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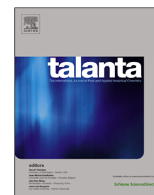

RP-HPLC × HILIC chromatography for quantifying ertapenem sodium with a look at green chemistry

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ABSTRACT

Ertapenem sodium is a polar and ionizable compound; therefore, it has little retention on traditional C_{18} columns in reverse-phase high-performance liquid chromatography, even using a highly-aqueous mobile phase that can result in dewetting in the stationary phase. Thus, the most coherent process for ERTM is to develop a method for Hydrophilic Interaction Chromatography. However, for the traditional methods in HILIC, the use of a highly organic mobile phase is necessary; usually an amount exceeding 80% acetonitrile is necessary. On the other hand, the RP-HPLC mode is considered for the analysis technique, which is more often used for quantification of substances, and new columns are often introduced to analyze different groups of compounds. Two new analytical methods have been developed for routine analysis. The proposed chromatographic method was adequate and advantageous by presenting simplicity, linearity, precision, accuracy, robustness, detection limits, and satisfactory quantification. Analytical methods are constantly undergoing changes and improvements. Researchers worldwide are rapidly adopting Green Chemistry. The development of new pharmaceutical methods based in Green chemistry has been encouraged by universities and the pharmaceutical industry. Issues related to green chemistry are in evidence and they have been featured in international journals of high impact. The methods described here have economic advantages and they feature an eco-friendly focus, which is discussed in this work. This work was developed with an environmental conscience, always looking to minimize the possible generated organic waste. Therefore, discussion on this aspect is included.

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1. Introduction

Ertapenem sodium (ERTM) is an extremely important antimicrobial agent for clinical practice nowadays, mainly because it remains active against aerobic and anaerobic microorganisms. This represents a breakthrough for this group. ERTM stands out among the other carbapenems that are currently available as the first with unique chemical characteristics, which makes it more resistant to β-lactamase enzymes [1,2]. Since approval by the FDA in 2001, ertapenem sodium (Fig. 1) has been used as a therapeutic agent for the treatment of various infectious diseases in several countries of the world [1]; however, there is still no monograph on it in any official compendium. Liquid chromatography is one of the main analytical techniques in the sciences because of its wide applicability.

The reverse-phase high-performance liquid chromatography is undoubtedly the most used in chromatography due to its numerous advantages, such as the separation capacity of substances of different polarities, the use of mobile phases with lower cost and low toxicity, and the rapid equilibrium of the column after altering the elution possibility in gradient in using the mobile phase, which leads to faster and better separation in the analysis [3].

RP-HPLC provides excellent retention for medium and low-polarity substances. However, most of the active pharmaceutical ingredients are high polar molecules, which often require a highly-aqueous mobile phase to obtain adequate retention by reverse phase using traditional C_{18} stationary phases. Nevertheless, this is an adverse condition, which can lead to a significant decrease in the lifetime of the chromatographic column. This is the case of ertapenem sodium, which is presented in the literature on its analytical methods as using highly-aqueous mobile phases in traditional columns [4].

The traditional stationary phases used in the RP-HPLC are constituted of a nonpolar organic layer, chemically bonded. The silica has many good features, such as having higher mechanical strength, having a high surface area, being energetically homogeneous, consisting of a narrow particle size and, being a highly

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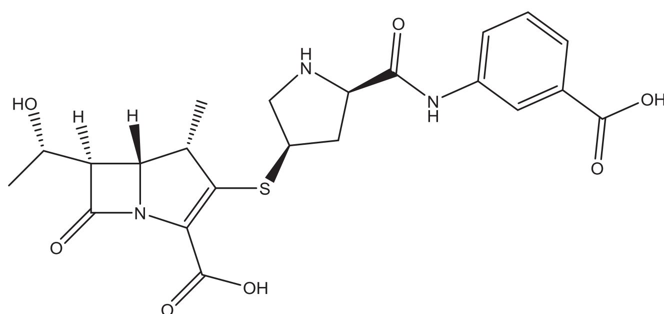

Fig. 1. Chemical structure of ertapenem sodium (CAS 1 53773-82-1).

reproducible synthesis method [5].

Despite its benefits, the use of silica as a chromatographic support does not present good chemical and thermal stability, so their use is limited to applications in which the mobile phase is at pH 2–8 with temperatures below 60 °C. At a pH above 8, dissolution of silica and the collapse of the stationary phase occurs. At pH lower than 2, there is the hydrolysis of siloxane bond (Si–O–Si–C), resulting in a continual loss linked phase, with subsequent analyte retention loss [6].

Currently, the focus on research and development of new stationary phases for RP-HPLC faces two main points: the stability and selectivity of the stationary phase. In other words, the two points are the increase in the lifetime of chromatographic columns in adverse conditions and the development of stationary phases having specific characteristics to a substance or class of substances, respectively [5].

New technologies in a stationary phase are developed every year. Thus, stationary phases with an embedded polar group, in which a polar group is inserted in the n-alkyl chain, the silica is often modified after the third methylene group is bonded to the active silicon organosilane atom and will be able to establish hydrogen bonding. Such groups may be carbamate, amide, amine, urea, and ether [5,7].

The stationary phases with embedded polar groups maintain reverse phase character, but they have different selectivity and can be used to separate polar and basic substances with highly-aqueous mobile phases. They may be used with 100% water without the occurrence of dewetting in the stationary phase, which means that there is no loss of phase support chains linked [5–7].

