DETERMINATION OF CEPHALEXIN LEVEL AND STABILITY IN HUMAN PLASMA BY FULLY VALIDATED RAPID HPLC ANALYSIS

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ABSTRACT

We optimized and validated a simple, precise, and rapid reversed phase high performance liquid chromatographic (HPLC) assay for cephalexin measurement in human plasma and applied it in a clinical and laboratory settings. Plasma samples containing cephalexin were spiked with cefuroxime (internal standard, IS), and precipitated with trichloroacetic acid in methanol. After centrifugation, the clear supernatant was injected into the HPLC system. The components of interest were efficiently separated on 4.6 x 150 mm, Atlantis dC18, 5 µm (particle-size) steel column, using a Guard Pak pre-column module with Nova-Pak C18, 4 µm insert. The mobile phase consisted of a mixture of equal volumes of 0.01 M of cetyltrimethylammonium bromide and 0.01 M dipotassium hydrogen phosphate (pH = 6.5, adjusted with phosphoric acid), acetonitrile, and triethylamine (60:40:0.001, v:v:v). The analytes were detected using Waters 2998 photodiode array detector set at 260 nm. The response was linear ($R^2 \geq 0.9951$) in the range of 0.5–120 µg/ml, the intra- and inter-day coefficient of variations (CV) were $\leq 3.1\%$ and $\leq 3.4\%$, respectively. Extraction recoveries for cephalexin and the IS were $\geq 94\%$ and 88%, respectively, whereas bias of cephalexin measurement was 1%-5% using quality control samples and 3%-12% as determined by back calculation from peak area ratios of calibration curves. In human plasma, cephalexin was stable ($\geq 93\%$) for at least 24 hours at room temperature in plasma; for at least 24 hours at room temperature ($\geq 96\%$) or 48 hours at –20°C ($\geq 98\%$) after processing; after 3 cycles of freeze at –20°C and thaw at room temperature ($\geq 92\%$); and for at least 8 weeks at –20°C ($\geq 99\%$). Stock solutions of
Cephalexin and the IS (1 mg/ml in methanol) were stable for at least 8 weeks at –20°C (101% and 89%). The method was successfully used to measure cephalexin level in samples obtained from a healthy volunteer.

**KEYWORDS:** Cephalexin, Cefuroxime, HPLC, Validation, Stability.

**INTRODUCTION**

Cephalexin is a semisynthetic cephalosporin antibiotic intended for oral administration. Its chemical structure is 7-(D-a-Amino-a-phenylacetamido)-3-methyl-3-cephem-4-carboxylic acid monohydrate, (CAS number: 23325-78-2).[^1] It is indicated for the treatment of certain bacterial infections such as respiratory tract infections, otitis media, and urinary tract infections.[^1]

Several analytical methods have been published for the quantitation of cephalexin level in different matrixes, including spectrophotometry,[^2] High Performance Thin Layer Chromatography (HPTLC),[^3] molecular imprinted solid phase extraction,[^4] High Performance Liquid Chromatography (HPLC),[^5-11] and Liquid Chromatography-tandem Mass Spectrometry (HPLC-MS-MS).[^12] Some of these methods suffered from a long processing time (liquid-liquid extraction,[^4-8] solid phase extraction[^4,7-8]), long chromatographic run time,[^7] or low recovery (82.3%).[^6] Other reported methods were developed to measure cephalexin level in pharmaceutical dosage form[^2,3,9] or bovine milk,[^11] or used equipment that may not be available in many pharmaceutical laboratories or need highly trained persons.[^12] Further, there are only limited data on cephalexin stability during the analytical process.[^6-7]

The aims of this study were to: 1) optimize and fully validate a rapid, simple, and sensitive HPLC method to determine cephalexin level in human plasma, and 2) determine the stability of cephalexin under various clinical laboratory conditions.

