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Short communication

Validated high performance liquid chromatography–UV detection method for the determination of daptomycin in human plasma

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ABSTRACT

Daptomycin is the first approved member of the new class cyclic lipopeptide antibiotic drugs, effective against a broad spectrum of Gram-positive bacteria. Here we present an HPLC method with UV detection capable to obtain pharmacokinetic data of daptomycin in human plasma, exemplarily shown in a critically ill patient with acute renal failure undergoing extended daily dialysis. Sample preparation consists only of protein precipitation with methanol. Chromatographic separation was achieved on a Zorbax Eclipse XDB-C8 column and daptomycin was detected at 224 nm. The calibration function was linear over the range from 3.5 to 350 μg/ml. The relative standard deviations were < 2% in the intra-day and < 6% in the inter-day measurements. The accuracy was always better than 7%. Daptomycin was stable in aqueous solutions for 2 months frozen at–20 °C. However, in plasma frozen at −20 °C a loss of 25% in 1 month was observed.

1. Introduction

Daptomycin (Cubicin®, Novartis Pharma, Germany) is the first approved member of the new class of cyclic lipopeptide antibiotic drugs with a broad spectrum of activity against Gram-positive bacteria [1,2]. The outcome of a therapy with daptomycin in clinical practice is comparable with standard antibiotic treatment [3]. However, daptomycin shows additional activity against multi-resistant bacterial strains like methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant enterococci or penicillin-resistant *Streptococcus pneumoniae* [4] and it is therefore a viable alternative for the treatment of persisting infections [5]. Because of its efficacy and safety in a variety of infectious conditions, this is especially true in critical care settings [6]. However, emerging resistances have been reported for daptomycin also [2]. The plasma half-life of daptomycin is relatively long with about 9 h and the excretion of the drug occurs mainly via the kidneys, requiring a dose adjustment in patients with chronic kidney disease stages 4 and 5, i.e. glomerular filtration rates of <30 ml/min [7]. Because of its high plasma protein binding of about 92% [8], extracorporeal clearance by dialysis eliminates only a small fraction (about 10–15%) of the drug, depending on the dialysis method applied [7]. However, reports on the pharmacokinetics of daptomycin in patients with renal and/or multi-organ failure, as they are common in critical care settings, are scarce [9]. To gain more insight in this important field, a simple and reliable method for the determination of daptomycin in human plasma is required. Besides a microbiological assay [8], some chromatographic methods comprising HPLC with UV detection are reported in the literature [10–12]. These methods rely on quite similar approaches, but unfortunately, in none of the publications the full validation data and detailed descriptions of the analytical procedures were provided. Here, we present for the first time a very easy, fully validated HPLC–UV method for the determination of daptomycin in human plasma, which requires only minor laboratory efforts and leads to reliable, precise and accurate results. Special attention has been drawn on the stability of daptomycin in the different stages of analysis and on minimizing laboratory workload.

2. Materials and methods

2.1. Instrumentation

The HPLC system consisted of an Agilent 1100 system (Waldbronn, Germany) comprising a binary pump, an autosampler, a thermostated column compartment and a diode array UV–vis detector. The analytical column was a Zorbax Eclipse XDB-C8 150 mm × 4.6 mm 5 μm particle size (Agilent Technologies, Böblingen, Germany), protected by a SecurityGuard system (Phenomenex,

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Aschaffenburg, Germany) equipped with a 4 mm × 3 mm C8 filter insert.

2.2. Chemicals

Pure daptomycin was purchased from Toronto Research Chemicals (North York, ON, Canada). Methanol, acetonitrile, trifluoroacetic acid and triethylamine were of analytical grade or better. Ultra pure water was produced in our laboratory by a Barnstead EASYpure UV-system (Werner, Leverkusen, Germany). Drug-free human plasma was obtained from the blood bank of the University Hospital Magdeburg (Germany).

