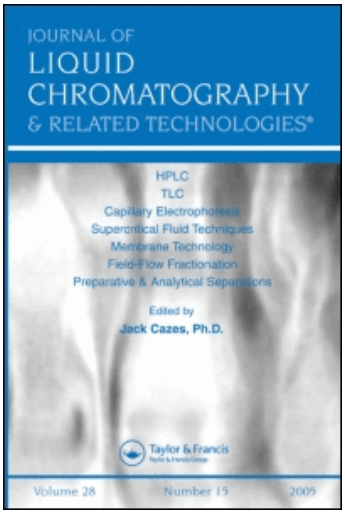

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Online publication date: 28 February 2001

To cite this Article Forlay-Frick, P. , Nagy, Z. B. , Fekete, J. , Kettrup, A. and Gebefugi, I.(2001) 'REVERSE PHASE HPLC METHOD FOR DETERMINATION OF VANCOMYCIN IN INFLUENZA VACCINE', Journal of Liquid Chromatography & Related Technologies, 24: 4, 497 — 507
To link to this Article: DOI: 10.1081/JLC-100103388
URL: http://dx.doi.org/10.1081/JLC-100103388

J. LIQ. CHROM. & REL. TECHNOL., 24(4), 497–507 (2001)

REVERSE PHASE HPLC METHOD FOR DETERMINATION OF VANCOMYCIN IN INFLUENZA VACCINE

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ABSTRACT

A simple and sensitive high-performance liquid chromatographic method for the determination of vancomycin in influenza-vaccine has been developed. The stationary phase was a Purospher RP-18e column (125 × 3.5 mm; 5 μm; Merck); the mobile phase consisted of acetonitrile-water-phosphoric acid (85%) (8.5 / 91.5 / 0.125 v/v/v %) and its pH was adjusted to pH 2.80 using distillated triethylamine immediately prior to use.

Separation was achieved using a flow rate of 0.5 mL/min at ambient temperature. The vancomycin was detected at 230 nm. The retention time for vancomycin was 7.20 ± 0.20 min. The limit of detection was 30 ng/mL; the limit of quantification was found to be 100 ng/mL.

INTRODUCTION

Vancomycin (Fig. 1), a glicopeptide antibiotic effective against all Gram-positive bacteria (i.e. *Staphylococcus epidermis* and *S. aureus*) is used in intraocular surgery to avoid bacterial endophtalmitis.[1] It is used if penicillins or other antibiotics cannot be used because of resistance or patient intolerance.[2] Due to its increased clinical significance, a number of high performance liquid chromatographic methods for the determination of vancomycin in biological fluids, i.e., in rabbit serum, vitreous, and aqueous humour[1] and in human plasma2,3 has been developed; on the other hand, drug residues in pharmaceutical products has not been investigated.

This paper describes a simple and sensitive procedure to evaluate residual amounts of drug in influenza-vaccine, as well as in other pharmaceutical products.

