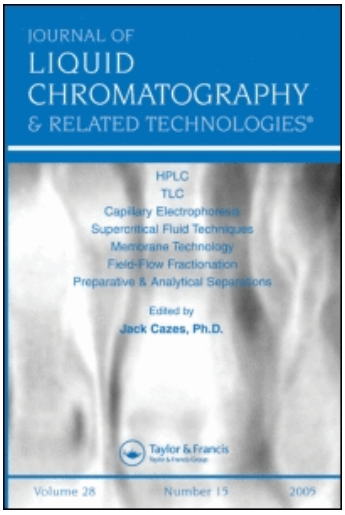

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Stability-Indicating HPLC Method for the Determination of Methicillin in Vials and Biological Fluids with Fluorometric Detection

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Abstract: A simple, stability-indicating liquid chromatographic method has been developed for the determination of methicillin sodium in the presence of its acid and alkaline-induced degradation products. A μBondapak–C_{18} column was used with a mobile phase composed of acetonitrile/2% v/v acetic anhydride (55:45 v/v) at a flow rate of 1.8 mL/min. The detection was accomplished fluorometrically using an excitation wavelength of 280 nm and emission wavelength of 360 nm. The peak area versus concentration plot was linear over the range 1–10 μg/mL with a limit of detection of 0.1 μg/mL (2.38×10^{-7} M). Between-day and within-day relative standard deviations were lower than 2%. The proposed method was successfully applied to the in-vitro determination of methicillin sodium in bulk material, dosage form, spiked urine, and spiked plasma, with minor modification of the mobile phase ratio in the case of spiked urine and plasma. The % recovery from human plasma (n = 9) was 99.04 ± 1.88 and from urine (n = 8) was 99.3 6 ± 1.77.

Keywords: Stability-indicating HPLC, Methicillin, Fluorometric detection

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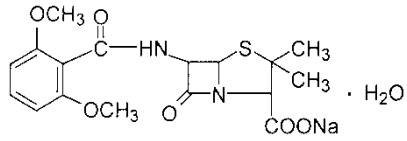
INTRODUCTION

Methicillin is a semisynthetic penicillin produced by substitution at the amino group of the naturally occurring 6-amino penicillanic acid. It is active against both penicillinase-producing and non-penicillinase-producing staphylococci. It exerts its action on growing and dividing bacteria by inhibiting the cell wall synthesis. It is inactivated by gastric acid and, therefore, must be administered parentally. Its use is associated with acute interstitial nephritis. Up to 80% of an injected dose had been detected unchanged in the urine.[1] It is used as methicillin sodium. The structural formula is given in Scheme 1.



The official method recommended for the assay of methicillin in the United States Pharmacopeia[2] is the HPLC method with UV detection. Other HPLC methods for determination of methicillin in bulk material,[3–5] as residue in milk[6] and in injections[7] were also reported. Gas chromatographic methods for the determination of penicillin residues (including methicillin) in milk and animal tissues were described by Meetschen and Petz.[8,9] Murillo and Allanon[10] described a spectrofluorimetric method for the assay of methicillin in dosage forms and also in bulk and in serum.[11] Other methods include charge-transfer spectrophotometry after reaction with dichlorodicyano benzoquinone or with tetracyanoquinodimethane, and measuring the coloured derivative at 460 nm or 842 nm, respectively.[12] An infrared spectroscopic method using a FTIR spectrometer to measure the β-lactam carbonyl band at 1767 cm^{-1} was also described by Wong and co-workers.[13] A relatively recent study of electrospray ionization mass spectrometric behaviour of several antibiotics including β-lactam antibiotics was performed.[14]

The majority of the reported methods are time-consuming, require post-column or precolumn derivatization, and are non-specific and/or not stability indicating.

The aim of the present work was to develop a simple stability-indicating HPLC method for the determination of methicillin in the presence of its degradation products. The method was successfully applied for the determination of the intact drug in bulk material, in vials for injection, and in spiked human plasma and urine.

