An advanced double column-switching technique (LC-LC) for liquid chromatography/electrospray ionisation tandem mass spectrometry for fully automated analysis of caspofungin

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Caspofungin (MK-0991; L-743,872) is the first representative of a new important class of antifungal agents, the glucan synthesis inhibitors. To the authors' best knowledge, to date only one high-performance liquid chromatography (HPLC) method has been published for the determination of caspofungin in serum. Severe difficulties with sorption were described. We developed a new method which addresses these difficulties using an advanced column-switching technique for fully automated analysis of caspofungin in serum without any pre-treatment. Extraction was performed automatically inline, using a diol column, followed by chromatography on a CN column. Detection was performed by electrospray ionisation tandem mass spectrometry (ESI-MS/MS) with isolation and fragmentation in the positive ion mode. Total analysis time was 30 min. Detection of caspofungin was achieved by retention time, isolation and fragmentation of the double positively charged caspofungin ion. This LC/MS assay was validated for between-run accuracy (max. 110%) and precision (max. CV 16.1%). The lower limit of quantification was 0.2 μg/mL. The analytical method with fully automated inline extraction of caspofungin described here removes the need for difficult and time-consuming sample pre-treatment. Sorption of caspofungin is not of importance. Additional advantages of the new method are that only a small quantity of serum (5 μL) is needed and that the method is very specific. Copyright © 2004 John Wiley & Sons, Ltd.
Applying a double column-switching technique we were able to analyse the caspofungin contained in serum without any pre-treatment. Combination of the inline extraction technique with double column-switching liquid chromatography (LC-LC) and electrospray ionisation tandem mass spectrometry (ESI-MS/MS) enabled us to detect caspofungin in low concentrations and with maximum specificity.

**EXPERIMENTAL**

**Chemicals**

Caspofungin was supplied by the University Hospital Pharmacy, Freiburg, Germany. We used the pharmaceutical preparation from MSD (Merck Sharpe Dome) Cancidas® 50 mg, due to the fact that pure caspofungin standard was not available. Excipients of Cancidas® 50 mg are: caspofungin acetate (55.5 mg) (is equivalent to 50 mg caspofungin), sucrose, mannitol, glacial acetic acid and sodium hydroxide. Acetonitrile (LiChrosolv®) and formic acid were purchased from Merck (Darmstadt, Germany). Acetonitrile was of gradient grade and formic acid was of analytical grade. HPLC-grade water was generated using a Milli-Q water-purification system from Millipore (Molsheim, France). Pooled blank serum samples were obtained from Freiburg University Hospital.

We prepared a stock standard solution of caspofungin in water at a concentration of 5000 μg/mL. Calibration standards at caspofungin concentrations of 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, and 20.0 μg/mL in pool serum were prepared by appropriate dilution of the stock solution with pool serum. Stock standard solution and the blank pool serum were stored at −20°C until analysis.

**LC/MS system**

An Agilent series 1100 LC system (Agilent Technologies, Wallbronn, Germany) equipped with two binary pumps, a degasser, two six-port switching valves and a column oven was used. Samples were injected via automatic sample injector. The Chemstation software (Agilent Technologies) was used for instrument control.

The MS system consisted of an Esquire 3000 plus (Bruker Daltonics, Bremen, Germany) with an orthogonal ESI source and an ion trap. The software used was Bruker Daltonics esquire 5.1 Build 230 (Bruker Daltonik GmbH, Bremen, Germany).

**Columns**

The configuration of the column-switching system is shown schematically in Fig. 2. The inline extraction column was a Vertex Eurospher 100 Diol 30 mm × 4 mm, 7 μm (Knauer, Berlin, Germany). Chromatography was performed on the analytical column Vertex Eurospher 100 CN 30 mm × 4 mm, 7 μm (Knauer). The column oven was set to 40°C.

**Analytical procedure**

**Sample preparation**

There was no sample pre-treatment. Serum samples were injected directly into the LC-LC/ESI-MS/MS system. The autosampler was set at an injection volume of 5 μL. Extraction

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*Figure 1. Chemical structure of caspofungin.*
Figure 2. Scheme of the HPLC-integrated sample preparation. (a) System in initial position, ready for sample injection: HPLC/MS circulation is isolated from extraction side. (b) Transfer and chromatographic separation step: the extraction column is connected with the analytical column.
and chromatographic separation of the analyte was carried out automatically by the LC-LC system.

