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Short communication

Determination of vancomycin in serum by liquid chromatography–high resolution full scan mass spectrometry

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Abstract

A liquid chromatography–mass spectrometry (LC–MS) method was developed for the analysis of vancomycin (VCM) in human serum. The method was based on full scan data with extracted ions for the accurate masses of VCM and the atenolol internal standard obtained by Fourier transform MS. VCM was extracted from serum using strong cation exchange (SCX) solid phase extraction (SPE). The method was found to be linear in the range 0.05–10 μg/ml, which was adequate for quantification of VCM in serum samples, with a limit of quantification (LOQ) of 0.005 μg/ml and a limit of detection (LOD) of 0.001 μg/ml. Intra-day precision ($n=5$) was ±3.5%, ±2.5%, ±0.7% at 0.05, 0.5 and 5 μg/ml, respectively. Inter-day precision ($n=5$) was ±7.6%, ±6.4%, ±3.9% at 0.05, 0.5 and 5 μg/ml, respectively. The process efficiency for VCM was in the range 89.2–98.1% with the recovery for the atenolol internal standard (IS) being 97.3%. The method was used to determine VCM levels in patients during peri-operative infusion of the drug, which was found to result in drug levels within the required therapeutic window.

Keywords: Vancomycin; Serum; Fourier transform mass spectrometry; Ion suppression

1. Introduction

VCM is a glycopeptide antibiotic that is often used in the prophylaxis and therapy against infections by many Gram-positive bacteria, including methicillin-resistant staphylococci [1,2]. Monitoring of serum concentrations is needed to ensure sufficient levels for therapeutic efficacy and avoid levels which are high enough to produce toxicity. Many methods have been reported for the determination of VCM in bio-matrices including immunoassays [3–7], high performance liquid chromatography (HPLC) [8–12] and liquid chromatography–tandem mass spectrometry (LC–MS–MS) [13,14]. Despite speed and ease of operation, immunoassays were found to demonstrate insufficient specificity and sensitivity for VCM and also require specific equipment [15]. Therefore, currently HPLC is a widely used method for the determination of VCM in bio-matrices. Although highly accurate and precise results were reported for HPLC analyses, the sensitivity of this method is limited by the fact that VCM lacks of strong absorption in the range of the longer wavelengths (>230 nm) of the UV spectrum. The lowest limit of quantification obtained by a HPLC method is reported to be 0.04 μg/ml in serum [8]. Recently quantification of VCM in plasma or serum samples using LC–MS–MS has been demonstrated on a triple quadrupole mass spectrometer. High sensitivity was achieved by careful choice parent and product ions in selected reaction monitoring mode and the limit of detection was shown to be 0.001 μg/ml in serum [13]. None of the previous publications on VCM determination by mass spectrometry have reported validation of methods with respect to the absence of matrix effects.

The aim of the current work was to develop highly sensitive method for determination of VCM concentration in serum by LC–MS exploiting a hybrid linear ion trap Fourier transform mass spectrometer (LTQ-Orbitrap) and to demonstrate at least comparable performance to tandem mass spectrometry. At the same time as providing sensitive analyses FT-MS produces

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data files which contain the full range of mass spectral data thus providing an information-rich sample archive which can be referred back to if required. Investigation of matrix effect for these samples was carried out by following the method demonstrated by Matuszewski et al. [16]. Finally, this study analysed human serum from a clinical trial aiming to validate the novel use of a continuous VCM infusion for antimicrobial prophylaxis in patients undergoing a vascular procedure requiring a prosthetic graft.

2. Experimental

2.1. Materials and reagents

Vancomycin hydrochloride, atenolol and drug-free horse serum were purchased from Sigma–Aldrich (Poole, Dorset, UK). HPLC grade water and acetonitrile for the mobile phase and methanol for SPE were purchased from Rathburn Chemicals Ltd. (Walkerburn, Scotland). HPLC grade formic acid and ammonium hydroxide were obtained from VWR International Ltd. (Leicestershire, UK). Sample clean-up solid phase extraction cartridges were carried out on 60 mg Strata-X-C cartridges from Phenomenex (Macclesfield, UK).

