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Simultaneous Determination of Acyclovir, Metoprolol and Phenol Red by a RP-HPLC Method for Intestinal Perfusion Studies

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Introduction

Acyclovir is an antiviral drug (Figure 1) used in the treatment of herpes simplex and varicella zoster infections[1]. Acyclovir shows its antiviral activity by competitively inhibiting the viral DNA[2]. It is also used in the treatment of cytomegalovirus and Epstein Barr virus infections[3]. According to Biopharmaceutics Classification System (BSC), acyclovir is a Class III (high solubility, low permeability) compound, and it is transported through paracellular route by passive diffusion[4-6].

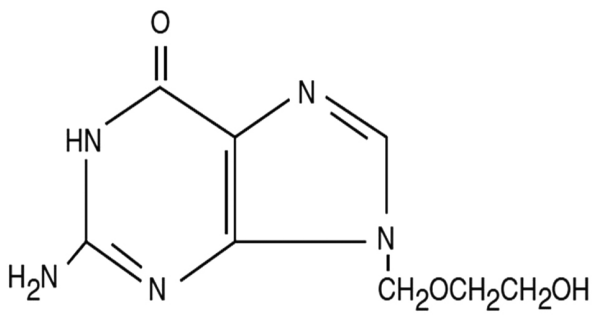

Figure 1
The chemical structure of acyclovir.

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Various HPLC methods have been described in the literature for separate determination of acyclovir[2,7-17] and metoprolol[18-28] in biological fluids. All these reported methods differ from each other with regard to the mobile phase, columns and detection methods used for the analysis of compounds (Table 1 and Table 2). However, to our knowledge, there is no study available in the literature for simultaneous determination of all these compounds. Therefore, the purpose of our study was to develop and validate a reversed-phase liquid chromatographic method for the simultaneous determination of acyclovir, metoprolol and phenol red to be used for intestinal perfusion studies. Acyclovir was selected as the model compound. In perfusion studies, metoprolol (as tartrate) is widely used as a reference standard to compare the permeability coefficient of the compound of interest (Figure 2a) and phenol red (Figure 2b) as a zero permeability marker for correction of permeability coefficient of the compound[29].

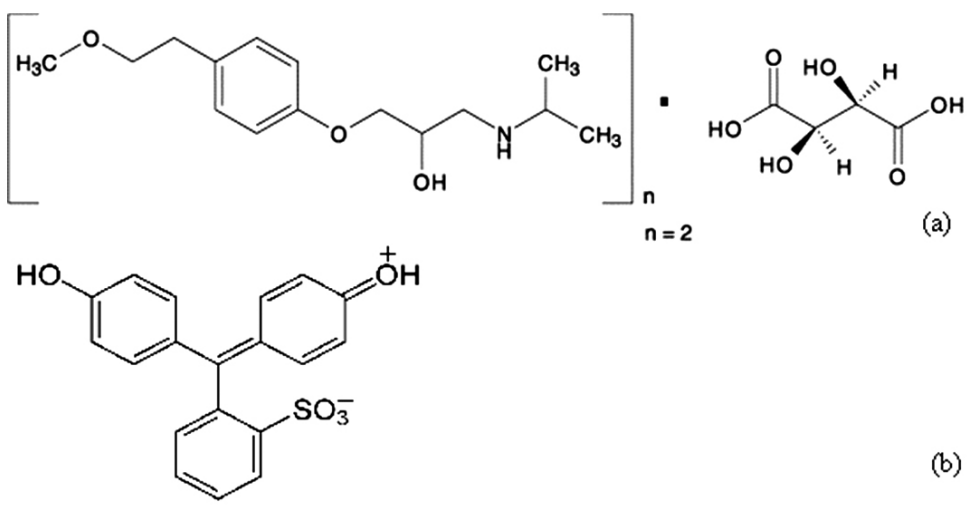

Figure 2
The chemical structures of metoprolol tartrate **(a)** and phenol red **(b)**.

TABLE 1

Reported HPLC methods for analysis of acyclovir.