**MATERIALS AND METHODS**

**Apparatus**

The liquid chromatography apparatus consisted of Waters Alliance e2695D Separations Module, an 4.6 x 150 mm, Atlantis dC18, 5 µm (particle-size) steel column, a Guard Pak pre-column module with Nova-Pak C18, 4 µm insert, and Waters 2998 photodiode array detector set at 260 nm (Waters Associates Inc., Milford, MA, USA). Data were collected with a
Pentium D computer using Empower Chromatography Manager Software (Waters Associates Inc., Milford, MA, USA).

Chemicals and reagents
Cephalexin hydrate (Figure 1-a) and cefuroxime sodium salt (Figure 1-b) standards were obtained from Sigma-Aldrich Co., St. Louis, MO, USA. Cetyltrimethylammonium bromide was purchased from Aldrich Chemical Company, Inc. USA. Dipotassium hydrogen phosphate, phosphoric acid, triethylamine, and acetonitrile (all HPLC grade) were purchased from Fisher Scientific, Fairlawn, NJ, USA. Trichloroacetic acid was purchased from BDH Chemicals Ltd, Poole, England. HPLC grade water was prepared by reverse osmosis and further purified by passing through a Synergy UV (Millipore, Bedford, MA, USA).

Chromatographic conditions
The mobile phase consisted of a mixture of equal volume of 0.01 M of cetyltrimethylammonium bromide and 0.01 M dipotassium dihydrogen phosphate (pH = 6.5, adjusted with phosphoric acid), acetonitrile, and triethylamine (60:40:0.001, v:v:v). It was filtered through a 0.45 µm size polyestersulfone membrane filter (Millipore Co., Bedford, MA, USA) and degassed under vacuum for 5 minutes. The analysis was carried out under isocratic conditions using a flow rate of 1.0 ml/min at room temperature and a run time of 10 minutes.

Preparation of stock and working solutions
Cephalexin stock solution (1 mg/ml) was prepared in methanol and used to study cephalexin stability and prepare cephalexin working solution. Cephalexin working solution (120 µg/ml in plasma) was prepared weekly to construct calibration curve and quality control (QC) samples. The internal standard (cefuroxime) working solution (100 µg/ml) was prepared weekly in methanol from a stock solution of 1 mg/ml in methanol.

Preparation of calibration standard and quality control samples
Calibration standards were prepared by mixing appropriate volumes of cephalexin working solutions with blank human plasma to produce final concentrations of blank, zero (blank plasma spiked with IS only), 0.5, 1.0, 5, 10, 20, 40, 80, 100 and 120 µg/ml. QC samples were prepared by mixing appropriate volumes of cephalexin working solution with blank human plasma to produce final concentrations of 0.5, 1.5, 60, and 108 µg/ml. Samples were vortexed
for 20 seconds, and 0.25 ml aliquots were transferred into 1.5 ml eppendorf microcentrifuge tubes (Fisher Scientific Co., Fairlawn, NJ, USA) and stored at –20°C.

**Sample Preparation**

Aliquots of 0.25 ml of calibration standard or QC samples in 1.5 ml eppendorf microcentrifuge tubes were allowed to equilibrate to room temperature, then, 180 µl of 100 µg/ml internal standard (IS) was added, and the mixture vortexed for 10 seconds. After the addition of 120 µl of 3% trichloroacetic acid in methanol, the mixture was vortexed again for 2 min and then centrifuged for 30 min at 16000 rpm at room temperature. The clear supernatant was transferred into an auto-sampler vial, and 50 µl were injected into the HPLC system.

**Stability studies**

Stability of cephalexin in plasma: Adequate numbers of aliquots of three QC samples (0.5, 60, and 108 µg/ml) were prepared. Aliquots were analyzed in 5 replicates immediately (baseline), after being processed and stored at room temperature for 24 h or at –20°C for 48 h (auto-sampler stability), after being allowed to stand on the bench-top for 8 or 24 h at room temperature before processing (counter stability), after being stored at –20°C for 8 weeks before processing (long term freezer stability), or after being repeatedly stored at –20°C for 24 h and then left to completely thaw unassisted at room temperature before processing (freeze-thaw stability).