In addition to the reverse stationary phases that contain embedded polar groups, there are sterically protected phases, which are commercially available and present beyond embedded polar groups; e.g., bulky groups such as isopropyl or isobutyl attached directly to the silicon atom. An example is the column ZORBAX, which is packed with a reverse-phase type with amide polar groups inserted in the n-alkyl chain of fourteen carbon atoms and isopropyl groups linked directly to the silicon atom [7].

Another attractive option for the analysis of polar compounds is hydrophilic interaction chromatography. In a simple way, HILIC could be described as an HPLC variant of a “normal phase” in which a hydrophobic column is used as a stationary phase and typical eluents are used as a “reverse phase”; they are used as a mobile phase (acetonitrile, methanol, and/or ethanol). A solvent mixture containing a high concentration of an organic solvent miscible with water is necessary for traditional methods in HILIC, which usually can be an amount exceeding 80% acetonitrile. Retention is directly proportional to the polarity of the solute and inversely proportional to the polarity of the mobile phase. The separation mechanism is multimodal, involving partition, electrostatic interactions, and hydrogen bonding.

McCalley presented another interesting contribution to HILIC elution mode in 2007 [8]. He showed important considerations on the interaction mechanism in HILIC and that, besides the hydrophilic retention by liquid-liquid partition achieved by traditional highly-organic mobile phases, it is also possible to retain ionizable substances that are moderately hydrophobic by siloxane interactions on the surface of the particle when using “HILIC reverse phase”; in other words, with a highly-aqueous mobile phase.

If the water content of the mobile phase exceeds 30%, siloxane bonds can occur, resulting in reverse-phase retention. Under these conditions, the retention is most notable when the water content of the mobile phase is higher than 90%, but polar compounds do not have to adequately retain in this kind of interaction. This leads to a retention-shaped curve “u”, which is very characteristic when it plots the retention of analytes versus a range of mobile phases containing 0–100% of an organic solvent [8].

The developments of new columns have strengthened the recognition of HILIC. HILIC was first proposed by Alpert [9] for the separation of polar solutes and has gained considerable attention in recent years, and the number of applications in the analysis of polar compounds has been increasing, especially in the academic field. However, HILIC mode is a powerful and versatile tool for the pharmaceutical industry, since the active pharmaceutical ingredients (API) are mostly small hydrophilic molecules. Therefore, HILIC has become an increasingly popular alternative method [8–11], and nowadays there are many types of HILIC stationary phases commercially available. The development of new columns and new applications in HILIC mode has contributed to its consolidation, making it globally recognized [12]. There are not any HILIC for ertapenem articles published in the literature to date.

The study for the development of the analytical method by liquid chromatography was based on studies available in the literature [13–33].

2. Experimental

2.1. Chemicals and reagents

The chemicals used were ertapenem sodium 99.2% (Lot no. EB004C1) and ertapenem sodium active pharmaceutical ingredient (API) in lyophilized powder for injection solution (Lot no. 2178140) both kindly donated by Merck Sharp & Dohme™ [https://ssl.gstatic.com/ui/v1/icons/mail/images/cleardot.gif]. All solutions and mobile phases used in this assay were prepared from ultrapure water obtained from a Milli-Q Plus (Millipore™, USA). The solvents used HPLC grade, acetonitrile J.T. Baker™, acetonitrile Tedia™, acetonitrile LiChorosov™, ethanol J.T. Baker™, ethanol Panreac™, ethanol Tedia™, methanol Vetec™ and all other Chemicals were of analytical grade: hydrochloric acid (Qhemis™), sodium hydroxide (Cinetica™), hydrogen peroxide (Vetec Química Fina™).

2.2. Apparatus

The RP-HPLC method was performed on a Waters LC system (Waters Corporation Systems, CA, USA) equipped with a Waters 1525 binary pump, a Rheodyne Breeze 7725i manual injector and a Waters 2487 UV detector. Empower software was used to integrate the peak areas automatically. The chromatographic separation was carried out under isocratic reversed phase conditions on an Agilent™ Zorbax Bonus-RP (4.6 × 150 mm, 5 µm particle size) column. While the HILIC method was performed on an ultra-high performance liquid chromatography (UHPLC), Shimadzu was equipped with two pumps LC-20CE, automatic injector model SIL-20-AHT, oven CTO-20A, column detector with variable wavelength UV/vis model SPD-20A Controller CBM-20A, computerized automatic integrator with LC Solution™ software. The stationary phase was Phenomenex™ HILIC Kinetex (4.6 × 100 mm, 2.6 µm particle

size) column. Another apparatus: nylon membrane with pore 0.45 μm and 47 mm diameter (Millipore™), ultrasonic bath USC2800A (Unique™); Q334M water-bath (Quimis™); H51 analytical scale (Mettler Toledo™); UV chamber with internal mirrors equipped with a UVC lamp (254 nm).

2.3. Solutions

In the RP-HPLC method, an ERTM RS stock solution was prepared by transferring 10 mg equivalent of ERTM RS to a 50 mL volumetric flask, which was filled with ultrapure water to obtain a concentration of 200 μg mL^{-1}. Aliquots from this solution stock were transferred to 10 mL volumetric flasks, the volumes of which were completed with ultrapure water to obtain working solutions with concentrations of 40, 60, 80, 100, 120, and 140 μg mL^{-1}. In the meantime, in the HILIC method, the ERTM RS stock solution was prepared by transferring 10 mg, the equivalent of ERTM RS, to a 10 mL volumetric flask, which was filled with ultrapure water to obtain a concentration of 1000 μg mL^{-1}. Aliquots from this solution stock were transferred to 10 mL volumetric flasks, the volumes of which were completed with methanol to obtain working solutions with concentrations of 70, 80, 90, 100, 110, and 120 μg mL^{-1}.

