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Development and optimization of a reversed-phase high-performance liquid chromatographic method for the determination of piperacillin and tazobactam in tazocin injectable powder

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Abstract

A reversed phase high-performance liquid chromatographic method with detection at 220 nm was developed and validated for the determination of piperacillin, **I**, and tazobactam, **II**, in Tazocin injectable powder. Acetaminophen was used as internal standard. A Hypersil BDS RP-C_{18} column (250 × 4.6 mm), 5 μm particle size, was equilibrated with a mobile phase composed of aqueous solution of sodium dihydrogenphosphate-dihydrate (20 mM)-acetonitrile-methanol (70:15:15, v/v/v) and pH 5.0. Its flow rate was 1.0 ml/min. Calibration curves were linear for **I** and **II** in the concentration ranges of $3.0 \times 10^{-7} - 2.0 \times 10^{-4}$ M and $7.0 \times 10^{-7} - 2.0 \times 10^{-4}$ M, respectively. Limits of detection and quantitation were 1×10^{-7}, 3×10^{-7} M for **I** and 2×10^{-7}, 7×10^{-7} M for **II**, respectively. Relative standard deviation, for **I** and **II** was less than 0.40 and 0.75%, respectively. Extensive recovery studies were also performed.

Keywords: Piperacillin; Tazobactam; Tazocin; Reversed-phase HPLC

1. Introduction

Tazocin is a new drug consisted of piperacillin sodium and tazobactam sodium. It is administered intravenously at a fixed ratio of its active ingredients (eight parts piperacillin to one part tazobactam).

Piperacillin [1], (2S,5R,6R)-6-[[(2R)-2-[[(4-ethyl-2,3-dioxopiperazin-1-yl)carbonyl] amino]-2-phenyl-acetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0.] heptane-2-carboxylic acid, **I** (Fig. 1), is a ureidopenicillin, a semisynthetic penicillin derivative that has been shown effective in the treatment of many serious infections associated with Gram-positive and Gram-negative organisms, including Pseudomonas aeruginosa, Proteus, Klebsiella pneumoniae and Serratia marcescens. It is a β-lactam antibiotic, susceptible to hydrolysis by a range of β-lactamases, includ-

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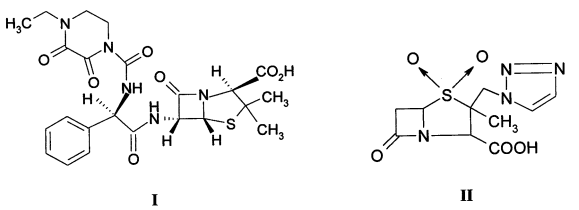
ing the plasmid-mediated enzymes. These enzymes inactivate β-lactam antibiotics by opening the β-lactam ring.

Tazobactam [1], [2S-(2a,3b,5a)]-3-methyl-7-oxo-3-(1H-1,2,3-triazol-1-ylmethyl)-4-thia-1-azabicyclo[3.2.0] heptane-2-carboxylic acid 4,4-dioxide, **II** (Fig. 1), is a potent and novel β-lactamase inhibitor belonging to a class of penicillanic acid sulfones. The combined use of tazobactam and piperacillin has been more effective against various β-lactamase-producing bacteria. Compound **II** has been shown to act synergistically with **I** and a variety of other β-lactam antibiotics against a broad spectrum of bacterial pathogens.

Piperacillin has been determined by many analytical methods, such as capillary zone electrophoresis [2], thin-layer chromatography [3], cyclic voltammetry [4], spectrophotometry [5], potentiometric titration [6] and especially by HPLC [7–13]. Tazobactam has been determined by high-performance liquid chromatography [14–17]. Simultaneous determination of piperacillin and tazobactam has been reported in biological fluids [18–20] and pharmaceutical preparations [21] by HPLC. These methods included gradient elution HPLC [19] and ion-pair HPLC [20,21]. The only suggested method for the determination of both piperacillin and tazobactam in pharmaceutical preparations was ion-pair HPLC [21] and the elution time was extremely long (≈ 40 min).

The purpose of the present work was to develop a new, reliable, reproducible, simpler, less expensive and less time-consuming reversed-phase HPLC method for the determination of piperacillin and tazobactam in intravenous injection solutions of Tazocin. In this paper, development, optimization and validation of such a method are presented.



I II

Fig. 1. Structures of piperacillin, **I**, and tazobactam, **II**.

