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Development and Validation of UV Spectrophotometric and RP–HPLC Methods for Determination of Ertapenem During Stability Studies

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Two novel analytical methods, spectrophotometric UV and reversed-phase high-performance liquid chromatography (HPLC), were developed for quantitative determination of ertapenem during studies of its stability in the preparation INVANZ. Both methods were validated and compared. In the HPLC method were used: a RP-column, a mobile phase – phosphate buffer 25 mmol L^{-1} (pH = 6.5) and methanol (85:15 v/v) with a flow rate of 1.2 mL min^{-1}, and diprophylline as internal standard. The detection wavelength was 298 nm. In the spectrophotometric method the subtraction technique was used to interpret the results, at λ = 294 nm. Both methods were validated with regard to linearity, limit of detection, limit of quantitation, selectivity and precision. Relative standard deviations for intra-day and inter-day precision were from 0.23% to 1.03%. Both methods proved to be suitable for kinetic studies of ertapenem in INVANZ. Although the UV method a faster, it requires the use of the subtraction technique to determine the observed rate constants.

Opracowano dwie nowe metody do oznaczania ertapenemu podczas badań trwałości w preparacie INVANZ: wysokosprawną chromatografię cieczową w odwróconym układzie faz (HPLC) i spektrofotometryczną UV. Obie metody zostały zwalidowane i porównane. W metodzie HPLC rozdział chromatograficzny prowadzono stosując kolumnę LiChrospher RP–18, z zastosowaniem fazy ruchomej – bufor fosforanowy 25 mmol L^{-1} (pH = 6,5) i metanol (85:15 v/v) o szybkości przepływu 1.2 mL min^{-1} i diprofiliny jako wzorca wewnętrznego. Chromatogramy rejestrowano przy długości fali 298 nm. W metodzie spektrofotometrycznej UV do interpretacji wyników (λ = 294 nm) zastosowano tzw. technikę odejmowania. Obie metody zostały zwalidowane w odniesieniu do liniowości, granicy

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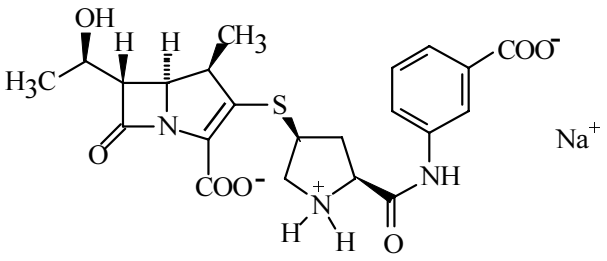
wykrywalności, granicy oznaczalności, selektywności i precyzji. Współczynnik zmienności dla precyzji bezpośredniej i pośredniej wynosił od 0,23% do 1.03%. Obie metody mogą być wykorzystane w badaniach kinetycznych trwałości ertapenemu w preparacie INVANZ. Metoda UV jest szybsza, ale wymaga zastosowania techniki odejmowania przy wyznaczaniu stałych szybkości reakcji rozkładu.

Ertapenem (Scheme 1) is a new parenteral intravenous and intramuscular carbapenem, antibiotic with a very broad spectrum of antimicrobial activity, against both gram-positive and gram-negative bacteria [1–5]. *In vitro* ertapenem is more active than imipenem and meropenem, against gram-negative bacteria, but less active against *Pseudomonas spp.*, *Acinetobacter* and *Enterococcus faecalis* [6].



Scheme 1. Ertapenem monosodium salt = ([4*R*-[3(3*S**,5*S**),4α,5β,6β(*R*)]]-3-[[5-[[(3-carboxyphenyl)--amino]carbonyl]-3-pyrrolidinyl]thio]-6-(1-hydroxyethyl)-4-methyl-7-oxo-1-azabicyclo--[3.2.0]hept-2-ene-2-carboxylic acid monosodium salt).

Ertapenem is a β-lactam, structurally different from imipenem and meropenem. Ertapenem contains in C4-position β-methyl group (similarly to meropenem), which provides stability against human dehydropeptidase, and a benzoate anionic side chain that contributes to high protein – binding (approx. 94%) and prolongs the $t_{0.5}$ thereby allowing one daily dosage [7, 8].