EXPERIMENTAL

Methicillin sodium working standard was obtained from the Drug Control Center, Riyadh, Saudi Arabia, and was used as received. Plasma was kindly

provided by King Khalid University Hospital, Riyadh, K.S.A., and was kept frozen until use, after gentle thawing. Urine was obtained from healthy male volunteers (around 40 years old). HPLC grade acetonitrile was purchased from BDH, Poole, UK. Acetic anhydride (purity >98%) was purchased from Fluka Chemica, Switzerland and was used as 2% v/v aqueous solution. Sodium hydroxide was purchased from BDH, Poole, UK, and was used as a 0.005 M solution. Hydrochloric acid was obtained from Winlab, Middlesex, England, and was used as 0.005 M solution. High purity helium gas was obtained from the local market. Other solvents and materials were HPLC grade or analytical grade.

Apparatus

The high performance liquid chromatograph used was model LC–10ADVP, Shimadzu, Japan, with a mixing chamber model FCV–10 AL VP and a system controller model SCL–10A VP. The injector used was a Rheodyne–7725i (USA). The detector was a fluorescence detector model RF–10A XL. The system was driven by a Pentium–IV Computer.

The HPLC column used was a μBondapak C_{18} (3.9 × 300 mm), Part No. WAT 027324, Waters (USA). Guard columns of the same type were used during the analysis of the biological fluids.

The mobile phase filtration unit was a product of Millipore, type XX10–047–04, with filter papers type GC–0.22 μm from the same company.

Standard Solutions

A stock solution containing 1.0 mg/mL of methicillin sodium was prepared in water. It is stable at room temperature for 24 hours, and stable for 4 days if kept in the refrigerator. Further dilutions were made with the same solvent as appropriate.

Calibration Curve

Aliquots of the stock solution of methicillin sodium, after the necessary dilutions, were transferred into a series of 10 mL volumetric flasks to obtain final concentrations ranging from 1.0–10 μg/mL and the volume was completed to the mark with water. Portions of each solution (20 μL) were injected in replicate into the chromatograph. The eluents were detected by the fluorescence detector with the wavelength of excitation fixed at 280 nm and that of emission fixed at 360 nm. The signals emerging from the detector were integrated as peak area, and a calibration graph of peak area against the concentration of methicillin sodium was plotted. Alternatively, the regression equation was derived.

Induced Degradation of Methicillin

Solutions of methicillin containing 10 μg/mL were prepared in either 0.005 M HCl or 0.005 M NaOH, and kept aside at room temperature for different time intervals. Starting at zero time, 20 μL portions of either of the solutions were injected into the chromatograph and the injection was repeated at ten minute intervals, each time recording the peak area of the intact methicillin. Figures 1 and 2 represent the acid and alkaline degraded methicillin solutions, respectively.

The area of the methicillin peak (a) at zero time represents the initial concentration, whereas the area obtained after time (t) i.e. (a − x) represents the remaining concentration after that time interval, where (x) represents the