2.4. Preparation of ERTM API solution

Five vials of ERTM powder (commercially available for the preparation of injection solutions) were weighed, and the average weight was calculated. The contents of these vials were mixed. The ERTM API stock solution was performed in the same way as the preparation of ERTM RS solutions previously described.

2.5. Methodology

In the RP-HPLC method, the mobile phase consisted of purified water and ethanol with 80:20 v/v with 0.1% formic acid and a flow rate of 1 mL min^{-1}; while in the HILIC method, the mobile phase consisted of acetonitrile and water, with 88:12 v/v with 0.1% formic acid and a flow rate of 0.5 mL min^{-1}. In both methods the procedure was performed isocratically. The volume of the injection was 20 μL, using UV detection at 297 nm. The solutions were filtered through a 0.45 μm filter before being injected, and the mobile phase was degassed in an ultrasound bath for 30 min before being used.

2.6. System suitability

The system suitability test was conducted to evaluate the system resolution and reproducibility to ensure that the complete testing system was suitable for the intended application. In order to obtain the required data, six solutions of ERTM reference standard at a concentration of 100 μg mL^{-1} were prepared and analyzed by HPLC. The parameters such as peak area, retention time (t_R), plate number (N), and asymmetry factor (A_S) were calculated according to the equations described here, and their respective relative standard deviations (RSD) were analyzed.

A non-retained substance passes through the column at a time t_0, called the void Time. The T_0 can verify experimentally by injecting a substance that has no affinity for the stationary phase or by injecting a solvent that is perceived by the detector in use or by the first disturbance that usually appears in the baseline preview with the inflection of the peak in the chromatogram. Some examples of substances commonly used for this purpose in the reverse mode include water, uracil, urea, and $NaNO_3$. Common substances in the normal mode are acetone, disulfide, and saturated or aromatic hydrocarbons. In HILIC mode, toluene is commonly used.

The T_0 can also be estimated theoretically based on the dimensions of the chromatographic column, and it is calculated at half the volume inside the column in mL, divided by the flow rate in mL min^{-1}, as shown in Eq. (1) [34].

Eq. 1 Theoretical calculations to estimate the void volume

$$T_0 = \frac{0.5(\pi \cdot r^2 \cdot L)}{flow\ \ rate} \tag{1}$$

where: T_0=non-retained substance "void time" and flow rate=In mL min^{-1}, volume inside the column=π3,14; r^2 column radius (cm); L column length (cm).

If substances are going to elute at T_0 or close to them, this substance does not have proper interaction with the stationary phase, so it cannot be quantified, because there this interaction lacks precision and accuracy.. The parameter that evaluates whether the interaction between the analyte and the stationary phase is suitable is the Retention Factor (k′) of an analyte. This may be measured experimentally as shown in Eq. (2) [34,35].

Eq. 2 Retention factor (k′)

$$k' = \frac{t_R - t_0}{t0} \tag{2}$$

where:

T_R= Retention time of peak [min]
t_0= Void time [min]

Number of plates (N) is defined as a measure of the efficiency of chromatographic conditions, based on the obtained retention times and the tailing factor. This parameter is normally used to evaluate the performance of the column. Eq. (3) shows the calculation for obtaining the number of plates [34,35].

Eq. 3 Number of Plates

$$N = 5.54\left(\frac{t_R}{W_{0.5}}\right)^2 \tag{3}$$

where:

T_R= Retention time of peak [min]
$W_{0.5}$=Peak width at half height

An ideal chromatographic peak should be of symmetrical Gaussian shape but, due to various factors, the shape often deviates. The peak asymmetry (A_s) is a measure of the proportion between the two parts of a chromatographic peak in the longitudinal direction where A and B are peak widths at 10% of the height for leading and the tailing ends of the peak, Eq. (4) [34].

Eq. 4 Peak asymmetry

$$A_s = \frac{b_f}{a_f} \tag{4}$$

where:

b_f =Distance from the midpoint to the tailing ends in peak widths at 10%.

a_f =Distance of the front point to the midpoint in peak widths

at 10%.

2.7. Method validation

This method was validated according to the International Conference on Harmonization guidelines ICH, 2005 and Harmonized Guidelines for Single Laboratory Validation of Methods of Analysis by IUPAC [36,37] for linearity, selectivity, accuracy, precision, robustness, detection limit, and quantification limit.

2.7.1. Linearity

The linearity was evaluated by regression analysis of ertapenem sodium. The analytical curve was constructed by plotting the concentration versus the average of the absorbance values with the average absorbance value of each ERTM – RS concentration. The assay was performed in triplicate on three different days. The regression lines were calculated by the least-squares method. Statistical evaluation was made by ANOVA. The values were reported as the average ± S.D. of the calibration curves.

2.7.2. Precision

Repeatability (Intra-day precision) and intermediate precision (inter-day precision). The repeatability was studied by the performance of seven determinations of the sample in the median concentration calibration curve. They were prepared and analyzed the same day under the same experimental condition. The intermediate precision was evaluated by the average percentage RSD obtained in three analytical curves performed on different days (interday) and by two different analysts (between analysts).

2.7.3. Selectivity by study of forced degradation

The method selectivity was determined by subjecting an ERTM API solution (100 µg mL^{-1}) in accelerated degradation by alkaline, acid, neutral, oxidative, and photolytic stress to evaluate the effect of degradation products in the quantization ertapenem sodium. The photodegradation was induced by exposing the samples in a photostability UV chamber with internal mirrors equipped with a UVC lamp (254 nm).

