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Short communication

Simultaneous determination of cefepime, vancomycin and imipenem in human plasma of burn patients by high-performance liquid chromatography

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Abstract

A liquid chromatographic method with UV detection for simultaneous determination of cefepime, vancomycin and imipenem has been developed. Cefuroxime was used as internal standard. After the clean up of samples by plasma protein precipitation, 5 μl of the extract were injected into the chromatograph and peaks were eluted from the Sulpelcosil™ LC-18 column using a mobile phase consisting of 0.075 M acetate buffer:acetonitrile (92:8, v/v), pH 5.0 at low rate (0.8 ml/min). The detection wavelength was 230 nm. The limit of detection was 0.4 μg/ml for cefepime and 0.2 μg/ml for vancomycin and imipenem. The method was applied to plasma samples of burn patients, and only small volumes of plasma were required for the simultaneous determination of those antimicrobial agents.
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1. Introduction

When sepsis is clinically diagnosed for the large burn patient, antibiotic therapy is initiated by prescription of a broad-spectrum sufficient to cover staphylococci, streptococci and gram-negative facultative anaerobes as well as *Pseudomonas aeruginosa* and *Acinetobacter* spp. [1]. More recently, antimicrobial agents as cefepime, a fourth generation cephalosporin, imipenem, a wide spectrum β-lactam antibiotic and vancomycin, glycopeptide antibiotic, often prescribed against gram-positive bacteria, including methycillin-resistant staphylococci, are commonly used in the intensive care unit in large burn patients with sepsis [2,3].

Although, several analytical methods using microbiological, spectrophotometric and chromatographic techniques were described to determine isolated antimicrobial agents in biological matrices, and the high-performance liquid chromatography is preferred due to its selectivity and specificity of the assay [4–6].

Since changes on the pharmacokinetics of these drugs should be expected and none HPLC method for the simultaneous determination of cefepime, vancomycin and imipenem in plasma was described previously, the purpose of the study was to develop a rapid, selective and sensitive method to measure simultaneously these antimicrobial agents for therapeutic drug monitoring in large burn patients.

2. Experimental

2.1. Reagents and chemicals

Standards of cefepime [Bristol Myers Squibb, Guayaquil, Ecuador], vancomycin [Eli Lilly, Sao Paulo, Brazil], imipenem [Merck Sharp & Dohme, Rio de Janeiro, Brazil] and also cefuroxime [GlaxoSmithKLine, Rio de Janeiro, Brazil] were kindly supplied by the Pharmaceutical Industries. The 3-[*N*-morpholino]propanesulfonic acid (MOPS) was purchased from

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Sigma (Steinheim, Germany). Solvents (HPLC grade) for the chromatographic assay and the rinsing of the chromatographic system were purchased from Carlo Erba (Rodano, MI, Italy). Purified water was obtained from Millipore Simplicity System (Milford, MA, USA).

2.2. Instrumentation

The chromatographic system consisted of a Shimadzu model LC-10AVP solvent delivery (Kyoto, Japan), an autosampler model SIL-10ADVP and a detector UV. The peak areas were integrated using a Shimadzu CR6A integrator. The analytical column was a Supelcosil™ LC-18 (25 cm × 4.6 mm × 5 μm, Supelco, Bellefonte PA, USA), with a C18 guard column (Waters Assoc., Milford, USA).

2.3. Chromatographic conditions

The mobile phase consisted of a mixture of 0.075 M acetate buffer, pH 5.0 and acetonitrile (92:8 v/v), was freshly prepared on the day of use, filtered trough a 0.45 μm filter and helium degassed for 3 min; the chromatographic analysis was performed in an isocratic system using a flow rate of 0.8 ml/min at room temperature. The injection volume was 5 μl and the effluent was monitored by an ultraviolet absorbance detector at 230 nm. A run time of 30 min was required to guarantee the selectivity of chromatographic analysis.