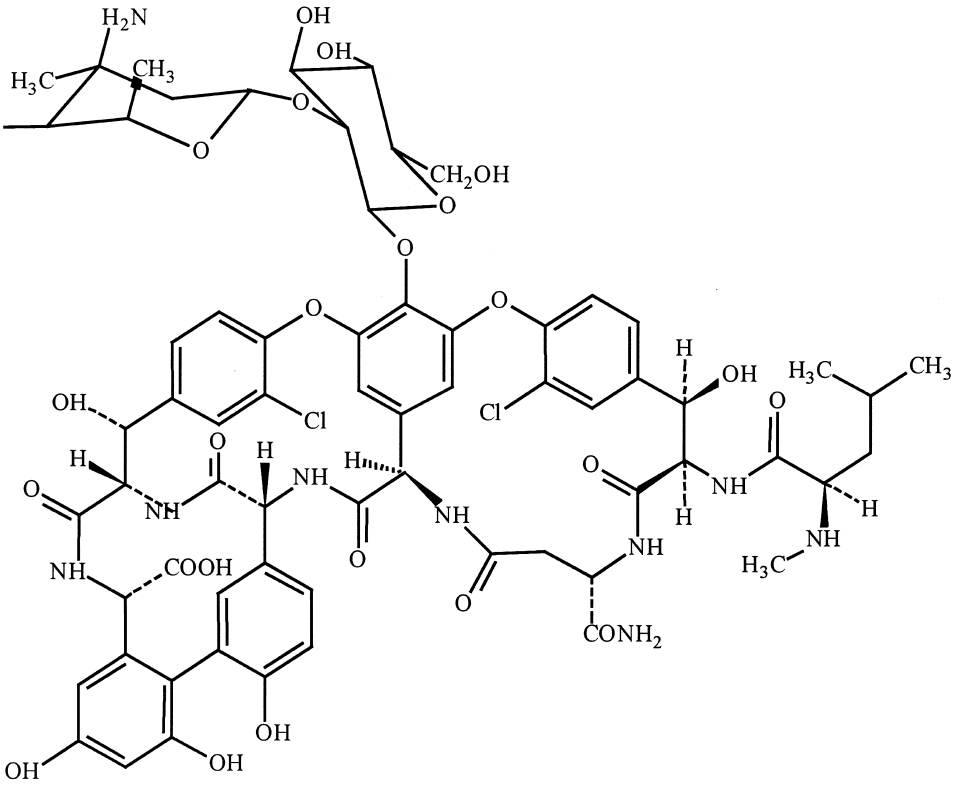

Figure 1. Structure of vancomycin.

EXPERIMENTAL

Chemicals

Acetonitrile, gradient grade; orthophosphoric-acid (85%), analytical grade were obtained from Merck (Darmstadt, Germany) and water was obtained from a Millipore Milli-Q system (Waters, Milford, USA). Triethylamine (Riedel-de Häen) was freshly distillated prior to use.

Vancocin® injection (S) (containing 50 mg/mL of vancomycin hydrochloride) was obtained from Eli Lilly and Company (Indianapolis, USA).

The 1000 mL of matrix-solution (M) consisted of the following: 10.40 g Na_3PO_4, 3.07 g NaCl, 0.07 g KCl, 0.57 g KH_2PO_4, 0.35 g Na_2HPO_4, 0.09 g merthiolate, and 5.40 g $AlCl_3$ diluted to 1000 mL with distilled water (Millipore).

Apparatus

The separation was performed using isocratic elution at a flow rate of 0.5 mL/min by a Series 200 LC pump (Perkin Elmer) attached to an ISS 200 autosampler (Perkin Elmer).

The stationary phase was a Purospher RP-18e column (125 × 3.5 mm, 5 μm, Merck).

The ultraviolet detection was carried out at 230 nm using a 235C diode array detector (Perkin Elmer). The software was Turbochrom Navigator from Perkin Elmer.

The mobile phase was ultrasonicated in a Realsonic 57 ultrasonic bath and its pH was adjusted using a Jenway 3020 pH-meter.

Sample Preparation

Sample preparation consisted of mixing equal volumes of influenza-vaccine solution ("Fluval") with the mobile phase, followed by filtering through a 0.45 μm filter (Millipore). The filtrate then was injected onto the chromatographic system. The stationary phase was a Purospher RP-18e column (125 × 3.5 mm, 5 μm, Merck); the mobile phase consisted of acetonitrile-water-phosporic acid(85%) (8.5/91.5/0.125 v/v/v %).

Since the retention time for vancomycin was found to be considerably affected by acetonitrile content of mobile-phase, the components were measured by mass. The pH was adjusted to pH 2.8 using distillated triethylamine immediately prior to use. The acetonitrile-content of the mobile phase is relatively low (8.5 v/v%), hence, the vaporisation of mobile phase causes significant error.

Therefore, the shift of retention time must be monitored, time to time, using one of the vancomycin standards.

Separation was achieved using a flow-rate of 0.5 mL/min at room temperature. Ultraviolet detection was carried out at 230 nm.

Validation

Linearity

Stock-solutions (T1, T2) were prepared from the Vancocin® solution (S) (containing 50 mg/mL vancomycin hydrochloride) by dilution with the eluent as follows:

T1 solution: 10 µL of S solution was diluted to 100 mL with the eluent (5 µg/mL vancomycin).

