# Determination of Daptomycin in human plasma and breast milk by UPLC/MS-MS

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## Abstract

During the lactation, the choice of a proper antibiotic is crucial since the drug can cross into breast milk causing toxicity to the infant. Therefore, an extraction protocol and LC/MS-MS method for the determination of daptomycin in human milk and plasma were developed, validated and applied to a case of a breastfeeding mother affected by a purulent acute soft skin infection treated with daptomycin. Because of daptomycin high protein binding and its high molecular weight, the optimisation of the extraction protocol and analytical conditions were deeply investigated, and several parameters were taken into account: in particular the type of extraction, internal standard, the type of organic modifier, pH of the aqueous solution, and gradient. The use of a protein precipitation protocol coupled to a C8-reverse phase LC-MS/MS allows for a reliable quantification of daptomycin in both plasma (in the range of 19-199 μg/ml) and breast milk (in the range of 0.12-0.32 μg/ml). The determination of milk/plasma (M/P) ratio, which ranged from 0.002 to 0.006, allowed to assess that daptomycin, effective for the mother, was contemporarily safe for the breastfed newborn.



## Keywords

Daptomycin, human milk, extraction, LC/MS-MS, soft skin infection.

## 1. Introduction

Daptomycin is a cyclic lipopeptide antibiotic with a molecular weight of 1620.67 Da, produced by *Streptomyces roseosporus*, with established activity against aerobic and anaerobic Gram-positive pathogens [1,2]. The daptomycin mechanism of action is unique since it binds to the bacterial cell membrane causing a potassium efflux which leads to its depolarization. Disruption of DNA, RNA and protein synthesis subsequently occurs provoking rapid concentration-dependent pathogen death [3,4]. Daptomycin is mostly distributed in the extracellular fluid since it cannot cross the cell membrane. The drug displayed an extensive (90-93%) but reversible bound to serum albumin and human plasma proteins in a concentration-independent manner [5].

Daptomycin was authorised in 2003 by the FDA for the treatment of 1) complicated skin and skin structure infections at a dose of 4 mg/kg every 24 hours intravenously, and 2) bloodstream infections (bacteraemia) at a dose of 6 mg/kg every 24 hours intravenously [6].

From an analytical point of view, in the literature, some HPLC methods to determine this antibiotic in biological fluids such as serum [7-9], plasma [10-13] and blood [14] are reported.



To the best of our knowledge, a unique case report has been published on daptomycin concentration in human milk after parenteral administration to the nursing mother [15]. Therefore, we developed and validated an extraction protocol and UPLC/MS-MS method for the determination of daptomycin in human milk and plasma, that was applied to a case of a breastfeeding mother affected by a purulent acute soft skin infection treated with daptomycin caused by methicillin-resistant *Staphylococcus aureus* (MRSA) [16]. Due to the increasing resistance of bacteria to these antimicrobial drugs, the choice of the appropriate drug can be crucial. The possible excretion of the antibiotic into milk, during breastfeeding, must be considered by clinicians to avoid side effects in the child [17]. Thus, the calculation of the milk/plasma (M/P) ratio was necessary to evaluate 1) the efficiency of daptomycin for the mother and 2) the Relative Infant Dose (RID) for the breastfed newborn.

## 2. Materials and methods

### *2.1 Reagents and chemicals*

Methanol, acetonitrile, formic acid, ammonium formate, reserpine (all analytical grade > 99%) were purchased from Sigma-Aldrich (St.Louis, USA). Standard solution of daptomycin and erythromycin were prepared using certified reference material purchased from Sigma-Aldrich (St.Louis, USA). Water (18.2Ω $cm^{-1}$) was prepared using a Milli-Q System (Millipore, Darmstadt, Germany).

### *2.2 Preparation of standard stock solutions*

Daptomycin standard stock solution was obtained dissolving 1 mg of daptomycin in 1 ml methanol. This solution was diluted in methanol 1:10, 1:100 or 1:1000 gaining working solutions 100 μg/ml, 10 μg/ml and 1 μg/ml respectively. Erythromycin standard stock solution was obtained dissolving 1 mg of erythromycin in 1 ml methanol. This solution was diluted in methanol 1:100 or 1:1000 gaining working solutions 10 μg/ml (IS used for plasma samples) and 1 μg/ml respectively (IS used for milk samples). Stock solutions were stored at 2-8°C, protected from direct light in amber glass containers.