The literature reports the use of HPLC method for the determination of ertapenem in biological fluids [9, 10], and for the study of the stability of ertapenem in sodium chloride solution injections, Ringer's, mannitol and dextrose solutions, at 25°C and 4°C [11].

Main products of degradation were indicated and identified as dimeric I–V in the pH range 4.5–8.5 with HPLC, LC–MS and NMR methods [12]. Dimers I + II are formed from opening of carbapenem ring in one ertapenem molecule and linkage with the proline amine group of another molecule. They are a pair of inter converting tautomeric isomers, which are in equilibrium in solution. Dimer III is formed from the carbapenem ring opening of one molecule by the meta aminobenzoic acid carboxylate (MABA) group from a second molecule, followed by acyl transfer. Dimers-H_2O *a* and *b* (dimers IV) are internal amides between a carboxylate group of one molecule

with the proline amine of a second molecule. Both carbapenem rings are intact. Dimer V is formed from the carbapenem ring opening of one molecule by the MABA carboxylate group of a second molecule. Acyl transfer to the secondary alcohol produces the ester.

The aim of the work was to develop and validate HPLC and UV methods to study the stability of ertapenem in aqueous solutions, in the pH range 7.62–10.05.

EXPERIMENTAL

Chemical and reagents

Ertapenem for injection – INVANZ (Merck) is a sterile, synthetic, white to colorless hygroscopic, weakly crystalline powder. Each vial contains 1.046 g of ertapenem sodium (equivalent to 1 g of ertapenem) and inactive ingredients: 175 mg of sodium bicarbonate and sodium hydroxide to adjust pH to 7.5.

Diprophylline (as specified in FP VI Poland) was used as an internal standard.

Phosphate buffer (pH = 6.5): 70.0 mL 0.5 mol L^{-1} KH_2PO_4 and 30.0 mL 0.5 mol L^{-1} Na_2HPO_4 in 2000 mL bidistilled water.

Other chemical substances and reagents were products of Sigma and all were of analytical purity grade.

The water used was distilled twice.

Apparatus and experimental conditions

The chromatographic separation and quantitative determination were performed using a high performance liquid chromatograph containing a Shimadzu pump, model LC–6A, a UV–VIS detector SPD–6AV (Shimadzu), a Rheodyne injection fitter with a 50 μL loop. As the stationary phase an analytical column was used (LiChrospher RP–18, 5 μm particle size, 250 × 4 mm I.D.). Ertapenem was eluted at a flow rate of 1.2 mL min^{-1} using a mobile phase consisting of 25 mmol L^{-1} (pH = 6.5) phosphate buffer and methanol at a volume ratio of 85:15. Determination wavelength of the UV–VIS detector was set at 298 nm.

For determination of ertapenem, a UV–VIS Lambda 20 (Perkin Elmer) spectrophotometer equipped with 1.0 cm-in-width quartz cells and controlled *via* UV WinLab software was utilized. Detection wavelength was at λ = 294 nm.

Chromatographic procedure

Standard solution was prepared as follows: 5.0 mg of INVANZ (equivalent to 3.025 mg of sodium ertapenem) was accurately weighted, transferred to a 10 mL-in-volume flask, and dissolved with doubly distilled water (solution A). Working solutions of the following concentrations: (1.06, 1.60, 2.14, 2.67, 3.21, 3.74, 4.28, 4.81, 5.34, 5.88, 6.41) × 10^{-2} mg mL^{-1} were prepared after appropriate dilutions of solution A.

Spectrophotometric procedure

Standard solution was prepared as follows: 12.5 mg of INVANZ (equivalent to 8.016 mg sodium ertapenem) was accurately weighted, transferred to a 25 mL-in-volume flask, and dissolved with doubly distilled water (solution B). Solution B was used to prepare more diluted solutions (in the concentration range: 0.012–0.10 mg mL^{-1} by diluting it with the approprate amount of distilled water.

Validation of the method

Both methods were validated according to International Conference on Harmonisation guidelines [13] for validation of analytical procedures and were compared using statistical analysis.