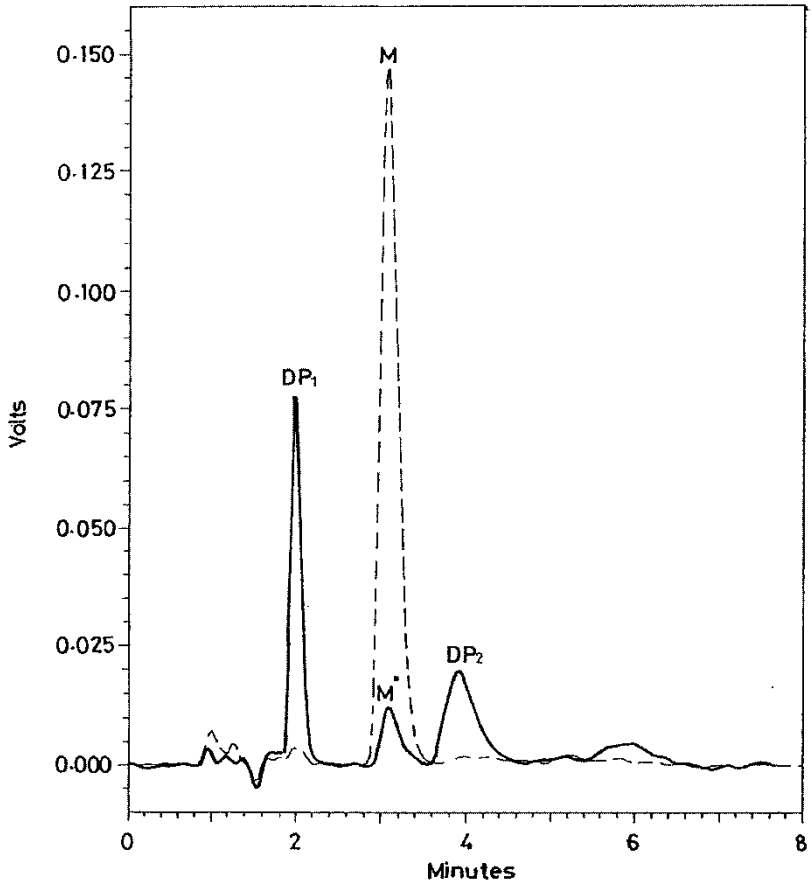


Figure 1. Typical chromatograms of methicillin in neutral solution (– – – – –) and in 0.005 M HCl (——). M and $M^{\bullet}$ are the peaks of intact methicillin. DP_1 and DP_2 are the peaks of the two major acid decomposition products.

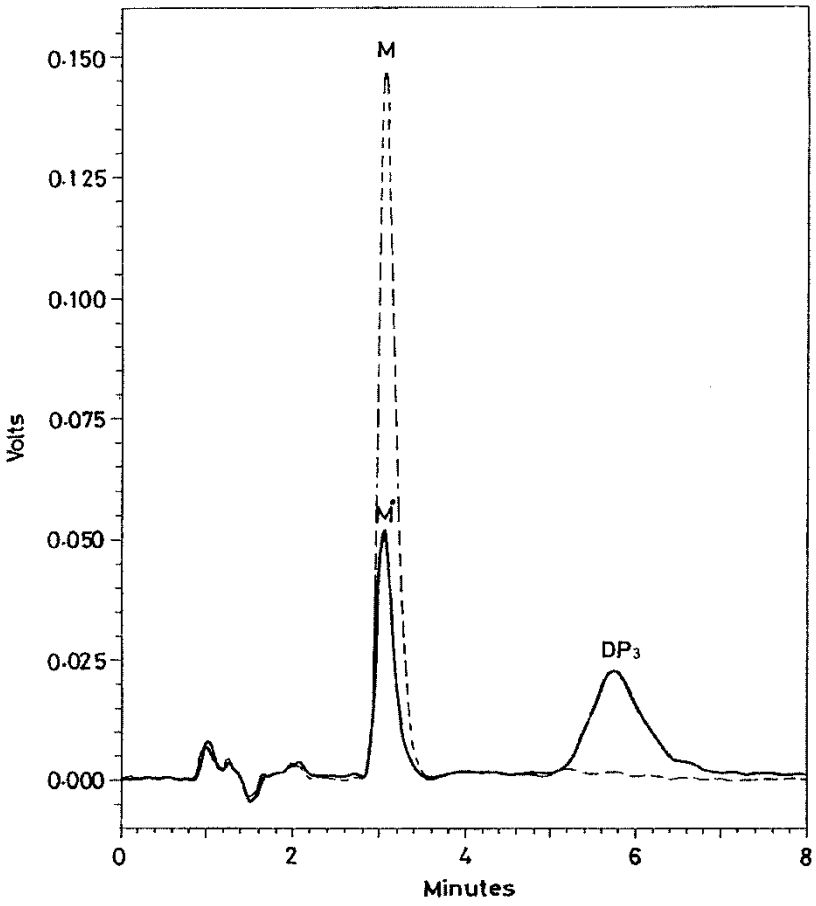


Figure 2. Typical chromatograms of methicillin in neutral solution (– – – – –) and in 0.005 M NaOH (———). M and $M^{\bullet}$ are the peaks of intact methicillin. DP_3 is the peak of the major alkaline decomposition product.

amount degraded by the acid or alkali. A graph of log $a/(a-x)$ versus time (t) was plotted (Fig. 3).

The apparent degradation rate constant and the half-life ($t_{1/2}$) in each case were calculated.

Application of the Proposed Method to the Determination of Methicillin in Pure Form

Solutions of the methicillin sodium in concentrations ranging from 3–9 μg/mL were assayed by the proposed method, and the actual concentrations were calculated using the regression equation or the standard calibration curve.