2.2. Standard and sample preparation

The standard solutions of VCM were prepared by dissolving an accurately weighed amount of VCM in 0.1% (v/v) formic acid in water and diluting to appropriate concentrations. The atenolol internal standard was also dissolved with 0.1% (v/v) formic acid and diluted to give a final concentration of 1 μg/ml. These solutions were stored in a refrigerator at 4 °C. For control serum samples 1 ml of a known concentration of VCM standard solution and 1 ml of IS standard solution were added to 200 μl of drug-free horse serum and for 200 μl human serum samples 1 ml of the VCM standard solution was replaced with 1 ml of 0.1% (v/v) formic acid. After SPE and evaporation to dryness the samples were reconstituted with 1 ml of 0.1% (v/v) formic acid and kept in a freezer at −20 °C until analysis was carried out.

2.3. Solid phase extraction

Strata-X-C SPE cartridges were conditioned, equilibrated and activated with 1 mL methanol, 1 ml water and 1 ml 0.1% (v/v) formic acid, respectively. The samples were then loaded at a flow rate of 1 ml/min followed by washing the SPE cartridges with 1 ml of methanol and 1 ml of water. VCM and internal standard were then eluted with 1.5 ml of 3% (v/v) ammonium hydroxide in methanol into vials and the eluates were evaporated by removing the solvent under a gentle stream of nitrogen gas at a temperature of 40 °C. The residues in the vials were then reconstituted by dissolving in 1 ml of 0.1% (v/v) formic acid.

2.4. Validation

The guidelines for proposed by the American FDA for validation of bioanalytical methods were followed [17]. A calibration series of VCM in serum was prepared in the range of 0.05–10 μg/ml. Spiked serum samples were prepared at three concentrations of 0.05, 0.5 and 5 μg/ml and were used to evaluate the accuracy, precision and recovery of this method. For intra-day precision and accuracy five sets of spiked serum samples at each level were prepared and assayed within 1 day. For inter-day precision test five sets of controlled serum samples at each level were prepared and assayed on five different days. Recovery was calculated by comparing the measured values of the spiked samples with those of standard aqueous solutions. The limit of quantification (LOQ) was defined as the lowest concentration in serum which was determined within <20% precision and the limit of detection (LOD) was defined as the minimum concentration that could be detected at a signal-to-noise ratio of 3:1. Matrix effects were assessed as described previously [16] by comparing serum extracts spiked before and after extraction and spiked standard solutions.

2.5. Instrumentation and chromatographic condition of LC–MS

The instrument consisted of a Finnigan Surveyor Plus HPLC System including an autosampler, a PDA detector and a MS pump and a Finnigan LTQ-Orbitrap hybrid mass spectrometer equipped with an electrospray ionization (ESI) probe as the interface. Chromatographic separations were performed on an ACE-3-C8 column (50 mm × 3.0 mm; 3 μm; HiChrom, Reading, UK) with a SecurityGuard C18 (4 mm × 3.0 mm; 5 μm; Phenomenex UK) guard column. The mobile phase containing 0.1% (v/v) formic acid and acetonitrile (9:1) was pumped through the column at a flow rate of 0.2 ml/min, and the temperature of the column was maintained at 40 °C. The ionization was generated in positive mode with the spray voltage of 5 kV and a temperature of 300 °C, respectively. Sheath and auxiliary nitrogen gas were applied to help the evaporation of the solvent at a flow rate of 60 and 20 arbitrary units, respectively. The instrument was operated in full scan mode from 200 to 800 amu.

2.6. Assay of samples

Serum sample collection was approved by an ethics committee and all patients provided written consent. Patients undergoing a vascular procedure requiring a prosthetic graft were given a peri-operative VCM infusion. Three intra-operative venous blood samples were taken, and immediately centrifuged. Serum was then decanted and stored at −20 °C prior to batch analysis. The samples were prepared on five consecutive days and one control serum sample at a concentration of 1 μg/ml was also prepared with the human serum samples everyday. Ninety human serum samples and five control serum samples were measured in one sequence run and quality control samples (QCs) at three concentrations (0.05, 0.5 and 5 μg/ml of serum) were also tested with each run. The set of samples were re-prepared if the accuracy of the control serum sample (1 μg/ml) deviated by more than 20% from its nominal value and the runs were repeated if more than two QC results deviated more than 20% from their expected values.

