DETERMINATION OF PENCYCLOVIR IN HUMAN PLASMA

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3.1 INTRODUCTION

Herpes virus infection is inhibited by the antiviral agent, penciclovir (2-amino-9-[4-hydroxy-3-(hydroxymethyl) butyl]-3H-purin-6-one), both in vitro and in vivo\textsuperscript{1-3}. Moreover, penciclovir is a potent and highly selective inhibitor of herpes viruses, such as herpes simplex virus types 1 and 2, varicella-zoster virus, Epstein virus and hepatitis B virus\textsuperscript{4-6}. It has been demonstrated that, in ocular herpes zoster infection, penciclovir reduced the incidence of ocular complications\textsuperscript{7}, but ocular penetration and pharmacokinetics of penciclovir in the human eye are still unknown. However, penciclovir has poor bioavailability when administered orally to human\textsuperscript{2-3}. To improve this poor oral bioavailability, the diacetate ester of the 6-deoxy derivative of penciclovir, famciclovir \{[2-(acetoxyethyl)-4-(2-aminopurin-9-yl) butyl] acetate\}, was developed as a prodrug of penciclovir\textsuperscript{8-10}. After oral administration, famciclovir is converted to penciclovir by the removal of two ester groups and 6-oxidation of the deoxyguanine ring. Four intermediate metabolites, depending on the rate of de-estereification and oxidation, are obtained \textsuperscript{9,11}, but pharmacokinetic studies have shown that penciclovir, and to a minor extent BRL42355, the desacetyl non-oxidized metabolite, are predominant in plasma and urine\textsuperscript{12,13}.

Penciclovir, like acyclovir and ganciclovir, is a nucleoside analogue that shares structural similarity with endogenous compounds. Therefore, selective analytical methods are required to analyze its levels and those of its analogs in biological fluids \textsuperscript{14}. In previous pharmacokinetic studies\textsuperscript{15-16}, penciclovir was analyzed by high performance liquid chromatography (HPLC) with UV\textsuperscript{15, 17-19} or fluorescence detection\textsuperscript{20-22}. However, these methods suffer from a number of disadvantages, including inadequate sensitivity [a lower limit of quantification (LLOQ) up to 0.1µg/mL], and require extensive sample preparation and large biological volumes (i.e. up to 0.5mL of plasma). Now a days, liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) has become a popular analytical technique for determining drug levels in biological fluids, since it can provide higher sensitivity and selectivity than other traditional methods. Till date, only two LC-MS/MS procedures have been reported for the quantitation of penciclovir\textsuperscript{23,24}, which employed protein precipitation to extract penciclovir from plasma. The method is sensitive but the sample preparation method is not preferred for massspectrometry due to the matrix effect; this may be a limiting factor when it is applied to
large number of subject samples. This section describes a novel and selective approach, which enables the determination of penciclovir with good accuracy at low drug concentrations in plasma using liquid chromatography coupled to tandem mass spectrometry (MS/MS).

3.2 EXPERIMENTAL

3.2.1 Study Objective

Purpose of the present study was to develop and validate an LC-MS/MS method for determination of penciclovir in human plasma for pharmacokinetic studies.

3.2.2 Reference Compounds: Penciclovir and Acyclovir (IS)

IUPAC Name: 2-amino-9-[4-hydroxy-3-(hydroxyl methyl)butyle]-6,9- dihydro-3H-purine-6-one ; 2-amino-9((2-hydroxyethoxy)methyl)-1H- purin- 6(9H)-one

Molecular Formula: C₁₀H₁₅N₅O₃ ; C₈H₁₁N₅O₃

Molecular Weight: 253.25 ; 225.20

Purity : 99.8% ; 99.6%

Supplier : Synchron Research Services Pvt. Ltd (Ahmedabad, India)

![Fig.3.1 Structure of Penciclovir (I) and Acyclovir (II)](image)

3.2.3 Chemicals, Reagents and Materials

3.2.3.1 Chemicals

All the solvents were of high purity, and all the glassware were carefully cleaned and rinsed with Milli Q water (Type-1 grade) before analysis. The reagents were obtained as stated:-

- Acetonitrile from J. T. Baker (USA)
- Methanol from J. T. Baker (USA)
Chapter 3

- Hydrochloric acid from J. T. Baker (USA)
- Formic acid from Merck (Germany)
- Water deionized and purified by a Milli-Q water purification system from Millipore (Bangalore, India).
- Drug-free (blank) human plasma was obtained from Prathma Laboratory (Ahmedabad, India)

3.2.3.2 Reagents

0.2% Formic Buffer: In a volumetric flask 200ml of water was taken, 1.0 ml formic acid was added and volume was made to 500ml with Milli Q water.

