Keywords:

vancomycin during TKA surgery. e use of the tissues was approved by the local Ethics Committee. All bone and fat samples were stored at -80°C until analysed. e human plasma used as the assay blank and for the preparation of standards was obtained from New Zealand Blood Services (Christchurch, New Zealand).

Instrumentation and analytical conditions

e LC-MS/MS system consisted of a Shimadzu LC-20AD HPLC

aliquots of vancomycin standard stock solutions to drug-free plasma, to give spiked vancomycin standards of 0.05, 0.1, 0.5, 1.0, 5.0, 10, 25 and 50.0 mg/L. Vancomycin plasma quality control (QC) standards were prepared in single 5 mL aliquots in concentrations of 0.05, 1.0, 10 and 50 mg/L and stored at -80°C until analysed.

For the analysis of vancomycin concentration in bone and fat, the supernatants of immersed blank bone and fat tissue suspensions for the preparation of standards were prepared. e blank bone samples were crushed with pliers, nely cut further with a scalpel, then weighed and immersed in the extraction solvent, phosphate bu ered saline pH 7.3, (the ratio of bone/ phosphate bu ered saline pH 7.3 was around 1:5, w/v) at 4°C overnight. e blank fat samples were nely cut with a scalpel, and then treated in a same way as the bone samples. e immersed bone or fat tissue suspensions were vortexed for 30 seconds and centrifuged at 15,000 g for 10 min to precipitate the tissue particles. e supernatants of the immersed blank bone and fat tissue suspensions were transferred into a clean tube, respectively. e standard curves for the vancomycin analysis in bone and fat were prepared by spiking appropriate aliquots of vancomycin standard stock solutions to the supernatants of immersed blank bone and fat tissue suspensions respectively, to give spiked vancomycin standards of 0.05, 0.1, 0.5, 1.0, 5.0, 10, 25 and 50.0 mg/L. e QC samples of vancomycin in the supernatants of immersed bone and fat tissue suspensions were prepared in single 5 mL aliquots in concentrations of 0.05, 1.0, 10 and 50 mg/L respectively and stored at -80°C until analysed.

e stock internal standard aminopterin solution (1.0 mg/mL) was prepared by dissolving 5.0 mg of aminopterin in 5.0 mL of 0.1 M NaOH. A working solution of the internal standard (0.25 µg/mL) was prepared by diluting the stock solution with water.

Sample preparation

For the analysis of vancomycin concentration in plasma: 50 µL of the internal standard, aminopterin, 0.25 µg/mL, was added to 50 µL of each of the blank, standard, QC and patient samples. e mixtures were vortexed brie y. Two hundred microliters of methanol was added to precipitate the proteins. A er centrifugation at 15,000 g for 5 min, a 50 µL aliquot of clear supernatant was mixed with 500 µL of water and transferred to the 96 well microtiter autosampler plate. A volume of 10 µL was injected into the LC-MS/MS system. All the samples were analysed in duplicate.

For the analysis of vancomycin concentration in bone and fat: Based on the method previously described by Graziani et al. [16], phosphate bu ered saline pH 7.3 (PBS) was selected as the extraction solvent to extract vancomycin from bone and fat tissue. e bone samples were crushed with pliers, nely cut further with a scalpel, then weighed and immersed in the extraction solvent PBS (the ratio of bone/ PBS was around 1:5, w/v) at 4°C overnight to extract vancomycin from the bone. e fat samples were nely cut with a scalpel, and then treated in the same way as the bone samples. e immersed bone or fat tissue suspension were vortexed for 30 seconds and centrifuged at 15,000 g for 10 min to precipitate the tissue particles. 50 µL of the supernatant was transferred to a clean tube and treated in a same way as the plasma sample.