**Double chromatography: LC-integrated extraction and chromatographic separation (LC-LC)**

The HPLC-integrated extraction procedure steps were: (i) sample application and extraction, (ii) transfer of the analyte fraction and chromatographic separation, and (iii) re-equilibration of the extraction column.

The configuration of the LC-LC/ESI-MS/MS system with the two switching valves is shown schematically in Fig. 2(a). The autosampler and pump 1 were used to load 5 µL of serum containing caspofungin onto the extraction column. The gradients of both pumps and the switching time of both valves are summarized in Fig. 3. The mobile phase of pump 1 was pure water for the first 4 min and was delivered to the extraction column at a flow rate of 0.8 mL/min. After this time a mobile phase gradient was started, changing from 0% acetonitrile to 100% acetonitrile over 2 min, then returning to 0% within 1 min. Caspofungin was retained on the extraction column, while matrix compounds were flushed to waste with the eluent. After 8 min, the matrix was fully washed out of the extraction column. The software time-schedule automatically switched the two high-pressure valves into transfer position and chromatographic separation position (Fig. 2(b)), thereby coupling the extraction column with the analytical column. The reservoir of pump 2A contained formic acid (0.1%) (v/v) and the reservoir of pump 2B contained acetonitrile. In this valve position the analytical mobile phase was delivered from pumps 2A and 2B in a ratio of 100:0 (v/v). The mobile phase gradient started after 8 min. After 14 min it reached its maximum with a ratio of 20% acetonitrile to 80% formic acid (0.1%). Within the time period from 8–14 min caspofungin was rapidly eluted from the extraction column by back flushing at a flow rate of 0.4 mL/min. The higher elution power of the formic acid (0.1%) desorbed the analyte from the extraction column and the caspofungin fraction adsorbed on the capillaries and on the autosampler. In addition, the formic acid transferred the caspofungin desorbed to the analytical column. The second chromatographic separation on the analytical column was accomplished by the mobile phase gradient: Pump 2 delivered 100% formic acid (0.1%) from 14–20 min. To clean the analytical column a mobile phase gradient at pump 2 started at minute 20 with 0% acetonitrile, reaching 100% acetonitrile at minute 24, and decreasing again to 0% acetonitrile within 1 min. Twenty-five minutes after starting the analysis, the high-pressure valves switched back to the initial position. The extraction column was re-equilibrated by pump 1 with pure water, while the disconnected HPLC circulation simultaneously re-equilibrated the analytical column. Total sample preparation time and analysis time was 30 min.

**Detection with mass spectrometry: isolation and fragmentation**

The HPLC column effluent was pumped to the ion-trap mass spectrometer equipped with an ESI source which was used in the positive ion mode. The instrument was tuned by direct injection of an aqueous solution of 20 ng/mL caspofungin at 5 µL/min. The following tune parameters were retained for optimum caspofungin detection: nebulising gas pressure 50.0 psi (1 psi = 6894.76 Pa); drying gas flow 10.0 L/min; drying temperature 350°C; spray voltage 4 kV; capillary exit 173.1 V; skimmer 40.0 V; octopole 1 DC 12.0 V; octopole 2 DC 2.95 V; octopole r.f. amplitude 159.5 V p.-p.; trap drive 89.9; lens 1 Voltage −5.0 V; lens 2 Voltage −60.0 V. The following optimum trap conditions are found: rolling, on; rolling averages 5;ts; scan begin 100 m/z; scan end 1500 m/z; maximal accumulation time 200 ms; ion charge control target 150 000; charge control, on. Optimum collision energy in the MS/MS mode, corresponding to nearly 100% fragmentation of the protonated molecule, was found to be 0.8 V. Further fragmentation parameters are: isolated specific mass 547.7 m/z with width 4.0 m/z; cut off 151 m/z; smartfrag on; smartfrag start amplitude 30%; smartfrag end amplitude 200%; fragmentation width 10.0 m/z; fragmentation time 40 ms; fragmentation delay 0 µs.

**Quantification and validation/qualification**

Chromatographic peaks of caspofungin were identified by their retention time and the detected mass of the fragment ion. Typically, the caspofungin product ion after fragmentation, i.e. MS/MS, was m/z 538.6. Quantification was carried out by peak area.