### *2.3 Clinical samples*

Five blood samples and nine breast milk samples were collected from a *neo*-mother hospitalized for complicated erysipelas of the left arm. Breast milk was obtained by breast milk pumping machine whereas plasma was harvested from blood by centrifugation (10 000 xg for 10 min). The therapeutic plan consisted of 14 days of intravenous administration of daptomycin 500 mg/day coupled with meropenem 1g/3 times a day. The pharmacological study was limited to 5 days: the last four days of treatment and the following one free of therapy. Each sample represents a different

time-point and they were referred as hours after the eleventh administration of daptomycin which was considered our time-zero point ($T_0$). Daptomycin was administered at 0, 24, 48 and 72 hours. Breast milk samples were collected at nine time-points: 0, 6, 12, 48, 72, 78, 84, 96 and 120 hours. Plasma samples were collected at five time-points: 72, 78, 84, 96 and 120 hours.

*2.4 Sample pre-treatment*

2.4.1 Plasma extraction

100 μL of plasma were diluted with 900 μL saline. 100 μL of diluted plasma was added with 100 μL of IS (erythromycin in methanol solution 1 μg/mL) and 200 μL of methanol. Samples were sonicated in an ultrasonic bath for 15 minutes and centrifuged for 10 min at 0.805 x g. The supernatant was withdrawn, centrifuged for others 15 min at 0.805 x g and filtered on a 0.45 μm nylon filter membrane. 10 μL were injected for LC/MS-MS analysis.

2.4.2 Breast milk extraction



200 μL of breast milk was added with 100 μL of IS (erythromycin in acetonitrile solution 10 μg/mL) and 300 μL of acetonitrile. Samples were sonicated in an ultrasonic bath for 15 minutes and centrifuged for 15 min at 0.805 × g. The supernatant was withdrawn, centrifuged for others 10 min at 0.805 x g and filtered on a 0.45 μm nylon filter membrane. 10 μL were injected for LC/MS-MS analysis.

*2.5 Preparation of sample for method validation*

Standard samples containing different daptomycin amounts (for breast milk 100, 250, 500 and 750 ng/ml and plasma 0.25, 5, 10 and 25 μg/ml) were prepared by adding suitable volumes of working solutions of daptomycin to drug-free plasma (100 μL) or milk (200 μL). The standard samples for method validation were treated as reported in the "sample pre-treatment" section.

*2.6 LC/MS-MS conditions*

The analytical system consisted of an HPLC coupled to a tandem mass spectrometer. The liquid chromatography system was an Acquity UPLC Class System (Waters Corporation, Manchester, UK) equipped with two chromatographic pumps and an autosampler. Separation was attained on a reversed-phase Agilent Poroshell 120 SB-C8 100 mm x 2.1 mm x 2.7 μm particle size (Santa Clara, USA) analytical column, preceded by a security guard cartridge. For plasma samples, the linear gradient was between eluent A (ammonium formate 10mM + 0.1% formic acid) and eluent B (acetonitrile). The column was equilibrated with 20% (B) for 2 min and increased to 80% (B) in 1 min, held for 5 min, back to the initial conditions in 1 min and kept for 1 min at 20% (B). The retention times were 3.30 (daptomycin) and 3.50 (IS) ± 0.3 min. For breast milk samples the linear gradient was between eluent A (water + 0.1% formic acid) and eluent B (acetonitrile). The column was equilibrated with 5% (B) for 3 min and increased to 99% (B) in 0.2 min, held for 3 min, back to the initial conditions in 1 min and kept for 2 min at 5% (B). The retention times were 4.30 (daptomycin) and 4.32 (IS) ± 0.3 min. The flow rate was 0.3 mL/min and the column oven was kept at 40°C. The tandem mass spectrometer was a triple quadrupole Acquity TQD by Waters Corporation (Milford, USA) with electrospray ionization (ESI) Z-spray ion source. Instruments were managed with the proprietary manufacturer's software MassLynx 4.1 according to the manufacturer's instructions. The capillary voltage was set at 1.5 kV and the source temperature was set at 150 °C. The dwell time was set at 0.3 s, and the MS analysis was performed in positive ion mode (ESI+). Nitrogen was used as desolvation and cone gas with a flow of 500 L/h at 350°C, whereas argon as collision ones. The product ion spectrum (MS-MS) of each analyte was generated *via* direct infusion to obtain optimised compound-dependent parameters (Table 1): cone voltage (V) and collision energies (eV). Multiple reaction monitoring (MRM) mode was used.



**3. Results and Discussion**

*3.1 Method development*

3.1.1 Internal standard

The choice of internal standard ranged between reserpine (from literature) and erythromycin. Reserpine was immediately discharged due to its strong insolubility in water. Despite the structural diversity, erythromycin and daptomycin showed a similar behaviour during extraction and chromatographic separation (see paragraphs below, Figure 1).