2.7.4. Robustness

The robustness of the method was evaluated by making small alterations to parameters simultaneously to show that the method validity is maintained. The obtained responses were evaluated according to Plackett-Burman factorial design in which an array of 15 experiments is held, ranging 7 parameters in the upper and lower levels. The results were calculated using the methodology proposed by Youden, Steiner, 1975. Table 1 shows the factorial combination used in the Plackett-Burman test, where the letters A to G represent the selected factors. 1–15 is the number of experiments (2n+1). Where n is the number of factors, the number (0) corresponds to normal pre-set parameters in the process and the number (1) and (−1) are the upper and lower levels to these parameters, respectively.

Table 1
Robustness test using the experimental model of Plackett-Burman.

Parameter analytical	Combination factorial														
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
A	1	1	1	0	1	0	0	0	−1	−1	−1	0	−1	0	0
B	0	1	1	1	0	1	0	0	0	−1	−1	−1	0	−1	0
C	0	0	1	1	1	0	1	0	0	0	−1	−1	−1	0	−1
D	1	0	0	1	1	1	0	0	−1	0	0	−1	−1	−1	0
E	0	1	0	0	1	1	1	0	0	−1	0	0	−1	−1	−1
F	1	0	1	0	0	1	1	0	−1	0	−1	0	0	−1	−1
G	1	1	0	1	0	0	1	0	−1	−1	0	−1	0	0	−1

A–G: selected factors; 1–15: N (number of experiments) =2n+1 where n=number of factors; −1, 0, +1: levels for the factors.

The robustness was determined in triplicate from injections of standard versus sample solutions containing 100 µg mL^{-1} ERTM under the same established experimental conditions. To determine the influence of changes in each parameter on the result, the average of the dosage performed in triplicate assays corresponding to normal ranges was compared to the average of the dosage corresponding to the modified levels. The average effect of each variable is the average difference among the observations made at the modified levels and those made at the optimum level. The deviation of each factor was calculated by using the methodology of Youden and Steiner. Eq. (5) illustrates the effect evaluation of the changing parameter A – brand of organic solvent. Likewise, the other parameters were also evaluated [38–40].

Eq. 5 Experimental model of Plackett-Burman calculated using the methodology of Youden and Steiner.

$$\sqrt{2}S > |DA| \tag{5}$$

where:

$$S = \sqrt{\frac{2}{7}\left(DA^2 + DB^2 + DC^2 + DD^2 + DE^2 + DF^2 + DG^2\right)}$$

Since the deviation of each factor changed (DA, DB, DC etc) must be less than the value resulting from $\sqrt{2}S$ to infer that the effects obtained with the variations of the parameters were not significant, the method is robust for all selected factors.

2.7.5. Accuracy

Accuracy was obtained via recovery assay in which a reference pattern of known quantities of ERTM was added to a known quantity of the sample. The recovery was performed in 3 different concentrations—R1, R2, and R3—equivalent to 80%, 100%, and 120% of the average concentration, respectively. Each simulated sample (R1, R2 and R3) was assayed on an independent trial. Accuracy was determined in triplicate and the values presented are the average content obtained with the standard relative deviation. The percentage of recovery (R%) was calculated by Eq. (6) determined by the Association of Official Analytical Chemists [41].

Eq. 6 Percentage of recovery calculation – R%

$$R\% = \frac{Cf - Cu}{Ca} x100 \tag{6}$$

Cf is the total API concentration measured after addition of the standard.
Cu is the total API concentration in the formulation.
Ca is the standard concentration added to the formulation.

3. Results and discussion

The results of system suitability are consistent with the recommended literature. It is considered adequate if the peak area and retention time vary less than 2.0% of the relative standard deviation, the retention factor is less than 2, and the plate number is considered suitable above 2000 plates; further, it is ideal to have a peak As=1, but values in the range 0.9–1.1 are acceptable. The results obtained for the parameters of System Suitability are

Table 2
Parameters evaluated in system suitability test in HILIC.

Parameters evaluated					
	Area	**t_r**	**k′**	**N**	**A_S**
	2,406,512	5.75	2.46	2101.20	1.08
	2,410,700	5.75	2.46	2100.47	1.04
	2,414,508	5.75	2.46	2101.93	1.07
	2,384,877	5.75	2.46	2101.93	1.03
	2,374,783	5.74	2.46	2093.91	1.09
	2,372,898	5.75	2.46	2100.47	1.07
	2,494,683	5.76	2.47	2110.71	1.05
	2,468,094	5.76	2.47	2107.78	1.06
	2,461,700	5.76	2.47	2106.32	1.05
	2,431,090	5.75	2.47	2104.86	1.05
Average	2,421,985	5.76	2.47	2102.96	1.06
RSD	1.71	0.11	0.16	0.22	1.80

RSD = Relative standard deviation.

Table 3
Parameters evaluated in system suitability test in RP-HPLC.

Parameters evaluated					
	Area	**t_r**	**k′**	**N**	**A_S**
	2,358,819	4.07	2.27	2052.52	1.05
	2,269,093	4.08	2.28	2055.54	1.07
	2,362,990	4.09	2.28	2065.63	1.02
	2,265,235	4.08	2.28	2057.56	1.05
	2,327,085	4.06	2.26	2041.47	1.02
	2,241,127	4.08	2.28	2061.59	1.05
	2,323,999	4.05	2.26	2033.44	1.05
	2,263,842	4.05	2.26	2032.44	1.05
	2,274,930	4.08	2.28	2058.57	1.02
	2,256,419	4.08	2.28	2059.58	1.07
Average	2,294,383	4.08	2.27	2051.83	1.04
RSD	1.94	0.29	0.01	0.58	1.59

RSD = Relative standard deviation.

presented in Table 2 for RP-HPLC and in Table 3 for HILIC. The proposed methods offer good selectivity and indicate that the selected chromatographic parameters are able to quantify the ERTM.

