



## Short Communication

## Validation of a HPLC-MS/MS assay for the determination of total and unbound concentration of temocillin in human serum



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## ABSTRACT

**Objectives:** The aim of this study was to develop and validate a HPLC-MS/MS assay to determine total and unbound concentrations of temocillin in serum samples.

**Design and methods:** Methanolic protein precipitation and ultrafiltration were used for total and unbound concentration extraction, respectively. Extract was injected into a LC-MS/MS system. Reversed phase chromatography was performed on a phenyl grafted column in gradient mode. Temocillin and internal standard (ticarcillin) were identified in positive electrospray ionization mode using ion transitions of  $m/z$  415.34 > 339.1 and 385.31 > 160.3, respectively.

**Results:** Temocillin total and unbound concentration quantification assays were linear over concentrations ranging from 1 to 500 mg/L and from 0.5 to 300 mg/L, respectively. Both assays presented acceptable intra and inter-assay precision and accuracy <13.9%. Limits of quantification and detection were of 1 and 0.10 mg/L, and 0.5 and 0.05 mg/L for total and unbound concentration respectively. Total temocillin concentration recovery ranged from 85.80 to 99.40%. Temocillin ion suppression effect was <36.2% in both assays.

**Conclusion:** The method described is fast, sensitive and selective, with no interferences. This method may be used for both pharmacokinetic studies and therapeutic drug monitoring purposes.

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## Introduction

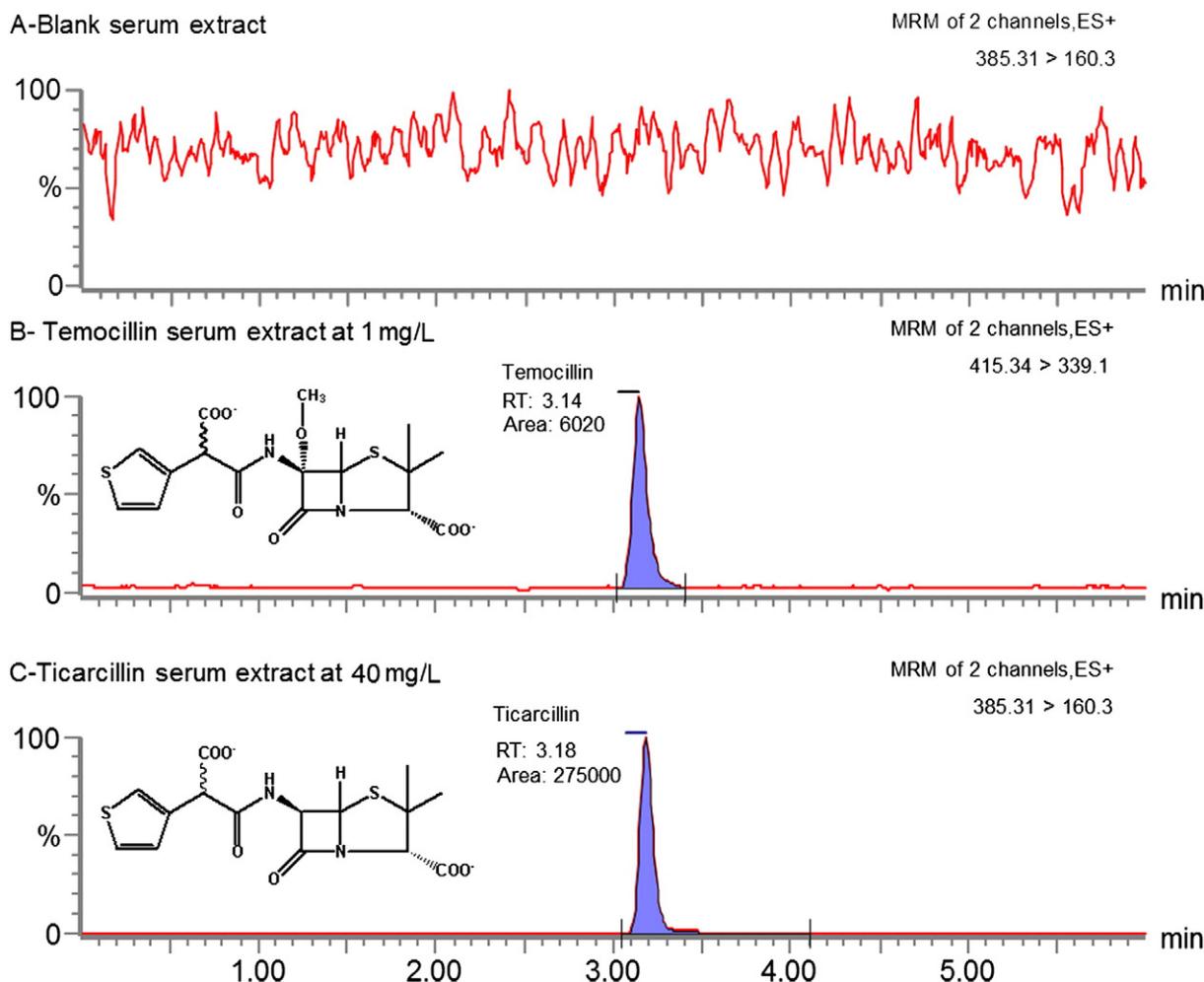
Temocillin is a  $\beta$ -lactam antibiotic active against gram-negative bacteria, including  $\beta$ -lactamase producers [1,2]. It is currently indicated for the treatment of septicemia, urinary tract infections, and lower respiratory tract infections [3]. In the recent years the interest on this drug has been greatly enhanced, due to the increased incidence of infections caused by microorganisms producing extended-spectrum  $\beta$ -lactamases. This makes temocillin a strategic and interesting alternative to carbapenems, especially with the current driving force to spare the use of these drugs [2].