2. Experimental

2.1. Instrumentation

The chromatographic system used, consisted of a Waters 600E Multisolvent Delivery System (a 600 Controller, a pump and a U6K Injector) and a Waters 486 Tunable Absorbance Detector (Waters, Milford, MA, USA). The above system was controlled by the software package Millennium 2010. The pH of the mobile phase was measured with a pH Meter 3310 Jenway Ltd. (Gransmore Green, England).

2.2. Chemicals and reagents

All chemicals were of analytical purity grade. Methanol (MeOH) and acetonitrile (ACN) of HPLC grade were purchased from E. Merck (Darmstadt, Germany). Piperacillin sodium and tazobactam sodium were of pharmaceutical purity grade and were kindly donated by Wyeth-Ayerst Research (Pearl River, N.Y., USA). Acetaminophen was donated by Rhone-Poulenc Hellas (Athens, Greece). Injection vials of Tazocin were commercially available. Water purified with a Milli-Q RG water purification system (Millipore Co., Bedford, MA, USA) was used in all procedures.

2.3. Chromatographic conditions

A Hypersil BDS-C18 column (250×4.6 mm), packed with silica, 5 μm particle size, was used. The mobile phase consisted of acetonitrile (ACN)-methanol (MeOH)-aqueous solution of sodium dihydrogenphosphate-dihydrate ($NaH_2PO_4 \cdot 2H_2O$), 20 mM (15:15:70, v/v/v). Its pH was adjusted to 5.0 with phosphoric acid or sodium hydroxide. The mobile phase was degassed for 10 min with Helium gas at a degassing rate of 20 ml/min. The flow-rate of the mobile phase was 1.0 ml/min. Injection volume was 20 μl. Experiments took place at room temperature. Absorption of **I** and **II** was measured at 220 nm.

2.4. Solution preparation

2.4.1. Stock solutions

Concentration of stock solutions of **I**, **II** and acetaminophen, **III** in purified water was adjusted to about 1.00×10^{-3} M, or 540, 324 and 152 ng/ml, respectively. Acetaminophen was used as internal standard. Tazocin lyophilized powder was reconstituted with 10.0 mL of purified water in each injection vial. Nominal concentrations of **I** and **II** after reconstitution were 0.3707 and 0.07757 M, respectively. Stock solutions were stored at -20 °C and were stable for at least a week.

2.4.2. Test solutions

Working standard solutions of **I** and **II** were prepared in concentration ranges of $3.0 \times 10^{-7} - 2.0 \times 10^{-4}$ M and $7.0 \times 10^{-7} - 2.0 \times 10^{-4}$ M, respectively and used for the establishment of the linearity ranges and construction of the corresponding calibration curves. Two series of solutions were prepared and used for evaluation of the precision and accuracy (% recovery) of the developed HPLC method. Each solution contained acetaminophen at a concentration of 5.0×10^{-5} M. All dilutions to volume were performed in mobile phase.

2.4.3. Sample preparation

Five Tazocin samples were analyzed. The lyophilized powder after reconstitution was diluted 1/5.000 v/v with mobile phase. Determination of **I** was performed by calibration curve and **II** by both calibration curve and the method of standard additions. In the latter procedure, three standard additions of 1.0×10^{-5}, 2.0×10^{-5} and 3.0×10^{-5} M were performed. All solutions contained constant concentration of acetaminophen equal to 5.0×10^{-5} M. Solutions after preparation were injected to the HPLC system.

2.5. Data analysis

For determination of piperacillin and tazobactam in injection solutions of Tazocin, peak-area ratios of **I** and **II** to internal standard were used to construct the calibration curves. Regression equations were obtained through unweighed least squares linear regression analysis, applied to peak-area ratios as a function of their concentration.

3. Results and discussion

3.1. Choice of mobile phase

First, an eluent mixture composed of acetonitrile and ammonium acetate (pH 5.0) was tried [18] but it resulted in an extremely unstable baseline, probably caused by the UV absorption of ammonium acetate. Then, several eluent mixtures based on acetonitrile, methanol and aqueous solution of NaH_2PO_4 20 mM were tried. It was noticed that the polarity of the mobile phase did not affect the retention time of **II**, drastically. However, the more polar the mobile phase, the longer the retention time of **I** was. Moreover, the retention time of **I** had a significant drift to longer times when the mobile phase used consisted of acetonitrile or methanol and aqueous solution of NaH_2PO_4. This drift was eliminated in the analysis time scale when methanol was added to mixtures of ACN and NaH_2PO_4. Thus, the mobile phase chosen was finally aqueous solution of NaH_2PO_4 (20mM): ACN: MeOH (70:15:15, v/v/v). Peaks under consideration were symmetric since calculated values of the asymmetry factor, $A_{s,f}$, for **I** and **II** were 1.10 and 1.19, respectively. Also, good resolution of **I** and **II** with coexisting by-products was observed since values of $R_s > 1.5$ were obtained.