Validation

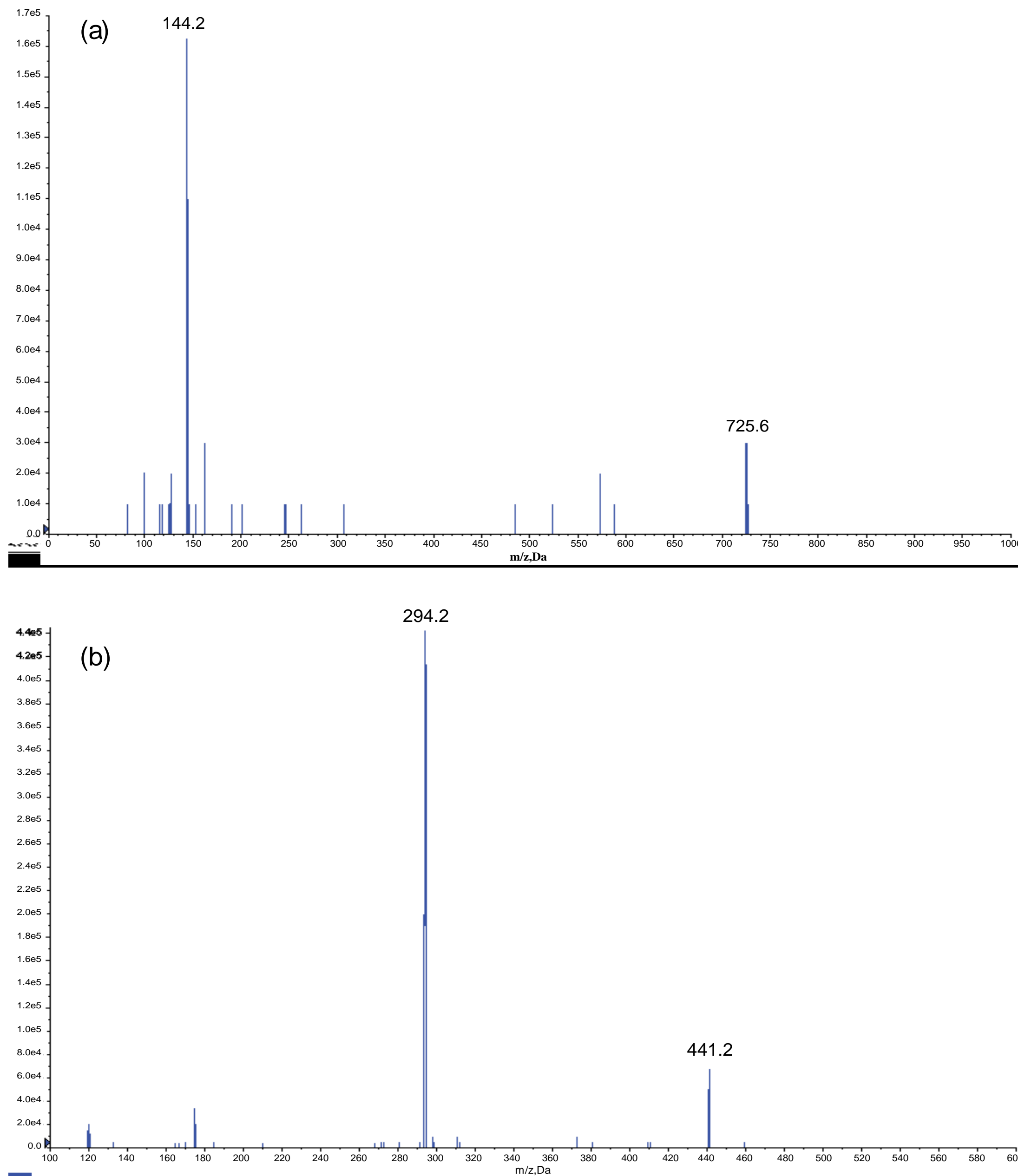
e standard curve was the plot of the peak area ratios (analyte/ internal standard) of vancomycin versus the corresponding concentrations of vancomycin. To evaluate the assay recoveries and matrix e ects, three sets of standards were prepared using a modi cation of the method of Matuszewski et al. [20] for vancomycin and the internal standard aminopterin. e standards of vancomycin were prepared at concentrations of 0.05, 1.0, 10 and 50 mg/L, and aminopterin at 0.25 µg/mL, the concentration used in the assay. e rst set was prepared in plasma or the supernatants of immersed blank bone and fat tissue suspensions, the second set t 1calnd