2.3. Patient samples

As part of a single shot prospective pharmacokinetic study samples of a 44-year-old male critically ill patient with acute renal failure who received 6 mg/kg daptomycin during a 30 min infusion were drawn into EDTA-plasma sampling tubes. Samples were obtained before infusion, after the end of the infusion and 0.5, 1, 2, 4, 6, and 8 h thereafter. Eight hours after the end of the infusion extended daily dialysis with a GENIUS batch dialysis system using a 1.3 m^2 polysulfone dialyser (F 60 S, Fresenius Medical Care, Germany) was performed. Samples were drawn at 0.5, 2, 4, 6 and 8 h during the dialysis, and afterwards at 0.5, 2.5, 7.5, 11.5, and 18.5 h. Informed consent was obtained prior to the blood sampling.

2.4. Calibration- and quality control samples

A daptomycin stock solution was prepared by dissolving 35 mg daptomycin in 10 ml water, leading to a concentration of 3500 μg/ml. From this stock solution 100 μl were diluted by 900 μl water, resulting in a working solution with a concentration of 350 μg/ml. From the working solution 10, 20 and 40 μl and from the stock solution 10, 20, 40 and 100 μl were added up with drug-free human plasma to 1000 μl to result in calibration samples with concentrations of 3.5, 7, 14, 35, 70, 140 and 350 μg/ml. In a similar manner, pools of quality control samples at the levels of 3.5, 35 and 350 μg/ml were prepared and aliquots were stored at −20 °C until usage.

2.5. Sample preparation

To 100 μl of calibration-, quality control- or patient samples, 200 μl methanol was added and the samples were mixed for 5 s on a vortex mixer. To complete protein precipitation, the samples were left at room temperature for 15 min. The samples were subsequently centrifuged at 9100 × *g* for 10 min and the clear supernatant was transferred into vials for the HPLC-autosampler.

2.6. Chromatography and detection

The mobile phase for the chromatographic separation of daptomycin was mixed from buffer solution and acetonitrile. The buffer solution consisted of 20 mM trifluoroacetic acid and 15 mM triethylamine, resulting in a pH of about 3.5. A shallow gradient starting at 30% acetonitrile, rising to 40% in 5 min, then constant until 11 min, was applied. After 11 min, the column was flushed for 3 min with 100% acetonitrile. The flow rate was constant at 1 ml/min and the column temperature was set to 30 °C. The injection volume was 50 μl. Detection took place at three different wavelengths: 224, 282 and 370 nm. For quantification, peak areas of daptomycin at 224 nm were evaluated.

2.7. Extraction yield

For the determination of the extraction yield six plasma samples from three different individual sources were spiked with 350 μg/ml daptomycin according to Section 2.4 and were extracted following the procedure described in Section 2.5. The mean peak areas ± S.D. of these samples were compared to those of six samples corresponding to 100% extraction yield, consisting of 350 μg/ml daptomycin in water, prepared in the same way as plasma samples.

2.8. Precision and accuracy

For the determination of the intra-day precision and accuracy, quality control samples underwent 10 times in each of the three concentration levels the whole sample preparation process and were quantificated in a single analysis batch. For the inter-day precision and accuracy the three levels of quality control samples were prepared and quantificated on six different days. Mean measured concentration levels, R.S.D.s and deviations from estimated concentrations (accuracy) were given in Table 1.

2.9. Stability of daptomycin

The stability of daptomycin in stock solutions was checked by comparing a freshly prepared stock solution with one stored for 2 months at −20 °C. For this purpose, three samples each of the new and old stock solutions were diluted with water by a factor of 10 and the samples were injected without further preparation into the HPLC system. Relative concentrations were computed by comparing the mean of the peak areas of each stock solution.

The stability of daptomycin in plasma samples was tested by repeated ($n = 6$) quantifications of quality control samples stored at −20 °C over a time range of 8 days. Stability over a longer time range was tested by reanalyzing patient samples ($n = 19$) after a storage time of 1 month. The stability of daptomycin in plasma at room temperature was tested by repeated processing and quantification of quality control samples ($n = 3$) after 1, 3 and 7 h.

Stability of daptomycin in processed samples was evaluated by a repeated measurement of sets of quality control samples ($n = 3$). The quantification results of the first injections were compared to those of a second injection after leaving the sample for 24 h in the autosampler at room temperature.

2.10. Statistical calculations

Statistical calculations were performed with the SPSS software package version 15.0. Regression analysis was performed to evaluate the significance of the calibration parameters and Spearman-Rho correlation analysis to evaluate significant correlations between daptomycin concentration vs. time in the stability testing. Significance was assumed at $p < 0.05$.

