CHAPTER - 4
ETOPOSIDE

TITLE: Rapid Quantification of Etoposide in Spiked Human Plasma by Ultra Performance Liquid Chromatography-Tandem Mass Spectrometry

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4.1 Abstract

A reliable, sensitive and rapid ultra performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) method has been developed for the determination of etoposide in human plasma. The method involved liquid-liquid extraction of etoposide together with its deuterated internal standard (IS) etoposide-d3 from 200 µL human plasma in methyl tert-butyl ether. The chromatographic separation was achieved on Waters UPLC BEH C18 (50 mm × 2.1 mm, 1.7 µm) analytical column using acetonitrile and 0.1% (v/v) formic acid in water (80:20, v/v) at a flow-rate of 0.300 mL/min under isocratic conditions. The precursor → product ion transition for etoposide ([m/z 589.2 → 229.0], and IS ([m/z 592.1 → 229.0]) were monitored on a triple quadrupole mass spectrometer, operating in the multiple reaction monitoring (MRM) and positive ionization mode. The method was validated over a wide dynamic concentration range of 5-8000 ng/mL in spiked human plasma. The recovery of etoposide and etoposide-d3 was greater than 90 % across quality controls. Relative matrix effect expressed as % CV of the slope of calibration curves obtained from eight plasma lots was 2.88 %. Stability was thoroughly investigated under different storage conditions. The method is rugged and rapid with a chromatographic run time of 2.0 min and can be highly useful for high throughput clinical studies.

4.2 Introduction

Etoposide (VP-16) is a semi synthetic derivative of podophyllotoxin, and is extensively used in anticancer therapy, both of solid tumors and hematological malignancies [1, 2]. It acts by inhibiting one of the most abundant nuclear proteins, deoxyribonucleic acid (DNA) topoisomerase. As etoposide is cell cycle specific, and leads to an accumulation of cells in G2/M, it has major effect on a large range of carcinomas, particularly gastric cancer cells, germ cell tumors, small cell lung carcinoma, hematologic malignancies, and childhood malignancies [3, 4]. However, etoposide has limitation of low and variable oral bioavailability. Several approaches are currently in progress to exhibit potential improvement in
the rate and extent of absorption of etoposide into the systemic circulation for oral therapy in clinical studies [5, 6].

Due to variable and low etoposide therapeutic levels it is essential to develop sensitive, rugged and rapid bioanalytical methods for its determination in biological fluids to minimize the risk of drug accumulation, for the optimization of therapy and to reduce the frequency of adverse effects. Determination of etoposide in biological samples has been a subject of many reports. Several methods based on liquid chromatography with electrochemical [7-9], fluorescence [10-12], UV [12-14] and mass detection [15-17] is reported in the literature for etoposide. In these methods etoposide has been estimated from different biological matrices like human serum, blood, plasma and leukemic cells. A UPLC-qTOF-MS method for simultaneous determination of etoposide and a piperine analogue from mouse plasma has also been described [18].

Liquid chromatography tandem mass spectrometry (LC–MS/MS) is an important tool in therapeutic drug monitoring as it offers increased sensitivity and specificity compared to other methods, and may be the only viable method for quantifying drugs without natural chromophores or fluorophores. The choice of suitable sample preparation method, column technology, internal standard and mass spectrometric conditions is important to ensure accurate drug measurement and to avoid interference from matrix effects and drug metabolites.

Ultra performance liquid chromatography (UPLC) is emerging as a superior device to separate complex mixtures in both isocratic and gradient modes. Column technology with sub-2 µm particle size has revolutionized the field of separation science during the last decade. With the advent of smaller particle size material as stationary phase, there has been a renewed interest and a step function change in the way liquid chromatography is conducted today. UPLC with sub-2 µm particle size has demonstrated enhanced efficiency, superior resolution, higher sensitivity and much faster throughput compared to conventional HPLC with 3 or 5 µm particles. This has provided a tremendous boost for analyzing complex mixtures and streamlining extensive analytical workflow.
Liquid chromatography has stood the test of time in the field of separation science since its introduction in the early 1900s. The major breakthrough towards enhancing the performance of LC was in the 1960s when high pressure (500 psi, 35 bar) was used to generate flow through packed columns with smaller stationary phase particle size (≤ 10 µm). This led to a new era of ‘high pressure liquid chromatography’ (HPLC). Thereafter, with significant advancement in column technology it was possible to achieve even higher pressure (6000 psi, 400 bar) to give much superior separation performance and thus replacing the prefix ‘high pressure’ with ‘high performance’ in conventional LC. Since then different approaches have been adopted to improve chromatographic performance especially for analysis time, resolution and sensitivity. Although conventional HPLC with 3 or 5 µm particles find widespread use in environmental, clinical, toxicology and pharmaceutical analysis, it has relatively moderate efficiency and requires long analysis time for separating complex mixture. Subsequently, several attempts were made to overcome these challenges by increasing the flow rates and reducing column lengths to enhance chromatographic efficiency [19]. Such modifications partially resolved the issues facing conventional HPLC, albeit with some limitations as they result in low phase ratio and small capacity factors. Another approach was also tried by increasing the column temperature, however it was largely ineffective especially for temperature sensitive compounds and could potentially damage the column material. Decreasing the particle size leads to significant increase in peak capacity and speed of analysis. **Figure 1** shows the timeline for evolution of stationary phase particle size for LC.