T2 solution: 100 µL of S solution was diluted to 100 mL with the eluent (50 µg/mL vancomycin).

Standard-solutions (0.1–10 µg/mL) were prepared from stock solutions (T1, T2), the eluent and the matrix-solution (M) in accordance with Table 1.

Every standard-solution was filtered through a 0.45 µm Millipore-membrane and then was injected onto the chromatographic system.

Specificity

1000 µL of matrix-solution (M) and 1000 µL of the eluent were mixed, filtered through a 0.45 µm Millipore-membrane, and then injected onto the chromatographic system (background).

Table 1. Calibration Standards for Vancomycin

T1 Solution (µL)	T2 Solution (µL)	Mobile Phase (µL)	M Solution (µL)	Concentration (ng/mL)
40	—	960	1,000	100
120	—	880	1,000	300
200	—	800	1,000	500
—	40	960	1,000	1,000
—	80	920	1,000	2,000
—	200	800	1,000	5,000
—	280	720	1,000	7,000
—	400	600	1,000	10,000

1000 μL of M solution, 280 μL T1 solution, and 720 μL eluent were mixed, filtered through a 0.45 μm Millipore-membrane, and then injected onto the chromatographic system (7 μg/mL vancomycin sample). A clear analytical window was expected on the background chromatogram at the retention time of ciprofloxacin.

The dead-time was determined using a mixture of 1000 μL distillated water and 1000 μL eluent. The first significant peak was accepted as dead-time. The net retention time was calculated as the difference between retention time and dead-time. For the characterisation of robustness the net retention time was used.

Repeatability

1000 μL of M solution, 40 μL of T2 solution, and 960 μL of the eluent, were mixed, filtered through a 0.45 μm Millipore-membrane, and then injected onto the chromatographic system five times, consecutively (1 μg/mL vancomycin sample). Mean retention time and peak area, as well as the precision for retention times and peak areas were calculated. The method is repeatable if the relative standard deviation for retention times is lower than 1%; for peak areas is lower than 10%.

Accuracy

1000 μL of M solution, 40 μL of T2 solution, and 960 μL of the eluent, were mixed, filtered through a 0.45 μm Millipore-membrane, and then injected onto the chromatographic system five times, consecutively (1 μg/mL vancomycin-matrix sample [No. 1]).

20 μL of T2 solution and 980 μL of the eluent were mixed and then injected onto the chromatographic system five times, consecutively (1 μg/mL vancomycin sample, [No. 2]). Recovery was calculated as the quotient of peak areas No. 1/No. 2. The method is reproducible if recovery(R%) is: 100 ± 5%.

Limit of Detection and Quantification (LOD/LOQ)

1000 μL of M solution and 40, 30, 20, 12, 10, or 6 μL of T1 solution were mixed, diluted to 2000 μL with the eluent (100, 75, 50, 30, 25, 15 ng/mL), filtered through a 0.45 μm Millipore-membrane, and then injected onto the chromatographic system.

LOD was determinated as the concentration where the peak height had a signal-to-noise ratio of 3; LOQ was determinated as the concentration where the signal-to-noise ratio was 10.

Robustness

The effect of acetonitrile-content and pH of the mobile-phase for the retention time was investigated. The acetonitrile-content was set 7.5 to 9.5 v/v% and net retention time for vancomycin was determinated. The pH of the mobile phase was adjusted to pH=2.0; 2.6; 2.7; 2.8; 2.9; 3,0; 4.0; 5.0; 6.0; consecutively, using distillated triethylamine, then retention times were measured.

RESULTS

Under the chromatographic conditions described above, the retention time for vancomycin was 7.20 ± 0.20min (Fig. 2). There was no interfering peak found on the background-chromatogram (Fig. 3) at the retention time of vancomycin; the determination is specific. The calibration curve (Fig. 4) showed good linearity in the concentration range of 0.1–10 μg/mL ($A = 94.906 \cdot c - 10711$; $R^2 = 0.9963$).