Samples	Column	Mobile Phase	Detection	Flow rate	Linearity Range	Reference
Human plasma	Novaflex C18 (300x4.6mm, 10 µm)	Methanol:octane sulfonic acid buffer (8:92, v/v, pH 2.5)	UV (254 nm)	1.5 mL/min	0.02-5.00 µg/mL	2
Human plasma	Diamonsil C18 (250x4.6 mm, 5 µm)	Gradient elution Solvent A: 0.08% [v/v] aqueous TFA solution (pH 2.30-2.35) Solvent B: Methanol	Fluorescence 260nm (excitation) 380 nm (emission)	1.5 mL/min	20-2000 ng/mL	7
Human plasma	Thermo C18 (250x4.6 mm, 5 µm)	5mM ammonium acetate (pH 4.0):acetonitrile (40:60, v/v)	UV (290 nm)	1 mL/min	25-150 ng/mL	8
Human plasma	Nucleosil C18 (250x4 mm, 5 µm)	Gradient elution Solvent A: 0.4% aqueous sodium heptanosulfonate solution (pH 2.6) Solvent B : Acetonitrile	Fluorescence 260 nm (excitation) 380 nm (emission)	1 mL/min	0.1-10.0 µg/mL	9
Human plasma	Hibar LiChrospher 100 RP8 (250x4.6 mm, 5 µm)	0.1% aqueous triethylamine solution (pH 2.5)	UV (255 nm)	1.2 mL/min	0.1-2.0 µg/mL	10
Human plasma	ERC-ODS (250x6.0 mm)	20 mM KH_2PO_4	Amperometric	1 mL/min	0.1-20 µg/mL	11
Maternal plasma Amniotic fluid Fetal tissues Placental tissues	Agilent Eclipse XDB C8 (150x2.1 mm, 5 µm)	For the plasma and amniotic fluid matrices: 10 mM acetate / citrate buffer:3.7 mM aqueous octanesulfonic acid (87.5:12.5, v/v, pH 3.08) For the placental and fetal tissue samples: 30 mM acetate / citrate buffer (pH 3.08):acetonitrile (99:1, v/v)	UV (254 nm)	0.2 mL/min	0.25-100 µg/mL	12
Human plasma	TSK gel ODS (150x4.6 mm, 5 µm)	Acetonitrile:20 mM phosphate buffer (2:70, v/v, pH 3.0)	UV (254 nm)	0.6 mL/min	0.063-2.080 µg/mL	13
Human plasma	Gemini C18 (150x4.6 mm, 5 µm)	0.1% formic acid:methanol (30:70, v/v, pH~3.0)	Mass spectrometry	0.8 mL/min	47.6-10225 ng/mL	14
Human plasma	Shimpack-CLC-ODS (150x6.0 mm, 5 µm)	Methanol:0.05 M phosphate buffer (5:95, v/v)	UV (250 nm)	2 mL/min	10-2560 ng/mL	15
Human plasma	LiChrospher RP18 (250x4.0 mm, 5 µm)	30 mM phosphate buffer (pH 2.6) containing acetonitrile 18%	UV (250-260 nm)	1.5 mL/min	0.05-1.80 µg/mL	16
Rat plasma Rat tissues	BS-5 silica (100x4.6 mm, 5 µm)	Acetonitrile:10 mM ammonium formate buffer (80:20, pH 3.0)	Mass spectrometry	0.7 mL/min	0.5-500 µg/mL	17

TABLE 2

Reported HPLC methods for analysis of metoprolol tartrate.

