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Journal of Chromatography B, 667 (1995) 277–281

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Rapid high-performance liquid chromatographic determination of vancomycin in human plasma☆

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First received 13 October 1994; revised manuscript received 3 January 1995; accepted 15 January 1995

Abstract

A rapid simple and robust reversed-phase HPLC method was developed for rapid screening in bioavailability studies or comparative bioequivalence studies. The method is specific for vancomycin as no interference from acetylsalicylic acid, paracetamol and caffeine was observed. The mean intra-day precision was from 11.7% (low concentration) to 0.3% (high concentration) and the within-day precision from 15.0 to 0.3%, determined on spiked samples. The accuracy of the method was 106.4–99.8% (intra-day) and 103.5–100.2% (inter-day).

1. Introduction

Vancomycin is a glycopeptide antibiotic that is primarily active as an inhibitor of cell-wall synthesis of susceptible organisms. It is used in the treatment of serious staphylococcal or other Gram-positive infections, where other agents such as penicillins cannot be used because of resistance or patient intolerance [1]. Methods reported for determination of vancomycin in biological fluids include microbiological assay [2], immunoreaction assays (fluorescence polarization immunoassay (FPIA) [3–5], radioimmunoassay (RIA) [4], radial partition immunoassay [6], enzyme immunoassay [7,8]) and high-performance liquid chromatography (HPLC) [9–16]. Biological determinations are time consuming and immunoassays require specific equipment. Hence HPLC is usually the method of choice. There are some comparisons of the above-mentioned methods for the determination of vancomycin in plasma in the literature which show good correlations, particularly between FPIA and HPLC [3,4]. As considerable inter-individual variations might occur in the pharmacokinetics of vancomycin [1], a large number of tests should be made in order to obtain reliable pharmacokinetic data. Therefore, there was a strong need for a rapid and simple method for screening vancomycin plasma levels in the development stage of pharmaceutical products and also in bioequivalence studies.

2. Experimental

2.1. Chemicals

Vancomycin working standard was produced

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☆ Presented at the International Symposium on Chromatographic and Electrophoretic Techniques, Bled, Slovenia, October 10–13, 1994.

0378-4347/95/$09.50
SSDI 0378-4347(95)00033-X

at Lek (Ljubljana, Slovenia). Dichloromethane and KH_2PO_4 of analytical-reagent grade from Kemika (Zagreb, Croatia), 60% perchloric acid of extra-pure grade and acetonitrile of LiChrosolv grade from Merck (Darmstadt, Germany) were used.

2.2. Apparatus

The equipment consisted of an HPLC system including an autosampler (Bio-Rad, Vienna, Austria), isocratic pump, thermostat, variable-wavelength UV absorbance detector and recorder (all from Thermo Separation Products, Darmstadt, Germany). The analytical column (150 × 4.6 mm I.D.) was packed with Nucleosil RP-18 (5 μm) (Macherey–Nagel, Düren, Germany).

2.3. Sample and standard preparation

Plasma samples and standard plasma samples were prepared by liquid–liquid extraction after acid precipitation of proteins. A 50-μl volume of perchloric acid was added to 1.0 ml of plasma sample and the proteins were precipitated and removed. The supernatant was extracted with 1.0 ml of dichloromethane. The aqueous phase was injected on to the analytical column.