Results and Discussion

Mass spectrometry and Chromatography

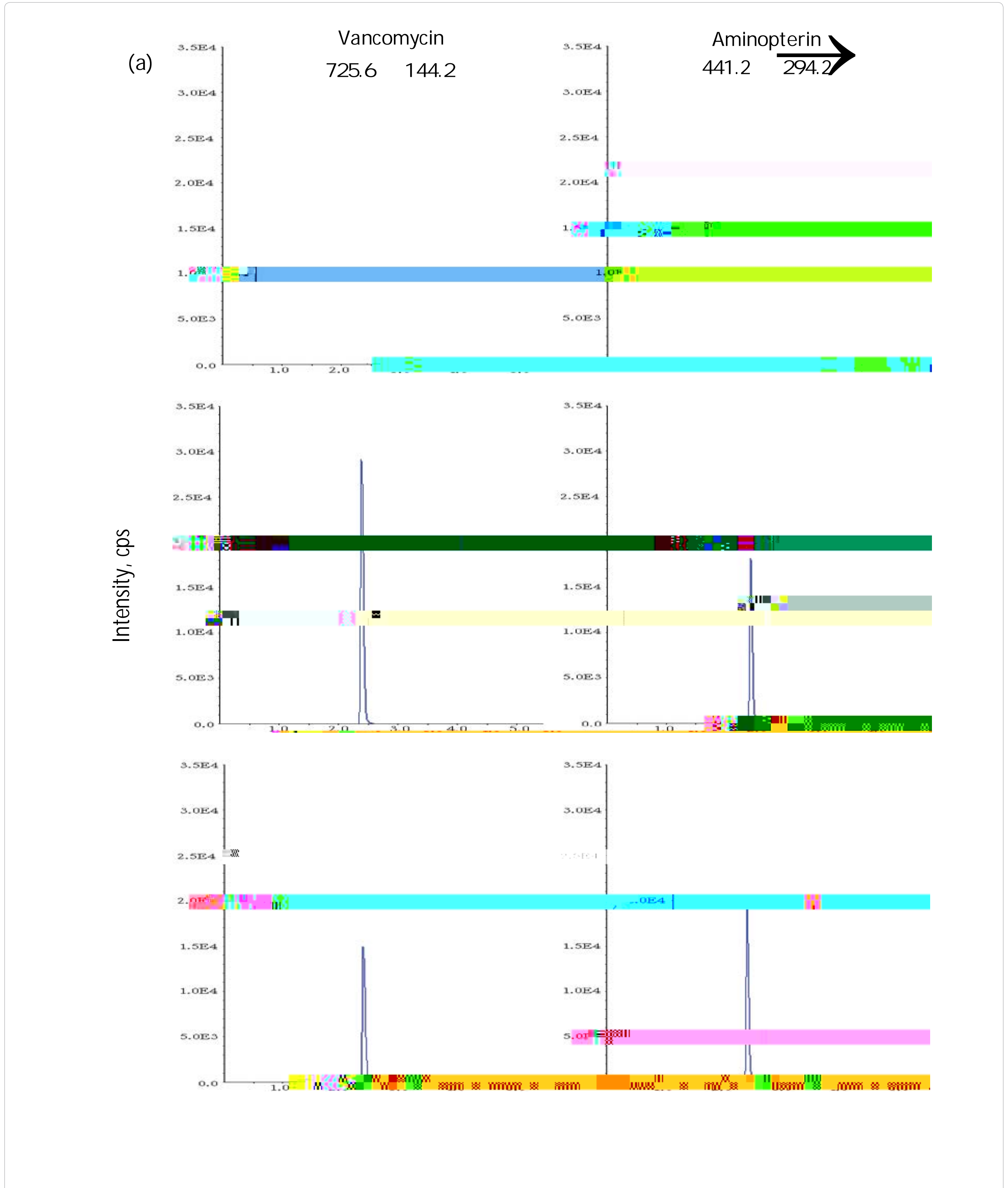
e MS/MS parameters were optimised to produce maximum responses for vancomycin and the internal standard aminopterin using electrospray ionisation in the positive ion mode. e protonated molecular ions of $[M + 2H]^{2+}$ for vancomycin and $[M + H]^{+}$ for aminopterin were *m/z* 725.6 and *m/z* 441.2, respectively. e transitions yielding the most abundant product ions were 725.6 → 144.2 for vancomycin and 441.2 → 294.2 for aminopterin. e product ion spectra of $[M + 2H]^{2+}$ for vancomycin and $[M + H]^{+}$ for aminopterin are shown in Figure 2.

Phenomenex Luna C18(2) and C8 columns gave the best chromatographic resolution and peak sharpness. Choosing the Phenomenex Luna C18(2) column over the Phenomenex Luna C8 column enabled this method to share the same column with another routine method in our laboratory, so that we could run both methods overnight without the need to change columns. e mobile phase consisting of 0.05% formic acid and methanol gave higher signal intensity. Gradient elution with increasing methanol decreased the retention of the components so that they eluted faster with sharper peaks, speeding up the analysis time for each sample. e optimized LC condition chosen was therefore a mobile phase consisting of 0.05%



Product ion mass spectra of $[M+2H]^{2+}$ for (a) vancomycin and $[M+H]^{+}$ for (b) aminopterin.