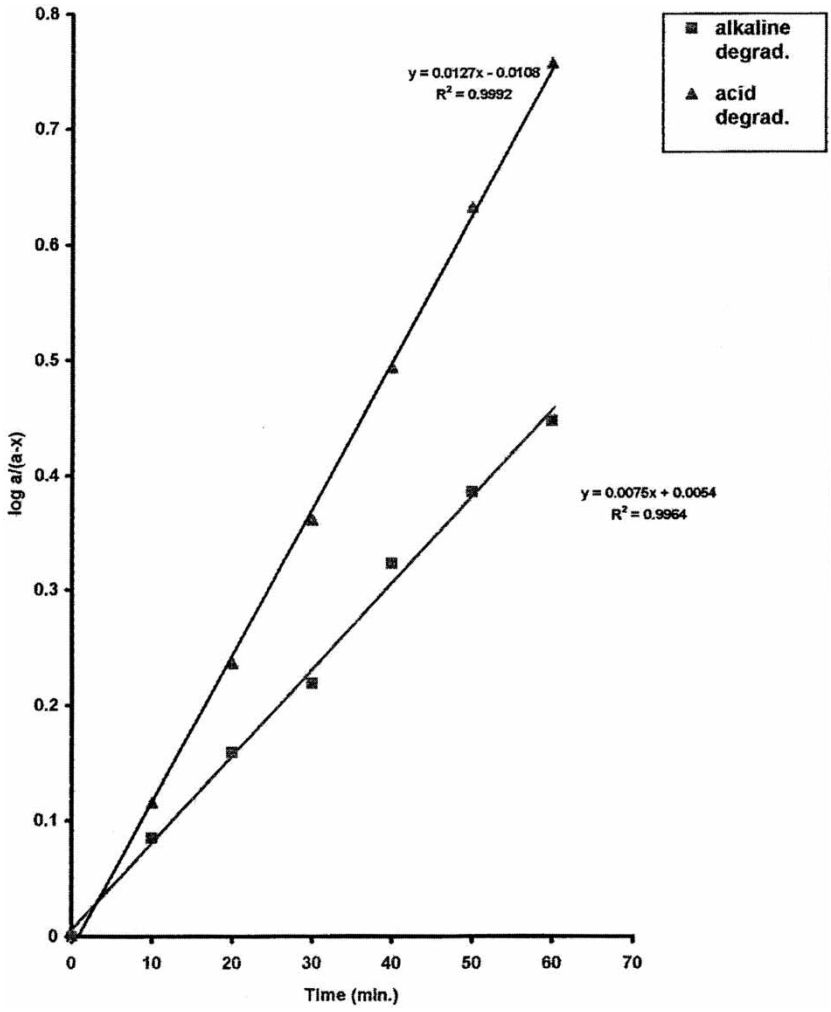


Figure 3. Relation between the amounts remaining of methicillin with time at room temperature. ■ in 0.005 M NaOH. ▲ in 0.005 M HCl.

Application of the Proposed Method to the Determination of Methicillin Sodium in its Vials for Injection

Vials containing methicillin sodium for injection were reconstituted with distilled water and diluted, as appropriate, to obtain expected concentrations of 5 μg/mL of methicillin sodium. Portions of 20 μL were injected into the chromatograph in replicates and the peak area obtained from each injection was compared to an average peak area of standard methicillin sodium

treated similarly. The mean value (as % of the labeled amount) was calculated using the regression equation.

Application of the Proposed Method to the Determination of Methicillin in Spiked Human Plasma and Urine

Aliquots of 0.5 mL of spiked human plasma or urine containing varying concentrations of methicillin sodium in the range 1.0–6.0 μg/mL were transferred into a series of 10 mL volumetric flasks containing 3.5 mL acetonitrile. The contents of the flasks were vortex-mixed and completed to volume with water. The contents were then transferred into a set of centrifugation tubes and centrifuged for 10 min at 3000 rpm. The 20 μL portions were treated similarly as described under the standard calibration curve, but using the mobile phase in the ratio of 45:55 (CH_3CN/2% v/v acetic anhydride). Samples of plasma or urine spiked with known amounts of methicillin sodium were treated in the same manner and the % recoveries were calculated from the corresponding regression equation.