Samples	Column	Mobile Phase	Detection	Flow rate	Linearity	Reference
Human urine	Purospher STAR RP-18 (250x4.0 mm, 5 µm)	Gradient elution Solvent A: 0.05% trifluoroacetic acid in water Solvent B: Acetonitrile Solvent C: Methanol	UV (227 nm)	1.0-1.4 mL/min	0.25-25 µg/mL	18
Rat plasma	Chiralpak AD (250x4.6 mm) (Chiral column)	Hexane: ethanol: Isopropanol: diethylamine (88:10.2:1.8:0.2, v/v/v/v)	Fluorescence 229 nm (excitation) 298 nm (emission)	1.2 mL/min	1-2500 ng/mL	19
Human plasma	Nucleosil RP18 (125x4 mm, 5 µm)	Acetonitrile: 1.2% triethylamine in water (20:80, v/v, pH:3.0)	Fluorescence 280 nm (excitation) 300 nm (emission)	1 mL/min	6.25-200 ng/mL	20
Dog plasma	Betabasic Cyano (250x4.6 mm, 5 µm)	Acetonitrile: 2 M sodium dihydrogen orthophosphate buffer: water (5:0.5:94.5, pH 2.6)	Fluorescence 275 nm (excitation) 300 nm (emission)	0.8 mL/min	0.047-1.5 µg/mL	21
Human plasma	Venusil MP-C18 (100x4.6 mm, 5 µm)	Methanol:10 mM ammonium acetate: formic acid (pH 3.4) (50:50:0.05, v/v/v)	Mass spectrometry	0.8 mL/min	3-1000 ng/mL	22
Human plasma (as tartrate)	Phenomenex C8 (50x3 mm, 3 µm)	Methanol:10 mM ammonium formate buffer (97:3, v/v)	Mass spectrometry	1 mL/min	5-500 ng/mL	23
Human urine	Phenomenex silica (250x4.6 mm) and Chiralcel OD (250x4.6 mm) were used as achiral column and chiral column, respectively	Mobile phase A n-hexane:ethanol:2-propanol:diethylamine (90:5:5:0.05, v/v/v/v) Mobile phase B n-hexane:ethanol: 2-propanol:diethylamine (85:7.5:7.5:0.05, v/v/v/v)	Fluorescence 276 nm (excitation) 309 nm (emission)	1 mL/min	0.1-2 µg/mL	24
Rat intestinal perfusate	Shimpack VP-ODS (250x4.6 mm, 5 µm)	Methanol: 0.05 M KH_2PO_4 (55:45, v/v, pH 6.0)	UV (227 nm)	-	-	25
Human urine	Waters Atlantis T3 (100x2.1 mm, 3 µm)	Gradient elution Solvent A: 10 mM ammonium formate (pH 3.5) Solvent B: Acetonitrile	Mass spectrometry	0.3 mL/min	-	26
Human plasma (as succinate)	Peerless C18 (33x4.6 mm, 5 µm)	Methanol: water containing 0.5% formic acid (80:20, v/v)	Mass spectrometry	1 mL/min	1.5-100 ng/mL	27
Human plasma Human urine	Ace C18 (250x4.6 mm, 5 µm)	Methanol: water (50:50, v/v)	Fluorescence 276 nm (excitation) 296 nm (emission)	1 mL/min	3-200 ng/mL (for plasma) 5-300 ng/mL (for urine)	28

Methods

Chemicals

Acyclovir was received as a gift from Nobel Pharma (İstanbul, Turkey). Phenol red was obtained from Merck (Darmstdt, Germany) and metoprolol tartrate from Novartis Pharma (İstanbul, Turkey). Furosemide was a generous gift from Sanofi Aventis (İstanbul, Turkey). Methanol was of HPLC grade, and all other reagents were of analytical grade.

Instrumentation and Chromatographic Conditions

A Hewlett-Packard 1110 series HPLC system with a ternary solvent pump, Rheodyne injection valve, equipped with a 20 µL loop and a diode-array detector was used. The separation of compounds was performed using a Fortis Technologies Ltd. (Chester, UK) C_{18} analytical reversed phase column (5 µm, 4.6 mm x 250 mm).

Isocratic chromatographic separation was accomplished. The mobile phase was a mixture of methanol and 0.0125 M KH_2PO_4 (55:45, v/v). For preparation of 0.0125 M KH_2PO_4 solution, 1.70 g KH_2PO_4 was dissolved in ultrapure water and then diluted to 1000 mL with the same solvent. pH of this solution was adjusted to 7.0 by using 0.3% (v/v) triethylamine. Detection was set at 254, 227 and 420 nm for acyclovir, metoprolol and phenol red, respectively. Analysis was run at a flow rate of 1 mL/min, and total analysis time was 10 minutes.

Preparation of Perfusion Medium

The perfusion medium was consisted of 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[30]. Perfusion medium was prepared freshly and then filtered through 0.45µm membrane filter before use.