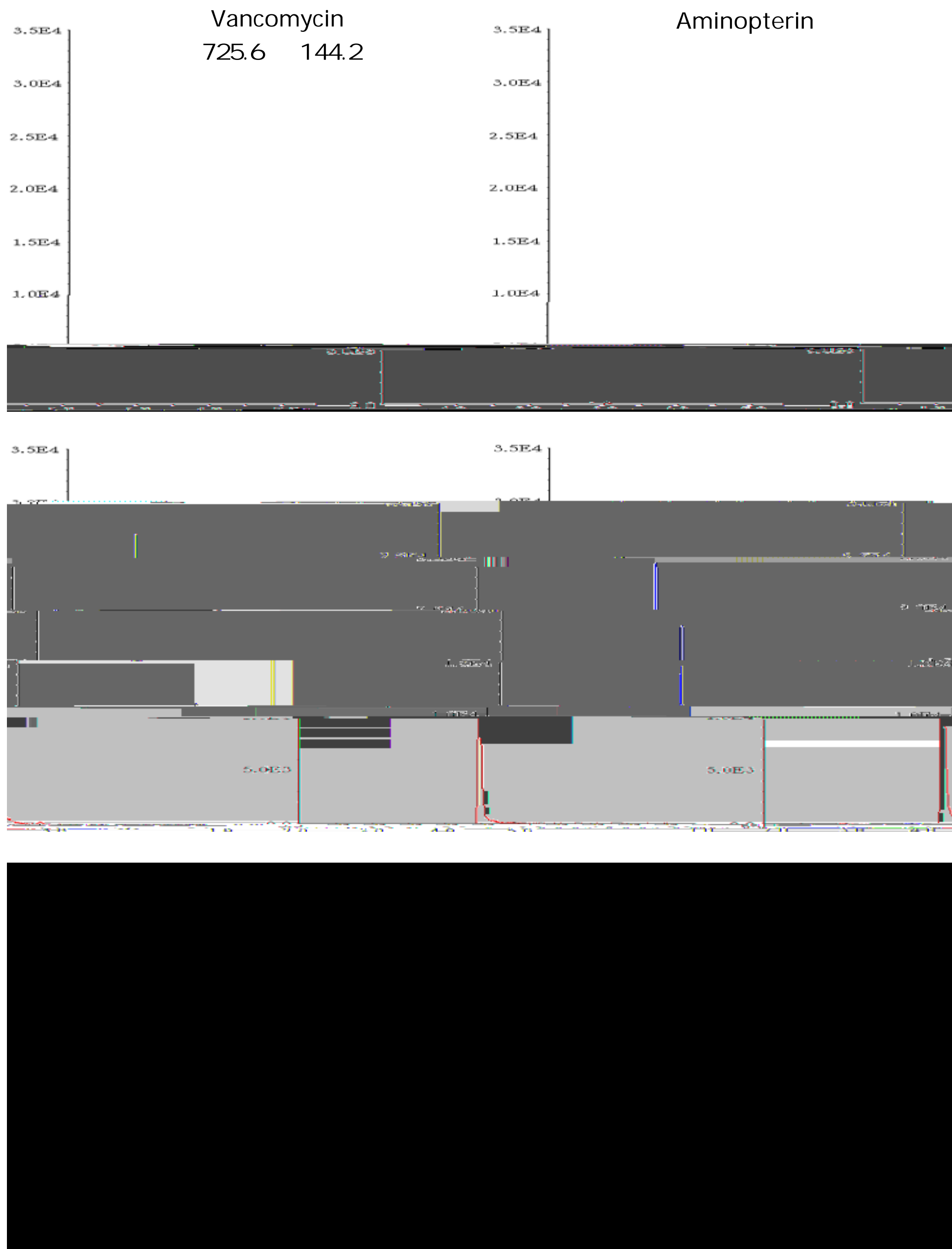
formic acid and methanol with gradient elution on a Phenomenex Luna C18(2) column. Under these conditions, the retention times were approximately 2.39, and 2.44 min for vancomycin and the internal standard aminopterin, respectively (Figures 3-5). Blank samples of



plasma, bone and fat from more than six di erent sources of the same matrix were tested for interference, and vancomycin and the internal standard peaks were free of interference from any other peaks present in the blanks (Figures 3-5). ere was no interference of cefazolin.

Sample preparation

Protein precipitation is the simplest and most rapid sample cleanup method of liquid sample preparation for the determination of drugs. Methanol is one of the most widely used precipitating agents.



of the spiked values (Tables 1-3). Imprecision was small, as indicated by both intra- and inter-day coe cients of variation of <10% at concentrations of LLOQ and QCs (Tables 1-3). e absolute recoveries of vancomycin from plasma and the supernatants of immersed bone and fat tissue suspensions at concentrations of 0.05, 1.0, 10 and 50 mg/L were similar and consistent, with mean values of around 100%. e absolute recoveries of the internal standard aminopterin from plasma and the supernatants of immersed bone and fat tissue suspensions at the concentration employed were similar with mean values of around 100%.

e matrix e ects (mean ± SD%) for plasma and the supernatants of immersed bone and fat tissue suspensions at concentrations of 0.05, 1.0, 10 and 50 mg/L were similar and consistent, with mean values of around 100%. e matrix e ects for the internal standard aminopterin for plasma and the supernatants of immersed bone and fat tissue suspensions at the concentration employed were similar with mean

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