3.1.2 Extraction optimisation

Different approaches were tried such as solid-phase extraction (SPE) and precipitation technique (using methanol and acetonitrile) for determination of daptomycin in biological matrices. SPE was performed using polymeric phase OASIS HLB cartridges (Waters Corporation, Manchester, UK). Before use, the cartridges were conditioned with 2 mL methanol and 2 mL deionized water. After sample loading, the cartridges were then rinsed with 2 mL deionized water with 5% methanol and vacuum-dried for 5 min to remove excess water. Then, the retained compounds were eluted with 2 mL of methanol and the eluate was evaporated until dryness. Finally, it was reconstituted with phase B. Captiva ND$^{lipid}$ filtration plates (Agilent Technologies, Santa Clara, CA) were evaluated in order to retain undesirable matrix interferences, lipids and proteins. 50-200 µL of the biological matrix was added with methanol acidified with 0.1% formic acid (1:3) and vacuum-filtered. The eluate was collected in a test tube and evaporated. These procedures demonstrated an incomplete recovery of analytes, due to their weak interactions with SPE phase and their high hydrophilic nature. Process efficiencies (calculated according to Matuszewski [18]) were for SPE about 40% and for filtration plates about 18%. A simple protein precipitation protocol (described in “sample pre-treatment”) fulfilled the expectation and was employed in the extraction of daptomycin in plasma and milk samples using as precipitation agents methanol and acetonitrile respectively. Process efficiencies [18] were evaluated in plasma 77±1.5% (*n*=3) for erythromycin and 81±2.7%

(*n*=3) for daptomycin respectively whereas for breast milk 79±3.5% (*n*=3) for erythromycin and 85±5.1.% (*n*=3) for daptomycin.

3.1.3 Chromatographic optimisation

To optimise the proposed LC–MS/MS method, the effects of several chromatographic parameters were investigated. These included the type of organic modifier, pH of the aqueous solution, and gradient. These parameters were optimised based on the peak shape, peak area, peak resolution and retention time for the analytes on Agilent Poroshell 120 SB-C8 (100 mm x 2.1 mm x 2.7 μm) column. Regarding organic modifier, methanol and ethanol were evaluated, but acetonitrile was found to have better performances in peak shape and resolution. Moreover, the introduction of ammonium formate (5-10 mM) and formic acid (0,1%) in organic phase did not provide any benefits. Importantly it has been noticed that the peak shape and the intensity of noise were matrix-dependent and correlated with % of eluent A during the chromatographic run. Thus, eluent A was changed between plasma and milk samples using ammonium formate 10mM + 0.1% formic acid and water + 0.1% formic acid respectively. In the same way, the gradient was adjusted as described in "LC/MS-MS conditions" (Figure 2).



*3.2 Method validation*

Validation protocol, adapted from EMEA guideline [19], included parameters such as specificity, precision, accuracy, linearity, limit of detection (LOD) and limit of quantification (LOQ). The specificity was assessed, in both plasma (*n*=6) and milk (*n*=6), by extracting blank control samples, control blank samples added in IS and solvents in each validation run. The lack of interfering peaks at the same analyte retention times was considered as an acceptable selectivity (Figure 3). LOQ is the lowest concentration that met at least five times the signal of a blank sample. LOD was not defined by EMEA guidelines, thus it was considered as the value that encounters a signal-to-noise (evaluated by MassLynx 4.1 software) ratio greater than 3 [20]. Validation parameters for precision

and accuracy were calculated using different replicates of samples in different working days. Accuracy was expressed as the percent recovery (REC%), while precision was measured as coefficient of variation (CV%). A CV% below 10% and REC% between 80-120% were considered suitable. Accuracy and precision were determined at four different concentration at LOQ, low, medium and high depending on matrixes: for breast milk 100, 250, 500 and 750 ng/ml and for plasma 0.25, 5, 10 and 25 μg/ml respectively. Five-point matrix-matched calibration curves were calculated by plotting peak area of quantifier ion of daptomycin/area IS (erythromycin) vs the total amount of daptomycin added in the range 0.25-200 μg/ml for plasma samples 100-1000 ng/ml for milk ones. The linearity was proven according to the regression line by the method of least squares and expressed by the coefficient of correlation ($R^2$). Method validation results were listed in Table 2.