3. Results and discussion

3.1. Sample preparation

The sample preparation procedure consisted only of protein precipitation by the addition of methanol to the plasma samples and subsequent centrifugation. Despite this simple procedure, sufficiently clean extracts were received (see next paragraph). The extraction yield was found to be quasi-quantitative with a recovery of 102.9 ± 1.3%, compared to aqueous samples in concentrations resembling 100% extraction yield. Other protein precipitation procedures such as the addition of perchloric acid or acetonitrile led

Table 1
Intra- and inter-day precision and accuracy

Level	Spike concentration (μg/ml)	Found concentration (μg/ml)	R.S.D. (%)	Accuracy (%)
Intra-day (n = 10)				
Low	3.5	3.45	1.15	−1.51
Medium	35	35.10	1.41	0.29
High	350	335.83	1.17	−4.05
Inter-day (n = 6)				
Low	3.5	3.74	5.69	6.93
Medium	35	35.22	3.37	0.64
High	350	359.99	2.07	2.86

to reduced recoveries, probably due to co-precipitation of protein bound daptomycin.

In our procedure, no internal standard was added, because it is not easy to find a molecule of similar molecular size, polarity and protein binding in comparison with daptomycin. Vancomycin could be a reasonable internal standard, albeit its less lipophilic behavior. However, we refrained from using it as I.S. because it is quite possible that unknown patient samples contain vancomycin as well as daptomycin, especially in patients with renal impairment. The compound ethylparaben, which was used as I.S. in previously reported methods for the determination of daptomycin [10–12], is, in our opinion, not an optimal choice because it shares practically no similarities in terms of molecular structure and size, protein binding and polarity. Due to such dissimilarities between ethylparaben and daptomycin, ethylparaben cannot safely correct for possible extraction losses of daptomycin in the protein precipitation step and provides a false sense of security about the performance of the sample preparation. The pipetting step required for the addition of the I.S. and the integration of its chromatographic peak are both additional sources of imprecision in the assay. Furthermore, ethylparaben is used in pharmaceutical preparations as a preservative and it can therefore not be excluded that it is present in unknown amounts in patient samples. Conclusively, ethylparaben only adds to the overall imprecision and inaccuracy of the procedure. On the other hand, a sample preparation procedure as the one here described with nearly quantitative recovery and stability between different individuals and the usage of modern HPLC equipment with an automatic sample injection device leaves the addition of an I.S. redundant.

3.2. Chromatography and detection

Under the described conditions, daptomycin resulted in a sharp and symmetrical peak at a retention time of 9.2 min, free from interferences by endogenous substances (see Fig. 1). This favorable result was achieved by the presence of trifluoroacetic acid and triethylamine to the mobile phase, which prevented second-order interactions with the stationary phase and therefore tailing of the daptomycin peak. The injection of 50 μl of samples containing two-thirds of methanol resulted in a slightly fronting peak shape, which could be refocused by the application of a shallow gradient profile to the mobile phase. To our experience, it is very advisable to flush the column with pure acetonitrile after each run to prevent buildup of strongly retained substances on the column. Without such a flushing step, the daptomycin peak shape deteriorates after a few injections considerably. Applying such a flushing step and exchange of the guard column every 70–100 sample injections, the separation quality of the analytical system remained constant over many hundreds of injections.

Daptomycin features a very distinctive UV-absorption spectrum with absorption maxima at 224, 264, 282 and 370 nm (see Fig. 2). It could be assumed that at the longest wavelengths the most selective chromatograms could be recorded. However, it was found that even at the shortest wavelength no interferences with the daptomycin peak appeared. On the other hand, at the shortest wavelength the absorption is most intensive, resulting in lower relative standard deviation in the validation measurements. Hence, only the absorption maximum at 224 nm was used for quantification, whereas the other wavelengths were used as peak identification qualifiers. Selectivity of the assay was proven by