Preparation of Standard Solutions

Primary stock solution was prepared by dissolving acyclovir, metoprolol tartrate and phenol red in ultrapure water to produce a final concentration of 1mg/mL. Seven standard solutions with different concentrations (1, 5, 10, 20, 30, 40, 60 µg/mL for acyclovir and metoprolol; 1, 2.5, 7.5, 12.5, 15, 20, 30 µg/mL for phenol red) were prepared by spiking in blank perfusion solution. Furosemide (IS; 20 µg/mL) was dissolved in methanol, and used as the internal standard.

Method validation

The developed HPLC method was validated as to selectivity, linearity, sensitivity (limit of detection and quantitation), accuracy- precision (intra-day and inter-day), and stability, according to the FDA Guidelines[31].

Selectivity of the method was assessed by injecting drug free perfusion solution into the HPLC system.

Linearity of the method was evaluated within the concentration range of 1-64 µg/mL for acyclovir, 2-100 µg/mL for metoprolol, and 4-100 µg/mL for phenol red. All calibration curves were constructed by plotting the peak area ratios of analyte to IS against the corresponding nominal concentrations. Linearity of the method was assessed by calibration equation which is characterized by determination coefficient, slope and intercept.

Sensitivity of the developed method was evaluated by determining the limit of detection (LOD) and lower limit of quantitation (LLOQ). The signal to noise ratios of 3:1 and 10:1 were taken as LOD and LLOQ, respectively.

Three different concentrations for each compound (5, 20, 40 µg/mL for acyclovir and metoprolol; 2.5, 12.5, 20 µg/mL for phenol red) were analyzed six consecutive days (inter-day) and six times within the same day (intra-day) to determine the precision of the method. Precision was expressed as the relative standard deviation (RSD) of the control sample concentrations, and accuracy was expressed as the relative error (RE).

For assessment of stability in perfusion solution, samples obtained from perfusion studies were frozen at –20°C for 24 hours. All samples were thawed at room temperature and then analyzed by the developed HPLC method. Results were compared with those of freshly collected perfusion samples.

System Suitability Parameters

System suitability test parameters must be checked to ensure that the system is working correctly during the analysis[32].

Capacity factor, k', is defined as the ratio of mole numbers of compounds (acyclovir, metoprolol, phenol red) in stationary phase and mobile phase. It is calculated as follows;

$$k'_A = \frac{t_A - t_0}{t_0} \qquad \text{(Equation 1)}$$

where, t_A is retention time of the compound, and t_0 is retention time of unretained compound (potassium bromide).

Selectivity parameter is a measure of separation of two compounds in the sample under given conditions. Selectivity parameter, α, is defined as;

$$\alpha = \frac{k'_A}{k'_B} = \frac{t_A - t_0}{t_B - t_0} \qquad \text{(Equation 2)}$$

Resolution (R) is a quantitative term that indicates to what extent components in the sample separated. It is expressed as;

$$R = \frac{2_A - t_B}{w_A + w_B} \qquad \text{(Equation 3)}$$

where w is the width of peak base.

In a particular separation, column efficiency refers to the performance of the stationary phase. The number of theoretical plates is used to determine chromatographic column efficiency. The number of theoretical plates was calculated using the following equation;

$$N = 16\left(\frac{t}{w}\right)^2 \qquad \text{(Equation 4)}$$

The height equivalent to theoretical plate (HETP) was expressed as HETP= L/N. In this equation, L was column length (250 mm).

Results And Discussion

Method optimization

An HPLC method was developed for simultaneous determination of acyclovir, metoprolol tartrate and phenol red for intestinal perfusion studies. Two different reverse-phase columns (ACE Ltd. (Aberdeen, Scotland) RP_{18}: 150 mm×4.6 mm, 5 µm; Fortis Technologies Ltd. (Chester, UK) C_{18}: 250 mm×4.6 mm, 5 µm) were used for separation of test (acyclovir) and reference compounds (metoprolol tartrate and phenol red). In terms of reproducibility, complete separation and peak shape, best results were obtained with Fortis Technologies Ltd. (Chester, UK) C_{18} (250mm×4.6mm, 5µm) column compared to the other column, and hence it was selected in our study. Feasibility of various solvent mixture(s) such as methanol and acetonitrile using potassium dihydrogen phosphate buffer with variable pH range of 2.5–7.0 was tested for complete chromatographic resolution of compounds. Good separation and short run time were obtained using a mobile phase consisting of

methanol:0.0125 M potassium dihydrogen phosphate buffer (55:45, v/v; pH 7.0). The compounds were analyzed at their respective lambda max values (acyclovir, metoprolol, phenol red at 254, 227 and 420 nm, respectively) to get adequate sensitivity.