The methods were validated following the guidelines for analytical methods validation according to the recommendation of the International Conference on Harmonization [36], the guidelines of Brazil, [42] and the FDA in 1994 [43]. The analyzed parameters were linearity, precision, accuracy, selectivity, and robustness. The chromatogram obtained by the proposed method, ERTM, demonstrated resolution and peak satisfactory symmetry plus adequate retention time (4 min for RP-HPLC and 5.7 min for HILIC), as illustrated in Figs. 2 and 3, respectively.

The selectivity was analyzed by studying forced degradation. The study of forced degradation of ERTM API was performed to verify the indicator stability properties of the proposed method. In all stress conditions in which the sample was submitted, there was a decrease from the peak of ERTM when compared to the peak of the ERTM at the time of preparation. In a typical degradation study, 10–30% degradation of the API is sufficient, but not so severe as to generate secondary products. Stress studies were carried out according to ICH guidelines Q1A (R_2), [36] RDC 58 [44], and Blessy [45]. The degradation has been confirmed by the decrease of the peak area, and significant additional peaks that could interfere with quantification of the molecule API were not observed. The ERTM with the β-lactam ring open is the main degradation product; however, this is not displayed because degradates of the API have very different UV response factors from the API. Nonetheless, it was decided to quantify the API around 300 nm because the drug presents maximum absorbance in this region, and the main goal is to quantify the API without interferences along with the band's API. This wavelength was chosen in most chromatographic methods available in the literature.

In the study of forced degradation, a higher quantity of degradation was observed in the alkaline condition; however, in HILIC mode there was a peak deformation, which is justified since it is known that under these conditions, the pH cell change interferes to ionize the molecule. Research on the forced degradation of an API is very important for understanding the molecule chemical behavior and stability. The degraded percentage is presented in Table 4.

The analytical curve for ERTM in elution modes RP-HPLC and HILIC was constructed by plotting the average values of the absolute areas obtained experimentally in the chromatographic peak of three analytical curves in relation to its respective concentrations (Figs. 4 and 5).

The analytical curve of ertapenem sodium RS was evaluated by ANOVA, and this is shown in Table 5 for RP-HPLC and Table 6 for HILIC.

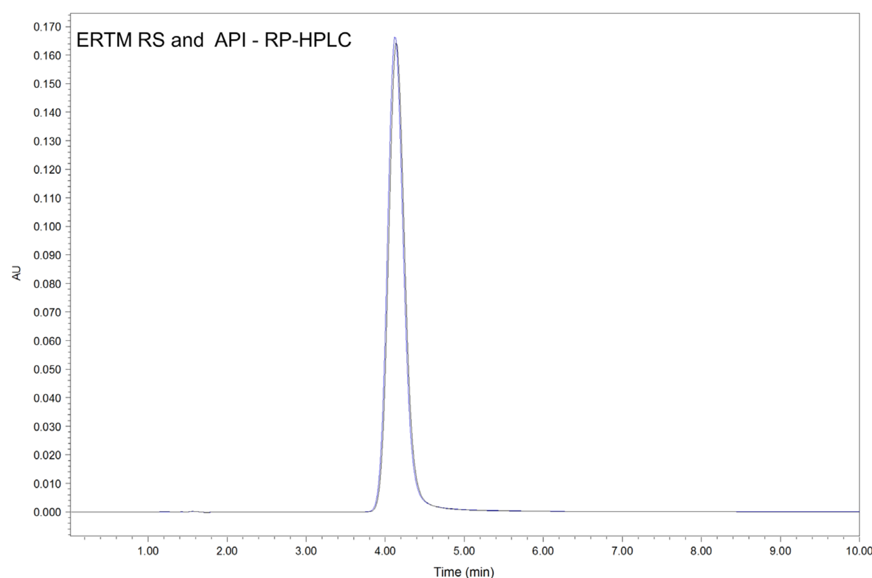

Fig. 2. Overlay of chromatogram ERTM RS and ERTM API, at a concentration of 100 μg mL^{-1} for RP-HPLC.

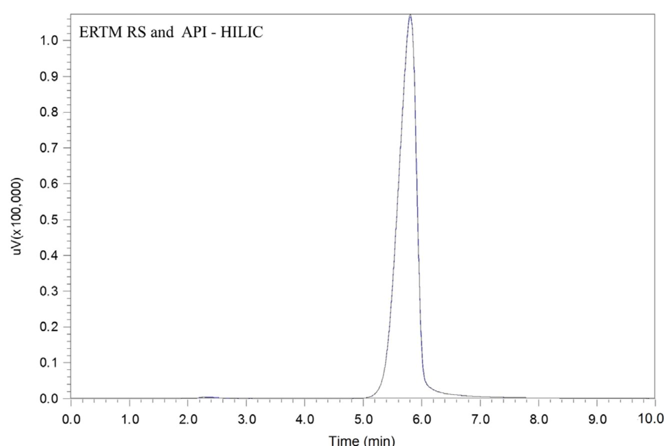

Fig. 3. Overlay of chromatogram ERTM RS and ERTM API, at a concentration of 100 (μg mL^{-1}) for HILIC.

Table 4
Study of forced degradation.