2.4. Preparations of standards and internal controls

Stock solutions of the antimicrobial agents were prepared to achieve 2 mg/ml for cefepime and 1 mg/ml for vancomycin and imipenem. Plasma standards containing a mixture of these agents for the calibration daily curve were prepared by adding of an appropriate volume from the stock solutions to drug-free plasma to obtain final concentrations equivalent to 200, 100, 50, 25, 12.5, 6.3, 3.1, 1.5 and 0.8 μg/ml of cefepime and 100, 50, 25, 12.5, 6.3, 3.1, 1.5, 0.8 and 0.4 μg/ml of vancomycin and imipenem, containing 0.25 ml of MOPS solution and stored at −80 °C until assay. Internal controls were prepared by specific dilution of the stock solution in drug-free plasma to obtain the following high, medium and low concentrations, respectively, 160, 80 and 3.2 μg/ml of cefepime or 80, 40 and 1.6 μg/ml of vancomycin and imipenem.

Cefuroxime (IS) stock solution was prepared to obtain 1 mg/ml and stored at −80 °C. The concentration of the working solution was of 40 μg/ml. The solution MOPS 10%, was prepared with ultrapure water, this solution was required to reduce degradation of antimicrobial agents.

2.5. Sample extraction procedure

Eppendorf tube was added of cefuroxime (100 μl), followed by plasma (200 μl) and 100 μl of MOPS. Mixture was vortexed for 10 s and the tube was added of acetonitrile (600 μl). Mixture was vortexed for 15 s, centrifuged at 6000 rpm at 4 °C for 40 min. Supernatant (400 μl) was transferred to a conic glass tube and the organic solvent was concentrated to dryness in a stream of purified nitrogen at 37 °C. Residue was dissolved with 200 μl of a mixture of acetonitrile:water (8:2, v/v) and a 5 μl volume were injected into HPLC.

2.6. Calibration curve and calculation procedures

The nominal value of cefepime, vancomycin and imipenem in plasma was plotted as a function of the peak area ratio obtained for each drug and its internal standard against the respective plasma concentration for each drug investigated. A linear regression line obtained and the estimated linear correlation coefficient was applied to each calibration curve prepared in duplicate for all standards (equation: $y = b + ax$, where x is the peak-height ratio, a the slope and b is the intercept). At least five from eight calibrators were considered for the construction of the daily calibration curve. The day curve was accepted, if at least 4/6 of the internal controls presenting systematic error lower than 15% (high, medium and low concentrations analysed in duplicate).

2.7. Accuracy, precision and recovery

Precision of a quantitative method is the degree of agreement among individual tests, when the procedure is applied repeatedly to analyse multiple replicates in three different concentrations, and expressed as coefficient of variation (CV%) from back calculated value subtracted from target value and divided by the target value, expressed as percentage. The intra-day precision was evaluated by analysis of 10 replicates of the high, medium and low concentrations, respectively, 160, 80 and 3.2 μg/ml of cefepime or 80, 40 and 1.6 μg/ml of vancomycin and imipenem. The inter-day precision was determined by the analysis of ten replicates of the high, medium and low concentrations, respectively, 160, 80 and 3.2 μg/ml of cefepime or 80, 40 and 1.6 μg/ml of vancomycin and imipenem, in 3 different days.

Accuracy was evaluated by analysis of multiple replicates ($n = 10$) in three different concentrations and expressed as percentage of inaccuracy, representing also the recovery of each drug expressed as systematic error. The parameter can be estimated by the value of the mean back-calculated concentrations divided by theoretical concentrations, expressed as percentage. The intra-day accuracy was evaluated by analysis of 10 replicates of the high, medium and low concentrations, respectively, 160, 80 and 3.2 μg/ml of cefepime or 80, 40 and 1.6 μg/ml of vancomycin and imipenem. The inter-day accuracy was determined by the analysis of 10 replicates of the high, medium and low concentrations of the antimicrobial agents in 3 different days.