3.2. Influence of pH of the mobile phase

The pH values of the mobile phase examined were 4.5, 5.5 and 6.5. The pH was adjusted with phosphoric acid or sodium hydroxide. The results showed that the retention times of **I** and **II** were not affected by the pH of the mobile phase. However, the drift of the retention time of **I** to higher times became significant at pH

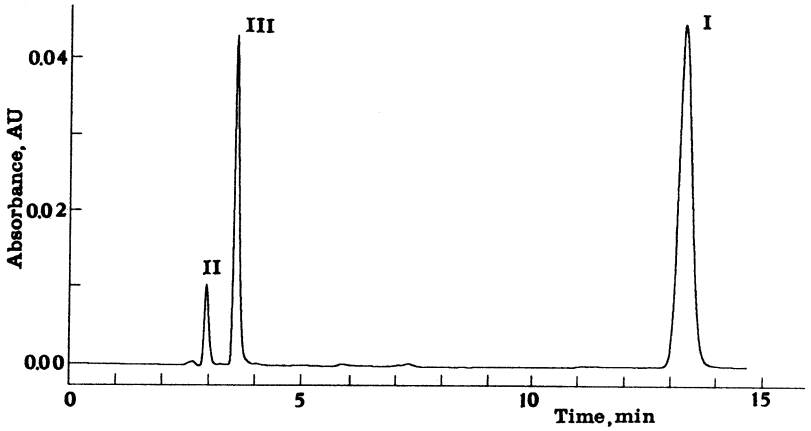


Fig. 2. A chromatogram of standard solution of piperacillin, **I**, tazobactam, **II** and acetaminophen, **III**, at a concentration of 5.0×10^{-5}, 1.0×10^{-5} and 5.0×10^{-5} M, respectively. The chromatographic conditions used were: BDS-C_{18} column, mobile phase of NaH_2PO_4 (20 mM)-MeOH-ACN (70:15:15, v/v/v), pH 5.0, flow rate of 1.0 ml/min, detection wavelength of 220 nm, room temperature.

Table 1
Analytical parameters of the calibration curves of piperacillin and tazobactam

Sample	Concentration range ($\times 10^5$ M)	Regression equation[a]		
		Intercept, a ± S.D.[b]	Slope, (b ± S.D.[b]) $\times 10^{-4}$	r (n)[c]
I	0.3–20.0	0.0002 ± 0.006	5.84 ± 0.01	0.999994(8)
II	0.3–20.0	0.001 ± 0.005	2.04 ± 0.01	0.99995(8)

The chromatographic conditions used were: BDS-C_{18} column, mobile phase of NaH_2PO_4 (20 mM)-MeOH-ACN (70:15:15 v/v/v), pH 5.0, flow rate of 1.0 ml/min, detection wavelength of 220 nm and room temperature.

[a] Linear unweighted regression analysis, with a regression equation $y = a + bx$, where x is concentration in M.

[b] S.D. is the standard deviation of intercept and slope.

[c] r is the correlation coefficient and n is the number of points in each calibration curve; each point is the mean of four experimental measurements.

6.5, while it was negligible at pH values 4.5 and 5.5. Thus, the pH value of 5.0 was chosen for the rest of the experiments.

3.3. Choice of internal standard

Thirteen substances were tried as internal standards. Acetaminophen, **III**, was chosen as the most appropriate one in the present analysis because it had good resolution with tazobactam ($R_s = 2.310 \pm 0.015$) and presented a symmetric peak ($A_{s,f} = 1.20$). A representative chromatogram of **I**, **II** and **III** is shown in Fig. 2.