Method validation

Selectivity

Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in a sample[33]. There was no interfering peak on the chromatogram following injection of blank perfusion solution into HPLC column (Figure 3). The chromatogram obtained from standard solution was identical with that obtained from spiked solution containing equivalent concentration of acyclovir (9 µg/mL), metoprolol tartrate (4 µg/mL), phenol red (10 µg/mL) and furosemide (20 µg/mL, IS) (Figure 3). Under optimum conditions, retention times were 2.8, 8.6 and 5.6 min for acyclovir, metoprolol tartrate and phenol red, respectively. Therefore, the proposed method was considered to be selective.

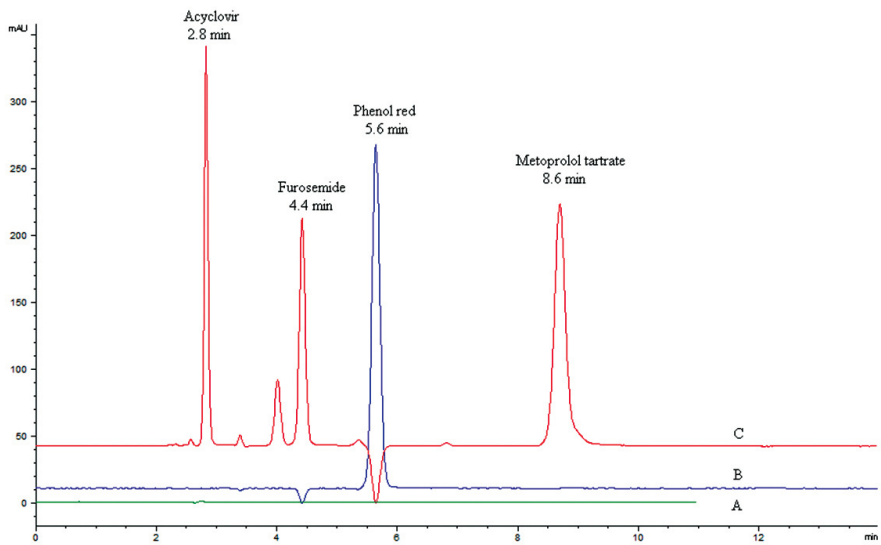

Figure 3

HPLC chromatogram of blank perfusion solution at 420 nm (A). The standard solution (20 µg/mL for acyclovir, metoprolol tartrate, furosemide and 12.5 µg/mL for phenol red) spiked in blank perfusion solution at 420 nm (B) and at 227 nm (C).

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. Quantifications of acyclovir, metoprolol and phenol red were based on the calibration curves constructed under optimum conditions as the ratio of the peak areas of analyzed substance to internal standard (y) against the corresponding concentrations (x). Linearity of the method was determined by performing injections at seven different concentration levels in the linear range over six different days. The calibration curves were found to be linear within in the concentration range of 1-64 µg/mL for acyclovir, 2-100 µg/mL for metoprolol tartrate and 4-100 µg/mL for phenol red (Table 3).

TABLE 3

The linearity data of developed HPLC method.

	Acyclovir	**Metoprolol**	**Phenol Red**
Regression equation	y= 0.0667x + 0.0089	y= 0.0749x – 0.0266	y= 0.1346x – 0.0837
Standard error of intercept	0.011	0.013	0.039
Standard error of slope	0.002	0.002	0.005
Determination coefficient (r^2)	0.9996	0.9997	0.998
Linearity range (µg/mL)	1-64	2-100	4-100
LOD (µg/mL)	0.010	0.002	0.003
LLOQ (µg/mL)	0.040	0.020	0.010

Sensitivity

Limit of Detection (LOD) and Lower Limit of Quantification (LLOQ) values were used to determine the sensitivity of the developed method. The LOD values were 0.01 µg/mL for acyclovir, 0.002 µg/mL for metoprolol tartrate and 0.003 µg/mL for phenol red. The LLOQ values were 0.04 µg/mL for acyclovir, 0.02 µg/mL for metoprolol tartrate and 0.01 µg/mL for phenol red (Table 3). Based on these results, we can conclude that our analytical method is sensitive enough for intestinal perfusion studies of selected test (acyclovir) and reference compounds (metoprolol tartrate and phenol red).