Reconstitute Solution: In a volumetric flask 200ml of acetonitrile was taken to which 50ml Milli Q Water and 5ml of formic acid was added and mixed.

Auto Sampler Needle Wash: 2.5ml formic acid in 500ml Milli Q Water was taken in a reagent bottle, mixed properly and sonicated.

Mobile phase: In a volumetric flask 800ml of acetonitrile and 200ml of water were taken, mixed thoroughly, 0.2% formic acid buffer added (80:20:0.2, v/v/v) was added and delivered with a flow rate of 0.5mL/min.

3.2.3.3 Materials

- Quattro Micro system from Waters corp., Milford, MA, USA
- HPLC system Alliance 2695 from Waters corp., Milford, MA, USA
- Purospher star 75 x 4mm, 3µm, C18 column from Merk, Germany
- Eppendorf centrifuge 5810R from Hamburg, Germany
- Micropipettes from Eppendorf, Hamburg, Germany
- Vortex mixer, Spinix, Tarson, India
- Analytical balance AB265-S form Mettler Toledo, Germany
- Micro balance MX-5 of Mettler from Toledo, Germany
- Sonicator Bandelin Sonorex from Zymark, Germany
- Class A calibrated glass ware from different supplier

3.2.4 Liquid Chromatographic Conditions

Waters Alliance HPLC system with C18 Purospher star column (75mm x 4mm, 3µ) that contains packing of octadecylsilane chemically bonded to porous silica was used for
chromatographic separation. The mobile phase was prepared with the combination of acetonitrile-water-formic acid (80:20:0.2, v/v/v). The flow rate of 0.5ml/min was used to carry out separation. The column temperature was set at 45˚C, the auto-sampler was conditioned at 10˚C and the injection volume was 5µL with a run time around 2.5min.

3.2.5 Mass Spectrometric Conditions and Data Processing

The Mass spectrometry was operated in positive ion detection mode. Nitrogen was used as nebulizing turboionspy. The source temperature was set at 120˚C, desolvation temperature was optimized at 350˚C and the ESI needle voltage was 3.2kV. The cone energy and collision energy were set at 18 volts. The mass spectrometer was operated at unit mass resolution with a dwell time of 500ms per transition. Quantification was performed using multiple reaction monitoring (MRM) of the transition m/z 254 (parent ion) → m/z 152 (product ion); m/z 226 (parent ion) → m/z 152 (product ion) for penciclovir and IS respectively. The analytical data were processed by Maslynx 4.0.

3.2.6 Standard Solutions (Calibration Standards and Quality Control Samples)

A stock solution (1mg/mL) was prepared by dissolving 10mg of pencyclovir in 10mL of water and 100µL of concentrated HCl. Spiking solution of pencyclovir was prepared from stock solution by serial dilution method in water. Nine levels of calibration curve standards were prepared by adding the spiking solution in plasma and concentration levels of 52.555, 105.110, 202.210, 323.417, 980.052, 3266.840, 4949.755, 5963.563 and 6626.181ng/mL were achieved. Three levels of quality control samples were prepared by adding the spiking solutions in plasma and concentration levels of 154.014, 2916.925 and 5303.501ng/mL were achieved.

