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Development and validation of an HPLC method for vancomycin and its application to a pharmacokinetic study

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ABSTRACT

A rapid and simple method of high performance liquid chromatography with UV detection for the quantification of vancomycin in artificial perfusion fluid and lung tissue samples has been developed and validated. Chromatographic separation was carried out in a Nucleosil 120 C_{18} 5 μm column (length, 15 cm; inner diameter, 0.4 cm) using a mixture of 0.05 M $NH_4H_2PO_4$ (pH 4)–acetonitrile (92:8, v/v) as the mobile phase at a flow rate of 1 mL/min, with UV detection at 220 nm. The method used for the vancomycin quantification showed linearity for concentration ranges of 0.1–2, 2–15 and 15–250 μg/mL, with r^2 = 0.9985, 0.9996 and 0.9985, respectively. The limit of quantification of the method was 0.1 μg/mL and the coefficients of variation of the between- and within-day precision showed values between 0.6% and 7.0%. The retention time of vancomycin was 8.5 min. The method was used successfully to study the pharmacokinetics of vancomycin in isolated rat lung after its administration through the systemic and inhalatory routes.

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1. Introduction

Vancomycin is a glycopeptide drug indicated for the treatment of serious infections caused by Gram-positive bacteria that are insensitive to other less potent antibiotics. Its main applications in the clinical field are for respiratory tract infections due to methicillin-resistant *Staphylococcus aureus* and *S. epidermidis* (SARM and SERM, respectively), pseudomembranous colitis. It is also used in prophylaxis against endocarditis, and in surgical prophylaxis during prosthetics implantation [1–4].

Among the methods developed to date for the quantification of vancomycin in biological fluids immunoenzymatic techniques (FPIA(TDx), EMIT and RIA) [5] and chromatographic methods are the most relevant. The former, although widely used in clinical practice for monitoring serum levels owing to their simplicity and speed, do have some drawbacks: the low precision of the EMIT assay for concentrations higher than 30 mg/L is an example. Furthermore, if the values obtained with EMIT are compared with those afforded by the fluorescence polarisation immunoassay (FPIA), a linear relationship can be seen between them but with values of the ordinate at the origin and slope significantly different from zero and one, respectively [EMIT] = (0.877) [FPIA(TDx)] + 0.435 mg/L (r = 0.971). This is more pronounced in individuals with high creatinine values, counselling great care in the use of immunoenzymatic assays for the quantification of vancomycin in samples from certain type of patients. Ackerman et al. compared radioimmunoassay (RIA) and FPIA methods, observing that the latter were very rapid and less expensive than RIA, as well as not demanding the use of radioactive reagents. There are also substantial differences between the coefficients of variation of both techniques, these being 9.5–12.2% and 1.5–4.1%, for RIA and FPIA, respectively [6]. Jandreski et al. determined vancomycin in cerebrospinal fluid (CSF) using FPIA (TDx) and obtained correlation coefficients for serum *versus* CSF of 0.999, the sensitivity in these assays being four times better for CSF than for serum [7]. Morse et al. demonstrated an overestimation of vancomycin concentrations on using FPIA in patients undergoing peritoneal dialysis. Those authors concluded that this was due to an accumulation of the degradation products of the antibiotic in these patients, and they obtained concentrations of 42.1 ± 9.1, 43.1 ± 8.7 and 45.6 ± 7.4 μg/mL in comparison with those analysed with HPLC: 36.3 ± 9.4, 32.2 ± 8.9 and 31.6 ± 9.1 μg/mL [8]. However, Sym et al. failed to find statistically significant differences between the vancomycin concentration values obtained with the EMIT and FPIA techniques and concluded that the increase in vancomycin levels in some patients was not due to an accumulation of the degradation products but to a change in vancomycin pharmacokinetics. Despite some pitfalls these techniques are used routinely in clinical practice for checking concentrations within the range considered to be therapeutic (20–5 μg/mL), with quantification limits of 2 and 5 μg/mL for the FPIA and EMIT techniques, respectively [9]. These drawbacks, which hamper some research studies into the pharmacokinetics of vancomycin, have propitiated

