fluid from the mucosal side (lumen) to the serosal side (blood) in the pure DMP group (-45.50±14.87mL/h/cm) (Table 6). In addition, we evaluated its permeability by comparing it with the Peff values of MPT, the reference material was used. MPT Peff value of 0.566±0.119 × 10^{-4} (cm/s) is considered normal permeability in this range [24]. On the other hand, the Peff value of DMP was found 2.270±1.46 × 10^{-4} (cm/s), which is higher than MPT in given Figure 3A. Furthermore, ka values of MPT and DMP was found to be 12.657±1.104 × 10^{-4} (cm/s) and 19.499±1.531 × 10^{-4} (cm/s), respectively. Calculated Human Peff showed similar results to Rat Peff values (Table 7, Figure 3B). Fa (%) values for MPT and DMP were found 87.725±4.687 x 10^{-2} (%) and 100.00±1.34 × 10^{-2} (%), respectively (Figure 3C). The animal experiments have been completed successfully. The amount of DMP was determined by the method that was developed. An exemplary sample chromatogram obtained during the analysis of in vivo samples is presented in Figure 4.

3. CONCLUSION

The SPIP model has a significant advantage as it offers close results compared to oral administration. Our study's goal was to create and validate a reversed-phase liquid chromatographic technique for the simultaneous detection of Domperidone maleate, metoprolol, and phenol red for use in intestinal perfusion studies. So, the current method is the first HPLC method in the literature that has fully optimized analytical properties; it was fully validated according to ICH guidelines, and perfusion analysis of SPIP was examined. Also, the developed method is easy to apply, fast, low consumable, has high accuracy, reproducible and precision, and is suitable for such perfusion studies and scientific work. To conclude, with the developed method, real-world application was made and the method's performance was demonstrated.