Stock solutions stability: five aliquots of the stock solutions of cephalexin and the IS were analyzed (after dilution to 100 µg/ml, and 10 µg/ml in mobile phase, respectively) at baseline, after storage for 48 h at room temperature, and after storage at –20°C for 8 weeks. Stability of the working solutions of cephalexin and the IS was evaluated after storage up to 2 weeks at –20°C.

**Assay validation method**

The method was validated according to standard procedures described in the US Food and Drug (FDA) bioanalytical method validation guidance.\[^{13}\] The validation parameter included: specificity, linearity, accuracy, intra-run and inter-run precision and accuracy, recovery, and stability.
RESULTS AND DISCUSSION

Optimization of chromatographic conditions

Different combination of the components of the mobile phase, pH, flow rates, and precipitation agents were investigated to optimize the separation of cephalaxin and the IS. A mobile phase of an equal mixture of 0.01 M of cetyltrimethylammonium bromide and 0.01 M dipotassium dihydrogen phosphate (pH = 6.5 ± 0.02, adjusted with phosphoric acid), acetonitrile, and triethylamine (60:40:0.001, v:v:v), delivered at a flow rate of 1.0 ml/min, gave the best result for best specificity and detection limit. A photodiode array detector set at 260 nm was found optimum. In regard to sample preparation, the best results were obtained when the sample was precipitated with 3% trichloroacetic acid in methanol. We used a Guard Pak pre-column module with Nova-Pak C18, 4 µm insert to reduce damage to the analytical column.

Under the described conditions, cephalaxin and IS were resolved within a run time of 10 min, with a retention time of 5.3 and 7.8 min, respectively.

Linearity

Linearity was determined in the range of 0.5 – 120 µg/ml using ten calibration curves. The data were analyzed by linear regression (Conc. = a + b (PAR), where Conc. is the concentration of cephalaxin, a is the intercept, b is the slope, and PAR is the peak area of cephalaxin divided by the peak area of the IS). Concentrations of the calibration standards of the ten calibration curves were back-calculated using individual regression lines. Linearity studies (n=10) showed mean (SD) R² of 0.9969 (0.0012), slope of 0.0151 (0.0008), and intercept of 0.0079 (0.0069). Figure 2 shows an overlay of chromatograms of a representative standard curve.

Limit of detection

The limit of detection, defined as three times the baseline noise, was 0.2 µg/ml.

Specificity

To evaluate specificity, we screened eight frequently used drugs and six different batches of human plasma. All batches of blank plasma were free from interfering components. None of eight drugs co-eluted with cephalaxin or the IS (Table 1).
Recovery
The extraction recovery of cephalexin was determined by dividing mean peak areas of five replicates of four QC samples (0.5, 1.5, 60, and 108 µg/ml) prepared in plasma (as described under sample preparation) by mean peak areas of five replicates of equivalent concentrations prepared in mobile phase. The recovery of the IS was determined similarly at a concentration of 18 µg/ml. The results are presented in Table 2. Recovery was ≥ 94% (mean 96%) for cephalexin and 88% for the IS.

Imprecision and bias
Imprecision was calculated as coefficient of variation (standard deviation divided by mean measured concentration x 100), and bias as the absolute value of (1 minus mean measured concentration divided by nominal concentration) x 100. The intra-run and inter-run imprecision and bias were determined by analyzing four QC samples: 0.5, 1.5, 60, and 108 µg/ml over three days (Table 3). Intra-run imprecision and bias (n = 10) ranged from 1.6% to 3.1% and from 1% to 5%, respectively. The inter-run imprecision and bias (n = 20) ranged from 1.7% to 3.4% and from 1% to 7%, respectively.