![Figure 1. Evolution of stationary phase particle size in the past four decades](image-url)
One major drawback though of reduced particle size is that it can induce high back pressure. As the column back pressure varies inversely with the square of the particle size at constant linear flow rate [20], the operating pressure can go up to 15000-20000 psi which is difficult to handle with conventional HPLC instrumentation. This increase in the back pressure (> 10000 psi, ~700 bar) has led to this technique to be referred as high or ultrahigh pressure liquid chromatography (UHPLC or UPLC). Apparently, some practical hitches in working with smaller particles took almost 4 decades to realize its full potential. The year 2004 can be considered as the defining moment in LC when the first UHPLC system with sub-2 µm particle size was commercialized.

To the best of our knowledge there are no reports on the use of UPLC-MS/MS technique for the determination of etoposide in human plasma. Thus, in the present work an accurate, simple and rapid UPLC-MS/MS method has been developed and fully validated for reliable measurement of etoposide in human plasma samples. The method requires only 200 µL human plasma sample for extraction and demonstrates excellent performance in terms of ruggedness and efficiency (2.0 min per sample).

4.3 Experimental and method optimization

4.3.1 Liquid chromatographic and mass spectrometric conditions

A Waters Acquity UPLC system (MA, USA) consisting of binary solvent manager, sample manager and column manager was used for setting the reverse-phase liquid chromatographic conditions. The analysis of etoposide and IS was performed on UPLC BEH C18 (50 × 2.1 mm, 1.7 µm) analytical column, maintained at 40 °C. The mobile phase consisted of acetonitrile and 0.1% (v/v) formic acid in water (80:20, v/v). The flow rate of the mobile phase was kept at 0.300 mL/min. The sample manager temperature was maintained at 5°C and the pressure of the system was 5000 psi. Quantitation was done using multiple reaction monitoring (MRM) for protonated precursor → product ion transitions, etoposide (m/z 589.2 → 229.0) and IS (m/z 592.1 → 229.0) on Quattro Premier
XETM mass spectrometer from Waters – Micro Mass Technologies (MA, USA), in the positive electro spray ionization mode.

The source dependent parameters maintained for both the compounds were, desolvation gas (heater gas): 900L/hr; capillary voltage: 3.50kV; desolvation temperature: 400°C; and cone gas flow: 100L/hr. The optimum values for compound dependent parameters like cone voltage, and collision energy were 22 V and 15 eV for etoposide and 25 V and 15 eV for IS respectively. Quadrupole 1 and 3 were maintained at unit mass resolution and the dwell time was set at 200 ms for both the drugs. Data collection, peak integration, and calculations were performed using Mass Lynx software version 4.1.

4.3.2 Preparation of stock solution, calibrators and quality control samples

The standard stock solution of etoposide (1000 µg/mL) was prepared by dissolving requisite amount in methanol. Calibration standards and quality control (QC) samples were prepared by spiking (2% of total plasma volume) blank plasma with stock solution. Calibration curve standards were made at 5.00, 10.0, 50.0, 100, 250, 500, 1000, 3000, 5000 and 8000 ng/mL concentrations respectively, while QC samples were prepared at four levels, 7000 ng/mL (HQC, high QC), 4000/800 ng/mL (MQC-1/2, medium QC), 15.0 ng/mL (LQC, low QC) and 5.00 ng/mL (LLOQ QC, lower limit of quantitation QC). Stock solution (1.0 mg/mL) of etoposide-d3 as internal standard was prepared by dissolving 2.0 mg in 2.0 mL of methanol. Its working solution (2500 ng/mL) was prepared by appropriate dilution of the stock solution in methanol. The stock solutions were stored at 5 °C, while calibration standards and quality control samples were stored at –70 °C until use.

4.3.3 Sample extraction protocol

Prior to analysis, all calibration standards and quality control samples were thawed and allowed to equilibrate at room temperature. To an aliquot of 200 µL of spiked plasma sample, 50µL of internal standard was added and vortexed for 15s.
Further, 100µL of 0.1N sodium hydroxide solution was added to the solution and vortex again for 10 s. Extraction was done with 1.5 mL methyl tert-butyl ether by first vortexing for 10 s and then keeping the samples on rotor for 15 min at 50 rpm followed by centrifugation at 4000 rpm for 5 min at 10°C. The supernatant was collected in pre labeled tubes and dried under a gentle stream of nitrogen at 40°C. The dried samples were reconstituted in 100 µL of mobile phase and 10 µL was used for injection in the chromatographic system.

4.3.4 Validation methodology

The method validation was performed as per the USFDA guidelines [21]. Details of validation procedure and acceptance criteria are given in Chapter-2.