	HILIC		RP-HPLC	
	Time (h)	Degraded (%)	Time (h)	Degraded (%)
HCl	12.00	17.57	3.00	15.80
NaOH	2.50	24.50	1.50	24.75
H_2O_2	12.00	22.76	1.50	21.13
UVC_{254} light	6.00	22.64	12.00	16.56

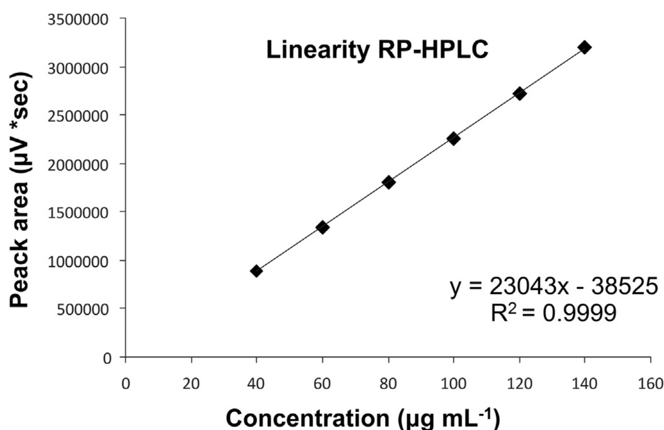

Fig. 4. Analytical curve of ERTM at concentrations of 40–140 μg mL^{-1} in RP-HPLC.

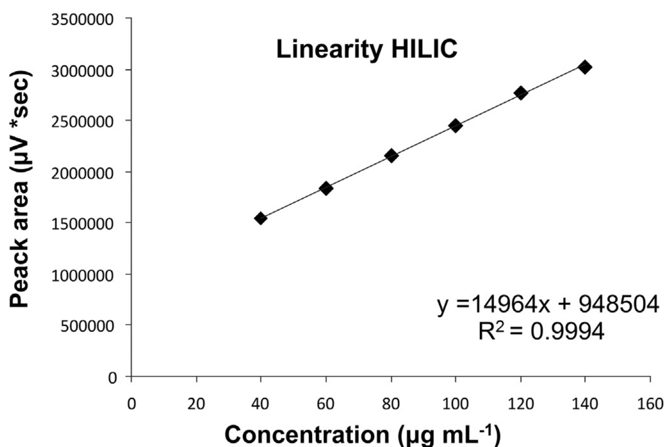

Fig. 5. Analytical curve of ERTM at concentrations of 70–120 μg mL^{-1} in HILIC.

The robustness was evaluated by the Plackett-Burman factorial matrix, in which 15 experiments were performed with simultaneous changes. The effects resulting from the changed parameters have been evaluated in comparison to the values obtained as reference for the test, calculated from Eq. (5). Concentrations found in normal conditions and changed for each factor are shown in Tables 7 and 8 for RP-HPLC and HILIC, respectively.

The robustness was performed with simultaneous changes. The effects resulting from the changed parameters have been evaluated in comparison to the values obtained as reference for the test, calculated in Eq. (5). No effect showed significant results, indicating that the proposed method is robust. The results indicated that the validity of the methods are maintained, even with small variations in its working conditions.

The method has proved to be accurate, robust, and exact as shown in Table 9. In other words, the proposed method has capacity to generate, for the same sample, reproducible results with low-response variation between independent assays.

The results of method validation for the analysis of ERTM showed that the developed high-performance liquid chromatography method is appropriate to quantify ERTM in powder for injection. ERTM is a polar and ionized molecule that has acid, basic, and amphoteric pKas. This factor makes it suitable to be used in the elution mode by liquid chromatography hydrophilic interaction—HILIC. Different mobile phases were evaluated by using solvents such as ethanol, methanol, and acetonitrile, with small proportions of water, because a minimum of 3% water in the mobile phase is required for the formation of an essential layer of water to promote interactions between analyte and the stationary phase.

Unfortunately for the ERTM analysis, tests performed using alcohols did not show adequate retention for quantification due to the high drag force in the mobile phase; that might happen because in HILIC the organic solvent is used as the weak eluent, and the alcohol's polarity is very close to the water polarity, which is used as a strong eluent. Thus, even with very high proportions of alcohol, a low retention factor was observed for ERTM. However, excellent results were achieved by using an acetonitrile (ACN) solvent as a weak eluent, which was chosen because of favorable conditions for validation. The strength of the mobile phase was adjusted to obtain a fast analysis to produce a higher retention factor 2. Thus, the chosen mobile phase was ACN: water (88:12 v/v) with 0.1% formic acid pH 2.5.

Traditionally, for the analysis of high polarity substances in HILIC, the use of highly organic mobile phases is required. ACN is preferred as it is a weak eluent and because it provides better retention of the analyte as compared to alcohol. However, the use of a mobile phase containing high concentrations of ACN in chromatographic methods divides opinions in the literature. On the one hand, it could be said that when ACN is incinerated, NO_2 wastes are generated, contributing to the formation of acid rain. On the other hand, the acetonitrile is a derivative by-product of the recycling of the residual acrylonitrile that is used in plastics manufacturing, which would be incinerated anyway. However, for the production of acetonitrile as a by-product of acrylonitrile synthesis, there must be much more energy than that in the production of HPLC grade ethanol. In 2008, at the height of the financial market economic crisis, the acetonitrile HPLC grade had an abnormal increase, and it ended up becoming very expensive. Thus, many researchers began to look for alternatives to change ACN by other solvents, such as acetone, ethanol and, methanol, for purely economic objectives and not for ecological reasons [46].

After that and with a “look outside the box” mentality due to the impact of the current environmental problems, authors considered “eco-friendly” engaged in the development of methods that are beneficial and sustainable to the environment and have

Table 5
Absorbance variance analysis, determined in obtaining the analytical curves ERTM using the RP-HPLC.