3.2.7 Extraction Procedure

All the calibration standards (250µL) or QC samples (250µL) were taken in polypropylene tubes, 25µL of internal standard (250µg/mL of acyclovir) was added and vortexed for 30s. The samples were transferred to a 1cm³/30 mg Oasis HLB SPE column, which had been conditioned with 1.0mL methanol, followed by 1.0mL water. After application of the samples, the SPE column was washed with 1.0mL of MilliQ water and dried for 1.0min by applying positive pressure at maximum flow rate. The column was eluted with 1.0mL of the reconstituted solution (acetonitrile - MilliQ water, 4:1, v/v buffered with 0.2% formic acid) and vortexed for about 10s. The SPE elutes were transferred into 1mL LC vials for injection of 5µL into the LC system.
3.2(a) PEN01 14 (0.050) Cm (1:74) Parents of 254ES+

3.2(b) PEN DAU03 2 (0.007) Cm (1:59) Daughters of 254ES+

3.2 (c) ACYCLOVIR PAR 20 (0.071) Cm (1:252) Parents of 226ES+

3.2 (d) ACYCLOVIR DAU 03 1 (0.004) Cm (1:18) Daughters of 226ES+

Figure 3.2 (a) Q1 Scan of Penciclovir and (b) MS/MS Scan of Penciclovir; (c) Q1 Scan of IS and (d) MS/MS Scan of IS
3.3 RESULTS AND DISCUSSION

3.3.1 Method Development

During the method development mass spectrometric conditions, extraction procedure and chromatographic conditions were optimized. The ideal condition of MS/MS detection was expected to be advantageous in developing a selective and sensitive method. Optimum mass acquisition parameters were obtained by direct infusion of 500ng/mL solution of both analyte and internal standard at a flow rate of 10µL/min. The mass spectrometer was operated in the MRM condition under positive ion mode. The transition of ions were monitored m/z 254 (parent ion) -152 (product ion) for pencyclovir and m/z 226 (parent ion) - 152 (product ion) for internal standard (acyclovir). An earlier report had suggested the source temperature 100°C and capillary voltage 3.5KV. However in the present study source temperature was 120°C and capillary voltage was 3.2KV which were optimized along with other tuning parameters for the enhanced sensitivity and reproducibility.

Zhou Q et al., Lee H.W et al., B. Nutley et al., Yi-jun D et al. have reported a protein precipitation method to separate penciclovir, the very polar drug from human plasma. The extraction was carried out initially via protein precipitation with common solvents like perchloric acid, acetonitrile, methanol, acetone, but the reproducibility was very poor due to lot of matrix interference, which resulted contamination of quadrapoles leading to lesser sensitivity.

Liquid-liquid extraction technique was not performed as penciclovir is a very polar (logP= -1.62) molecule. Based on earlier reports extraction method in solid phase using Water Oasis MCX, Waters Oasis HLB, Waters Oasis WAX, and Phenomenex Strata cartridges was evaluated. Waters Oasis HLB and Waters Oasis MCX (cation exchange) produced repeatable and reproducible results. Based on the cost factor Waters Oasis HLB cartridge has been chosen for the extraction of penciclovir and IS.

It was essential to have a chromatographic separation of the drugs and to minimize any interference during quantitation. Chromatographic analysis of penciclovir and IS was initiated under isocratic conditions to obtain adequate response, sharp peak shape and a shorter run time. The separation was attempted using various combinations of methanol/acetonitrile, acidic buffers and additives like formic acid on different reversed-phase columns [ACE C18 (50×4.6mm, 5µm), Gemini C18 (50mm×4.6mm, 5µm),...
Thermo C18 (50mm×4.6mm, 5µm), Purospher star C18 (75mm x 4mm, 3µm), Kromasil C18 (50mm×4.6mm, 5µm), and Chromolith RP18 (100mm×4.6mm, 5µm)]. Best results in terms of reproducibility, complete separation and peak shape without any interference were obtained with Purospher star C18 (75mm x 4mm, 3µm) column compared to others and hence was selected for further study. Earlier reports \(^{20, 21}\) used the ion pairing agents for the mobile phase, resulting in slow equilibration of the column and fluctuation of retention times. In this method, a mobile phase consisting of acetonitrile-water-formic acid (80:20:0.2, v/v/v) without any ion pairing agent was found most suitable for eluting penciclovir and internal standard at around 1.0min. A flow-rate of 0.5mL/min produced good peak shapes and permitted a run time of 2.5min per analysis. Earlier publications \(^{19, 21, 24}\) have reported longer run times (3min to 15min) when compared with present chromatographic condition. By virtue of its similarity in therapeutic group, chromatographic behaviour and ionization pattern, acyclovir was selected as internal standard.