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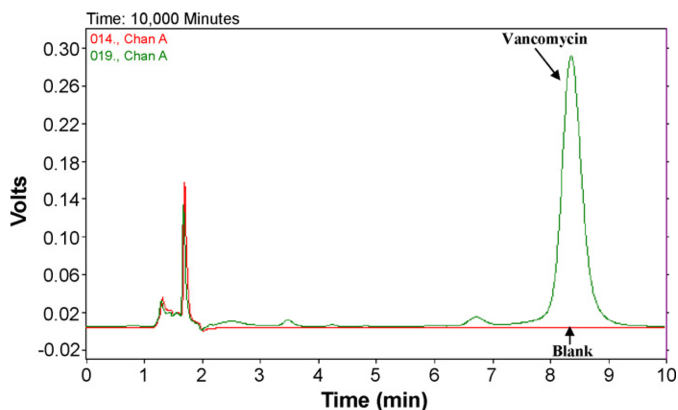

Fig. 1. Specificity chromatogram showing a blank of a Krebs–Henseleit medium sample and a Krebs–Henseleit medium sample spiked with vancomycin.

the development of different HPLC techniques for quantification of the antibiotic in biological fluids with greater accuracy and precision and greater sensitivity. Some of these techniques use complex procedures for sample preparation, including liquid–liquid extraction (LLE) [10–13] or solid-phase extraction (SPE) [14,15]. In the case of Luska et al, whose sample treatment is simple acid precipitation and liquid extraction, a limit of quantification of the order of 1 μg/mL is achieved, which is insufficient in certain cases [16].

More recently, methods involving liquid chromatography coupled with mass spectrometry (LC–MS) have been developed for the analysis of vancomycin in human serum. In one instance [17], an injection system with thermal fragmentation was added to the mass spectrometer, achieving a limit of detection of 1 ng/mL. In another case, SPE extraction was performed using a cation exchanger, attaining a quantification limit of 0.005 μg/mL [18]. There is thus no doubt that owing to their high sensitivity and specificity these techniques offer optimum methodology for the quantification of vancomycin in samples of any origin; however, often they may not be feasible because of the expensive technology required for their implementation.

The present work reports the setting up and validation of an HPLC method for the quantification of vancomycin concentrations in biological fluids in a rapid, simple way, using a basic HPLC system. The method shows higher sensitivity than other techniques of similar complexity described to date. The application of the method to a pharmacokinetic study of vancomycin in isolated rat lung is also described.

2. Experimental

2.1. Materials and reagents

Vancomycin was provided by Combino Pharm (Barcelona, Spain). HPLC grade acetonitrile was used to prepare the mobile phase and was supplied by Merck (Darmstadt, Germany). Ultrapure water was obtained with a MilliQ Millipore system. All other chemical reagents were of analytical grade and were used as received: 60% perchloric acid (Panreac Química S.A., Barcelona, Spain), Triton X-100 (Merck, Darmstadt, Germany) and $NH_4H_2PO_4$ (Sigma–Aldrich Química S.A. Madrid, Spain).

2.2. Instruments

The Shimadzu HPLC system (SCL-10A × L) comprised a pump (model LC-10AD), a Kontron a variable-wavelength UV detector (Groβ-Zimmern, Germany) and a Kontron column oven. For data processing, a Class VP Data system (Shimadzu, Duisburg, Germany) was used.

2.3. Standard solutions

A 500 μg/mL stock vancomycin solution was prepared in ultrapure water. Standard vancomycin solutions (0.1–250 μg/mL) were obtained by serial dilutions using Krebs–Henseleit medium (pH 7.4) containing 0.7% NaCl, 0.04% KCl, 0.03% $CaCl_2$, 0.02% KH_2PO_4, 0.03% $MgSO_4{\cdot}7H_2O$, 0.2% HCO_3Na, 3% bovine albumin and 0.09% of glucose. Since this medium was used in the pharmacokinetic study for lung perfusion perfusate samples were assessed against this standard solutions.