Stability

The freeze-thaw stability of the analytes were determined in rat intestinal perfusate by comparing initial concentrations of freshly prepared spiked samples and perfusion samples stored at -20 ^{0}C for 24 hours. The difference between nominal and measured concentrations of all compounds was less than 2.1% 24h after storage indicating that test and reference compounds are stable for at least during HPLC analysis (Table 4).

TABLE 4

The stability results.

	Concentration (µg/mL)	
	Fresh perfusion samples	After freeze-thaw cycle
Acyclovir	19.95 ± 0.35	20.19 ± 1.35
Metoprolol tartrate	19.86 ± 1.11	19.97 ± 0.42
Phenol red	11.98 ± 1.09	12.23 ± 1.42

Precision and accuracy

Precision represents degree of proximity between successive measurements under the same analytical conditions, whereas accuracy is the measure of how close the experimental value is to the true value.

The intra-assay (intra-day) and between- assay (inter-day) precision and accuracy results for acyclovir, metoprolol tartrate and phenol red in standard solution at low, medium and high concentrations were summarized in Table 5. According to the 'Bioanalytical Method Validation' of FDA Guidance, the coefficient of variation (CV) of inter-day and intra-day precision values should not exceed 15% for each concentration level. For determination of accuracy of a developed HPLC method, the mean values of each concentration level should be within 15% of the actual value[31]. Based on the results, the method was judged to be accurate and precise for intended purpose and minor variations did not affect the analysis.

TABLE 5

The intra-assay (intra-day) and between- assay (inter-day) precision and accuracy results.

	Added (µg/mL)	**Intra-day Precision CV %**	**Inter-day Precision CV %**	**Accuracy RE %**
Acyclovir	5	4.56	2.96	4.54
	20	6.97	2.00	6.97
	40	7.65	1.75	7.65
Metoprolol	5	5.80	0.93	5.80
	20	5.79	2.13	5.79
	40	6.51	1.89	6.51
Phenol Red	2.5	12.68	13.88	12.68
	12.5	9.71	7.69	9.71
	20	8.46	4.32	8.46

In this study, an HPLC method for simultaneous determination of acyclovir, metoprolol tartrate and phenol red was developed and validated according to the FDA Guideline[31]. When we compared with the analytical methods reported in the literature[34-40], the prominent advantage of our HPLC method is simultaneous analysis of these compounds indicating that it is a more economical method because of shorter analysis time and hence use of less mobile phase.

System Suitability Tests

Capacity Factor

The capacity (or retention) factor (k') is a means of measuring the retention of an analyte on the chromatographic column. For an optimum separation, capacity factor should be in the range of $0.5 < k' < 10$ [41]. The capacity factors estimated for acyclovir, metoprolol and phenol red (0.58, 1.5 and 2.56, respectively) indicate that all these k' values were ideal for optimum separation[41].

Selectivity Factor (α)

Selectivity factor,α, is a measure of seperation of two components. High α values indicate good separating power and a good separation between the apex of each peak. The selectivity parameters calculated were 4.41 for separation of acyclovir-phenol red, 2.59 for separation of acyclovir-metoprolol, and 1.71 for separation of phenol red –metoprolol. These results indicate that our chromatographic system has a good separating power[41].

Resolution (R)

Resolution is a measure of separation between adjacent peaks. A resolution value of 1.5 or greater between two peaks will ensure that the sample components are well separated to a degree at which the area or height of each peak may be accurately measured[41]. The resolution values estimated for separation of acyclovir- phenol red, acyclovir-metoprolol, metoprolol-phenol red were 7.95, 5.06, 4.16, respectively, indicating that all component were well separated from each other.