Selectivity. The selectivity was determined only for the HPLC method and was examined for non--degraded and degraded samples (borate buffer, 303 K). Non-degraded sample: the solution of ertapenem powder injection (4.26×10^{-2} mg L^{-1}) in borate buffer (pH = 7.6, degraded sample: the solution of ertapenem powder injection (4.26×10^{-2} mg L^{-1}) in borate buffer (pH = 7.6) after incubation 10 min at 303 K.

Linearity. For the HPLC method the calibration plot $P_E/P_{IS} = f(c)$ was obtained in the concentration range $(1.068–6.41) \times 10^{-2}$ mg mL^{-1}, where P_E/P_{IS} is the ratio of peak area of ertapenem and diprophyline (internal standard). Calibration plot A = f(c) was obtained by plotting the measured absorbance values against the corresponding ertapenem concentration in the range 0.012–0.1 mg mL^{-1}.

Precision. Precision of the assay was determined in relation to repeatability (intra-day) and intermediate precision (inter-day) for the HPLC and UV methods. In order to evaluate the repeatability of the methods eight samples were determined during the same day for three concentrations in HPLC method (2.14×10^{-2} mg mL^{-1}, 4.27×10^{-2} mg mL^{-1}, 5.34×10^{-2} mg mL^{-1}) and for four concentrations in the UV method (1.61×10^{-2} mg mL^{-1}, 4.27×10^{-2} mg mL^{-1}, 6.41×10^{-2} mg mL^{-1}, 8.82×10^{-2} mg mL^{-1}). For both methods the intermediate precision (at the ertapenem concentration 4.27 mg mL^{-1}) was studied by comparing the assays performed on two different days.

Limit of detection and limit of quantitation. The LOD and LOQ parameters were determined from the regression equation, where: $LOD = 3.3\ S_y/a$, $LOQ = 10\ S_y/a$, where S_y is standard deviation and a is the slope of the corresponding calibration curve.

Stability of ertapenem in aqueous solutions

Stability of ertapenem in aqueous solutions was studied by HPLC and UV methods in borate buffer (pH = 7.6–10.5) at 303 K. Accurately weighed 5.0 mg (for HPLCmethod) or 10.0 mg (for UV method) of INVAZ were dissolved in 25 mL (for HPLC) or 50 mL (for UV) of borate buffer solution, of pH (7.6–10.5) and ionic strength of 0.5 mol L^{-1} and heated up to 303 K. The samples of reaction mixtures (1.0 mL for HPLC and 2.0 mL for UV) were collected at time intervals depending on reaction rates at a given pH. They were instantly cooled with a mixture of ice and water and neutralized with 1.0 mL (for HPLC) or 2.0 mL (for UV) of appropriately concentrated HCl solution and finally they were analysed. In case of HPLC method, 2.0 mL of the internal solution (0.4 mg mL^{-1} diprophylline) were added to each such sample.

RESULTS AND DISCUSSION

HPLC and UV methods have been found suitable for evaluation of stability of ertapenem in aqueous solution. HPLC method has been also proven appropriate for determination of ertapenem in pharmaceutical form (INVANZ).

Validation of the method

Only HPLC method was found selective for determination ertapenem (E) in the presence of its degradation products (P) and diprophylline (internal standard, IS), see chromatograms in Figure 1. As shown in the chromatograms, the ertapenem formed a symmetrical peak, clearly separated from the peak of degradation products and that of the internal standard. Calibration plots for the ertapenem were linear in the following concentration ranges: $(1.06–6.41) \times 10^{-2}$ mg mL^{-1} (HPLC, n = 11, r = 0.9988) and 0.012–0.10 mg mL^{-1} (UV, n = 23, r = 0.9997). Parameters of the regression were calculated for f = n–2 degrees of freedom with $\alpha = 0.05$. The calibration curves for both methods are described by the equation y = ax; y = (46.44 ± 1.70)x (for HPLC method) and y = (24.59 ± 0.28)x (for UV method).