Table 5. Robustness data *(n = 3)*

		Retention time (min)		Peak area		Resolution		Tailing factor	
		Observed value	Difference(%)	Observed value	Difference(%)	Observed value	Difference(%)	Observed value	Difference(%)
MTP									
Column temperature °C	27	2.9±0.1	2.1±0.1	901617±1050	-8.6±0.1	9.2±0.2	2.0±2.6	1.3±0.1	-9.8±0.2
	33	2.8±0.1	-1.5±0.6	968554±5545	-1.7±0.6	8.7±0.1	-3.7±1.6	1.5±0.0	6.5±0.2
Flow rate (mL/min)	1.1	3.2±0.1	10.9±0.1	984694±2469	-0.1±0.3	9.5±0.4	5.7±4.9	1.3±0.0	-8.6±0.1
	1.3	2.6±0.1	-9.4±0.2	812741±2245	-17.5±0.2	8.9±0.4	-1.6±4.1	1.3±0.0	-11.4±0.2
organic phase (%)	38.7	2.4±0.1	-16.3±0.1	980849±776	-0.5±0.1	6.4±0.2	-29.4±2.0	1.4±0.1	15.8 ±0.1
	47.3	3.5±0.1	-24.7±0.1	918073±1024	-6.9±0.1	12.4±0.4	37.9±4.2	1.5±0.0	-9.0±0.1
pH of the buffer	3.0	2.8±0.1	-1.8±0.1	920442±1017	-6.6±0.1	8.7±0.1	-3.4±1.6	1.3±0.0	-5.6±0.1
	3.2	2.8±0.1	1.5±0.1	964826±2285	-2.1±0.2	8.7±0.2	-3.3±1.6	1.3±0.1	-5.6±0.1
Detector wavelength (nm)	202 nm	2.8±0.1	0.13±0.1	1637099±668	-66.0±0.1	8.8±0.1	-2.9±0.1	1.3±0,1	-3.6±0.6
	210 nm	2.8±0.1	0.13±0.1	850605±840	-13.7±0.1	8.6±0.1	-4.6±0.4	1.3±0.0	-4.6±2.2
DMP									
Column temperature °C	27	5.5±0.1	5.3±0.3	2019546.0±3503	1.1±0.2	9.6±0.1	6.4±0.6	1.1±0.00	-1.1±0.4
	33	4.9±0.1	-5.7±0.3	2006246.3±18979	0.4±1.0	58.6±0.1	-5.0±0.9	1.2±0.01	0.5±0.1
Flow rate (mL/min)	1.1	5.8±0.1	10.4±0.1	2199612.7±5996	10.1±0.3	9.2±0.1	2.1±0.5	1.2±0.01	1.8±0.2
	1.3	4.7±0.1	-10.5±0.2	1787103.7±9324	-10.5 ±0.5	8.8±0.1	-1.9±0.4	1.3±0.01	-1.6±0.3
organic phase (%)	38.7	3.7±0.1	-29.5±0.02	2001625.3±561	0.2±0.1	5.8±0.1	-35.1±0.1	1.1±0.01	5.8±0.1
	47.3	7.9±0.1	52.1±0.1	1990773.0±13081	-0.3±0.7	13.1±0.1	46.3±0.2	1.2±0.01	-8.9±0.1
pH of the buffer	3.0	5.1±0.1	-2.5±0.5	1976852.0±218	-1.0±0.1	8.8±0.1	-1.9±0.4	1.1±0.01	0.8±0.4
	3.2	5.1±0.1	-3.2±0.3	1986850.0±187	-0.5±0.1	9.0±0.1	-0.6±1.7	1.2±0.01	1.0±0.9
Detector wavelength (nm)	202 nm	5.2±0.1	0.4±0.1	1784907.7±3030	-10.7 ±0.2	9.3±0.3	3.0±3.5	1.2±0.01	1.6±0.1
	210 nm	5.2±0.1	0.4±0.2	1604958.7±1338	-19.7±0.1	9.2±0.2	1.7±2.0	1.2±0.01	1.8±0.1
PR									
Column temperature °C	27	9.8±0.1	6.2±0.1	1664307.3±2375	-0.4±0.1	10.2±0.1	2.6±0.4	1.2±0.01	0.2±0.4
	33	8.7±0.1	-6.1±0.6	1667678.0±7532	-0.2±0.5	9.8±0.1	-1.9±0.7	1.2±0.0	0.1±0.4
Flow rate (mL/min)	1.1	10.3±0.1	10.6±0.1	1856383.7±10068	11.1±0.6	10.3±0.0	3.6±0.4	1.2±0.0	1.8±0.2
	1.3	8.3±0.1	--10.1±0.2	1518093.7±12865	-9.1±0.8	9.8±0.0	-1.1±0.0	1.2±0.0	-0.3±0.5
Organic phase percentage(%)	38.7	6.0±0.1	-35.8±0.1	1685535.0±10912	-0.9±0.7	7.6±0.0	23.9±0.0	1.2±0.01	1.2±0.1
	47.3	15.5±0.1	-67.5±0.1	1657064.3±6889	-0.8±0.4	12.7±0.0	27.9±10.0	1.2±0.0	1.5±0.2
pH of the buffer	3.0	9.4±0.1	0.9±0.1	1663889.7±3712	-0.4±0.2	10.5±0.1	16.2±1.0	1.2±0.0	1.7±0.1
	3.2	9.3±0.1	-0.3 ±0.1	1658034.3±5481	-0.7±0.3	8.7±0.1	-3.4±1.7	1.2±0.0	1.2±0.3
Detector wavelength (nm)	202 nm	9.4±0.1	1.3±0.0	2184558.3±3568	-30.8±0.2	10.0±0.1	10.4±0.1	1.2±0.0	0.6±0.1
	210 nm	9.4±0.1	1.3±0.1	1306304.0±3119	-21.8±0.2	9.7±0.3	7.3±3.7	1.2±0.0	1.0±0.1

Table 6. The net water flux (NWF) in each group (n = 6)

Time (min)	DMP
10	82.76±17.06
20	-44.38±55.44
30	-70.81±28.51
40	-78.25±27.91
50	-88.17±17.32
60	-74.14±16.93
Mean±SD	-45.50±14.87

Table 7. Permeability of MPT and DMP (n = 6)

Drug	Rat P_{eff} $(x10^{-4}, cm/s)$	k_a $(x10^{-4}, 1/s)$	Human P_{eff} $(x10^{-4}, cm/s)$
MPT	0.566±0.119	12.657±1.104	1.930±0.428
DMP	2.270±1.46	19.499±1.531	8.200±1.460