Pharmacodynamic studies predict that therapeutic success with  $\beta$ -lactams depends on the time during which their unbound

concentration in serum remains above the MIC of the offending organism [4]. Dosage should thus be adapted to take into account not only the bacterial susceptibility, but also the rate of elimination of the drug. There is evidence of a wide interindividual variability in the temocillin serum levels so that standard dosing regimens may not be adequate in special patient populations such as intensive-care and end-stage renal disease patients [5,6]. These patients may benefit from individualized dose regimens based on therapeutic drug monitoring.

Two validated assays (HPLC-UV) have been described in the literature to quantify temocillin. The first one was used for controlling purity and detecting related substances in temocillin batches [7], and the other one, for quantifying the drug in serum [8]. The later method involves a laborious sample preparation with solid-phase extraction to eliminate possibly interfering substances and was validated for the total concentration only. The present work aims at validating a new, rapid, sensitive and selective HPLC-MS/MS method for determination of both total and unbound concentrations of temocillin in serum.

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**Fig. 1.** Typical chromatograms. Ion chromatograms of serum extracts obtained either with a blank sample (A) or with a sample spiked simultaneously with temocillin at 1 mg/L (B) and with ticarcillin as IS at 40 mg/L (C).

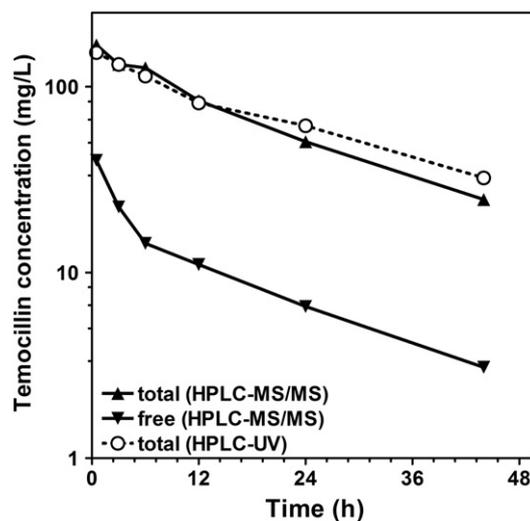
## Materials and methods

Temocillin (~68% R and ~32% S isomers; NEGABAN®) was obtained from Eumedica s.a. (Brussels, Belgium). Ticarcillin disodium (~55% R and ~45% S isomers; internal standard, IS) was from Sigma-Aldrich Inc. (St. Louis, MO, USA). Both molecules are chemically stable for at least 24 h at 37 °C [9,10] and much longer at 4 °C [11,12]. HPLC/MS-grade methanol and acetonitrile were purchased from J.T. Baker (Deventer, Netherlands). Formic acid was obtained from Merck KGaA (Darmstadt, Germany). Ultrapure water was obtained from MEDICA-R 7/15 water purification system (Veolia Water Systems, Bucks, UK) and Milli-Q Academic apparatus (Millipore Corporation, Billerica, MA, USA). Human drug-free serum was obtained from healthy volunteers, in agreement with local ethics guidelines.