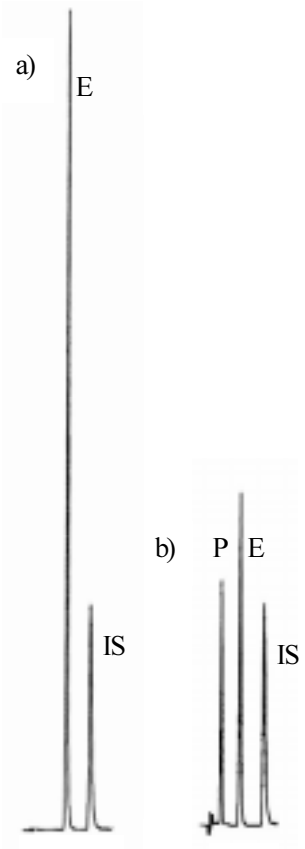


Figure 1. HPLC chromatograms of: (a) the ertapenem powder injection of concentration 4.26×10^{-2} mg mL^{-1}; (b) solution of ertapenem and its degradation products in borate buffer (pH = 7.6) obtained after incubation at 303K after 10 min

Both methods had good intra-day repeatability (RSD from 0.42% to 1.03%) and inter-day repeatability (RSD = 1.16% – for HPLC and RSD = 1.71% – for UV methods) (Tab. 1). Under the applied chromatographic conditions, LOD was 2.80×10^{-2} mg mL^{-1} (0.14 μg of the ertapenem injected on the column), the LOQ was 8.44×10^{-3} mg mL^{-1} (equivalent to 0.42 μg of the ertapenem injected on the column). In the UV method LOD and LOQ were 2.0×10^{-3} and 6.06×10^{-3} mg mL^{-1}, respectively.

Table 1. The statistical analysis of the results of the determination of ertapenem by two proposed methods (n = 8)

Methods	HPLC, λ = 298 nm			UV, λ = 294 nm			
Concentration of ertapenem, mg mL^{-1} 10^2	2.14	4.27	5.34	1.60	4.10	6.41	8.81
Parameters							
Mean value (P_E/P_{IS}) or (A_i)	0.9903	1.9913	2.8959	0.4234	1.0053	1.6668	2.2280
Variance (SD^2)	8.18×10^{-6}	2.76×10^{-4}	4.77×10^{-4}	6.84×10^{-6}	2.79×10^{-6}	1.45×10^{-7}	1.04×10^{-7}
Standard deviation (SD)	4.09×10^{-3}	1.02×10^{-2}	2.37×10^{-2}	3.42×10^{-3}	1.04×10^{-2}	7.27×10^{-3}	5.18×10^{-3}
Relative standard deviation RSD	0.42%	0.52%	0.82%	0.81%	1.03%	0.44%	0.23%

Stability of ertapenem in aqueous solutions

The degradation of ertapenem in INVANZ is a pseudo-first-order reaction described by the following equations:

$$\ln (P_E/P_{IS}) = \ln (P_E/P_{IS})_0 - k_{obs}\, t \qquad \text{(HPLC)}$$

$$\ln (A_i - A_\infty) = \ln (A_i - A_\infty)_0 - k_{obs}\, t \qquad \text{(UV)}$$

In HPLC method the semilogarythmic plot $\ln (P_E/P_{IS}) = f(t)$, obtained according to the above equation, was linear and its slope was equal to the rate constant of the reaction with the negative sign ($-k_{obs}$). However, in the UV method, absorption of the analysed samples of the ertapenem decreases from A_i to $A_\infty > 0$ over a period of time from t_0 to t_∞, (Figs. 2 and 3a). Within the same time period, reaction reached steady state and the plots: $\ln (A_i - A_\infty) = f(t)$ were linear (Fig. 3b). Catalytic rate constants

(k_{obs}) and coefficients (α) for degradation of ertapenem in borate buffer (pH = 7.6–10.3) determined by both methods are presented in Table 2.