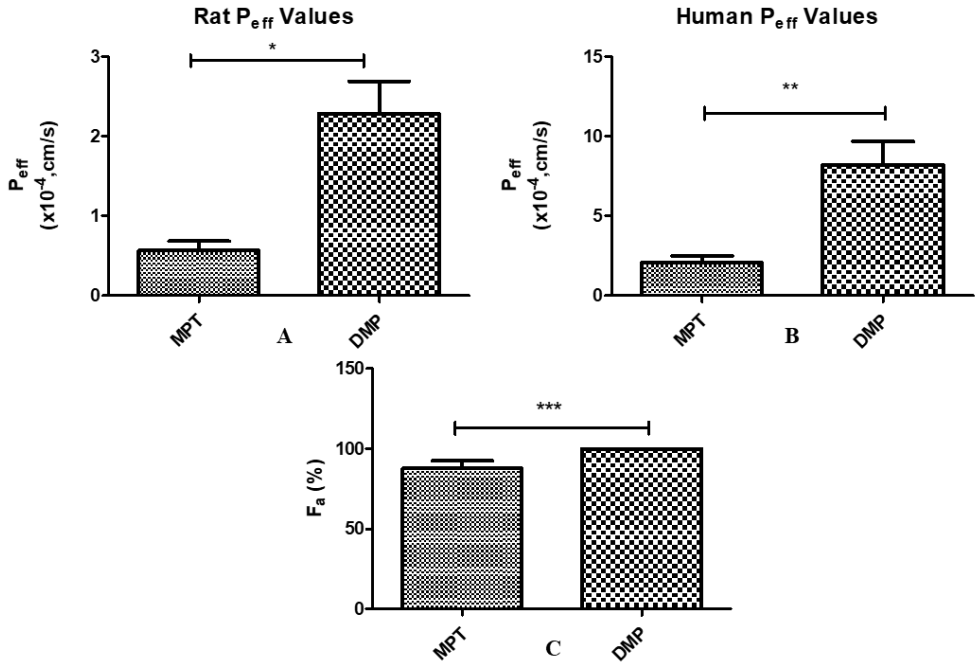

Figure 3. The permeability coefficients of MPT and DMP obtained from perfusion of rat ileum (mean SD, n=6) A: Rat P_{eff} values (cm/sec), B: Calculated Human P_{eff} values (cm/sec), and C: Calculated fraction absorbed values (Fa %) *, **, *** p<0.05, MPT vs DMP