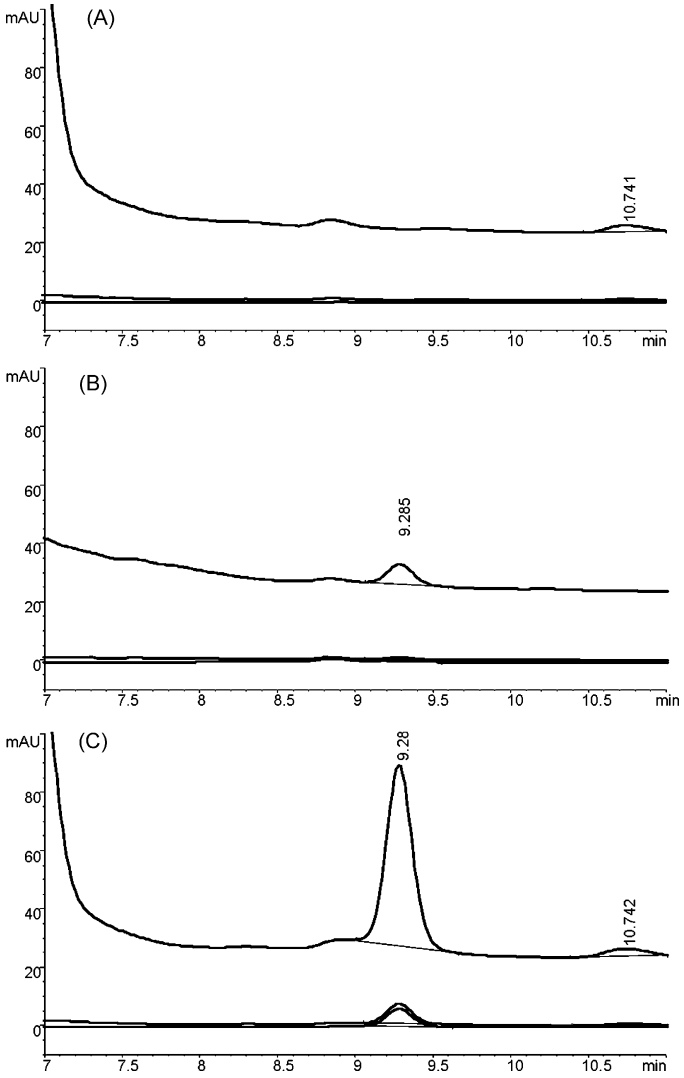

Fig. 1. Typical chromatograms obtained from human plasma. The three chromatographic traces in each chromatogram refer to 224, 282 and 370 nm, from top to bottom. (A) Blank patient sample before the administration of daptomycin; (B) calibration level 3.5 μg/ml daptomycin; (C) patient sample obtained 2 h after the end of a 6 mg/kg daptomycin infusion containing 28.8 μg/ml daptomycin.

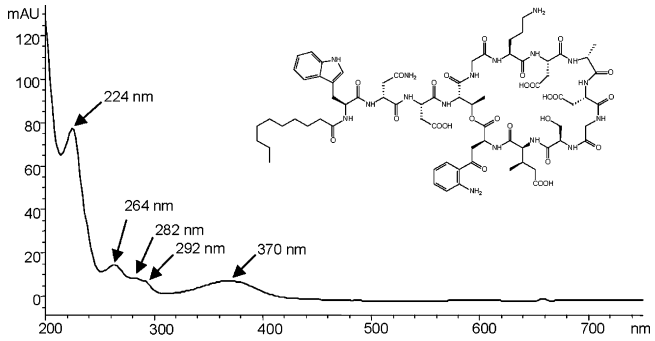

Fig. 2. UV-absorption spectrum and molecular structure of daptomycin. The maxima in the UV-spectrum are marked with their respective wavelengths.