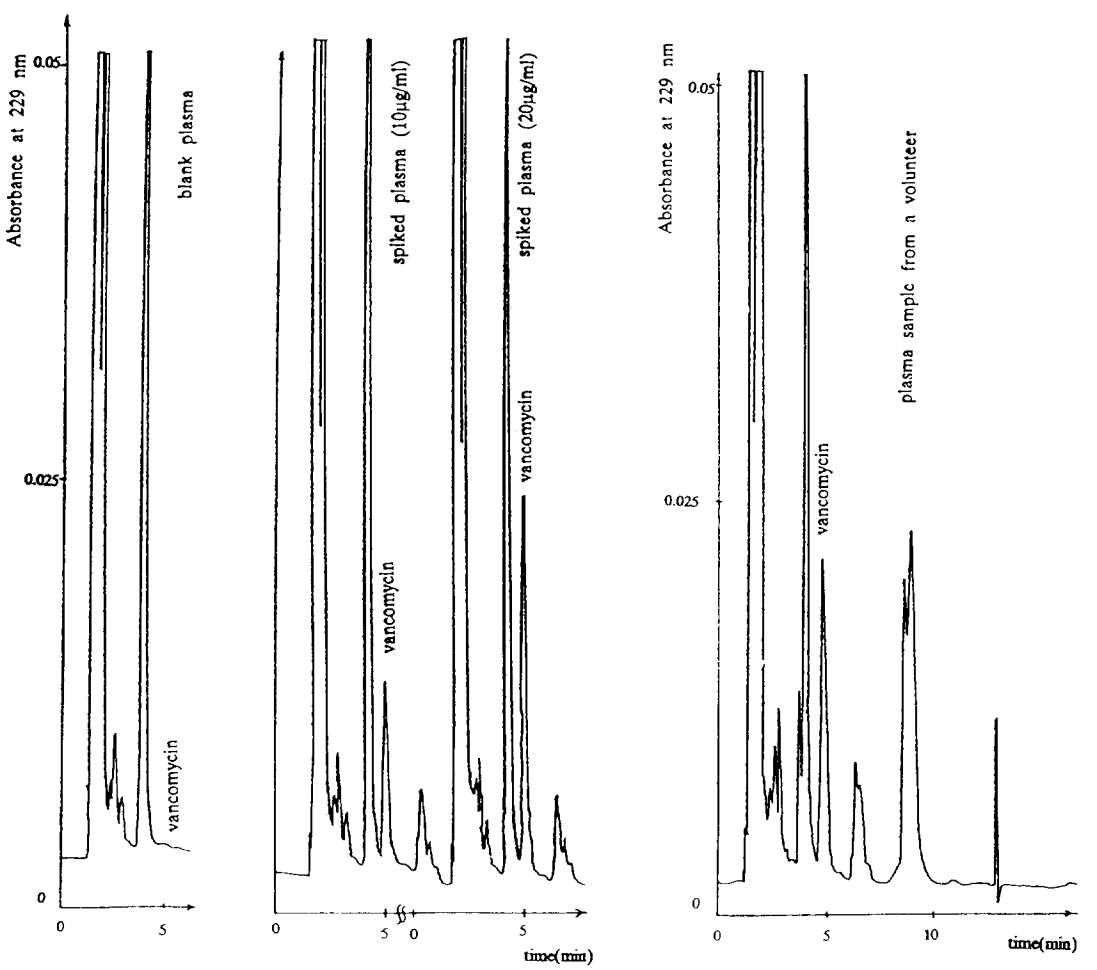

Fig. 1. Chromatograms of blank plasma, spiked plasma samples (10 and 20 μg/ml) and plasma sample from a healthy volunteer. Sample preparation and chromatographic conditions as described under Experimental.

2.4. High-performance liquid chromatography

The mobile phase was prepared by premixing 0.005 *M* KH_2PO_4 (pH 2.8) and acetonitrile in a 90:10 (v/v) ratio, degassed and pumped through the column at a flow-rate 1.0 ml/min. A 50-μl aliquot of sample extract was applied by means of an autosampler. The temperature of the column was maintained at 30°C. Separated components were detected at 229 nm and peak heights were measured. The concentration of vancomycin was calculated from the calibration graph (height of vancomycin peak *versus* concentration).

2.5. Validation

Validation procedures, parameters and acceptance criteria were based on USP [17] guidelines and recommendations in the literature [18]. The method was validated regarding accuracy and precision (intra- and inter-day), concentration range, linearity, limit of quantification (LOQ) and limit of detection (LOD). LOD was determined as the concentration where the peak height had a signal-to-noise ratio of 3:1 LOQ was determined as the concentration where the precision of measuring the peak-height response was lower than 20%. The stability of vancomycin is spiked plasma samples was studied after three consecutive freeze–thaw cycles, after storage for 24 h at room temperature and after storage for 4 days in a refrigerator at 4°C.

3. Results and discussion

The sample preparation method is rapid and robust, as only two simple steps of acid precipitation and dichloromethane extraction are needed. The chromatographic peaks are sharp (Fig. 1), with no tendency towards tailing, as demonstrated in Table 1 for system suitability tests. Peak-height measurements were therefore used for quantification. The calibration graph was linear in the range 1–100 μg/ml of plasma. The mean linear regression equation ($n = 18$) was $y = 1.0205\ (\pm 1.2915) + 4.2588\ (\pm 0.4846)X$

Table 1
System suitability tests during validation period

N	R.S.D. (%) (*n*)	*T*
1975	1.2 (5)	1.2
1783	0.6 (7)	1.1
2330	0.5 (7)	1.3
2162	0.4 (6)	1.1
1975	0.9 (6)	1.1
2095	1.0 (6)	1.2
2262	0.3 (7)	1.2
2756	0.5 (6)	1.2
2007	1.0 (6)	1.0
2395	0.5 (6)	1.0
2478	1.7 (6)	1.0
2075	0.3 (6)	1.1

Parameters: *N* = number of theoretical plates of the column; R.S.D. = relative standard deviation of vancomycin peak height; *T* = tailing factor of vancomycin peak (all calculated according to USP [17]).

and the correlation coefficient (r) was 0.99994 ± 0.00006. The LOD was 0.2 μg/ml of plasma at a signal-to-noise ratio of 3:1. This LOD was comparable to those reported for HPLC methods (from 3 μg/ml [11] to 0.01 μg/ml of plasma [14]). The LOQ of 1 μg/ml and the LOD of 0.2 μg/ml of plasma are adequate for pharmacokinetic purposes. Acceptable variations in intra-day precision and accuracy testing were observed as well as in inter-day determinations (Table 2).