This LC-LC/ESI-MS/MS assay was validated for linearity of calibration, inter-assay accuracy and precision, quantification limit and specificity of the methodology. The accuracy and precision of the method were assessed by analysing replicates of pool serum samples spiked at different concentrations (see Table 1). The accuracy of the caspofungin serum assay was determined by calculating the mean percentage differences between nominal and measured concentrations. The assay precision was characterised by mean value and coefficient of variation (CV) from six replicates of spiked serum samples. Between-run precision and accuracy was studied by analysing peak areas of spiked serum samples from eight different runs (= inter-run precision). The lower limit of quantification (LLOQ) was defined as being the lowest quantity of analyte determined with a precision and accuracy equal to or better than 20% and an analyte signal of at least 5 times the signal compared to blank (noise). An analyte signal at least 3 times the noise of the
Table 1. Statistics of spiked caspofungin serum samples

<table>
<thead>
<tr>
<th>Nominal conc. (µg/mL)</th>
<th>Precision</th>
<th>Accuracy</th>
<th>Precision</th>
<th>Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Within-run</td>
<td></td>
<td>Between-run</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>Mean (µg/mL)</td>
<td>CV (%)</td>
<td>N</td>
</tr>
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<tr>
<td>1.0</td>
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<td>0.93</td>
<td>11.11</td>
<td>8</td>
</tr>
<tr>
<td>5.0</td>
<td>6</td>
<td>5.33</td>
<td>5.99</td>
<td>8</td>
</tr>
</tbody>
</table>

CV: coefficient of variation; N: number of samples tested.

RESULTS AND DISCUSSION

LC-LC

Figure 2 shows a special column-switching configuration. By using two switching valves we have the opportunity to choose two completely different analytical settings. The first valve (valve 1) offers two different modes for analysis: the first is an analysing mode with column switching, the other without column switching, i.e. an analysing mode like a HPLC system without a switching valve. Caspofungin presented difficulties because it is not adequately analysable when it is dissolved in water only, since it shows strong sorption on all materials, including HPLC capillaries. Schwartz et al.19 showed that albumin (a serum matrix component) as add-on is necessary to analyse urine samples. To circumvent these difficulties the other switching valve (valve 2) couples the extraction column with the analytical column. These two valves are necessary for analysis of caspofungin by online extraction. Caspofungin sorbed on all capillary and other HPLC components. The elution power of water was too low to prevent this retention on HPLC components. In contrast to water, 0.1% formic acid partially elutes caspofungin. Sufficient elution was achieved with a combination of acetonitrile and formic acid only. If we use just one switching valve, as is common practice, we detect an intensive carry-over effect (>20%). Application of the second valve solved this problem, because all columns between autosampler and extraction column and especially the autosampler could be washed with the mobile phase, which consisted of formic acid and acetonitrile during the analysis. All the gradients and washing steps cleaned the surfaces which were in contact with caspofungin and all of the caspofungin was desorbed. The carry-over effect was reduced to 1%. To clean the analytical column, we used a mobile phase gradient with 100% acetonitrile for the same reason.

By using 0.1% formic acid, we obtained narrow, well-shaped peaks. Therefore, it was possible to extract and detect with the same mobile phase. By using a CN column we did not need any ion-pairing reagents, in contrast to the method presented by Schwartz et al.19 This is important because triethylamine impairs the extraction power of the diol column.

Another important parameter for inline extraction is the transfer time, i.e. the time needed to transfer the substance from the extraction column to the analytical column. A short transfer time is favourable for sharp peaks and fast analysis. We were able to realise a short transfer time of 2 min. A typical chromatogram of pooled serum spiked with caspofungin compared with a blank pooled serum sample is shown in Fig. 4.

The analytical LC-LC/ESI-MS/MS method with inline extraction of caspofungin described here removes the need for the time-consuming and difficult procedures involved in the method reported previously,19 such as SPE conditioning, washing, sample evaporation, etc. No manual step for sample preparation in the method described here is required. The serum assay is fully automated and allows the determination of caspofungin levels within 30 min. Another advantage of the new method is the low demand on sample quantity because one analysis requires 5 µL of serum only. Schwartz et al. used 1000 µL of serum samples. In some special cases, such as pharmacokinetic studies involving small children, use of small sample amounts is beneficial to the patients. Another advantage is that no internal standard is required. Currently it is difficult to obtain a reasonable internal standard.