Likewise, lung tissues samples were assessed against vancomycin calibration curves prepared with lung tissue from untreated rats. One gram of tissue was homogenised in 1 mL of 50 mM phosphate buffer/Triton X-100, pH 7.4, centrifuged at 10,900 rpm for 5 min and 100 μL of the supernatant was injected into the HPLC system. The standard calibration curve for tissue samples was prepared in the 0.1–2 μg/g concentration range. All samples were stored at −20 °C.

2.4. Chromatographic conditions and sample preparation

Separation was accomplished using a Nucleosil 120 C_{18} 5 μm column with a length of 15 cm and an inner diameter of 0.4 cm. The following different chromatographic conditions were assessed:

- Mobile phases of different compositions (KH_2PO_4 and $NH_4H_2PO_4$), different pH (3 and 4), different buffer/organic phase ratios (85/15–95/5, v/v) and different molarities (5 and 50 mM).
- 40% trichloroacetic acid and 60% perchloric acid as precipitating agents.
- Column temperatures ranging between 22 and 40 °C.
- Flow rate: 1–1.5 mL/min.
- Injection volumes: 50, 100 and 150 μL.

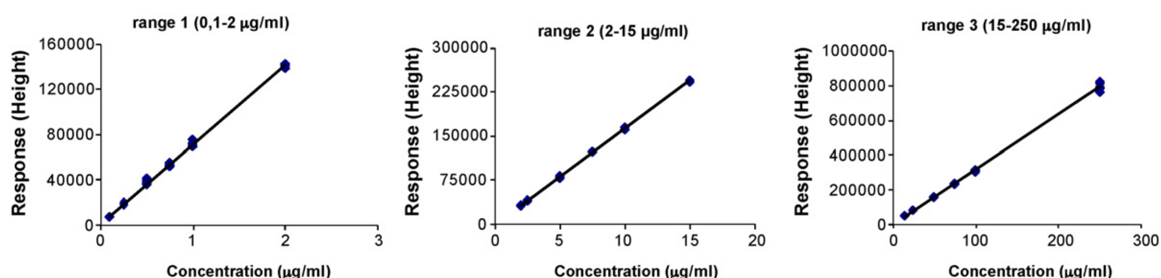

Fig. 2. Linear relationship between peak heights and concentrations for the three calibrations ranges used.

Table 1
Linear regression and statistical analysis

Concentration range (μg/mL)	Slope	*p* slope	Intercept	*p* intercept	Determination coefficient (r^2)
0.1–2	70128.30	2.72E−41	1411.06	0.0087	0.9985
2–15	16369.28	2.86E−49	−179.18	0.7276	0.9996
15–250	3171.73	5.02E−41	−3985.30	0.1541	0.9985

2.5. Validation

Validation was performed following the FDA guidelines for bioanalytical methods [19]. The method was validated as regards its specificity, linearity, accuracy, precision (within- and between-day), sensitivity and stability.

The stability of vancomycin samples analysed was investigated after three consecutive cycles of freezing–thawing.

2.5.1. Calibration

Linearity was studied for a concentration range of 0.1–250 μg/mL since the type of pharmacokinetic assay to which the technique was intended to be applied involved the existence of concentrations within the broad range quoted. When a loss of linearity for the extreme concentration values was observed, narrower ranges were analysed until those for which the linear character persistence was found. For the perfusion fluid samples, three calibration straight lines were used, the concentration ranges for each of them being 0.1–2, 2–15 and 15–250 μg/mL.

These standard curves were analysed by linear regression of peak height *versus* vancomycin concentration. UV detector was set-up at higher sensitivity as the concentration range decreases. Six standards from each straight line in five assays were carried out on the same day. Following FDA recommendations the correlation coefficient calculation and the regression analysis were performed without applying any type of mathematical transformation or data weighting.