*3.3 Application on clinical samples*



The validated method was successfully applied to quantify daptomycin in plasma and milk of a patient. To ensure a reliable instrumental performance clinical samples were run with day quality control samples (LOQ, low, medium and high) and blank samples. The concentration of daptomycin in these clinical samples fall within the linearity range of the method in both matrixes: plasma samples ranged between 19 to 199 μg/ml whereas milk ones between 0.12 to 0.32 μg/ml (Table 3). The aim was to identify its accumulation in milk and the related toxicity for the child after breastfeeding. From obtained results, daptomycin excretion in milk was confirmed and marked differences in concentration were observed between the two different biological matrices (Figure 4). $C_{max}$ was found to be, at 78h after the eleventh administration, with a concentration of 199 μg/mL in plasma and 0.329 μg/mL in milk. Moreover, milk/plasma concentration ratio (M/P) was calculated in order to investigate an eventual bio-distribution in milk and a maximum of 0.05 was found [16]. These M/P ratios (<<1) indicate an irrelevant accumulation in milk with no significant toxicity for

the child. AUC calculated in the final region of the pharmacokinetics profile ($AUC_{72\text{-}120h}$) agrees with previous findings:  3315 for plasma vs 11.3 for milk. For ethical reasons, daptomycin could not be quantified, as a direct evaluation, in blood samples from the child. However, the theoretical absolute infant dose (AID) was calculated as the product of the median milk concentration and assumed milk intake of 0.15 l $kg^{-1}$ $day^{-1}$ resulting 36.5 µg $kg^{-1}$ $day^{-1}$. The relative infant dose estimated as AID expressed as a percentage of the maternal dose in µg $kg^{-1}$ $day^{-1}$, was 0.50%, well below the most common accepted cut-off of 10% of the weight-adjusted maternal dose [16]. Overall, these data suggested that daptomycin is excreted into human milk at a low concentration due to daptomycin's high protein binding and its high molecular weight.

The determination of milk/plasma (M/P) ratio, which ranged from 0.002 to 0.006, allowed to assess that daptomycin was effective for the mother, in accordance with the study by Dvorchik et al. [10] in which safety and tolerability of daptomycin were evaluated by monitoring adverse events (AEs) and laboratory parameters. Daptomycin pharmacokinetics was linear through 6 mg/kg. Daptomycin protein binding (mean amount bound, 91.7%) was independent of the drug concentration. No gender effect was observed and there were no serious AEs and no pattern of dose-related events. Daptomycin was well tolerated when it was administered once daily at a dose as high as 8 mg/kg for 14 days. Daptomycin was contemporarily safe for the breastfed newborn as highlighted in the study by Buitrago et al. [15], in which a case of pelvic inflammatory disease (PID) caused by methicillin-resistant Staphylococcus aureus, in a breastfeeding mother who was successfully treated with daptomycin is described. Daptomycin concentrations in her breast milk were measured to determine potential exposure to her infant. These concentrations were extremely low, with an estimated milk:plasma ratio of 0.0012, in accordance with the values calculated in the present study. So, as the authors conclude, although additional confirmatory studies are needed, daptomycin may be a reasonable option in the treatment of PID caused by gram-positive organisms that are resistant to other antibiotics.

## 5. Conclusions

An extraction protocol and UPLC/MS-MS method for the determination of daptomycin in human milk and plasma was developed, validated and applied to a case of a breastfeeding mother affected by a purulent acute soft skin infection treated with daptomycin. Daptomycin excretion in milk was confirmed, but marked differences in concentration were observed between the two different biological matrices. M/P was calculated and a maximum of 0.05 was found. These M/P ratios (<<1) indicate an irrelevant retaining in milk with no significant toxicity for the child [16].

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**Tables**

**Table 1.** Mass spectrometry conditions for each analyte, in **bold** transition used for quantification

| Analytes | Transition | Cone (V) | Collision (eV) |
|---|---|---|---|
| Daptomycin | **811.10 > 341.00** | **36** | **56** |
| | 811.10 > 159.13 | 36 | 34 |
| | 811.10 > 313.26 | 36 | 26 |
| Erythromycin (IS) | **733.00 > 158.09** | **34** | **32** |