3.3.2 Method Validation

3.3.2.1 Selectivity

Selectivity was carried out by analyzing six blank plasma samples spiked with penciclovir and IS. Possible interferences at the retention times of penciclovir (LLOQ level) and IS from endogenous compounds were checked during the validation by testing six different batches of K\(_3\)EDTA human plasma (Fig.3.1).

3.3.2.2 Linearity

Linearity of the method was evaluated using bulk spiked plasma samples in the concentration range as mentioned above using the method of least squares. Three such linearity curves were analyzed. Each calibration curve consisted of a blank sample, a zero sample (blank + IS) and nine concentrations. The standard curves were linear over the concentration range of 52.555 - 6626.181ng/mL. Samples were quantified using the ratio of peak area of analyte to that of IS. A weighting factor linear regression (1/concentration) was performed with the nominal concentrations of calibration levels. Peak area ratios were plotted against plasma concentrations, the limit of quantitation was 52.555ng/mL.
3.3.2.3 Recovery

The recovery of drug and IS were evaluated at three concentration levels namely low, medium and high quality control. Recovery was calculated by comparing its response in replicate samples with that of neat standard solution responses. Analyte recovery from a sample matrix (extraction efficiency) is a comparison of analytical response from an amount of analyte added to that determined from sample matrix. Extraction was carried out using ethyl acetate as organic solvent. Experiments with spiked compounds resulted mean recovery of 54.0% and 47.8% for penciclovir and acyclovir, respectively (Table 2.1).
3.3.2.4 Precision and Accuracy

Intra-day accuracy and precision were evaluated from replicate analyses (n = 6) of quality-control samples containing penciclovir at different concentrations on the same day. Inter-day accuracy and precision were also assessed from the analysis of the same QC samples on separate occasions in replicate (n = 6). QC samples were analyzed against calibration standards.

All calibration curves were found to be linear over the range of 52.555 - 6626.181 ng/ml. The mean correlation coefficient was 0.9992. The precision for the six plasma samples spiked at LOQ concentration was 2.8% with a mean accuracy of 102.87% (Table 3.2). The inter-batch assay accuracy ranged between 93.72 - 97.62%, whereas intra-batch accuracy ranged between 89.45 - 94.93%. The inter-batch precision ranged between 8.8 - 11.7%. Intra batch precision ranged between 0.7 - 4.9%. The results are presented in Table 3.3. All the results were found within the acceptable limit of precision not more than 15.0% and accuracy 85.0 - 115.0%, except LLOQ for which precision was not more than 20% and accuracy was between 80.0 - 120.0%.

3.3.2.5 Matrix factor

The matrix of plasma constituents over the ionization of analyte was determined by comparing the response of post-extracted plasma standard QC samples (n = 6) with the response of analyte from neat samples at equivalent concentrations\textsuperscript{26-28}. The matrix effect intended method was assessed using chromatographically screened human plasma. Precision (%CV) is 5.0% and 2.6% for penciclovir and IS respectively (Table 3.4).

<table>
<thead>
<tr>
<th>Nominal concentrations (ng/mL)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Penciclovir</td>
</tr>
<tr>
<td>5303.501</td>
<td>53.81</td>
</tr>
<tr>
<td>2916.925</td>
<td>53.94</td>
</tr>
<tr>
<td>154.014</td>
<td>54.40</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td><strong>54.05</strong></td>
</tr>
</tbody>
</table>
Table 3.2 Precision and Accuracy Data of Back-Calculated Concentration of Calibration Samples for Penciclovir in Human Plasma