3.4. Calibration curves of piperacillin and tazobactam

Under the experimental conditions described above, linear calibration curves for both **I** and **II** were obtained throughout the concentration ranges studied. Regression analysis was done on the ratios of peak-areas of **I** and **II** to that of the internal standard (y) versus concentration (x). The results are tabulated in Table 1. Preparing and measuring standards of the same concentrations of **I** and **II** four times each, relative standard deviation (RSD) was calculated and found less

than 0.39 and 0.15% respectively in the whole concentration range.

3.5. Precision and accuracy

Injection vials of Tazocin did not contain any excipients, as it was written on the package material. To verify precision and accuracy of the proposed HPLC method, an extensive interference study between **I** and **II** was conducted. Intra-and inter-day mean recovery of **I** was calculated and found not lower than 99.1 and 98.8% and not higher than 100.7 and 100.3%, respectively. Its relative standard deviation was not higher than 0.40 and 1.6% for within day and between day measurements, respectively. It was also observed that intra-day recovery of **II** was in the range of 99.1–103.0% when the concentration ratio of **I** to **II** was from 1:10 to 5:1. There was a slight increase in the % value of recovery when concentration ratio of **I** to **II** was higher than 8:1. This may be due to some degradation product of piperacillin or impurities in its raw material. Intra-and inter-day relative standard deviation of **II** was not higher than 0.75 and 2.8%, respectively. Results of accuracy, intra-and inter-day precision

Table 2
Intra-and inter-day precision and accuracy of the HPLC method for the determination of **I**

Concentration of **I**, $\times 10^5$ M[a]	Concentration of **II**, $\times 10^5$ M[a]	Intra-day mean % recovery $\pm$ S.D.[b]	Inter-day mean % recovery $\pm$ S.D.[b]
1.0		99.3 ± 0.3	100.0 ± 1.5
3.0		99.1 ± 0.2	98.8 ± 1.5
5.0	1.0	99.1 ± 0.4	99.6 ± 1.6
8.0		100.0 ± 0.4	99.8 ± 1.4
10		100.7 ± 0.2	100.3 ± 1.5
	3.0	99.8 ± 0.2	–
1.0	8.0	100.7 ± 0.2	–
	10	100.2 ± 0.1	–

The chromatographic conditions used were: BDS-C_{18} column, mobile phase of NaH_2PO_4 (20 mM)-MeOH-ACN (70:15:15 v/v/v), pH 5.0, flow rate of 1.0 ml/min, detection wavelength of 220 nm and room temperature.

[a] Standard solutions were prepared and measured four times each.

[b] S.D. is the standard deviation of the intra- and inter-day mean % recovery.

Table 3
Intra-and inter-day precision and accuracy of the HPLC method for the determination of **II**

Concentration of **I**, $\times 10^5$ M[a]	Concentration of **II**, $\times 10^5$ M[a]	Intra-day mean % recovery $\pm$ S.D.[b]	Inter-day mean % recovery $\pm$ S.D.[b]
	1.0	99.1 ± 0.2	101.2 ± 2.8
	3.0	101.8 ± 0.1	102.2 ± 2.4
1.0	5.0	99.8 ± 0.3	100.1 ± 2.6
	8.0	99.2 ± 0.1	100.4 ± 2.5
	10	99.3 ± 0.2	100.0 ± 2.7
3.0		101.8 ± 0.6	–
5.0	1.0	103.0 ± 0.4	–
8.0		104.4 ± 0.4	–
10		106.2 ± 0.8	–

The chromatographic conditions used were: BDS-C_{18} column, mobile phase of NaH_2PO_4 (20 mM)-MeOH-ACN (70:15:15 v/v/v), pH 5.0, flow rate of 1.0 ml/min, detection wavelength of 220 nm and room temperature.

[a] Standard solutions were prepared and measured four times each.

[b] S.D. is the standard deviation of the intra- and inter-day mean % recovery.

for **I** and **II** are presented in Tables 2 and 3, respectively.

3.6. Limits of detection (LOD) and quantitation (LOQ)

The LOD was defined as the analyte concentration that gives a signal equal to $y_b + 3.3s_b$, where y_b is the signal of the blank and s_b is its standard deviation. Similarly, the LOQ was defined as $y_b + 10s_b$. In the unweighted least-squares method is quite suitable in practice to use $s_{y/x}$ [22] instead of s_b and the value of the calculated intercept a instead of y_b. Thus,

$$\text{LOD} = \frac{3.3s_{y/x}}{b} \text{ and } \text{LOQ} = \frac{10s_{y/x}}{b}$$

where, b is the slope of the regression line. Based on the above equations, the calculated LOD values were 1×10^{-7} M (54 ng/ml) for **I** and 2×10^{-7} M (64 ng/ml) for **II** while the LOQ ones were 3×10^{-7} M (164 ng/ml) and 7×10^{-7} M (195 ng/ml), respectively.