Absolute recovery of antimicrobial drugs from plasma was estimated by the peak area integrated for each drug in plasma assayed, accordingly the procedure *versus* the peak area integrated for each drug, after direct injection of the same concentration in purified water; expressed as percentage. The efficiency of relative recovery was estimated by the peak area ratio integrated for each drug in plasma related to its internal

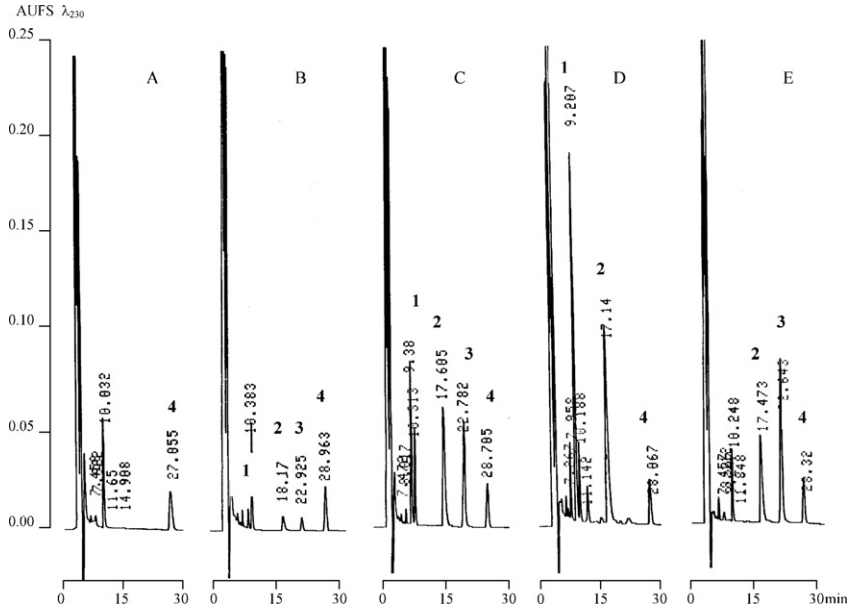

Fig. 1. Chromatographic profile of three antimicrobial agents in biological matrices, run time 30 min: (A) blank plasma with internal standard (IS); (B) spiked blank plasma/LOQ (cefepime 0.8 μg/ml, vancomycin and imipenem, 0.4 μg/ml); (C) spiked blank plasma (cefepime 8 μg/ml, vancomycin and imipenem, 4 μg/ml); (D) patient A treated with cefepime plus vancomycin; (E) patient B treated with vancomycin plus imipenem retention times (min) of peaks eluted were expressed as mean values: 9.3 min (cefepime) [1], 17.4 min (vancomycin) [2], 22.7 min (impenem) [3] and 28.4 min (IS: cefuroxime) [4].

standard, assayed accordingly the procedure *versus* the peak area ratio integrated for each drug after direct injection.

2.8. Specificity and selectivity

The specificity of an analytical method is its ability to measure accurately an analyte in the presence of endogenous compounds. The specificity was evaluated by the analysis of samples drug-free plasma (normal, hemolised, lipemic and icteric sample biological matrices) applying the analytical procedure, the retention times of endogenous compounds were compared with those obtained for cefepime, vancomycin, imipenem and the internal standard.

The selectivity of the method was investigated by testing several samples obtained from large burn patients receiving antimicrobial therapy with cefepime, vancomycin, imipenem plus ciprofloxacin, polymixine B, oxacillin, fluconazole and also other drugs as omeprazole, diazepam, dipirone, tramadol, hydrochlorothiazide and amitriptyline.

2.9. Limit of detection and limit of quantification

The limits of detection (LOD) and of quantification (LOQ) were determined based on the analysis of 10 replicates. The LOQ was defined as the lowest drug plasma concentration of the calibration daily curve which can be determined with an accuracy of 80–120% and precision lower than 20%. The LOD was defined as 0.5 times of the limit of quantification. In addition, the LOD presents a peak signal to noise of baseline ratio equivalent to 3:1, while the LOQ shows a ratio of 6:1.

2.10. Stability study

Short-term stability was performed at room temperature by repetition of several times of a sequence of injections up to 24 h; the study was done by testing a sequence of microvials on the rack of the autosampler containing plasma extracts in three different concentrations determined on the basis of a day curve.