The HPLC-MS/MS equipment consisted of a Quattro micro tandem mass spectrometer (Micromass UK, Ltd., Manchester, UK) fitted with a Z-spray ion source. The instrument was operated in electrospray positive ionization (ESI) and coupled to a Waters 2795 HT, Alliance HPLC system, with an integrated auto sampler (Waters, Mildford, MA, USA). Data were recorded in the multiple reaction monitoring (MRM) mode.

For determination of total temocillin concentrations, aliquots of stock solutions prepared in water (10 mg/mL) were diluted with drug-free serum to prepare 7 calibrators (CS) in the 1 to 500 mg/L range. Quality control (QC) samples (5, 250, and 450 mg/L) were prepared from an independent stock solution. Then, 200 µL of each CS or QC were treated with 600 µL of methanol after addition of 32 µL of IS

(1 mg/mL). After a 5 s vortex step, samples were centrifuged at 11,000 g. Ten microliters of the supernatant were injected into the HPLC/MS-MS.



**Fig. 2.** Concentration–time profile for temocillin in the serum in a representative patient (with end-stage renal disease) after administration of a 2 g dose IV. The graph shows the total and unbound concentrations as determined by HPLC-MS/MS and the total concentration determined by HPLC-UV using a previously described method [8].

For determination of unbound temocillin concentrations, drug-free serum was added to Amicon® Ultra-15 device, Centrifugal Filters (NMWL 30 K; Merck Millipore Ltd.) and submitted to ultrafiltration (2000 g, 25 °C, 20 min). The ultrafiltrate was used to dilute aliquots of the temocillin stock solution in order to prepare 8 CS (0.5 to 300 mg/L), and QC (0.75, 75, and 200 mg/L). Twenty microliters of IS solution (0.2 mg/mL) were added to 100 µL of CS or QC. Ten microliters were injected into the HPLC/MS-MS.

The analytical column was an XBridge® phenyl, 2.1 × 50 mm, 3.5 µm (Waters, Milford, MA, USA) maintained at 40 °C. The mobile phase consisted of a mixture of solvents A (aqueous solution of 0.1% formic acid) and B (0.1% formic acid in acetonitrile), under gradient conditions and 0.3 mL/min flow rate. The auto sampler used a rinse solution of 0.1/50/50 formic acid/water/acetonitrile (v/v/v). The total run time was 6 min. The transitions  $m/z$  415.34 > 339.1 and  $m/z$  385.31 > 160.3 were used for temocillin and IS quantification, respectively. The cone voltages (CoV) and collision energies (CE) optimized for the method were 20 V and 25 V, and 12 and 14 eV, respectively for temocillin and IS. Ion transitions  $m/z$  415.34 > 172.2 (CoV = 20 V, CE = 35 eV) and  $m/z$  385.31 > 243.3 (CoV = 20 V CE = 23 eV) were used for temocillin and IS qualification, respectively.