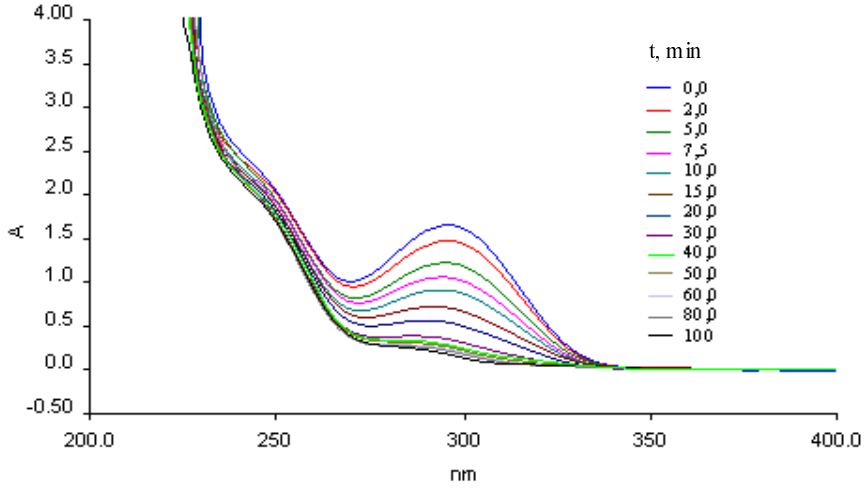


Figure 2. UV spectrum of ertapenem in borate buffer (pH 7.6) after heating to 303 K (during stability studies)

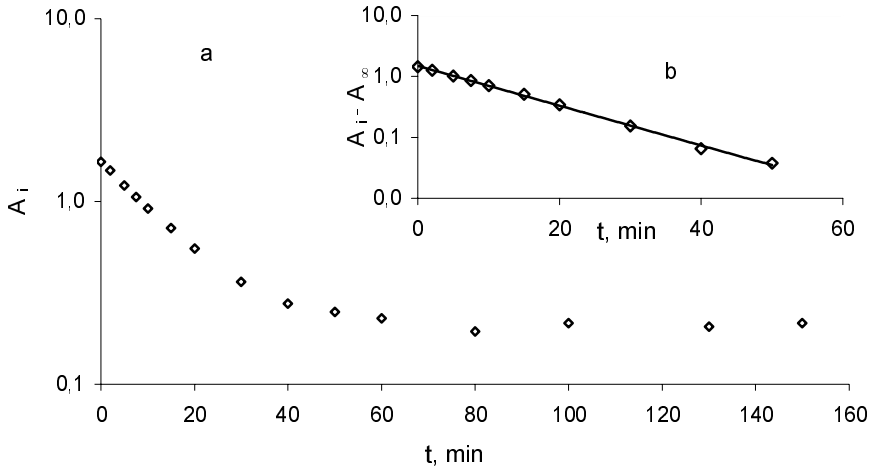


Figure 3. Semilogarithmic plots ln A_i = f(t) (a) and ln(A_i – A_∞) = f(t) (b); both plots characterize degradation kinetics of ertapenem in borate buffer (303 K)

Comparison of the methods

The proposed analytical methods were compared using statistical analysis. The precision of both methods was evaluated using F-Snedecor test. Experimental F values did not exceed theoretical ones referring to P = 95% for n_1, n_2 = 7 ($F\alpha_{(7,7)}$ 1.0414 < $F\alpha_{(7,7)}$ 3.79) for concentration 4.17×10^{-2} mg mL^{-1}. Thus, the results obtained during the succeeding days did not differ significantly with respect to variability. To verify that k_{obs} determined with both methods were statistically insignificant the parallelism test was used (Tab. 2).

Table 2. Catalytic rate constants and coefficients α for degradation of ertapenem in borate buffer of the pH range 7.62–10.05 at 303 K (B_T – total concentration of buffer)