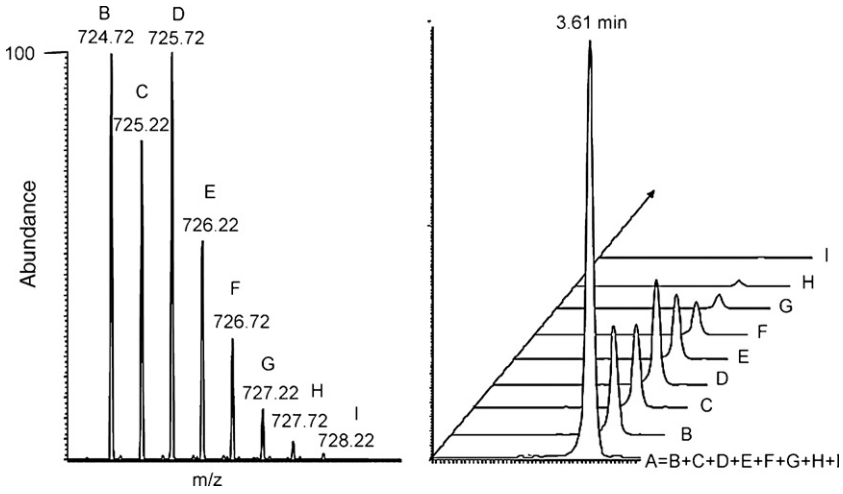

Fig. 1. A molecular ion cluster for VCM B chromatographs of VCM (concentration = 10 μg/ml in aqueous solution) obtained at each mass-to-charge ratio (B–I) in the molecular ion cluster and their sum A.

3. Results and discussion

Since high resolution extracted ion chromatograms were to be used for quantification of vancomycin it was necessary to determine the accurate masses of the analyte and internal standard. Standard mass spectra in the full scan range of 200–2000 *m/z* were obtained by individual infusion of standard VCM and atenolol (10 μg/ml) in a mixture of water and acetonitrile (1:1, v/v). As expected, high intensity of signal at *m/z* 267.17 was observed for atenolol as the molecular ion $[M+H]^+$. However, instead of the molecular ion a group of ions for VCM were observed at around *m/z* 725.72 (Fig. 1). The absence of the molecular mass of VCM (1449.22) indicated that VCM was diprotonated [13,14] and the multiple signals were due to a combination of the isotope pattern for two chlorine atoms and the corresponding ^{13}C isotope peaks. It was found that the best sensitivity was obtained by summing the peak areas for the molecular ion cluster (Fig. 1). Fig. 2 shows the chromatograms of VCM standard at the concentration of 10 μg/ml in aqueous solution (Fig. 2A), blank drug-free horse serum sample (Fig. 2B) and serum spiked with 10 μg of VCM (Fig. 2C). The background interference in the drug-free horse serum was negligible. The scan range was set up from 200 to 800 amu and extracted ions were set up in the range of 267.16–267.18 *m/z* for atenolol and a group of extracted ions as described above were used for VCM determination. Good separation of atenolol (1.94 min) and VCM (3.75 min) was obtained in 5 min and no interfering peaks resulting from the serum or extraction procedure were observed.

The calibration curves were made by plotting peak area ratios of VCM and atenolol versus spiked concentrations of VCM in the range 0.05–5 μg/ml in serum. The mean linear regression equation ($n = 5$) was $y = 0.0428\ (\pm 0.00247) + 0.2843(\pm 0.0165)x$ and the correlation coefficient (r) was 0.998 or higher. The results of intra- and inter-day accuracies and precisions for VCM in serum at three different concentrations are shown in Table 1. The percentage accuracy in VCM control samples at 0.05, 0.5