For the 1 μg/mL vancomycin-sample, the mean peak area calculated from five parallel measurements was 92951 with a relative standard deviation of 6.2%; the retention time calculated from five measurements was 7.18 with a relative standard deviation of 0.6%, hence, the method is repeatable. Since the recovery for the 1 μg/mL vancomycin-samples calculated from five measurements was found to be 95.3 ± 5%, the method is accurate.

The limit of detection (LOD) at a signal-to-noise ratio of 3 was 30 ng/mL; the limit of quantification (LOQ) at a signal-to-noise ratio of 10 was found to be 100 ng/mL. The retention time for vancomycin was found to be considerably affected by acetonitrile content of mobile phase (Fig. 5) ($t_N = -4.374c + 43.011$; $R^2 = 0.9939$), therefore, components must be measured by mass and shift of retention time must be monitored, time to time, using one of the vancomycin standards.

The effect of pH for the retention time is also significant The t_N vs. pH curve (Fig. 6) is minimum at pH=4. In lower pH, the ionization of acidic groups is suppressed in higher pH, the protonisation of basic groups is prevented. These non-ionic forms are responsible for increased retention. In the range of pH=2.6–3.0 the pH dependence was found to be linear (Fig. 7) ($t_N = -4.5pH + 18.362$; $R^2 = 0.990$). Because of the retention time's significant pH dependence,