Biological matrices spiked blank plasma, were analysed after three thawing cycles by HPLC in the same sequence after the clean up of plasma samples as detailed above, using three different concentrations (triplicate) during three consecutive periods. Data were expressed as percentage by the systematic error. The acceptance criterion for all concentrations studied was adopted as less than 10% variation.

2.11. Robustness

The robustness of the method was determined, by using two different chromatographic system connected to two different Supelcosil^{TM} LC-18 columns, small changes in the proportion of acetonitrile in the mobile phase and in the flow rate. The study was developed using two different concentrations (three replicates/each). Data were expressed by systematic error, as percentage.

2.12. Therapeutic drug monitoring of antimicrobial agents in burn patients

Two burn patients with wound infection were treated: patient (A) was a female, 17-year-old, burned raw area on 80% of his total body surface, vancomycin was administered by 1 h infu-

sion every 12 h at a dose of 1 g and cefepime 10 min infusion, 2 g every 8 h. Patient (B) was a male, 47-year-old, with burned raw area on 40% of his total body surface, also vancomycin was administered and imipenem infusion, 1 g every 6 h. Blood samples (2 ml/each) at the peak and trough of both patients were collected from femoral catheter, plasma was obtained by centrifugation and transferred to an Eppendorf tube containing MOPS, then samples were stored at −80 °C.

3. Results

Typical chromatograms of a blank, spiked and extracts obtained from the large burn patients are shown in Fig. 1. A good selectivity was demonstrated, since any peak of all drugs tested, were co-eluted in the retention times of cefepime, imipenem, vancomycin and also cefuroxime (IS).

A good linearity was obtained after the simultaneous analysis of all antimicrobial agents in plasma. Data for linearity studies was expressed by the intercept and also the slope of the linear function as mean, standard error of the mean (SEM) and the linear correlation coefficient (r^2) as follows: intercept 0.0284 (SEM: 0.0011) and slope 0.0587 (SEM: 0.0022) for cefepime (r^2: 0.996) over the 0.8–200 μg/ml; intercept 0.0296 (SEM: 0.0007) and slope 0.0944 (SEM: 0.0039) for vancomycin (r^2: 0.999) and also the intercept 0.0196 (SEM: 0.0007) and slope 0.0841 (SEM: 0.0029) for imipenem (r^2: 0.999), both over 0.4–100 μg/ml concentration range.

The LOQ was 0.8 μg/ml for cefepime and 0.4 μg/ml for vancomycin and imipenem. The LOD was 0.4 μg/ml for cefepime and 0.2 μg/ml for vancomycin and imipenem. Good results were obtained for accuracy, intra-day and inter-day precision and recovery presented in Table 1.

Short-term stability performed at room temperature of the cefepime, imipenem and vancomycin in the plasma extracts on the rack of autosampler was guaranteed up to 5 h. Three consecutive freeze–thaw cycles showed good stability for antibiotics from biological matrices stored at −80 °C in the ultra low freezer, Table 1.

Specificity of analytical method was confirmed, since no endogenous peaks were co-eluted with antimicrobials agents after extraction followed by chromatographic analysis of blank normal, lipemic, hemolised and icteric plasma samples. In addition, the selectivity of the method was investigated by testing several drugs commonly prescribed to the large burn patient; only hydrochlorothiazide and dypirone were eluted in the chromatographic run, but they did not interfere with the antibiotics investigated, Table 2.