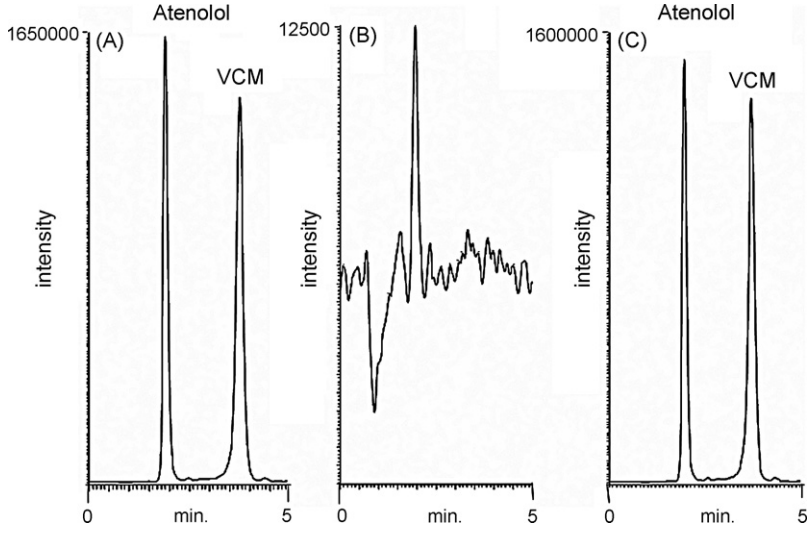

Fig. 2. Representative chromatographs of (A) 1 μg/ml of atenolol and 10 μg/ml of VCM in aqueous solution, (B) drug-free horse serum and (C) 1 μg/ml of atenolol and 10 μg/ml of VCM in horse serum.

Table 1
Results of accuracy, intra- and inter-day precision of controlled serum samples at three concentrations

Nominal concentration (μg/ml)	0.05	0.5	5
Calculated concentration (μg/ml) $n=5$	0.048 ± 0.004	0.486 ± 0.02	5.02 ± 0.09
Accuracy (%)	95.5	97.2	100.4
Intra-day R.S.D. (%) $n=5$	3.5	2.5	0.7
Inter-day R.S.D. (%) $n=5$	7.2	6.4	3.9

and 5 μg/ml were 95.5%, 97.2% and 100.4%, respectively. The limit of quantification (LOQ), which is defined as the lowest concentration determined within less than 20% precision, was 0.005 μg/ml and the limit of detection (LOD) was 0.001 μg/ml at a signal-to-noise ratio of 3:1. The results of recovery tests, conducted as described previously [16], are shown in Table 2. Slight ion enhancement rather than ion suppression was indicated by the fact that the values representing the matrix effect when calculated according to peak areas gave more than 100% recovery for VCM as well as for atenolol. This explains why the values representing overall process efficiency are higher than the ones representing "true recovery". It should be noted that the all values in this study were corrected since peak area ratio was used for calculations and the small matrix effects on VCM were mirrored by the atenolol internal standard. Since the data was collected in full scan mode it was possible to observe that there was little evidence of additional interfering peaks in processed serum samples compared to the spiked unextracted standard. There was no evidence for phospholipid peaks which occur in the range 700–900 amu and are likely to produce ion suppression. The only background ion enhanced in the extracted samples was at *m/z* 242, which is a background ion that we commonly observe, having an elemental composition $C_{16}H_{36}N$ thus corresponding to an aliphatic amine; it is not clear from where this compound originates.

Considering that the sample preparation and assay was not performed on the same day the effects of freeze–thaw on the stability of VCM was investigated by running control serum samples at concentrations of 0.05, 0.5 and 5 μg/ml during three such freeze–thaw cycles. The mean accuracies obtained were 93.8%, 96.3% and 99.4% with R.S.D. < 3.6% and indicated that the influence of thawing and freezing could be ignored.

In order to determine the stability of VCM during the assay sequence time (maximum = 8 h) one 1 μg/ml serum sample was measured at the beginning and the end of each sequence in replicate. The mean peak area of VCM decreased by 3.3% from the first to the last replicate injection suggesting acceptable stability of VCM during the running time.