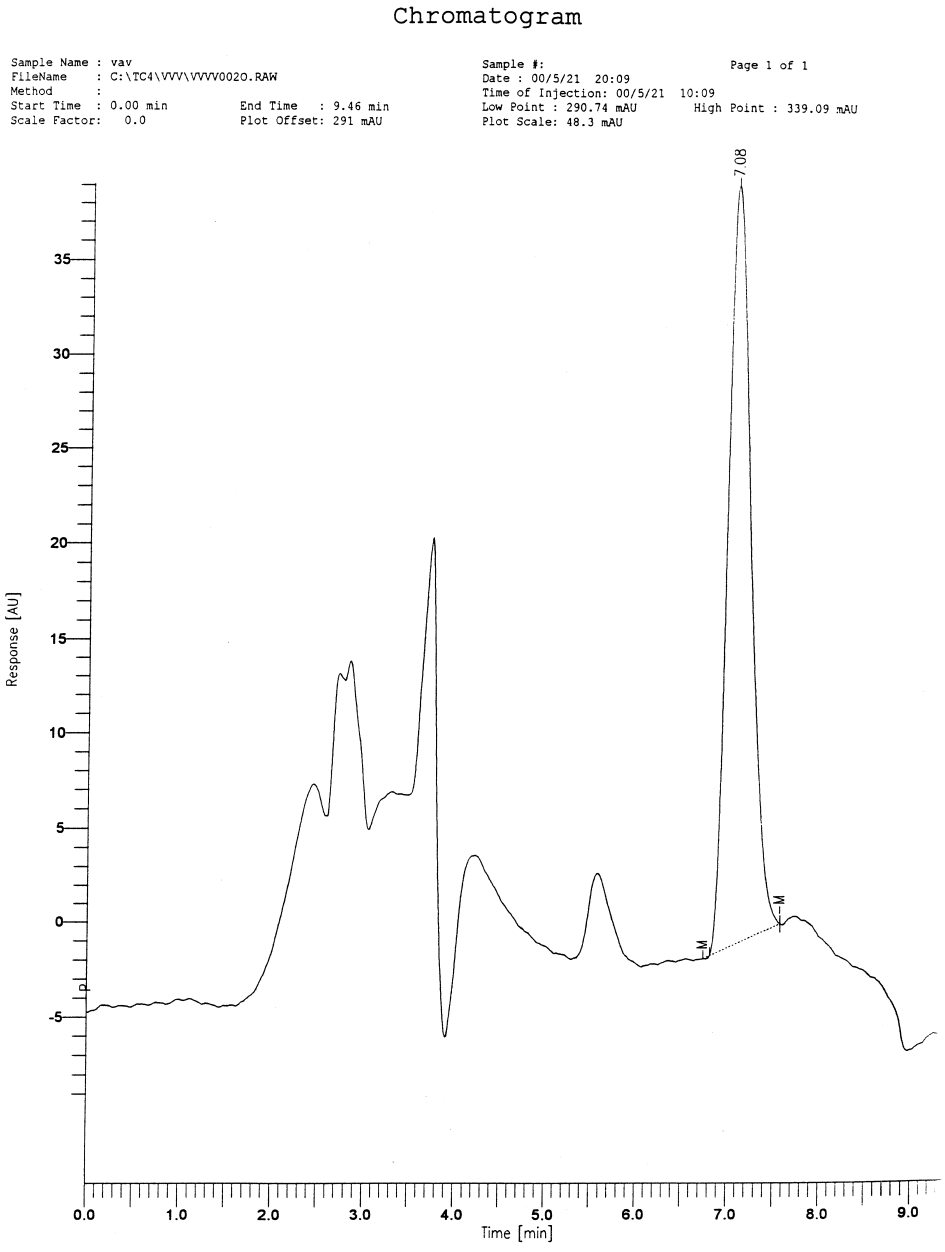

Figure 2. Chromatogram for the 7 μg/mL vancomycin sample.