<table>
<thead>
<tr>
<th>Concentration added (ng/mL)</th>
<th>Concentration determined (mean ± S.D) (ng/mL)</th>
<th>Precision (%)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6626.181</td>
<td>6844.98 ± 513.374</td>
<td>7.5</td>
<td>103.302</td>
</tr>
<tr>
<td>5963.563</td>
<td>6088.45 ± 200.919</td>
<td>3.3</td>
<td>102.094</td>
</tr>
<tr>
<td>4949.757</td>
<td>5029.63 ± 357.104</td>
<td>7.1</td>
<td>101.608</td>
</tr>
<tr>
<td>3266.840</td>
<td>3012.08 ± 108.435</td>
<td>3.6</td>
<td>92.201</td>
</tr>
<tr>
<td>980.052</td>
<td>992.19 ± 116.087</td>
<td>11.7</td>
<td>101.239</td>
</tr>
<tr>
<td>323.417</td>
<td>349.40 ± 22.012</td>
<td>6.3</td>
<td>108.034</td>
</tr>
<tr>
<td>202.210</td>
<td>197.64 ± 11.266</td>
<td>5.7</td>
<td>97.743</td>
</tr>
<tr>
<td>105.111</td>
<td>99.45 ± 5.370</td>
<td>5.4</td>
<td>94.618</td>
</tr>
<tr>
<td>52.555</td>
<td>54.07 ± 1.514</td>
<td>2.8</td>
<td>102.87</td>
</tr>
</tbody>
</table>

Table 3.3 Precision and Accuracy of the Method for Determining Penciclovir Concentration in Plasma Samples

<table>
<thead>
<tr>
<th>Conc. added (ng/mL)</th>
<th>Intra-batch (n=6)</th>
<th>Inter-batch (n=18)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conc. determined (mean ± S.D) (ng/mL)</td>
<td>Precision (%)</td>
</tr>
<tr>
<td>5303.501</td>
<td>5034.84 ± 35.244</td>
<td>0.7</td>
</tr>
<tr>
<td>2916.925</td>
<td>2609.27 ± 117.417</td>
<td>4.5</td>
</tr>
<tr>
<td>154.014</td>
<td>142.87 ± 7.001</td>
<td>4.9</td>
</tr>
</tbody>
</table>

Table 3.4 Matrix Effect of Penciclovir

<table>
<thead>
<tr>
<th></th>
<th>HQC</th>
<th>LQC</th>
<th>Internal Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Processed (A)</td>
<td>Aqueous (B)</td>
<td>Matrix Factor (A/B)</td>
<td>Processed (A)</td>
</tr>
<tr>
<td>3489848</td>
<td>3486221</td>
<td>1.001</td>
<td>1260436</td>
</tr>
<tr>
<td>3537572</td>
<td>3495995</td>
<td>1.012</td>
<td>1326926</td>
</tr>
<tr>
<td>3263890</td>
<td>3472935</td>
<td>0.940</td>
<td>1196900</td>
</tr>
<tr>
<td>3131518</td>
<td>3605106</td>
<td>0.869</td>
<td>1165605</td>
</tr>
<tr>
<td>3476562</td>
<td>3455926</td>
<td>1.006</td>
<td>1286950</td>
</tr>
<tr>
<td>3218336</td>
<td>3447795</td>
<td>0.933</td>
<td>1238300</td>
</tr>
<tr>
<td>Mean</td>
<td>3493996</td>
<td>0.96</td>
<td>1265950</td>
</tr>
<tr>
<td>SD</td>
<td>0.056</td>
<td>0.047</td>
<td>0.076</td>
</tr>
<tr>
<td>% CV</td>
<td>5.9</td>
<td>4.8</td>
<td>7.5</td>
</tr>
</tbody>
</table>
3.3.2.6 Dilution Integrity

During the course of study, probability of encountering samples with concentrations above the upper limit of quantitation (ULOQ) could not be ruled out. Therefore dilution with drug free plasma is necessary to bring them within the calibration range. To establish the effect of dilution on the integrity of samples, six aliquots of 10772.736ng/mL of penciclovir were prepared. The samples were subjected to two-fold dilution (n = 6) and five-fold dilution (n = 6) with drug free human plasma to bring them within the calibration range. The samples were processed, analyzed and the concentrations obtained were compared with theoretical values. The precision for dilution integrity standards at 1:2 and 1:5 for penciclovir was 0.5 and the mean accuracy was 9.6% for penciclovir (Table 3.5).