2.5.2. Accuracy

To check the accuracy of the method, three standard samples with low, intermediate and high concentrations for each range (calibration 1: 0.1–2 μg/mL; calibration 2: 2–15 μg/mL; calibration 3: 15–250 μg/mL) were analysed; the concentrations were recalculated from the corresponding calibration straight line (experimental concentration) and were compared with the theoretical concentrations. Recovery was estimated as the relationship between the experimental concentration and the theoretical concentration expressed as a percentage ((C_{exp}/C_{teo}) × 100), after which it was analysed, together with its coefficient of variation. A *t* test for paired samples was also performed, assuming unequal variances between the theoretical and experimental percentages of recovery.

Table 2
Accuracy

Concentrations (μg/mL)	% Recovery (C_{xp}/C_{eo}) × 100	S.D.
0.25	100.67	6.37
1	100.09	3.59
2	100.00	1.17
Mean ± C.V.	100.20 ± 3.96	
2	98.83	2.11
5	99.40	2.46
15	99.92	0.62
Mean ± C.V.	99.38 ± 0.61	
15	104.35	3.09
50	99.60	0.91
250	100.35	3.04
Mean ± C.V.	101.43 ± 1.43	

Table 3
Repeatability (within-day precision)

Concentrations (μg/mL)	Mean response ± S.D.	C.V. (%)
0.1	7369.40 ± 348.83	4.73
0.5	37894.20 ± 2253.64	5.95
2	141437.60 ± 1630.43	1.15
2	32176.40 ± 692.62	2.15
5	81179.00 ± 2015.32	2.48
15	245165.60 ± 1533.34	0.62
15	46896.00 ± 531.49	1.13
50	153964.20 ± 1449.34	0.94
250	791759.60 ± 24138.54	3.05

2.5.3. Precision

To evaluate the within- and between-day precision, five replicates of standard solutions at three different concentrations for each validation range were assayed on the same day and on 5 different days, calculating the response obtained and its coefficient of variation.

3. Results and discussion

From all the conditions assayed, the best results were obtained with a mixture of 50 mM $NH_4H_2PO_4$ (pH 4)–acetonitrile (92:8, v/v) as the mobile phase at a flow rate 1 mL/min and a column temperature of 40 °C. Regarding sample treatment, the following procedure proved to be optimum: a mixture of 300 μL of sample with 15 μL of 60% perchloric acid was vortexed for 30 s, followed by centrifugation at 10,900 rpm to separate proteins, after which the supernatant was collected. Of this, an aliquot of 100 μL was injected into the chromatographic system. With this simple, fast and inexpensive procedure an excellent chromatographic specificity was obtained (Fig. 1), with no interferences due to the components of the biological samples analysed (perfusion fluid and lung tissue homogenate). The method was checked with respect to 6 blanks of different origins, as recommended by the FDA.

Within the concentration range initially considered (0.1–250 μg/mL) linearity was not maintained. A regression analysis was performed, with the observation of the good linearity of the technique in the following concentration ranges: 0.1–2, 2–15 and 15–250 μg/mL (Fig. 2).

Table 4
Reproducibility (between-day precision)

Concentrations (μg/mL)	Mean response ± S.D.	C.V. (%)
0.1	7479.50 ± 523.56	7.00
0.5	37439.90 ± 2483.30	6.63
2	140891.20 ± 2932.47	2.08
2	31137.20 ± 1076.36	3.46
5	79853.40 ± 2722.85	3.41
15	242816.40 ± 2902.91	1.20
15	48442.10 ± 1012.33	2.09
50	152963.70 ± 2592.60	1.69
250	797772.50 ± 24833.92	3.11

Table 5
Freeze and thaw stability

Concentrations (μg/mL)	Basal	Mean ± S.D.	Third cycle	Mean ± S.D.	Recovery as referred to basal level (%)
0.25	100.52 94.83 101.10	98.82 ± 3.46	95.85 90.10 106.88	97.61 ± 8.52	98.78
5	97.12 100.04 99.14	98.77 ± 1.49	98.90 96.18 95.06	96.71 ± 1.97	97.92
100	97.88 96.35 96.23	96.82 ± 0.92	97.46 99.15 95.66	97.42 ± 1.74	100.62

Table 1 summarizes the results of the regression analysis and shows the slope and ordinate values for each calibration, coefficients of correlation higher than 0.99 being obtained in all ranges. From this data equations to estimate drug concentration according to the corresponding range are defined.