Column Efficiency (Number of Theoretical Plates)

The number of theoretical plates were determined to measure the column efficiency. The plate number (N) is a measure of the peak dispersion on the HPLC column. The effectiveness of chromatographic column increases with the number of layers[41]. Calculated N values were 1277, 2822 and 1942 for acyclovir, metoprolol and phenol red, respectively. The height equivalent to theoretical plate (HETP) values were found to be 0.196 mm, 0.089 mm and 0.129 mm, respectively.

Conclusions

In our study, a simple, precise and accurate HPLC method using a C_{18} column was developed and validated for the simultaneous determination of acyclovir, metoprolol tartrate and phenol red in a biological matrix (blank perfusion medium). The method showed a good performance with respect to linearity, accuracy, precision, selectivity. This newly developed and validated method can be readily used on a routine basis for the standardization of *in situ* intestinal permeability experiments.

Acknowledgement

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Abstract

Intestinal perfusion (SPIP) technique is one of the most commonly used techniques to determine the intestinal permeability of a drug. In perfusion studies, metoprolol (as tartrate) and phenol red are widely used as a reference compounds to evaluate the permeability coefficient of the compound of interest (acyclovir in this study). The aim of our study was to develop and validate a reversed-phase liquid chromatographic method for the simultaneous determination of acyclovir, metoprolol and phenol red for use in intestinal perfusion studies. The analysis was performed on a C_{18} column (4.6 mm x 250 mm, 5 µm) using a mobile phase consisting of methanol:0.0125 M potassium dihydrogen phosphate buffer (55:45, v/v; pH 7.0). Method was validated according to the FDA guidelines for selectivity, sensitivity, linearity, precision, accuracy, stability. All calibration curves were linear (r^2> 0.999). Lower limit of quantitation was 0.04 µg/mL for acyclovir, 0.02 µg/mL for metoprolol, 0.01 µg/mL for phenol red. Detection limit was 0.01 µg/mL for acyclovir 0.002 µg/mL for metoprolol, 0.003 µg/mL for phenol red. Precision and accuracy results of the method fulfilled the required limits. This newly developed and validated method can be readily used on a routine basis for the standardization of *in situ* intestinal permeability experiments.

Keywors: Acyclovir, metoprolol, phenol red, HPLC, in situ intestinal perfusion

Özet

Barsak perfüzyon tekniği, bir ilacın barsaklardan permeabilitesini tayin etmek amacıyla en çok kullanılan tekniklerden biridir. Perfüzyon çalışmalarında incelenen maddenin (bu çalışmada asiklovir) permeabilite katsayısını değerlendirmek amacıyla metoprolol ve fenol kırmızısı referans maddeler olarak sıklıkla kullanılır. Bu çalışmanın amacı, barsak perfüzyonu çalışmalarında kullanılmak için asiklovir, metoprolol ve fenol kırmızısının eş zamanlı olarak tayin edileceği bir ters faz sıvı kromatografisi yöntemi geliştirmek ve valide etmektir. Analiz bir C_{18} kolonunda (4.6 mm x 250 mm, 5 µm) metanol:0.0125 M potasyum dihidrojen fosfat tampon (55:45, h/h; pH 7.0)' dan oluşan mobil faz kullanılarak gerçekleştirilmiştir. Yöntem FDA kılavuzuna göre seçicilik, duyarlılık, doğrusallık, kesinlik, doğruluk ve stabilite için valide edilmiştir. Tüm kalibrasyon eğrileri doğrusaldır (r^2> 0.999). Kantitatif olarak tayin edilebilir en küçük değer asiklovir için 0.04 µg/mL, metoprolol için 0.02 µg/mL, fenol kırmızısı için 0.01 µg/mL'dir. Saptayabilme sınırı asiklovir için 0.01 µg/mL, metoprolol için 0.002 µg/mL, fenol kırmızısı için 0.003 µg/mL'dir. Kesinlik ve doğruluk sonuçları talep edilen sınırları karşılamıştır. Yeni geliştirilen ve valide edilen bu yöntem in situ barsak permeabilite deneylerinde rutin olarak kolayca kullanılabilir.

Anahtar kelimeler: Asiklovir, metoprolol, fenol kırmızısı, HPLC, in situ barsak perfüzyonu

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