Stability
The stability of cephalexin in plasma and extracted samples was investigated under common laboratory storage conditions. The results are presented in Table 4. The data indicate that cephalexin is: 1) stable in plasma for at least 24 h at room temperature (93% to 101%) or 8 weeks at −20°C (99% to 106%), and after at least three cycles of freeze at −20°C and thaw at room temperature (92% to 108%), 2) stable in processed samples for at least 24 h at room temperature (96% to 101%) or 48 h at −20°C (98% to 101%), and 3) stable in methanol (1 mg/ml) for at least 48 h at room temperature (99%) or 8 weeks at −20°C (101%).

The IS stock solution (1 mg/ml) in methanol was also stable for at least 48 h at room temperature (99%) or 8 weeks at −20°C (89%). Further, the working solutions of cephalexin (120 µg/ml in plasma) and the IS (100 µg/ml in mobile phase) were stable for at least 2 weeks at −20°C (103% and 100%, respectively).

Robustness of the method
We evaluated the effects of changing acetonitrile proportion in the mobile phase from 40% to 35% or 45% on the retention time, peak shape, or peak area of cephalexin and the IS. Acetonitrile content in the mobile phase was found to be critical to the separation and of
symmetry of peaks. With 35% acetonitrile, the retention time of cephalexin and the IS was increased to 6.3 and 9.2 min, respectively, and the peaks became asymmetric. On the other hand, with 45% acetonitrile, the retention time of cephalexin and the IS decreased to 3.5 and 5.2 min, respectively, and the peaks remained symmetric but co-eluted with unknown peaks from blank plasma. We also evaluated the influence of the length of the analytical column (packed with the same stationary phase) at the same flow rate of 1.0 ml/min. Retention times for cephalexin and the IS were delayed to 8.7 and 13.2 min, respectively by increasing the column length to 250 mm. In contrast, slightly altering the flow rate (range of 0.9-1.1 ml/min) or the pH (range of 6.4-6.6) did not significantly affects the retention time, peak shape, or peak area of cephalexin or the IS. Further, the chromatographic resolution and peak responses were stable over about 2500 injections of processed plasma samples using a single column.

Application to A volunteer sample

Figure 3 depicts an overlay two chromatograms of volunteer plasma samples collected before, 0.5, and 1 hour after oral administration of a single 500 mg regular release cephalexin tablet. The measured concentrations of cephalexin were zero, 0.47, and 9.3 µg/ml, respectively.

Table 1. Specificity of Cephalexin Assay

<table>
<thead>
<tr>
<th>Drug Name</th>
<th>Retention Time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cephalexin*</td>
<td>5.3</td>
</tr>
<tr>
<td>Cefuroxime*</td>
<td>7.8</td>
</tr>
<tr>
<td>Aspirin*</td>
<td>ND</td>
</tr>
<tr>
<td>Acetaminophen*</td>
<td>2.3</td>
</tr>
<tr>
<td>Ranitidine*</td>
<td>2.2</td>
</tr>
<tr>
<td>Nicotinic Acid**</td>
<td>4.5</td>
</tr>
<tr>
<td>Ascorbic Acid**</td>
<td>3.7</td>
</tr>
<tr>
<td>Caffeine**</td>
<td>2.4</td>
</tr>
<tr>
<td>Diclofenac*</td>
<td>ND</td>
</tr>
<tr>
<td>Omeprazole*</td>
<td>4.3</td>
</tr>
</tbody>
</table>

1 mg/ml in methanol* or water** solutions were diluted to 10 µg/ml in mobile phase and 100 µl were injected into the HPLC system.