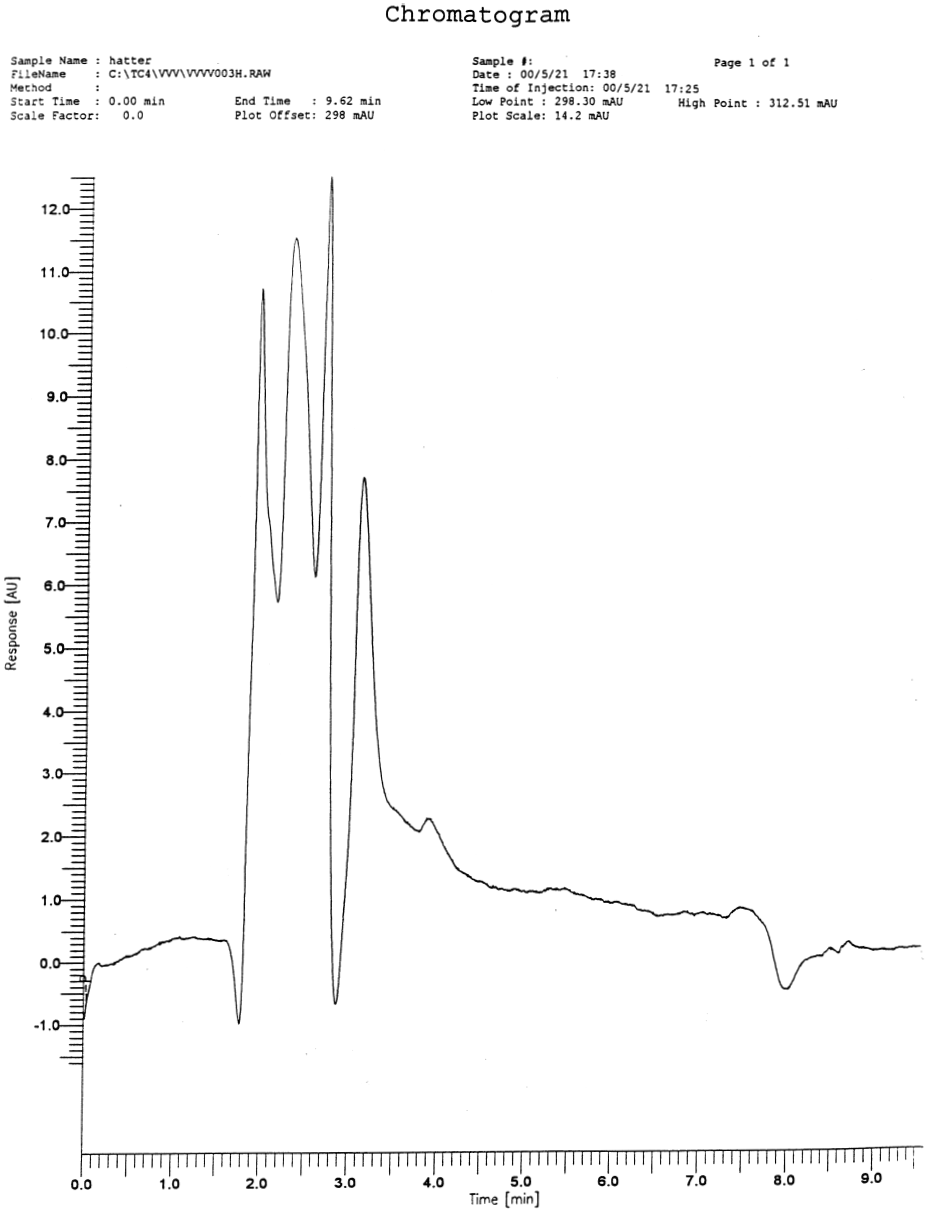

Figure 3. Background chromatogram for vancomycin.

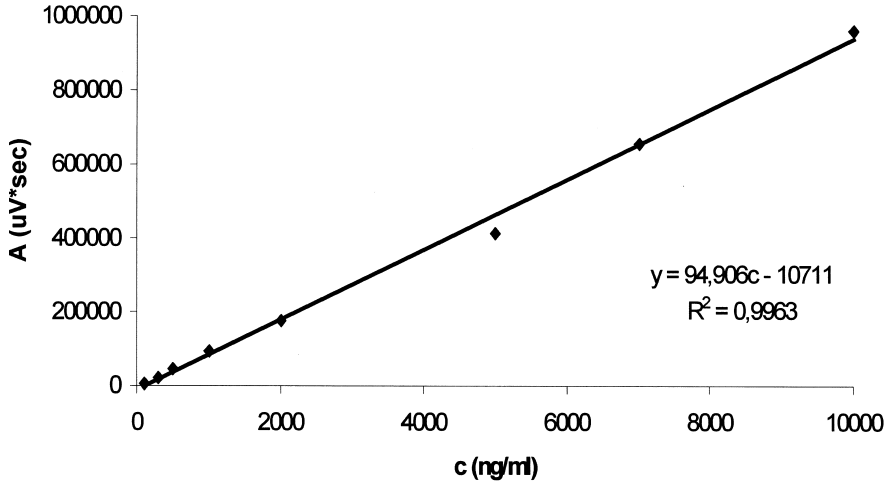

Figure 4. Calibration curve for vancomycin.

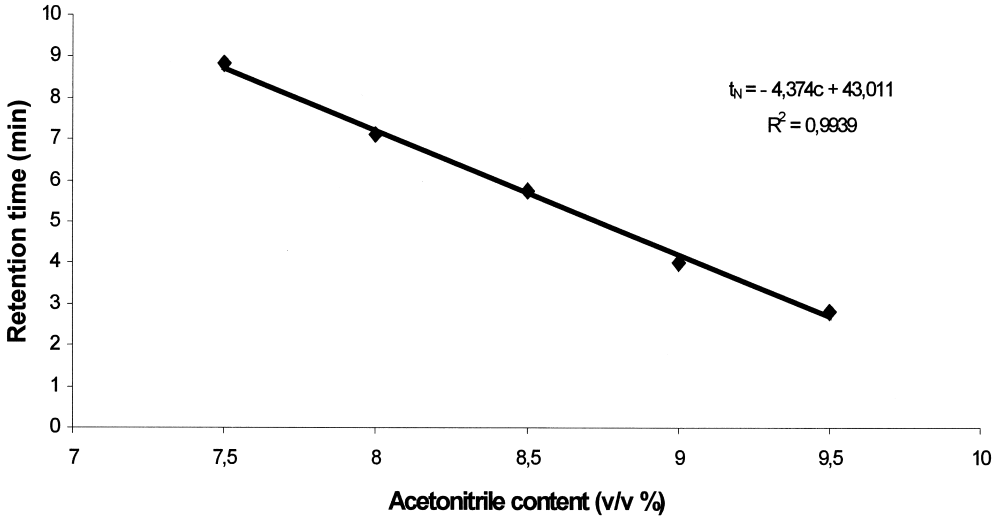

Figure 5. The effect of acetonitrile-content of mobile phase for the retention time.