3.1. Drug plasma monitoring

Data obtained by therapeutic plasma drug monitoring showed peak vancomycin concentration 11 and 35 μg/ml at the 1st h (20–40 μg/ml, reference) and trough concentrations of 2.0 and 3.7 μg/ml at 12th h (5–10 μg/ml, reference) for patients A and B, respectively. Cefepime plasma concentration in patient (A) was 3.8 μg/ml at 8th h (trough) lower than expected (10 μg/ml), while imipenem plasma concentration in-patient

Table 1
Validation of analytical method of cefepime, vancomycin and imipenem in plasma using the liquid chromatography

Parameter	Cefepime	Vancomycin	Imipenem
Linearity (μg/ml)	0.8–200	0.4–100	0.4–100
Linear correlation coefficient	$r^2 = 0.9994$	$r^2 = 0.9986$	$r^2 = 0.9996$
LOD (CV%)	0.38 ± 0.02	0.17 ± 0.01	0.21 ± 0.01
LOQ (CV%)	0.76 ± 0.04	0.40 ± 0.02	0.42 ± 0.01
Absolute recovery (%)	98.6	95.7	96.8
Relative recovery (%)	104.2	101.6	102.9
Precision/within-day (CV%)			
160 μg/ml	0.04		
80 μg/ml	0.46		
3.2 μg/ml	0.45		
80 μg/ml		0.99	1.36
40 μg/ml		2.40	1.40
1.6 μg/ml		0.91	2.18
Precision/between-day (CV%)			
160 μg/ml	0.55		
80 μg/ml	1.67		
3.2 μg/ml	2.53		
80 μg/ml		1.39	1.55
40 μg/ml		1.36	1.61
1.6 μg/ml		1.68	1.97
Accuracy/within-day (%)			
160 μg/ml	0.86		
80 μg/ml	1.58		
3.2 μg/ml	3.81		
80 μg/ml		1.37	1.45
40 μg/ml		2.39	1.27
1.6 μg/ml		3.37	2.10
Accuracy/between-day (%)			
160 μg/ml	1.89		
80 μg/ml	2.12		
3.2 μg/ml	4.95		
80 μg/ml		1.27	0.55
40 μg/ml		2.01	1.94
1.6 μg/ml		4.79	1.85
Stability/thawing cycles (%)	1.49	1.93	4.42

LOD: limit of detection; LOQ: limit of quantification; CV%: coefficient of variation; accuracy (SE): systematic error expressed as mean ± S.D. Recovery expressed as mean ± S.D. Thawing cycles (SE) systematic error expressed as mean values.

Table 2
Drugs commonly prescribed with the vancomycin, imipenem and cefepime—study of the specificity of the analytical method by HPLC-UV

Drug	Retention time (min)
Amitriptyline	N.D
Cefepime	9.3
Diazepam	N.D
Dipirone	26
Fluconazole	N.D
Hydrochlorothiazide	19
Imipenem	22.7
Omeprazole	N.D
Oxacillin	N.D
Polymixine B	N.D
Tramadol	N.D
Vancomycin	17.4

N.D: not detected in the run time of 30 min.

B was 2.3 μg/ml at 6th h (trough). Trough plasma levels of imipenem obtained for patient B reached the effective concentration recommended (1.0–1.7 μg/ml).

4. Discussion

Various methods to determine antimicrobial agents alone in plasma or serum by HPLC-UV have been reported in the literature, but any analytical procedure was described for the simultaneous analysis of cefepime, imipenem and vancomycin in plasma.

Biological matrices as serum or plasma [4,6–11], even urine [12] was described for cefepime, also for vancomycin [5,13–19] or even imipenem measurements [20–23].

The volume required in the assay for the quantification of drugs in biological matrices is a decisive factor to be considered for the choice of the analytical method. Our method and also some procedures described for vancomycin alone [5,17,19] or for cefepime alone [7] require lower volumes of plasma for the clean up of biological matrix. In addition, lower volumes of biological matrix were necessary by ultrafiltration of imipenem [23]. Concerning the validation of analytical methods reported previously, despite the small blood volume required, the method described by Valassis et al. showed low linearity and problems of sensitivity for cefepime and the method reported by Favetta et al. required an electrochemical detection.

Some procedures require also an oven at 35–40 °C [8,10] for the chromatographic measurements in biological matrices of humans.

Compared to other procedures reported in the literature, the present study describes an analytical method using HPLC-UV for the simultaneous determination of antimicrobial agents in plasma, which was found to be advantageous in terms of robustness, selectivity and sensitivity, guaranteeing stability, precision and accuracy.