Table 2. Extraction Recovery of Cephalexin and Cefuroxime

<table>
<thead>
<tr>
<th>Nominal Concentration (µg/ml)</th>
<th>Plasma</th>
<th>Mobile Phase</th>
<th>** Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>*Mean Peak Area</td>
<td>SD</td>
<td>*Mean Peak Area</td>
</tr>
<tr>
<td>Cephalexin 0.5</td>
<td>9786</td>
<td>313</td>
<td>10144</td>
</tr>
<tr>
<td>1.5</td>
<td>28512</td>
<td>2456</td>
<td>29846</td>
</tr>
</tbody>
</table>
Table 3. Intra-run and Inter-run Imprecision and Bias of Cephalexin Assay

<table>
<thead>
<tr>
<th>Nominal Concentration (µg/ml)</th>
<th>Intra-run (n=10)</th>
<th>Inter-run (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean Measured Concentration (µg/ml)</td>
<td>SD</td>
</tr>
<tr>
<td>0.5</td>
<td>0.5243</td>
<td>0.0162</td>
</tr>
<tr>
<td>1.5</td>
<td>1.5222</td>
<td>0.0365</td>
</tr>
<tr>
<td>60</td>
<td>62.0310</td>
<td>1.6840</td>
</tr>
<tr>
<td>108</td>
<td>108.8980</td>
<td>1.6969</td>
</tr>
</tbody>
</table>

* Coefficient of variation (CV) = standard deviation (SD) divided by mean measured concentration x 100. **Bias = absolute value of 1 minus (mean measured concentration, divided by nominal concentration) x 100.

Table 4: Stability of Cephalexin in Plasma Samples and Stock Solution

<table>
<thead>
<tr>
<th>*Plasma Samples</th>
<th>Unprocessed</th>
<th>Processed</th>
<th>Freeze-thaw</th>
<th>**Stock solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nominal Concentration (µg/ml)</td>
<td>8 h RT</td>
<td>24 h RT</td>
<td>8 wks –20°C</td>
<td>24 h RT</td>
</tr>
<tr>
<td>0.5</td>
<td>100</td>
<td>93</td>
<td>99</td>
<td>101</td>
</tr>
<tr>
<td>1.5</td>
<td>103</td>
<td>100</td>
<td>104</td>
<td>100</td>
</tr>
<tr>
<td>108</td>
<td>98</td>
<td>101</td>
<td>106</td>
<td>96</td>
</tr>
</tbody>
</table>

Stability (%) = mean measured concentration (n=5) at the indicated time divided by mean measured concentration (n=5) at baseline x 100. * Spiked plasma samples were processed and analyzed immediately (baseline, data not shown); after 8 or 24 hours at room temperature (8 h RT and 24 h RT), or after 8 weeks at –20°C (8 wks –20°C); or processed and analyzed after 24 hours at room temperature (24 h RT) or 48 hours at –20°C (48 h -20°C); or after 1 to 3 cycles of freezing at –20°C and thawing at room temperature (freeze-thaw). ** Cephalexin, 1 mg/ml in methanol.
Fig. 1: Chemical structures of cephalixin (a) and cefuroxime (b).

Fig. 2: Overlay of cephalixin calibration curve chromatograms spiked with cefuroxime. The insert is a blow up of the lower concentrations.
CONCLUSION

We optimized and fully validated a simple, precise, and rapid assay for measurement of cephalexin level in human plasma following FDA guidelines,[13] and applied the optimized assay to study cephalexin stability under various laboratory conditions and determine cephalexin level in plasma samples of a healthy volunteer. The described assay has the following overall advantages over previously published assays: the use of one-step protein precipitation and simple equipment, high sensitivity (0.5 µg/ml), high extraction recovery (96%), and short run time (10 min). Together with the information obtained from stability studies, the validation data indicate that the described assay is suitable for cephalexin level monitoring and bioequivalence studies in humans.

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REFERENCES
11. Regina VO, Angela CD, Quezia BC. Quantification of cephalexin as residue levels in bovine milk by high-performance liquid chromatography with on-line sample cleanup. Talanta. 2007; 71 (3): 1233–1238.
12. Melvin ST, Christopher JL. Buggé, Cedra Corporation. Quantitative Analysis of Cephalexin in Human Plasma by LC-MS-MS.