RESULTS AND DISCUSSION

The proposed method proved to be selective by the virtue of the fluorescence detector used. The chromatograms obtained from a series of standard solutions and the calibration graph of the peak area versus the concentration of methicillin using the proposed method, were linear over the range 1.0–10.0 μg/mL with a detection limit of 0.1 μg/mL (2.38×10^{-7} M). The linear regression analysis of the data gave the following equation:

$$P\ (\text{peak area} \times 10^6) = 0.205\ C + 200 \qquad (r = 0.9996)$$

where C is the concentration of methicillin in μg/mL.

Development and optimization of the method were achieved through several trials involving different columns including μPorasil column, amino column, Micropak CN column, symmetry C_{18} column, and μBondapak C_{18} column, all with different lengths and internal diameters. The polar columns, i.e., the μPorasil and the amino-column, resulted in retention times less than 3 minutes but with poor resolution between the parent peak and the peaks of the decomposition products. The Micropak CN column resulted in a retention time of about 3 minutes for the parent peak, but the decomposition products were not resolved and one of them appeared as a shoulder with the parent peak. The symmetry C_{18} (3.9 × 150) column improved the results but the best results were obtained by using a μBondapak C_{18} (3.9 × 300) column. Also a variety of mobile phase compositions were tried. Acetonitrile, methanol, water, acetic acid, phosphate buffer, and acetic anhydride (as 1% and 2% v/v) were used. The combinations were

investigated to separate methicillin from its degradation products. Increasing the polarity of the mobile phase increased the retention of methicillin in the μBondapak column. A mixture of 0.01 M KH_2PO_4 and CH_3CN (9:1) was tried and the resolution between the peaks was poor. Introduction of methanol into the mobile phase system did not improve the results, even after increasing the ratio of CH_3CN to be CH_3CN:CH_3OH:KH_2PO_4 (50:40:10). Methanol was omitted and 1% aqueous acetic anhydride was mixed with CH_3CN (50:50). The results were better but the resolution was still not satisfactory. Aqueous acetic anhydride (2%) mixed with CH_3CN in the ratio of 45:55, respectively, resulted in reasonable retention times for the parent methicillin and the decomposition products, with best resolution between the peaks.

To obtain possible degradation products, methicillin was kept in 0.005 M HCl or 0.005 M NaOH at room temperatures for different time intervals. The chromatogram obtained for the acid degraded methicillin solution, showed two major degradation products besides the parent compound (Fig. 1), whereas the alkaline degradation showed one major degradation product (Fig. 2). The best column, mobile phase, flow rate, etc, to resolve the parent peak from both acid and alkaline decomposition products were found to be as described under standard calibration curve. System suitability criteria[2] and performance data of the proposed method are presented in Table 1.

Table 1. System suitability criteria[2] and performance data for the proposed HPLC method

Parameter	DP_1[a]	M[b]	DP_2[a]	DP_3[a]
Retention time (min)	2.0	3.1 (3.9)[c]	3.9	5.7
Capacity factor K^-	1.1	2.3	3.1	5.0
Resolution	3.13	1.41		
Tailing factor	—	1.43	—	—
Number of theoretical plates	—	3916	—	—
Height equivalent to theoretical plate (HETP)	—	2.66×10^{-4} (m)	—	—
Slope consistency (as RSD%)	—	1.75	—	—
Correlation coefficient (r)	—	0.9995	—	—
Linearity range	—	1.0–10.0 (μg/mL)	—	—
Limit of detection	—	0.1 μg/mL (2.38×10^{-7} M)		