ESI-MS/MS

Caspofungin is detected by isolation and fragmentation of the double positively charged caspofungin molecule. Its specific mass is m/z 547, the specific fragment mass is m/z 538, which results from loss of one molecule of water (m/z 18, i.e. (547 × 2) – 18 = 538 × 2). Caspofungin also shows other peaks in the mass spectrum (see Fig. 5). The relative mass of unloaded caspofungin is 1093 calculated by using the chemical structure. We detected three different specific masses (365, 547 and 1094). The lowest specific mass was m/z 365, this is a threefold charged caspofungin (= (1093 + 3)/3). The triple charge is not only due to the MS/MS but the molecule itself is somehow charged. Probably it is still existent as a salt on the LC-LC columns. Among the other charged caspofungin molecules, this ion shows a different behaviour on the LC-LC columns. It was impossible to retain this caspofungin fraction (about 5%), which shows a specific mass of 365 in the MS, either on the diol column or on the CN column, in contrast to the main caspofungin fraction (about 95%). The singly charged caspofungin ion behaved similarly on the columns to the doubly charged molecule. However, detection with MS/MS of the singly charged ion did not resemble that of the doubly charged ion. The trapping in the ion trap did not work well in this case. Obviously, effective isolation and fragmentation was not possible. Reasons for this can be many. The one
of importance here could not be identified. Because of these facts we used the doubly charged caspofungin molecule for detection. Balani et al.\textsuperscript{21} showed that caspofungin has different metabolites. After a dose of 70 mg plasma, urine samples collected around 24 h post-dose contained predominantly unchanged caspofungin, together with trace amounts of a peptide hydrolysis product.\textsuperscript{21} Due to the fact of specific mass isolation we could separate the unchanged caspofungin from the metabolites.

Quantification and statistics

Spiked pool serum standards were tested in a concentration range from 0.2–20.0 μg/mL. There are two different linear calibration ranges. From 0.2–2.0 μg/mL the equation of the calibration curve was $y = 141370x + 17435$, from 2.0–20 μg/mL the equation was $y = 174992x/52464$, respectively. The term $x$ represents the analyte concentration in μg/mL and $y$ the corresponding peak area. The linear regression coefficients were $R^2 = 0.999$ and 0.997, respectively.

The linearity range achieved for this assay (0.2–2.0 and 2.0–20.0 μg/mL) effectively covers the therapeutic range of caspofungin, which, for fungal infections, is between 1.2 and 10.4 μg/mL in serum.\textsuperscript{3} The precision and accuracy of the caspofungin assay using spiked serum standards is summarized in Table 1. The lower limit of quantification (LLOQ) of the assay is 0.2 μg/mL. The limit of detection (LOD) is 0.1 μg/mL.

CONCLUSIONS

The new LC-LC/ESI-MS/MS assay presented here allows automated determination of caspofungin in serum without deproteinisation or any other form of pre-treatment. Automated inline extraction chromatography shows good performance and precision. In addition, the method is very specific. The first step is an intensive sample cleanup, the second step chromatographic separation, and the third step mass-sensitive selection and fragmentation followed by detection and quantification of the specific caspofungin.
fragment mass. These three steps ensure specific caspofungin detection and effective elimination of potential contaminations and circumvent difficulties due to sorption. No internal standard and only very small quantities (5 μL) of serum are required. It is suitable for use in pharmacokinetic studies.

Acknowledgement

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REFERENCES


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Figure 5. (a) Mass spectrum of caspofungin obtained by direct injection of aqueous caspofungin standard performed with a syringe pump. The MS ion mode is positive. m/z 1093 is the relative mass of caspofungin only with a single charge [M+H]+, m/z 547 describes the relative mass of caspofungin with two H+ charges [M+H2]+, and m/z 365 is caspofungin threefold charged [M+H3]+. A degradation product from caspofungin is the specific mass m/z 1033. Maybe it is caspofungin without its diaminoethane structure. (b) Mass spectrum of the fragmented caspofungin after isolation of the specific mass m/z 547 and fragmentation with an amplitude of 0.8 V. The major caspofungin ion is the specific mass m/z 538.6. This is the doubly positively charged caspofungin molecule after the cleavage of one molecule of water. Specific masses m/z 392.7 and 684.3 represent two fragmentation products from the relative mass 1077 (= 392.7 + 684.3) and this is the doubly charged caspofungin ion with its specific mass m/z 538.6.