**Table 2.** Method validation results in both plasma and breast milk samples.

| **Paramet**ers | Plasma | Breast milk |
|---|---|---|
| LOD | 0.10 µg/ml | 50 ng/ml |
| LOQ | 0.25 µg/ml | 100 ng/ml |
| Precision | | |
| Intraday (overall) | CV%=3.5 (*n*=12) | CV%=3.7 (*n*=12) |
| LOQ | CV%=6.1 (0.25 µg/ml, *n*=3) | CV%=7.4 (100 ng/ml, *n*=3) |
| Low | CV%=1.9 (5 µg/ml, *n*=3) | CV%=5.2 (250 ng/ml, *n*=3) |
| Medium | CV%=0.2 (50 µg/ml, *n*=3) | CV%=1.4 (500 ng/ml, *n*=3) |
| High | CV%=5.7 (100 µg/ml, *n*=3) | CV%=1.1 (750 ng/ml, *n*=3) |
| Interday (overall) | CV%=6.0 (*n*=24) | CV%=4.8 (*n*=24) |
| LOQ | CV%=7.5 (0.25 µg/ml, *n*=6) | CV%=7.4 (100 ng/ml, *n*=6) |
| Low | CV%=4.2 (5 µg/ml, *n*=6) | CV%=4.1 (250 ng/ml, *n*=9) |
| Medium | CV%=5.7 (50 µg/ml, *n*=6) | CV%=5.4 (500 ng/ml, *n*=9) |
| High | CV%=6.7 (100 µg/ml, *n*=6) | CV%=4.8 (750 ng/ml, *n*=9) |
| Accuracy | | |
| Intraday (overall) | REC%=105 (*n*=12) | REC%=108.5 (*n*=12) |
| LOQ | REC%=105.0 (0.25 µg/ml, *n*=3) | REC%=113.2 (100 ng/ml, *n*=3) |
| Low | REC%=98.5 (5 µg/ml, *n*=3) | REC%=112.4 (250 ng/ml, *n*=3) |
| Medium | REC%=111.1 (50 µg/ml, *n*=3) | REC%=104.4 (500 ng/ml, *n*=3) |
| High | REC%=105.6 (100 µg/ml, *n*=3) | REC%=104.2 (750 ng/ml, *n*=3) |
| Interday (overall) | REC%=108.9 (*n*=24) | REC%=113.3 (*n*=24) |
| LOQ | REC%=108.0 (0.25 µg/ml, *n*=3) | REC%=113.2 (100 ng/ml, *n*=3) |
| Low | REC%=109.2 (5 µg/ml, *n*=6) | REC%=114.6 (250 ng/ml, *n*=6) |
| Medium | REC%=111.1 (50 µg/ml, *n*=6) | REC%=117.0 (500 ng/ml, *n*=6) |
| High | REC%=106.5 (100 µg/ml, *n*=6) | REC%=108.3 (750 ng/ml, *n*=6) |
| Linearity | | |
| range | 0.25-200 µg/ml | 100-1000 ng/ml |
| slope | 0,07347 ± 0,002129 | 2.518e-005 ± 8.908e-7 |
| y-intercept | 0,1221 ± 0,05125 | 0.01141 ± 0.0005317 |
| coefficient of correlation | $R^2$=0.9967 | $R^2$= 0.9963 |

[1] LOD: limit of detection [2]LOQ: limit of quantification [3] low, medium and high referred to the daptomycin concentration in the quality control samples which are indicated in brackets.

**Table 3.** Daptomycin concentrations in biological samples: comparison between plasma and breast milk concentrations [16]

| Sample | Administration time | Plasma (µg/ml) | Breast milk (µg/ml) | M/P ratio |
|---|---|---|---|---|
| T0 | 500 mg | - | 0.229 | |
| T6 | - | - | 0.270 | |
| T12 | - | - | 0.269 | |
| T24[1] | 500 mg | - | - | |
| T48 | 500 mg | - | 0.209 | |
| T72 | 500 mg | 44.4 | 0.186 | 0.004 |
| T78 | - | 199.07 | 0.329 | 0.002 |
| T84 | - | 65.85 | 0.329 | 0.005 |
| T96 | - | 64.21 | 0.243 | 0.004 |
| T120 | - | 19.89 | 0.121 | 0.006 |

[1]no sample was collected at T24, but it has been included in the table as a time of administration.

**Figure captions**

**Figure 1.** Chemical structure of erythromycin and daptomycin.

**Figure 2.** An example of two chromatograms from plasma (sx) and milk (dx) showing both daptomycin and erythromycin.

**Figure 3.** Analysis of daptomycin in blank matrix: plasma (A) and milk (B). The lack of interfering peaks at the retention time of daptomycin (depicted by black arrows) was considered as an acceptable selectivity.

**Figure 4.** (A) Concentration profile of daptomycin vs time in both plasma and breast milk samples. Red arrows indicate the time in which daptomycin 500 mg was intravenously administered.
(B) Ratio milk/plasma profile vs time, a ratio > 1 shows a prevalence distribution and accumulation in milk.