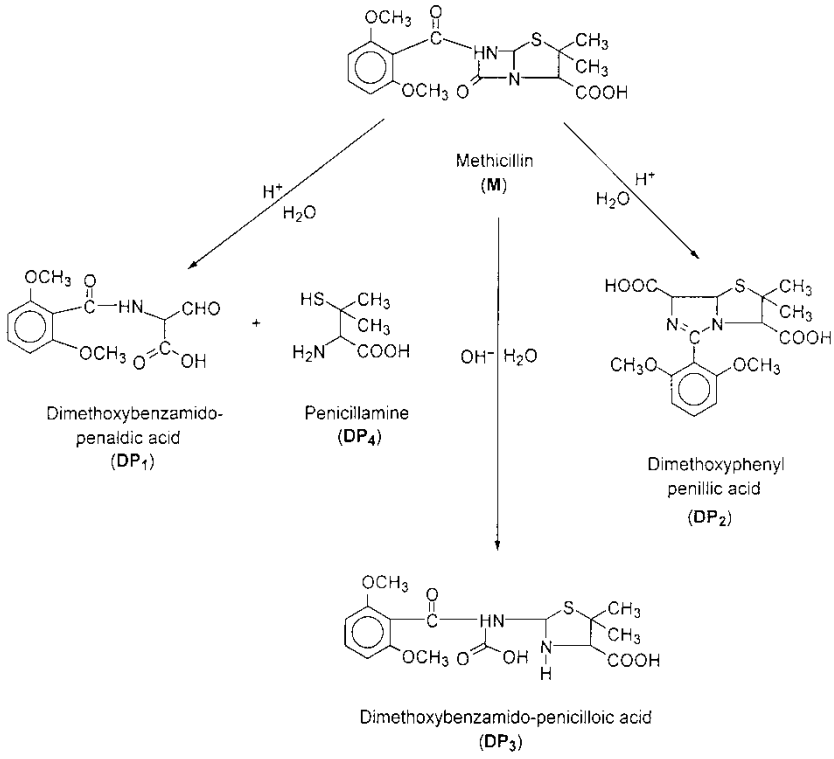
[a]DP_1, DP_2 are the major acid degradation products of methicillin; DP_3 is the major alkaline degradation product of methicillin.

[b]M is the intact methicillin sodium.

[c]Retention time for methicillin in case of spiked plasma and urine analysis using the modified mobile phase ratio.

Both the acid and alkaline degradation of methicillin followed pseudo-first order kinetics (Fig. 3). The apparent first-order degradation rate constant K and the half-life $t_{1/2}$, in each case were calculated from the slope of the straight line obtained and were found to be $0.0297\,min^{-1}$ and 23.33 min, respectively, in acid medium. In alkaline medium the values were found to be $0.0177\,min^{-1}$ and 39.15 min, respectively.

The main cause of deterioration of penicillins (including methicillin) is hydrolysis. The acid and alkaline hydrolysis are suggested to proceed as in Scheme 2.[15]



Compounds DP_1 and DP_2 are the major acid degradation products whereas compound DP_3 is the major alkaline degradation product. Penicillamine (DP_4) is non-fluorescent and, therefore, could not be detected in the chromatogram. This is in accordance with the chromatograms found in Figures 1 and 2. Compound DP_1, looking more polar, could be the one having retention time of 2 min, compound DP_2 with retention time of 3.9 min and compound DP_3 with retention time of 5.7 min. All the degradation products are well resolved from the parent methicillin peak (M), which has a retention time of 3.1 min.

APPLICATIONS

The validity of the proposed method was assessed by the determination of methicillin in pure form. The within-day and between-day precisions were studied by analyzing varying concentrations of methicillin during the same day and for four consecutive days. The results are abridged in Table 2. In all cases, the mean values and the standard deviations were satisfactory.

The high sensitivity and selectivity of the method allowed the determination of methicillin in its vials. The mean % content and the standard deviation are presented in Table 3.

The proposed method was successfully applied for the in-vitro determination of methicillin in spiked plasma and urine. Methicillin is administered intramuscularly in a dose of 1 g and 75–80% of the dose is detected in urine unchanged.[1] Therefore, the anticipated level of concentration will be around 8 μg/mL, which is within the working range of the proposed method. Percentage recoveries from added amounts in plasma and urine are abridged in Table 4. Precipitation of soluble proteins in the biological fluids was best achieved by using trichloroacetic acid. The disadvantage of this reagent was that being a strong acid, it resulted in the rapid decomposition of methicillin; and therefore, acetonitrile was used instead. When the spiked plasma and urine were analyzed using the same mobile phase described under the standard calibration curve, peaks arising from the plasma and also the urine constituents could not be resolved from those of methicillin and/or its decomposition products. However, simple reversing of the ratio of acetonitrile and the 2% v/v of acetic anhydride, i.e., 45:55 instead of 55:45, showed good separation of the methicillin peak, with a retention

Table 2. Within-day and between-day recovery (%) of methicillin using the proposed method