**Table 3.5 Dilution Integrity for Penciclovir**

<table>
<thead>
<tr>
<th>Description</th>
<th>Concentration Obtained for 1/2 Dilutions (ng/mL)</th>
<th>Concentration Obtained for 1/5 Dilutions (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sample conc.</td>
<td>With dilution factor</td>
</tr>
<tr>
<td>Aliquot 1</td>
<td>5937.681</td>
<td>11875.362</td>
</tr>
<tr>
<td>Aliquot 2</td>
<td>5936.182</td>
<td>11872.363</td>
</tr>
<tr>
<td>Aliquot 3</td>
<td>5891.728</td>
<td>11783.456</td>
</tr>
<tr>
<td>Aliquot 4</td>
<td>5867.618</td>
<td>11735.236</td>
</tr>
<tr>
<td>Aliquot 5</td>
<td>5899.289</td>
<td>11798.578</td>
</tr>
<tr>
<td>Aliquot 6</td>
<td>5881.012</td>
<td>11762.024</td>
</tr>
<tr>
<td>Mean</td>
<td>5902.252</td>
<td>11804.503</td>
</tr>
<tr>
<td>SD</td>
<td>28.902</td>
<td>57.804</td>
</tr>
<tr>
<td>% CV</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>% Mean Accuracy</td>
<td>109.6</td>
<td>109.6</td>
</tr>
</tbody>
</table>

3.3.2.7 Stability Study

Evaluation of the stability of samples was based on the comparison of various samples against freshly prepared sample of the same concentration. Percentage difference between the back calculated concentrations obtained for the sample under investigation and freshly prepared sample was evaluated. Four aliquots, each of LQC and HQC concentrations were used for stability study.
The bench top stability (at room temperature) was determined for 6h by comparing the ratio of means of the concentrations for the low and high QCs which were found to be 99.8 and 92.1% respectively. The freeze-thaw stability was determined by measuring the assay precision and accuracy of the LQC and HQC samples, which underwent three freeze thaw cycles. In each freeze thaw cycle, the frozen plasma samples were thawed at room temperature for 2-3 h and refrozen for 12-24h. After completion of each cycle the samples were analyzed and the results were compared with that of zero cycle. The results showed that the analyte was stable in human plasma through freeze thaw cycles. The ratio of means of concentrations for the low and high QC was 93.2 and 98.7%. This was within the acceptable range of 85-115%. The results demonstrated that human plasma samples could be thawed and refrozen without compromising the integrity of the samples. Auto sampler stability of the plasma samples were over 46h. All the stability results tabulated in Table 3.6, were found within the acceptable limits.

**Table 3.3 Stability Results for Penciclovir**

<table>
<thead>
<tr>
<th></th>
<th>Mean calculated comparison sample conc. (ng/mL) (n=6)</th>
<th>Mean calculated stability sample conc. (ng/mL) (n=6)</th>
<th>Precision (%)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bench top stability (10h)</strong></td>
<td>167.705</td>
<td>166.344</td>
<td>2.3</td>
<td>99.2</td>
</tr>
<tr>
<td></td>
<td>5165.130</td>
<td>5548.362</td>
<td>1.8</td>
<td>107.4</td>
</tr>
<tr>
<td><strong>Freeze thaw stability (after 4 cycles)</strong></td>
<td>167.705</td>
<td>167.415</td>
<td>1.2</td>
<td>99.8</td>
</tr>
<tr>
<td></td>
<td>5165.130</td>
<td>4756.449</td>
<td>3.6</td>
<td>92.1</td>
</tr>
<tr>
<td><strong>Auto sampler stability (9h at 10°C)</strong></td>
<td>171.113</td>
<td>153.371</td>
<td>3.9</td>
<td>93.2</td>
</tr>
<tr>
<td></td>
<td>5317.914</td>
<td>4788.219</td>
<td>2.5</td>
<td>98.7</td>
</tr>
</tbody>
</table>
3.4 CONCLUSION

A simple, specific, rapid (2.5min) and sensitive (LLOQ 52.555ng/mL) LC-MS/MS method has been developed for the determination of penciclovir in human plasma and fully validated according to FDA guidelines. Smaller injection volume (5µL) is of particular advantage with our method which is most important for the LCMS/MS analysis to reduce the matrix effect and increase the sensitivity. We can conclude that the present method can be useful for clinical pharmacokinetic studies.
3.5 REFERENCES