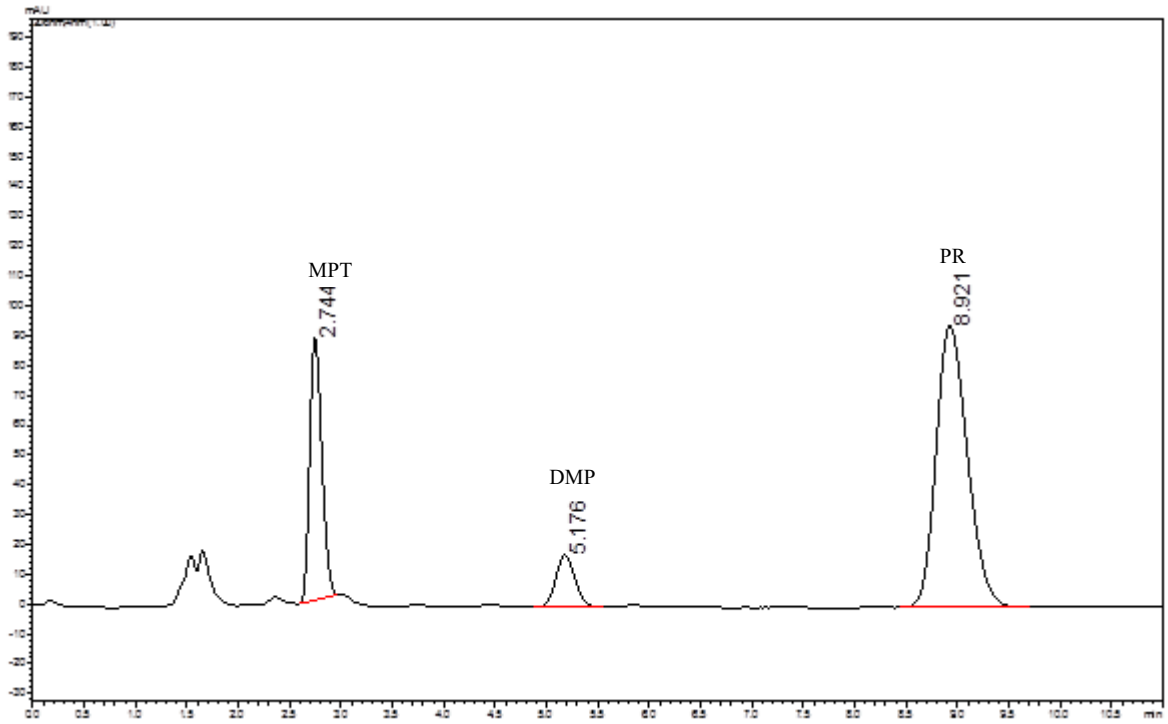

Figure 4. Assay chromatograms of SPIP studies

4. MATERIALS AND METHODS

4.1. Chemicals and reagents

DMP was gifted by Deva Holding, Turkey. HPLC grade sodium dihydrogen phosphate (99.8%, w/w), phosphoric acid (86%, w/w), sodium hydroxide (pure), methanol, NaCl, KCl, Na_2SO_4, $NaHCO_3$, phenol red (PR) and mannitol were purchased from Sigma-Aldrich (Missouri, ABD). Metoprolol tartrate (MPT) was donated from Novartis (Novartis Drug Co., Turkey).

4.2. Instruments

All analyses were carried out using a Prominence series of HPLC systems from Shimadzu Co. (Kyoto, Japan). The instrument equipped with an LC-20AT tandem dual-plunger pump equipped with a low-pressure gradient unit, SIL-20AC HT auto-sampler, CTO-10ASVP column oven, DGU-20A5R online degasser, CBM-20A communications bus module, and SPD-M20A photodiode array detector (PDA). The dwell volume of the liquid chromatography system was determined as 500 µL. All standard and sample solutions were prepared using an RK 100 H model ultrasonic bath from Bandelin (Berlin, Germany), a pH meter from Mettler-Toledo (Greifensee, Switzerland), a Rotina 380 R model centrifuge from Hettich (Tuttlingen, Germany) and a Heidolph Reax Top model vortex mixer (Schwabach, Germany).

4.3. Instrumental conditions

The chromatographic separation was achieved on a Intersil ODS-3 column C18, 150 × 1.5 mm, 5.0 µm particle size, from GL Sciences (GL Sciences, Tokyo, Japan) with isocratic elution. In addition, two different reversed-phase columns, i.e. Ascentis Express Phenyl Hexyl (100 × 4.6 mm, 2.7 µm ID) and Ascentis Express F5 (100 × 4.6 mm, 2.7 µm ID) were tested for performance, system suitability and specificity comparison. The mobile phase was composed of methanol: phosphate buffer (20 mM, pH 3.1) (43:57, v/v). The injection volume was 5 µL, and the column oven temperature was set at 30 °C. The flow rate of the method was chosen 1.2 mL/min. The PDA detector was set at 206 nm wavelength obtained maximum absorbance from MTT, PR and DMP. The real-time spectra were recorded at 0.240 msec data sampling and 0.640 sec time constant.

4.4. Preparation of solutions

4.4.1. Reference solutions

Separately, 6.2 mg MTT, 2.4 mg PR, and 1 mg DMP were precisely weighed and added to a 10 mL volumetric flask. The MTT and PR substances were dissolved in water, DMP was dissolved in methanol. All working and calibration solutions were prepared with these stock solutions and diluted to volume with water.

4.4.2. Perfusion solution preparations

The perfusion medium consisted on 25 mM NaCl, 10 mM KCl, 40 mM Na_2SO_4, 20 mM $NaHCO_3$ and 80 mM mannitol. The pH of the perfusion medium was adjusted to 7.4 by ortho-phosphoric acid. Perfusion medium was prepared freshly and then filtered through 0.45µm non-sterile cellulose acetate membrane filter before use.

4.4.3. Sample perfusion solution preparations

The solutions passed through the rat intestinal region were centrifuged during 10 min, at approximately 5180 rpm, at 15 °C. and then diluted with acetonitrile 1:2 and injected.

4.4.4. Mobile phase solutions

In the mobile phase, the buffer was set up as follows: In 1 L mL of water, 2.7 g of sodium dihydrogen phosphate and 420 µL of phosphoric acid were dissolved, then the mixture was sonicated for 10 minutes. The buffer solution and the other mobile phase component, methanol, were degassed before use and filtered.

4.5. In-situ SPIP assays and surgical procedure

All experiments involving animals were carried out in accordance with protocols approved by the Anadolu University's Committee on the Use and Care of Animals; the number of ethical approvals was 2020–41. The female Sprague Dawley rats (weighing 200-250 g) used in all in-situ intestinal perfusion experiments were 200-250 g in weight. Prior to each experiment, the rats were fasted for approximately 12 hours with free access to tap water overnight before beginning.

For the in-situ SPIP research, the experimental approach was carried out in accordance with the previously published studies that had been previously described [22, 25-29]. Ketamine-xylazine mixtures (90 and 10 mg/kg, respectively) were used to anesthetize the rats, which were administered intraperitoneally to ensure complete anesthesia.