When some unusual values are obtained, repeated analyses should be carried out and in that case the sample may undergo one or more freeze–thaw cycles. The stability of vancomycin at concentrations of 5, 20 and 50 μg/ml (in three replicates) during three such cycles was therefore examined. The results were 98%, 101% and 104% with R.S.D. 2.4% or less, which confirmed the absence of an influence of thawing and freezing.

Although the sample preparation time is very short, it is necessary to know for how long samples can be exposed to room temperature (time of sample handling) and a short period of storage in a refrigerator (4°C). The stability results for samples kept for 0, 1, 2 and 4 h at room temperature (each in three replicates, $n = 12$) varied with R.S.D. 3.8% for a 5 μg/ml

Table 2
Intra- and inter-day precision and accuracy

Spiked concentration (μg/ml)	Intra-day (n = 7)			Inter-day (n = 11)		
	μg/ml	Precision R.S.D. (%)	Accuracy (%)	μg/ml	Precision R.S.D. (%)	Accuracy (%)
0.5				0.70	27.3	140.0
1.0				1.18	15.0	116.4
2.0	2.13	11.7	106.4	2.08	6.6	103.5
5.0	4.93	3.0	98.1	4.95	2.0	97.9
10.0	9.98	1.0	99.3	9.89	1.3	98.4
20.0	19.87	1.1	98.9	19.87	2.1	98.9
50.0	50.65	1.1	100.8	50.40	1.3	100.3
100.0	100.30	0.3	99.8	100.67	0.3	100.2

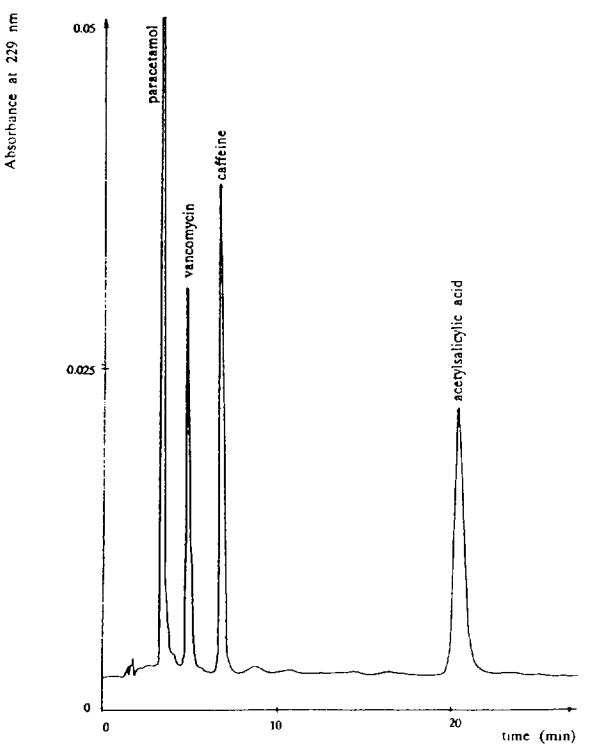

Fig. 2. Specificity of the method: chromatogram of vancomycin, paracetamol, caffeine and acetylsalicylic acid each at an absolute concentration of 10 μg/ml of mobile phase. Chromatographic conditions as described under Experimental.

concentration level and 3.9% for a 20 μg/ml concentration level. There was a decrease in the concentration of vancomycin to 92% (R.S.D. 1.2%, n = 3) after 24 h. Separate samples of 5 and 20 μg/ml were kept in the refrigerator and analyses were carried out after 0, 1 and 4 days in three replicates (n = 9). The variations were 2.5% and 4.8%, respectively. Adequate stability for 4 h at room temperature and 4 days at 4°C was demonstrated, as the recoveries and R.S.D.s of the results are comparable to the intra-day variations in Table 2.

Specificity of the chromatographic separation was examined with respect to some possible exogenous compounds, such as paracetamol, caffeine and acetylsalicylic acid. These substances are well resolved from vancomycin (retention times 4.8 min for vancomycin, about 3.4 min for paracetamol, about 6.6 min for caffeine and about 22 min for acetylsalicylic acid) (Fig. 2). Hence the method is specific.

4. Conclusions

The method reported here meets validation criteria such as precision, accuracy, linearity in the examined concentration range, sensitivity and specificity. The sample preparation is simple and chromatography is reproducible and reliable. The overall procedure is rapid compared with the previously reported methods [2–16]. No

special equipment as in FPIA or RIA [3–5] is needed. There is one HPLC method that is comparable regarding sample throughput [14], but it has a longer retention time (retention time of vancomycin *ca.* 5 min versus 8 min). This is very important when pharmacokinetic studies are performed with a large number of samples. The commonly used reversed-phase (C_{18}) column employed in our chromatographic system is also advantageous, as these columns are usually in stock in any analytical laboratory, in contrast to some normal-phase columns, such as aminopropyl [14]. The method is easily applicable in every analytical laboratory and is suitable both for rapid screening of vancomycin plasma levels in the development stage of pharmaceutical products and in bioequivalence studies.

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