Analytical method validated was applied for drug plasma monitoring of two large burn patients that showed subtherapeutic plasma levels for vancomycin and cefepime, once only imipenem trough levels were in the recommended range. Additionally, it was registered high loss of drugs dissolved in the vascular fluid through the area submitted to surgical debridement.

5. Conclusion

The advantage of the method described in the present study consists in the simultaneous determination of cefepime, vancomycin and imipenem in just one run, requiring low plasma volume, simple mobile phase and robust chromatographic assay; the short run time permits its application clinical studies including therapeutic drug monitoring and also pharmacokinetic studies in burn patients.

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References

[1] M.J. Weinbren, J. Antimicrob. Chemother. 44 (1999) 319.
[2] C.M. Bonapace, R.L. White, L.V. Friedrich, E.D. Norcross, Antimicrob. Agents Chemother. 43 (1999) 2848.
[3] E. Dailly, M.F. Kergueris, M. Pannier, P. Jolliet, Fundam. Clin. Pharmacol. 17 (2003) 645.
[4] R.H. Barbhaiya, S.T. Forgue, W.C. Shyu, E.A. Papp, Antimicrob. Agents Chemother. 31 (1986) 55.
[5] H. Elkhaili, L. Linger, H. Monteil, F. Jehl, J. Chromatogr. B 690 (1997) 181.
[6] M. Ip, C. Au, S.W. Cheung, C.Y. Chan, A.F.B. Cheng, J. Antimicrob. Chemother. 42 (1998) 121.
[7] I.N. Valassis, M. Parissi-Poulou, P. Macheras, J. Chromatogr. B 721 (1999) 249.
[8] B. Calahorra, M.A. Campanero, B. Sadaba, J.R. Azanza, Biomed. Chromatogr. 13 (1999) 272.
[9] D. Breilh, C. Lavallee, A. Fratta, D. Ducint, P. Cony-Mayhoul, J. Chromatogr. B 734 (1990) 121.
[10] N. Cherti, J.M. Kinowski, J.L. Lafrant, F. Bressolle, J. Chromatogr. B 754 (2001) 377.
[11] J.A. Ocaña González, F.J. Jiménez Palacios, M. Callejón Mochón, F.J. Barragán, J. Pharm. Biomed. Anal. 36 (2004) 117.
[12] A. Isla, A. Arrzuada, J. Maynar, A.R. Gascón, M.A. Solinis, E. Corral, J. Pharm. Biomed. Anal. 39 (2005) 996.
[13] F. Jehl, C. Gallion, R.C. Thierry, H. Monteil, Antimicrob. Agents Chemother. 27 (1985) 503.
[14] S.V. Green, T. Abdalla, S.L. Morgan, J. Chromatogr. 417 (1987) 121.
[15] H. Hosotsubo, J. Chromatogr. 487 (1989) 421.
[16] D. Farin, G.A. Piva, I. Gozlan, R. Kitzes-Cohen, J. Pharm. Biomed. Anal. 18 (1998) 367.
[17] D.W. Backes, H.I. Aboleneen, J.A. Simpson, J. Pharm. Biomed. Anal. 16 (1998) 1281.
[18] Furata, T. Kitahashi, T. Kuroda, H. Nishio, C. Oka, Clin. Chim. Acta 301 (2000) 31.
[19] P. Favetta, J. Guitton, N. Bleyzac, C. Dufresne, J. Bureau, J. Chromatogr. B 751 (2001) 377.
[20] D.A. Gravallese, D.G. Musson, L.T. Pauliukonis, W.F. Bayne, J. Chromatogr. 310 (1984) 71.
[21] C.M. Myers, J.L. Blumer, Antimicrob. Agents Chemother. 26 (1984) 78.
[22] G. Carlucci, L. Biordi, C. Vicentini, M. Bologna, J. Pharm. Biomed. Anal. 8 (1990) 283.
[23] L. Garcia-Capdevila, C. López-Calull, C. Arroyo, M.A. Moral, M.A. Mangues, J. Chromatogr. B 692 (1997) 127.