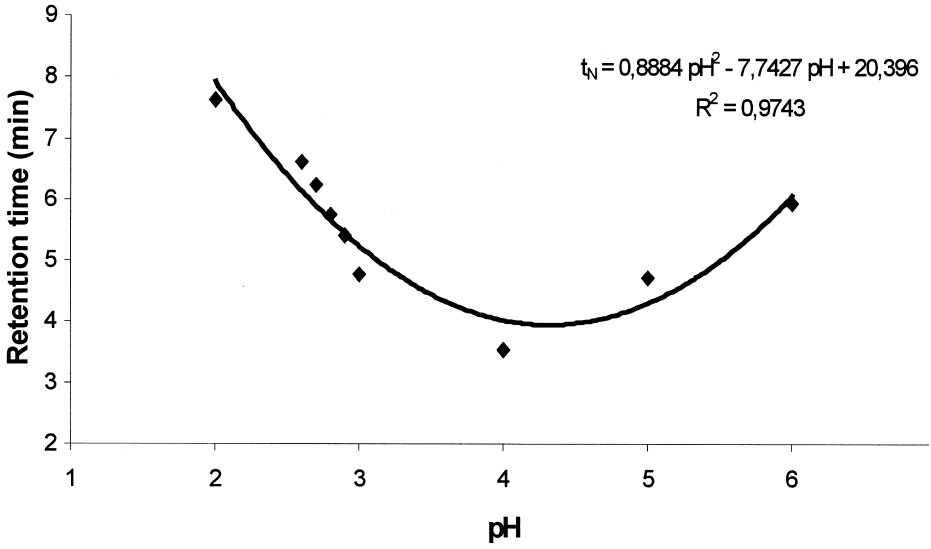

Figure 6. The effect of pH of mobile phase for the retention time.

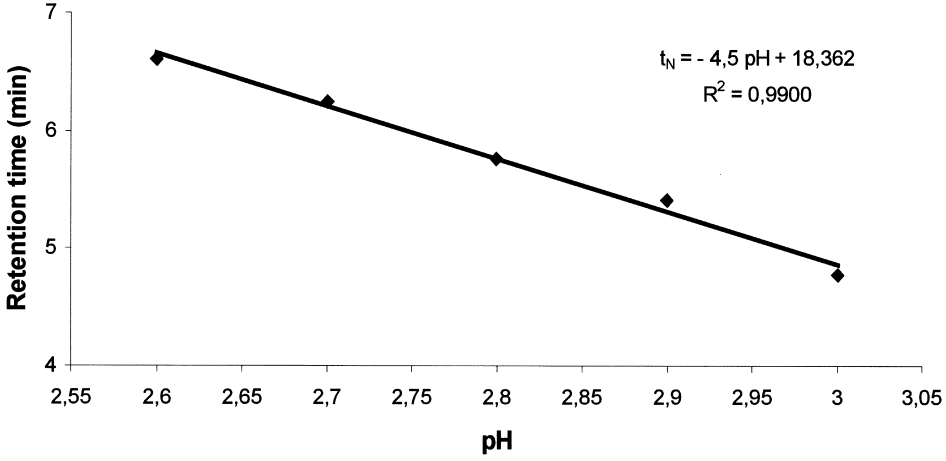

Figure 7. The effect of pH of mobile phase for the retention time in the range of pH=2.6–3.0.

the pH of the eluent must be adjusted exactly to pH=2.8 using distillated triethylamine immediately prior to use.

DISCUSSION

A specific, repeatable and accurate method for the detection of vancomycin-residues has been developed. This highly sensitive procedure is suitable for the rapid and simple determination of low concentrations of vancomycin in influenza-vaccine or in other pharmaceutical products. Accurate mixing of mobile phase-components by mass, monitoring of vancomycin's retention time's shift; and careful adjusment of pH, is essential in order to guarantee robustness.

ACKNOWLEDGMENT

The authors express their appreciation to the Omninvest Oltóanyagtermel és Kutatásfejleszt Kereskedelmi Ltd., for supporting our work.

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Received July 24, 2000 Manuscript 5363
Accepted August 20, 2000