The assay was fully validated according to the U.S. Food and Drug Administration (FDA) guidelines [13]. Statistics were performed using JMP software (SAS Institute, Cary, NC, USA). The linearity has been assessed over the 7 and 8 calibrators (total and unbound concentration, respectively), in 5 replicates over 3 days. The limit of quantification (LOQ) was calculated as the minimum concentration at which temocillin can be reliably quantified with a precision ≤20% and accuracy within –20–20%. The limit of detection (LOD) was calculated as the smallest detectable peak above baseline noise (signal-to-noise ratio >3:1). Inter-assay precision and accuracy were assessed on QC samples and the LOQ, in replicates of 5 for 3 days. To determine intra-assay accuracy and precision, the same samples were analyzed 5 times. Extraction efficiency of temocillin from serum was performed by comparing concentrations recovered from samples treated as described above for the determination of total concentration with unextracted calibrators (6 replicates for the 3 QC concentrations). Matrix effect was assessed against the QC samples using the post-extraction addition technique using 6 different drug-free serum samples for each aliquot [14]. The suitability of IS to minor matrix effect was assessed by comparing calibration curves made in HPLC solvent A and in 6 different serum samples with and without IS.

## Results

The correlation coefficient ( $R^2$ ) for each calibration curve was >0.99, with a mean value of 0.993 and 0.995 for the total ( $n = 15$ ) and unbound ( $n = 15$ ) temocillin concentration, respectively. LOQ and LOD were found to be 1 and 0.10 mg/L (total concentration) and 0.50 and 0.05 mg/L (unbound concentration). As shown in Fig. 1, the retention times (RT) were 3.14 and 3.18 min for temocillin and IS, respectively.

For the total concentrations, inter-assay (3 days;  $n = 15$ ) precision and accuracy ranged from 6.48 to 8.88% and from 2.26 to 11.64% respectively, while the intra-assay (3 days;  $n = 5$ ) precision and accuracy ranged from 7.14 to 9.18% and from 6.86 to 13.91% respectively. For the unbound concentrations, inter-assay (3 days;  $n = 15$ ) precision and accuracy ranged from 3.4 to 4.8% and from 3.3 to 7.6%, with an intra-assay (3 days;  $n = 5$ ) precision and accuracy ranging from 1.1 to 6.7% and from 8.4 to 12.5%, respectively.

Temocillin extraction recovery ranged from 85.8 to 99.4% with a precision ranging from 7.5 to 11.9%, and appeared concentration independent. No interfering peak greater than 20% of the response at the LOQ was detected at the  $m/z$  transitions and RT of temocillin and IS. Typical ion chromatograms of extracts from blank serum sample, serum spiked with temocillin (1 mg/L) and ticarcillin (40 mg/L) are shown in Fig. 1.

At the three QC levels, matrix effect was acceptable, ranging from –36.2 to –17.3% ion suppression for both temocillin and IS. This effect was significantly corrected using ticarcillin as IS. Indeed, the function slope between calibration curves made in HPLC solvent A and different serum samples was comparable (mean = 0.571; CV = 4.8%;  $n = 7$ ). The results of these experiments suggested that matrix effects have minimal influence on the results.

This method was then applied to determine the concentration–time profile of total and unbound temocillin for temocillin in the serum of an end-stage renal disease patient, having received 2 g of temocillin by IV route. As shown in Fig. 2, total concentration matched those determined by a previously validated HPLC-UV method [6]. Unbound concentrations oscillated between 24% at 0.5 h and 13% at 44 h. Unbound AUC represented 14% of the total value. These observations are in line with what is stated in the summary of product characteristics, namely that the unbound fraction depends on the total concentration due to saturation of binding sites on serum proteins and that the protein binding is about 85% for total concentrations around 16 mg/L [3].

## Conclusion

The HPLC-MS/MS method described in this work for the quantification of total and unbound temocillin in human serum proved rapid, sensitive, and selective, with a LOQ of 1 mg/L. As MS/MS detection is highly specific, this method has probably a wider applicability to different clinical situations than the previously developed HPLC-UV assay [8], like in patients receiving other medications or in hemodialysis patients who accumulate acidic metabolites [15].

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