Source of variation	Degree of freedom	Sum of squares	Quadratic means	F calculated	F critical
Between concentrations	5	11,151,186,532,014.60	2,230,237,306,402.91	5816.65*	3.11
Linear regression	1	11,149,758,817,751.90	11,149,758,817,751.90	29,079.50*	4.75
Linear deviation	4	1,427,714,262.70	356,928,565.67	0.93	3.26
Residue	12	4,601,079,492.95	383,423,291.08	–	–
Total	17	11,155,787,611,507.50	–	–	–

* Significant at $p < 0.05\%$.

Table 6
Absorbance variance analysis, determined in obtaining the analytical curves ERTM using the HILIC.

Source of variation	Degree of freedom	Sum of squares	Quadratic means	F calculated	F critical
Between concentrations	5	4,705,291,573,109.66	941,058,314,621.93	1192.67*	3.11
Linear regression	1	4,699,193,364,056.96	4,699,193,364,056.96	5955.62*	4.75
Linear deviation	4	6,098,209,052.69	1,524,552,263.17	1.93	3.26
Residue	12	9,468,418,919.41	789,034,909.,95	–	–
Total	17	4,714,759,992,029.06	–	–	–

* Significant at $p < 0.05\%$.

Table 7
Results for ERTM method robustness RP-HPLC.

Fator	(−1)	Teor (%)[a,b]	(1)	Teor (%)[a,b]
Ethanol brand	A=J.T. Baker	99.75−99.63=0.12	A=Tedia	97.39−96.65=0.74
Mobile phase flow rate (mL min^{-1})	B=0.99	100.06−98.72=1.34	B=1.01	97.55−96.49=1.05
Additive amount in mobile phase	C=0.09	98.48−100.30=−1.82	C=0.11	97.88−96.18=1.73
Water source	D= Lab. 2	99.46−99.32=0.14	D=Lab. 3	97.32−96.72=0.60
Proportion of FM v/v (%)	E=79:21	98.85−99.94=−1.09	E=81:19	96.47−97.57=−1.10
Wavelength (nm)	F=296	99.65−99.13=−0.52	F=298	97.32−96.72=0.60
Room temperature (°C)	G=19	100.55−98.24=2.31	G=21	98.04−96.00=2.04

[a] Subtraction of average contents in normal conditions and average contents in the altered conditions.
[b] Reference criteria calculated: 2.61 (Test 1) and 2.48 (Test −1).

Table 8
Results for ERTM method robustness HILIC.

Fator	(−1)	Teor (%)[a,b]	(1)	Teor (%)[a,b]
Acetonitrile brand	A= LiChorosov	101.13−101.00=−0.13	A=tedia	100.77−101.22=−0.46
Mobile phase flow rate (mL min^{-1})	B=0.49	101.00−101.12=0.11	B=0.51	101.04−100.95=0.09
Additive amount in mobile phase	C=0.09	100.50−101.62=−1.12	C=0.11	100.69−101.30=−0.62
Water source	D= Lab. 2	101.28−100.84=0.44	D=Lab. 2	101.06−100.93=−0.14
Proportion of FM v/v (%)	E=87:13	101.13−100.99=0.14	E=89:11	100.87−101.12=−0.25
Wavelength (nm)	F=296	100.81−101.31=−0.50	F=298	100.97−101.02=0.04
Oven temperature (°C)	G=24	100.59−101.53=−0.95	G=26	100.65−101.34=0.69

[a] Subtraction of average contents in normal conditions and average contents in the altered conditions.
[b] Reference criteria calculated: 0.81 (Test 1) and 1.23 (Test −1).

prioritized the use of ethanol as phase mobile if possible. The research group led by Professor Pat Sandra from the University of Gent in Belgium also proposed some methods in HILIC elution mode using 100% aqueous phase, which he called “Per Aqueous LC – PALC” or “normal-phase LC Aqueous”. Another contribution given by this group was the development of methods for subcritical fluid chromatography using CO_2 and ethanol as mobile phase, in an attempt to replace ACN [47,48].

Thereafter, another research group also proposed carbonate-ethanol propylene mixtures instead of acetonitrile organic modifiers in the mobile phases [49]. Research groups continue to work with the development of methods, replacing ACN by solvents that are considered environmentally friendly, which can bring environmental benefits and prevent problems if a new financial crisis arises.

Nowadays, the analytical methods classified as methods of green chemistry prioritize the low consumption of organic solvents or their replacement with low-toxicity solvents. This has been a highly relevant topic since sustainability has been discussed and encouraged worldwide, not only at the University but also by laboratories and pharmaceutical industries.

Among the many proposals to find a solvent that can replace ACN, the most discussed, undoubtedly, is the constant attempt to use ethanol. However, replacing the ACN by ethanol is not always possible. ACN is the most used solvent in chromatography due to unique characteristics such as low viscosity and, consequently, low pressure in the equipment; low acidity; high miscibility when mixing the nonpolar solvents and water; low absorption of ultraviolet light; and mainly, improved selectivity when it is in ACN in the mobile phase. Therefore, an ethanol solvent may not always be interchangeable.

In contrast, ethanol is derived from renewable and

Table 9
Results of method validation using the chromatography method.