Since vancomycin exhibits time-dependent killing of susceptible bacteria, the concentration of the drug must be maintained above the minimum inhibitory concentration (MIC) during peri-operative time. Fig. 3 shows the average concentration of VCM in the three serum samples collected during the vascular surgery for each patient who received peri-operative VCM infusion. The R.S.D. is 5.6% or less for each patient

Table 2
Results of recovery tests based on peak area and peak area ratio

	Peak area ($n=2$)						Peak area ratio ($n=2$)					
	ME = *B/A* (%)	R.S.D. (%)	RE = *C/B* (%)	R.S.D. (%)	PE = *C/A* (%)	R.S.D. (%)	ME = Br/Ar (%)	R.S.D. (%)	RE = Cr/Br (%)	R.S.D. (%)	PE = Cr/Ar (%)	R.S.D. (%)
Atenolol	112.7 ± 2.6	2.3	92.2 ± 1.5	1.6	96.7 ± 2.3	2.4						
VCM 5 μg/ml	109.3 ± 3.8	3.5	89.2 ± 4.2	4.7	94.2 ± 3.8	4.0	102.2 ± 2.4	2.4	96.2 ± 2.9	3.0	97.4 ± 3.0	3.1
VCM 0.5 μg/ml	114.9 ± 6.8	5.9	87.6 ± 5.8	6.6	92.5 ± 4.3	4.7	100.5 ± 4.2	4.2	94.8 ± 4.3	4.5	95.7 ± 3.4	3.6
VCM 0.05 μg/ml	111.5 ± 10.5	9.4	84.6 ± 7.4	8.7	89.5 ± 7.5	8.4	97.3 ± 6.0	6.2	90.2 ± 6.8	7.6	92.4 ± 4.8	5.2

ME = matrix effect, RE = recovery of extraction, PE = process efficiency, A = peak area obtained from the sample without extraction, B = peak area obtained from the sample spiking after extraction, C = peak area obtained from the sample spiking before extraction and Ar, Br and Cr was the peak area ratio obtained under each the above conditions, respectively.

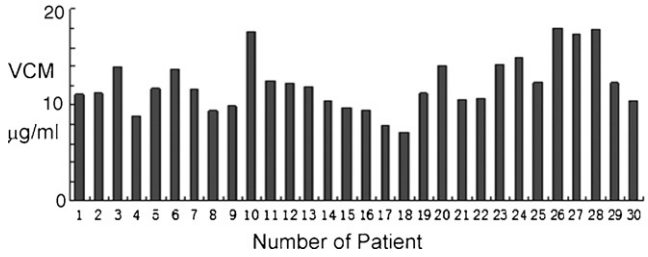

Fig. 3. Mean levels of VCM in 30 patients sampled at three time points intra-operatively.

indicating a steady concentration of VCM in blood circulation system during the operation period. No patient was found to be out of the recommended "therapeutic range" of VCM (5–20 μg/ml) [18] suggesting that this procedure for administration of VCM improves efficacy and reduces toxicity.

In conclusion, the LC–MS method developed was rapid in terms of machine time and provided high sensitivity quantification of VCM in serum samples. Although two methods of comparable sensitivity have been reported previously the advantage of high resolution full scan mass spectrometry is that all the mass spectral information, available in positive ESI mode, in the sample is collected. Greater confidence in identification of the drug results from the ability to monitor isotopes of the molecular ion and metabolites can be monitored and identified in the same run if required. In effect the runs are an archive of a range of metabolic events in the sample since effects a drug on overall metabolism can also checked and this information is retained for as long as the data is retained. The ThermoFinnegan Orbitrap is a new instrument and the current paper demonstrates that the Orbitrap can deliver comparable sensitivity and dynamic range to more established tandem instruments as well as potentially providing information-rich data. With a move to the use of chemometric methods in analysis it may in the future not be simply enough to report drug levels since the data available through using full scan FT-MS has the potential to paint a more global picture of biological response to treatment. The purpose of the current paper was not to demonstrate such an approach to treatment monitoring but rather to confirm the suitability of the Orbitrap for traditional pharmacokinetic studies. However, it is important to draw attention to the other data available from the sample runs carried out in this study which can be re-examined if it is deemed medically relevant. Another consideration is that, since data is obtained in full scan mode, it is also possible to directly monitor interfering ions in the sample matrix that could produce ion suppression. Each individual is different so even if ion suppression studies are carried out for standard samples there is nothing to guarantee that this applies to profile of a given individual's plasma. In the current study it was possible from the ion suppression studies using spiked serum to observe that there were few additional interfering ions present in extracted serum samples. Phospholipid ions were completely absent from the samples but this is not surprising since Strata mixed mode SCX cartridges are highly retentive so that phospholipids would not be eluted even in 100% methanol.

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