All the animals were placed on a heated surface that was maintained at 37.5% of their body temperature. It was necessary to open the abdomen by making a midline incision (3-4 cm) in order to expose the small intestine. Using flexible PVC tubing (inlet tubing with an internal diameter (id) of 0.76 mm and outlet tubing with an internal diameter (id) of 1.70 mm), a 10 cm segment of the jejunum was carefully cannulated and then connected to the perfusion system. Care was taken to ensure that the segment's blood supply remained intact, and the exposed segment was kept moist with a 37°C saline solution throughout the procedure. Perfusion medium was incubated in a water bath at 37.5°C of its maximum temperature. The surgical area was then coated with parafilm in order to limit the evaporation of fluid from the exposed segment surface. For approximately 20 minutes, a peristaltic pump (Minipuls-3, Gilson, France) pushed into the colon at a flow rate of 0.5 mL/min via a perfusion solution that was free of medication and 37.5°C (blank perfusion solution) to clean up any leftover debris. A perfusion solution containing the marker phenol red was administered for 60 minutes at a flow rate of 0.2 mL/min after the cannulated intestinal section had been cleaned and disinfected. At 10-minute intervals (10, 20, 30, 40, 50, and 60 minutes), samples were obtained from the distal region of the jejunum and placed in tubes. The samples were immediately frozen at -20°C to analyze with using HPLC. Animals were euthanized with cervical dislocation at the conclusion of each experiment, in accordance with standards for euthanasia in experimental animals. After each trial, the intestinal segment was measured to determine its length. According to the previously mentioned equations, Net Water Flux (NWF), effective permeability for rats (P_{eff}) and calculated effective permeability for humans (Human P_{eff}), as well as fraction absorbed predicted for humans (F_a, %), were all determined [22, 30-35].

4.6. Analytical method validation

This optimized new method was fully validated according to the ICH (Q2)R1 guideline and investigated each validation parameter was given above [17].

4.6.1. System suitability testing

For system suitability testing, resolution (R_s), capacity factor (k'), tailing factor (T), USP width and the height equivalent to a theoretical plate (HETP) were calculated via Shimadzu LC LabSolutions 1.24 SP1 data integration software.

4.6.2. Specificity

For the method specificity, the signals of MTT, PR and DMP were examined, and peak purities were investigated using PDA detector.

4.6.3. Linearity and range

The linearity solutions were prepared to cover eight different concentration levels for both types of working solutions, between 15.4-100.0 ng/mL for matrix-matched and 94.2-18839.2 ng/mL for solvent-based. The range of the method corresponded to 20–200% estimated concentration of the test solutions. The calibration curves of the method were plotted as being analyte concentration versus peak area. Slope, intercept, certain intervals of the slope, the intercept at 95% confidence level, and correlation coefficient were calculated.

4.6.4. Accuracy

Recovery experiments were used to test the accuracy. The recovery tests were carried out at 3 different concentration levels, corresponding to low (80%), medium (100%), and high (120%) fortification. For each level, 3 parallel sets have been prepared. Following a reanalysis of the spiked samples, the average recovery was estimated with a 95 % confidence level using standard deviation, RSD percent, and confidence levels.

4.6.5. Precision

The results of intraday and interday (intermediate) experiments used to determine precision tests were analyzed along with sample and reference solutions. Using the given methodology, 6 repeating analyses were carried out on the same day and 3 consecutive days. Statistics were used to assess the results, including mean, standard error of mean, standard deviation, RSD%, and confidence interval at a 95% level of assurance. A one-way ANOVA test was also used to assess differences between inter-day groups using Prism 6.0 software (GraphPad, CA, USA).

4.6.6. Limits of detection and quantification

LOD and LOQ values of the method were calculated according to the signal/noise ratio according to the ICH recommendations; signal/noise ratio was equalized to 3 for LOD, and to 10 for LOQ. Then, MPT, PR and DMP solutions at the LOD and LOQ were injected six times for each, and the responses in the chromatograms were evaluated.

4.6.7. Robustness

The robustness was examined by applying deliberate changes in the methodological parameters by ±10% and analyzing calibration solutions at 100% level for both calibration curves. The effects of each parameter on the method were examined separately and compared with the optimum conditions. The results were calculated to recover system suitability tests such as retention time, and peak area.

4.6.8. Statistical Analysis

The two-tailed non-parametric Mann-Whitney U test was employed to compare the two experimental groups and find any differences. A *p* value of less than 0.05 was regarded as significant.

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