Parameters	Results
Linearity[a]	y= 23,043x − 38,525, R^2=0.9999 RP-HPLC y= 29,928x − 547,879, R^2=0.9994 HILIC
Intra-day precision[b]	Peak area 2,076,598 ± 2.20% RP-HPLC Peak area 2,465,068 ± 1.80% HILIC
Inter-day precision[a]	1st day=99.86%; 2nd day=98.71% and 3rd day=100.05% ± 0.73% RP-HPLC 1st day=99.14%; 2nd day=98.04% and 3rd day=100.11% ± 1.04% HILIC 1st analyst=102.98% and 2nd analyst=100.05% ± 2.04% RP-HPLC 1st analyst=100.11% and 2nd analyst=100.02% ± 0.06% HILIC
LOD[a]	3,11 μg mL^{-1} RP-HPLC and 7.80 μg mL^{-1} HILIC
LOQ[a]	9,41 μg mL^{-1} RP-HPLC and 23.63 μg mL^{-1} HILIC
Accuracy[a]	99.81%, ±.66% RP-HPLC 99.18%, ± 2.01% HILIC

[a] Average of three determinations.
[b] Average of seven determinations.

biodegradable sources, and with the new "UHPLC" technologies, the high viscosity of ethanol that is responsible for the significant increase in pressure in the equipment is no longer a problem. Ethanol residues are easily removed without the need for incineration. As Brazil is one of the largest producers of ethanol from sugarcane in the world, it also allows some groups to refine their own to obtain quality ethanol to be used in chromatography, thereby reducing the cost of acquisition from this solvent. In addition, ethanol is classified as a class III solvent, which is potentially less toxic, as well as acetic acid, acetone, ethyl acetate, heptane, and dimethyl sulfoxide, according to the classification from The Center for Drug Evaluation and Research (CDER) and the Food and Drug Administration (FDA) [50].

Even so, ethanol has been used as a weak eluent in HILIC, and retention of substances has not been a problem, so the work can continue with ethanol instead of ACN, resulting in the proposal of methods considered "environmentally friendly". In this regard, the study for research and quantification of hydrazines can be cited, which are substances that, although they are used in the synthesis of pharmaceuticals, are carcinogens and require monitoring. The hydrazines have a high polarity substance, and therefore are weakly retained in the reverse phase. In this study, excellent results have been obtained using alcohol as an organic modifier associated with the HILIC stationary phase. The authors have found that the retention time for hydrazines increases proportionally to the size of the alcohol alkyl chain (methanol < ethanol < isopropanol), because by increasing the alkyl chain, a decrease in the polarity of the alcohol occurs, which makes hydrazines have a greater affinity for the aqueous layer on the surface of the stationary phase, providing greater retention. In this work, the mobile phase elution strength containing proportions of water and ethanol was studied [51].

However, despite being HILIC, the most suitable elution mode for polar and ionized molecules such as the ERTM. Nowadays, new columns RP-HPLC are often introduced to promote separation of different groups of substances. Thus, it was also possible to develop a method by RP-HPLC to show excellent results in terms of resolution, peak symmetry, and a 4 min-retention time, thereby providing a rapid API determination, which is a key attribute for the method applicability on quality control in the pharmaceutical industry. Furthermore, ethanol was used as the organic solvent. Ethanol was chosen to be a "green", "eco-friendly" solvent and because of its excellent results in this way. In the chromatographic method proposed in this work, we sought to reduce operating costs in order to avoid damage to the chromatographic column, decrease analysis time, and reduce the production of toxic waste.

Different additives were tested and it was observed that the most acidic additives had better chromatographic peak resolution so that, in the mobile phase with acidic pH, the molecule is in its less ionized form. Moreover, varying only the type of additive under the same chromatographic conditions, revealed that API retention is inversely proportional to the acidity of the additive (retention: acetic acid > formic acid > trifluoroacetic acid). Although higher retention has been reached, the acetic acid was discarded due to inadequate resolution, and the tail of the chromatographic band was observed. Excellent results have been achieved using trifluoroacetic acid, and having lower retention would not be a problem because the retention could be adjusted by increasing the proportion of weak eluent of the mobile phase (water).

On the other hand, the use of TFA was avoided because it is corrosive, cytotoxic, and it persists in the environment. Finally, formic acid showed adequate results, and it was chosen because of its low toxicity, and it is not harmful to the environment since it readily decomposes to CO_2 and H_2O. The ZORBAX Bonus-protected RP™ column is a sterically different and suitable stationary phase for polar substances requiring a highly aqueous mobile phase (pH range: 2–9).

Thus, two analytical methods have been proposed in this paper. Using a mobile phase of simple preparation, they do not need buffer preparation as a constituent and the method presents rapid analysis, It provides the decrease in consumption of organic solvents by reducing operating costs and contributing to the environment. Analytical methods are constantly undergoing changes and improvements. Green chemistry is rapidly being adopted. The development of new pharmaceutical methods based in Green chemistry has been encouraged by universities and the pharmaceutical industry. It is up to the researcher to evaluate the possibilities based on the characteristics of each substance.

4. Conclusion

Considering the importance of ERTM in clinical practice nowadays and considering that there is still no official monograph in the pharmacopoeia to analyze ERTM, two new analytical methods have been developed for routine analysis. The selected conditions were chosen for greater enjoyment of useful life in the chromatographic column and to avoid problems arising from the precipitation of buffers, which are common concerning chromatography equipment. Thus, the methods described here have economic advantages and an eco-friendly focus that was discussed in this role, which is of the utmost importance to the environment. The proposed chromatographic method was adequate and advantageous, presenting simplicity, linearity, precision, accuracy and robustness, detection limits, and satisfactory quantification. Further, it can be used for quantitative analysis of ertapenem sodium in the pharmaceutical industry, contributing to improved quality control and being an excellent alternative for the environment.

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