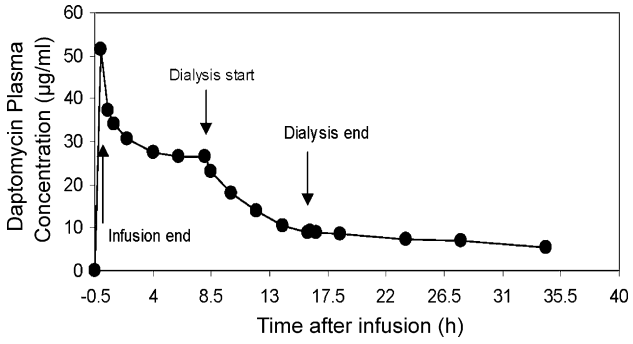

Fig. 3. Concentration–time curve of daptomycin in a critically ill patient with acute renal failure. Infused dose was 6 mg/kg. The marks depict the beginning and end of the 8 h extended daily dialysis.

the fact that in a large number of samples from different patients the daptomycin peak always remained undisturbed and that its UV-spectrum always was in accordance to the reference spectrum. Quantification errors by carry-over effects were ruled out by the fact that the injection of a blank sample directly after the injection of the highest calibration level ($n = 3$ different plasma sources used) resulted in the non-detection of the daptomycin peak.

3.3. Calibration

The calibration samples covered the concentration range from 3.5 to 350 μg/ml. This range was sufficient to quantify clinical samples from intensive care patients over a time course of more than 24 h after administration. The calibration function was linear with an insignificant ($p = 0.422$) intercept of -0.468 ± 1.416. The slope was calculated to 2.161 ± 0.010. Owing to the concentration dependently increasing standard deviation (see Table 1), weighing of the calibration levels with a factor of 1/concentration was applied. The limit of quantification was set to the lower limit of the calibration range, i.e. 3.5 μg/ml. The limit of detection was found to be about 0.5 μg/ml (S/N ratio 3).

3.4. Precision and accuracy

In Table 1 the precision and accuracy data for the intra-day and inter-day validation measurements are summarized. These measurements were accomplished using quality control samples, which were prepared prior to all measurements and were stored at −20 °C. As it can be seen, all parameters are satisfying the acceptance criteria for biochemical assays [13].

3.5. Stability of daptomycin

Daptomycin is known to be susceptible to hydrolysis in alkaline solutions [14]. Therefore, we investigated the stability of daptomycin under various conditions.

Stock solutions of daptomycin in water were stored at −20 °C until usage. After a storage time of 2 months, the concentration of a stock solution was compared with a freshly prepared one ($n = 3$). The difference between these two stock solutions was found to be 1.1%. Therefore, the stock solutions can be regarded as stable for at least 2 months at −20 °C. The stability of the prepared samples in the autosampler was investigated by comparing the results of the immediate determinations with measurements of the same samples after 24 h at room temperature. Under these conditions, a loss in daptomycin concentration of 2.2% was found. Hence, it is possible to do overnight measurements without special precautions such as sample cooling. However, in plasma the stability of daptomycin is limited. Plasma samples left over a time period of 7 h at room temperature showed no significant concentration loss. However, over the course of 8 days, the concentrations of daptomycin in samples frozen at −20 °C declined significantly ($p < 0.05$) for about 5%. A longer storage time at −20 °C for 4 weeks resulted in an average loss of 25% of the originally determined daptomycin concentration. It is therefore very advisable to measure plasma samples as quickly as possible to obtain reliable results.

3.6. Clinical application of the method

The described method is currently used to obtain pharmacokinetic data of daptomycin in critically ill patients with acute renal failure undergoing various modes of renal replacement therapy including intermittent hemodialysis, extended daily dialysis and continuous venous hemofiltration. As an example, in Fig. 3 the time–concentration course of daptomycin after a single dose of 6 mg/kg in a patient with acute renal failure is depicted. As can be seen, after a dispersion phase directly after the infusion, a slow elimination phase follows. The elimination rate of daptomycin is accelerated during the dialysis session, and slowed down again afterwards. Such a pharmacokinetic pattern demonstrated the possibility of extracorporeal elimination of daptomycin during extended daily dialysis.

4. Conclusion

The here presented method for the determination of daptomycin is precise, reliable and occupies only very little laboratory workforce. It is capable to measure pharmacokinetic pattern of daptomycin over a time course of more than 30 h. It is currently used to monitor critically ill patients who undergo various modes of renal replacement therapy.

The full validation of the method with focus on the stability of daptomycin in the different stages of the analytical process revealed that daptomycin is not stable in plasma samples over prolonged time courses. A loss of about 25% in 1 month can be estimated in plasma samples stored at −20 °C. Therefore, it is very advisable to analyze plasma samples as quickly as possible to get reliable results.

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