Accuracy was determined using standard calibration curves of vancomycin within the ranges of 0.1–2, 2–15 and 15–250 μg/mL for each day. The data on accuracy are shown in Table 2, with coefficients of variation <7%, a value below the limit accepted by the FDA (15%).

The means ± S.D. of the recoveries were 100.20 ± 3.96; 99.38 ± 1.83; 101.43 ± 3.16 for standard curves 1 (0.1–2 μg/mL), 2 (2–15 μg/mL) and 3 (15–250 μg/mL), respectively. In the 0.1–2 range, accuracy was calculated for concentrations of 0.25, 1.0 and 2.0 μg/mL instead of the lower limit −0.1 μg/mL, considering this as the quantification limit and hence admitting greater variability in it.

For the 0.1 μg/mL concentration, the results were: mean ± S.D. = 84.96 ± 4.97; C.V. = 5.85; error = 15.04%. A slight underestimation can be seen in the results for concentrations close to the limit of quantification, with an error of 15.04%; i.e., lower than that stipulated by the FDA (20% for the limit of quantification).

The values of the coefficients of variation of the response obtained in the three validation ranges were less than 6% in the studies on repeatability and within-day precision and less than 7% in those on reproducibility or between-day precision, as may be seen in Tables 3 and 4, respectively.

The stability of the drug at three concentration levels (0.25, 5 and 100 μg/mL) (in three replicates) during three cycles of freezing–thawing was assessed. The results obtained, shown in Table 5, showed recoveries with respect to the baseline of 98.78%, 97.92% and 100.62% for 0.25, 5 and 100 μg/mL, respectively, confirming the absence of any effect of three thawing and freezing cycles.

The limit of quantification was <0.1 μg/mL for vancomycin, since this concentration can be determined with an error of 15.04% and hence less than the 20% limit permitted by the FDA. The next concentration assayed 0.05 μg/mL afforded a higher error, such that it could not be quantified according to these criteria.

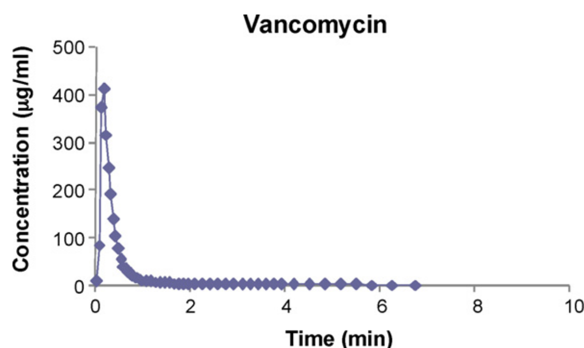

Fig. 3. Vancomycin concentrations curve in efferent fluid from isolated rat lung quantified with the method developed.

The analytical method proposed and validated here was applied in a pharmacokinetic study of the pulmonary distribution of vancomycin in isolated rat lung [20]. This type of study requires the quantification of vancomycin in a large number of effluent fluid (perfusate) samples, whose concentrations decrease rapidly from very high maximum values. Here the availability of a rapid, simple and inexpensive method of analysis that allows precise and accurate quantification of the drug across a broad range of values facilitated the characterisation of the kinetic profile in all its phases and the mechanisms involved in vancomycin disposition in the pulmonary system. Additionally, the method allowed the quantification of lung homogenate samples, which is a further advantage for this type of study and also for experimental and clinical assays requiring drug tissue level quantification. Fig. 3 shows a curve of vancomycin concentrations in isolated lung effluent fluid quantified using the proposed validated method.

4. Conclusions

The proposed analytical methodology developed and validated for vancomycin quantification in biological samples constitutes an additional technique that, in comparison with previously reported methods of similar complexity and equipment requirements, provides a lower quantification limit together with a shorter sample analysis time. These characteristics are very interesting and helpful for research studies related to drug pharmacokinetics involving a high number of samples within a broad concentration range and tissue levels information.

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