Amount expected (μg/mL)	Within-day recovery (%)				Between-day mean recovery (%) + S.D.	
	Day 1	Day 2	Day 3	Day 4	$\overline{X}$	S.D.
3.0	103.67	99.00	102.9	100.53	101.53	1.86
4.0	101.50	98.50	98.38	103.02	100.40	2.04
5.0	98.20	98.20	102.56	103.5	100.62	2.43
6.0	99.33	100.17	96.48	99.23	98.80	1.39
7.0	98.71	97.14	102.66	99.51	99.51	2.01
8.0	100.00	100.50	99.21	99.31	99.76	0.53
9.0	99.11	98.78	99.98	99.55	99.36	0.45
$\overline{X}$	100.07	98.90	100.31	100.66	99.99	
S.D.	1.76	1.07	2.29	1.69	0.85	

Table 3. Assay of methicillin sodium for injection (final solutions were expected to contain 5 μg/mL)

Amount found	Recovery (%)
4.88	97.60
4.92	98.40
4.93	98.60
5.01	100.20
4.99	99.80
4.93	98.60
4.99	99.80
4.88	97.60
$\overline{X}$	98.83
S.D.	0.94

time of 3.9 min compared to less than 3 min for those of the plasma and urine constituents. Typical chromatograms obtained from standard series in plasma and urine are shown in Figures 4 and 5, respectively. This later ratio of 45:55 of the mobile phase was tried for the separation of methicillin from its decomposition products but there was an overlapping between the methicillin

Table 4. % Recovery of methicillin sodium from human plasma and urine using the proposed HPLC method

Sample	Amount added (μg/mL)	Amount found (μg/mL)	Recovery (%)	Mean ± S.D.
Plasma	2.00	2.04	102.00	$\overline{X} = 99.04$
	2.00	1.99	99.50	S.D. = 1.88
	2.00	1.93	96.50	
	3.00	2.97	99.00	
	3.00	2.89	96.33	
	4.00	4.04	101.00	
	5.00	5.04	100.80	
	5.00	4.88	97.60	
	5.00	4.93	98.60	
Urine	2.00	2.01	100.50	$\overline{X} = 99.36$
	2.00	1.96	98.00	S.D. = 1.77
	3.00	2.97	99.00	
	3.00	3.09	103.00	
	4.00	4.0	100.00	
	4.00	3.91	97.75	
	5.00	4.85	97.00	
	5.00	4.98	99.60	