Parameters B_T, mg mL^{-1}	UV method		HPLC method		t_0*
	$10^2 \times \alpha$, min^{-1}	k_{obs}, s^{-1}	$10^2 \times \alpha$, min^{-1}	k_{obs}, s^{-1}	
pH = 7.62					
0.221	–1.91 ± 0.06	(3.18 ± 0.01) 10^{-4}	–1.94 ± 0.01	(3.22 ± 0.31) 10^{-4}	0.2217
0.166	–1.41 ± 0.02	(2.35 ± 0.06) 10^{-4}	–1.71 ± 0.02	(2.85 ± 0.48) 10^{-4}	0.7886
0.110	–1.91 ± 0.02	(2.18 ± 0.01) 10^{-4}	–1.40 ± 0.02	(2.33 ± 0.27) 10^{-4}	0.9841
0.055	–9.36 ± 0.79	(1.56 ± 0.01) 10^{-4}	–9.54 ± 1.58	(1.59 ± 0.26) 10^{-4}	0.3922
pH = 7.91					
0.195	–4.05 ± 1.57	(6.75 ± 0.01) 10^{-4}	–4.12 ± 1.87	(6.87 ± 0.05) 10^{-4}	0.2498
0.169	–3.60 ± 0.06	(6.00 ± 0.06) 10^{-4}	–3.75 ± 1.81	(6.26 ± 3.03) 10^{-4}	0.6658
0.147	–3.11 ± 0.03	(5.19 ± 0.01) 10^{-4}	–3.08 ± 1.12	(5.12 ± 1.87) 10^{-4}	0.2667
0.098	–2.34 ± 0.02	(3.90 ± 0.01) 10^{-4}	–2.42 ± 0.08	(4.03 ± 1.33) 10^{-4}	0.8652
pH = 8.87					
0.162	–1.00 ± 0.14	(1.67 ± 0.01) 10^{-3}	–9.27 ± 1.60	(1.54 ± 0.26) 10^{-3}	1.7936
0.122	–7.49 ± 0.09	(1.25 ± 0.06) 10^{-3}	–7.94 ± 0.20	(1.32 ± 0.30) 10^{-3}	1.2075
0.101	–7.32 ± 1.19	(1.22 ± 0.01) 10^{-3}	–6.55 ± 2.52	(1.09 ± 0.42) 10^{-3}	1.9040
0.081	–5.93 ± 1.61	(9.89 ± 0.01) 10^{-4}	–5.52 ± 0.01	(9.21 ± 1.60) 10^{-4}	1.5484

(Continuation on the next page)

Table 2. (Continuation)

Parameters B_T, mg mL^{-1}	UV method		HPLC method		t_0^*
	$10^2 \times \alpha$, min^{-1}	k_{obs}, s^{-1}	$10^2 \times \alpha$, min^{-1}	k_{obs}, s^{-1}	
pH = 9.76					
0.126	–15.80 ± 1.80	(2.64 ± 0.01) 10^{-3}	–1.50 ± 0.38	(2.50 ± 0.63) 10^{-3}	1.2976
0.094	–12.9 ± 0.60	(2.15 ± 0.99) 10^{-3}	–1.21 ± 0.27	(2.01 ± 0.45) 10^{-3}	0.8605
0.063	–87.80 ± 24.0	(1.46 ± 0.01) 10^{-3}	–8.21 ± 2.13	(1.37 ± 0.35) 10^{-3}	0.2400
0.032	58.90 ± 16.3	(9.82 ± 0.01) 10^{-4}	–5.27 ± 2.45	(8.79 ± 4.09) 10^{-4}	1.0478
pH = 10.05					
0.122	–16.70 ± 2.70	(2.79 ± 0.45) 10^{-3}	–1.74 ± 0.24	(2.91 ± 0.41) 10^{-3}	0.7251
0.099	–14.22 ± 1.00	(2.36 ± 0.17) 10^{-3}	–1.44 ± 0.14	(2.40 ± 0.23) 10^{-3}	0.7836
0.076	–10.80 ± 1.91	(1.79 ± 0.82) 10^{-3}	–1.11 ± 0.28	(1.84 ± 0.47) 10^{-3}	0.7111
0.054	–90.50 ± 23.72	(1.51 ± 0.40) 10^{-3}	–8.67 ± 1.53)	(1.44 ± 0.25) 10^{-3}	0.5916

t_0^* – Comparison methods.

CONCLUSIONS

HPLC and UV methods used to evaluate the stability of ertapenem in INVANZ were found simple, rapid, precise, sensitive. However, only the HPLC method is selective. When the UV method is applied, it is necessary to use subtraction technique and then it can be used for the stability studies.

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