3.7. Determination of *I* and *II* in Tazocin

Mass ratio of **I** to **II** in Tazocin vials was 8:1 (2 g of **I** and 0.25 g of **II**). After reconstitution with 10.0 mL of purified water in each vial, concentration ratio of **I** and **II** was 4.8:1 (0.3707 M of **I** to 0.07757 M of **II**). Five different injection vials of Tazocin were analyzed. Concentration of **II** in Tazocin was calculated not only by its calibration curve but also by the standard addition method. This happened because the previous recovery study for **II** showed a systematic error, probably caused by piperacillin's by-products. The slope of the standard addition plot was $(2.05 \pm 0.01) \times 10^4$ M^{-1}. Compared with the slope of the conventional calibration curve (Table 1), it was statistically the same; this meant that the recovery of analyte **II** was complete [23]. Concentration of **I** in Tazocin was calculated only by its calibration curve since recovery experiments performed previously did not show any systematic error. The results of these determinations are presented in Table 4. It is obvious that the direct method gave

Table 4
Determination of **I** and **II** in Tazocin vials

Tazocin vial	Nominal concentration, M		Concentration found ± S.D., M				
			Our method			Other method [21]	
			Direct method		Standard addition method		
	I	**II**	**I**	**II**	**II**	**I**	**II**
1			0.3738 ± 0.0007	0.08015 ± 0.00020	0.07805 ± 0.00011	0.3507 ± 0.0003	0.07597 ± 0.00042
2			0.3731 ± 0.0005	0.08005 ± 0.00017	0.07799 ± 0.00008	0.3561 ± 0.0015	0.07741 ± 0.00046
3	0.3707	0.07757	0.3692 ± 0.0006	0.07955 ± 0.00003	0.07789 ± 0.00002	0.3470 ± 0.0004	0.07596 ± 0.00049
4			0.3661 ± 0.0004	0.07973 ± 0.00008	0.07796 ± 0.00004	0.3428 ± 0.0013	0.07428 ± 0.00009
5			0.3701 ± 0.0003	0.07893 ± 0.00083	0.07786 ± 0.00034	–	–
3[a]			0.3743 ± 0.0008	0.07995 ± 0.00004		–	–

Chromatographic conditions used were: BDS-C_{18} column, mobile phase of NaH_2PO_4 (20 mM)-MeOH-ACN (70:15:15 v/v/v), pH 5.0 (in our method), ODS-C_{18} column, mobile phase of 10 mM tetra-n-butylammonium hydroxide and 5 mM Na_2SO_4 (pH 4.1)-ACN-MeOH (1000:300:25, v/v/v) (in the other method) and flow rate of 1.0 ml/min, detection wavelength of 220 nm and room temperature for both methods.

[a] Measurement from the same injection vial, kept in −20 °C, two weeks after reconstitution.

values for **II** approximately 3% above its nominal concentration in Tazocin, while the standard addition method gave values not higher than 0.6% of its nominal concentration in each of the five injection vials of Tazocin that were analyzed. In the determination of **I** in the same samples, differences were not greater than 1.0%.

The proposed method was also compared with that found in the literature for the determination of **I** and **II** in pharmaceutical preparations [21]. These results are also included in Table 4. The latter method involved ion-pair HPLC and very long total elution time of the chromatograms. Another drawback was that the internal standard was eluted very late with a retention time of approximately 40 min and the peak of piperacillin was broad with a retention time of approximately 30 min. These facts were, probably, reasons of the large deviation observed in calculation of the concentration of **I** in Tazocin, by that method. Values of **I** were from 3.9 to 7.5% lower than the nominal, while those of **II** were from 0.2 to 4.2% lower than its nominal concentration in Tazocin.

4. Conclusion

In this work, a reliable, fast and simple reversed-phase HPLC method for the determination of piperacillin and tazobactam in intravenous injection vials of Tazocin was developed, optimized and validated. Application of the standard addition method was suggested for accurate determination of **II** in order to eliminate any interference from piperacillin by-products.

This method compared to the one existed in the literature [21] is much simpler, faster, less expensive and more precise and accurate as it has been already shown in the previous section.

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