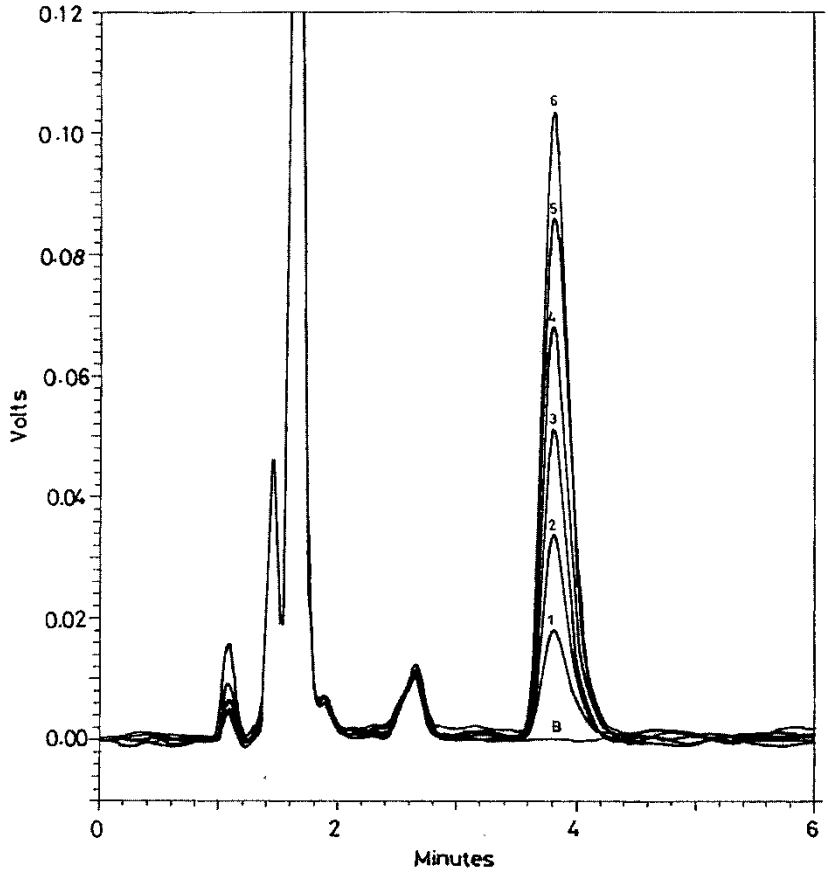


Figure 4. Typical chromatograms of plasma spiked with varying amounts of methicillin. B: blank plasma; 1, 2, 3, 4, 5, and 6 are standard series containing 1.0, 2.0, 3.0, 4.0, 5.0, and 6.0 μg/mL, respectively.

peak and the peak of one of the acid decomposition products (DP_2). For this reason, as mentioned above, the two ratios were used as appropriate.

The linearity in both the spiked plasma and urine was excellent over the range 1.0–6.0 μg/mL in each case. The regression equation obtained from a standard series of spiked plasma was:

$$P = 0.2793\,C - 6.5 \times 10^3 \qquad (r = 0.9998)$$

where P is the peak area ($\times 10^6$) and C is the concentration of methicillin in μg/mL in the spiked plasma.

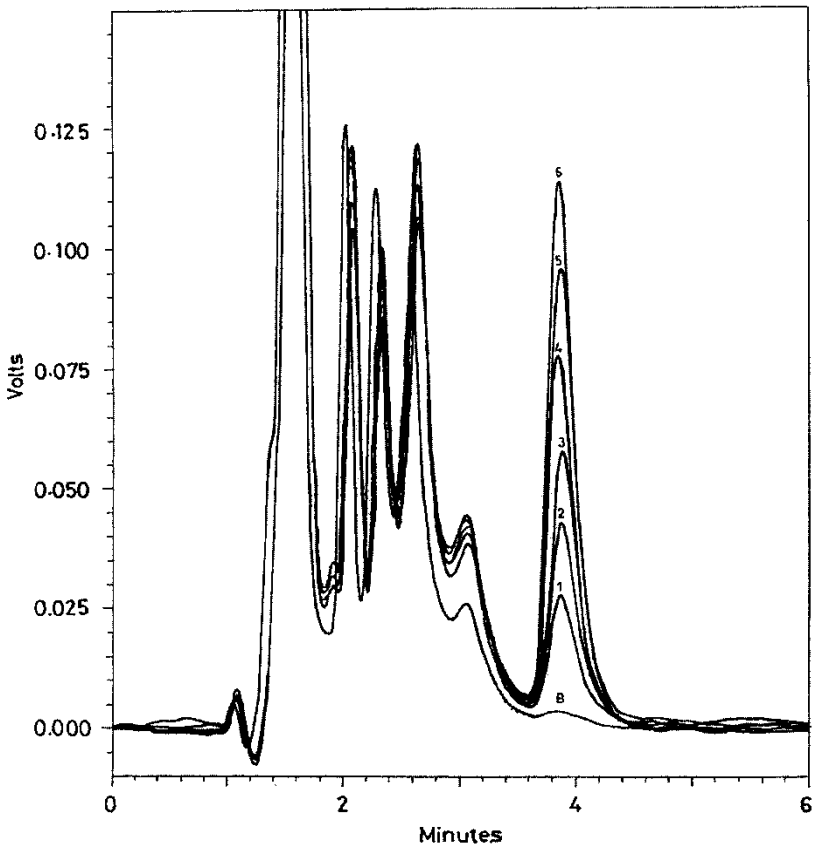


Figure 5. Typical chromatograms of urine spiked with varying amounts of methicillin. B: blank urine; 1, 2, 3, 4, 5, and 6 are standard series containing 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 μg/mL, respectively.

Similarly, the regression equation obtained from a standard series of spiked urine was:

$$P = 0.2876\,C + 4.78 \times 10^4 \qquad (r = 0.9987).$$

CONCLUSION

A simple, specific, and precise HPLC method has been developed, using a single isocratic system, for the determination of methicillin in bulk material, in vials and in spiked plasma and urine. Sample preparation in all cases is simple and does not require any special treatment. No interference was

encountered from the possible degradation products and, therefore, it could be used as the stability-indicating method.

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