4.4 Results and discussion

4.4.1 Method development

Mass spectrometry

The present study was conducted using electrospray ionization (ESI) for MRM UPLC-MS/MS analyses to attain high sensitivity and a good linearity in regression curves. Q1 MS full scan spectra for etoposide and IS predominantly contained protonated precursor [M+H]⁺ ions at m/z 589.2 and 592.1 respectively. The most abundant product ions in Q3 MS spectra for etoposide and IS were observed at m/z 229.0 at 15 eV collision energy. This product ion can be attributed to the fragment formed due to the elimination of beta-D-glucopyranose and 4-hydroxy-3,5-dimethoxyphenyl moieties from the protonated precursor ion as shown in Figure 2. The fragments corresponding to other less intense product ions are also shown for etoposide and IS. The source dependent and compound dependent parameters were suitably optimized to obtain a consistent and sufficient response for the analyte. A dwell time of 200 ms for etoposide and IS was adequate and no cross talk was observed between their MRMs.
Figure 2. Product ion mass spectra of (a) etoposide (m/z 589.2 → 229.0) and (b) etoposide-d3, IS (m/z 592.1 → 229.0) in the positive ionization mode.
Optimization of chromatographic conditions

Chromatographic analysis was initiated on Waters Acquity UPLC BEH C18 (50 × 2.1 mm, 1.7 µm) column to attain higher response, good peak shape, minimum matrix interference and short run time. Initially, different combinations of acetonitrile/methanol and 0.1 % formic acid (90:10, 80:20, 70:30, v/v) were evaluated having apparent pH between 2.8 and 3.0. However, the best chromatographic conditions with respect to symmetrical peak shape, adequate retention and response was obtained using acetonitrile and 0.1% (v/v) formic acid in water (80:20, v/v) as the mobile phase. Acetonitrile was selected as an organic modifier because of its low ionic suppression effect, is volatile, and therefore compatible with MS detection. This ensured a total run time of 2.0 min with retention time of 1.15 and 1.14 min for etoposide and etoposide-d3 respectively at a flow rate of 0.300 min. Under the optimized conditions, the values of capacity factor and theoretical plates for etoposide were 2.83 and 1079 respectively. Use of deuterated internal standard, etoposide-d3 adequately compensated for any variability during extraction and UPLC-MS/MS analysis in the present work. Representative MRM ion chromatograms in Figure 2 of extracted blank human plasma (double blank), blank plasma fortified with IS, etoposide at LLOQ and ULOQ levels demonstrates the selectivity of the method.

Optimization of sample extraction

The extraction procedure together with mass detection gave very good selectivity for the analysis of etoposide and IS in the blank plasma. Results of post-column infusion experiment indicated no ion suppression or enhancement at the retention time of etoposide and IS. The average matrix factor value calculated as the response of post spiked sample/response of neat solutions in mobile phase at the HQC, MQC and LQC levels was 0.97, which indicates a minor suppression of 3%.
Figure 2. Representative MRM chromatograms of (a) in double blank plasma (without analyte and IS), (b) blank plasma and etposide-d3, IS (m/z 592.1 → 229.0), (c) etoposide (m/z 589.2 → 229.0) at LLOQ and IS, (d) etoposide (m/z 589.2 → 229.0) at ULOQ and IS
For plasma extraction, initially protein precipitation was tried in acetonitrile and methanol; however, this resulted in poor recovery for etoposide possibly due to high plasma protein binding (94 %). Thus, liquid-liquid extraction was investigated as etoposide is a lipophilic drug with poor solubility in water (app. 0.03 mg/L). Different extraction solvents like chloroform, diethyl ether, and dichloromethane and methyl tert-butyl ether were tested for quantitative and precise recovery. As etoposide has a pKₐ value of 9.8, addition of sodium hydroxide prior to extraction rendered the –OH group unionized for quantitative recovery. Amongst the four solvents tested, methyl tert-butyl ether gave highly consistent recovery at all QC levels and hence was selected in the present work.

4.4.2 Assay performance and validation results

System suitability, system performance and carry-over test

The precision (%CV) of system suitability test was observed in the range of 0.06 to 0.23 % for the retention time and 0.85 to 2.31 % for the area response of etoposide and IS, which is within the acceptance criteria of 4 %. The signal to noise ratio for system performance was > 42 % for etoposide and IS. Auto-sampler carry-over evaluation was performed in each analytical run so as to ensure that it does not affect the accuracy and the precision of the proposed method. The experiment showed negligible carry over in blank plasma (≤ 0.14 % of LLOQ sample) after subsequent injection of ULOQ sample. There was no enhancement in the response in double blank plasma (without analyte and IS) following injection of highest calibration standard at the retention time of etoposide and IS respectively.

Linearity, intra- & inter-batch accuracy and precision

All five calibration curves were linear over the concentration range of 5.00-8000 ng/mL ($r^2 \geq 0.9985$). The mean linear equation for calibration curve concentrations was $y = (0.00234 \pm 0.000058)x + (0.00543 \pm 0.00115)$ where $y$ is the peak area ratio of the etoposide/IS and $x$ the concentration of etoposide.
accuracy and precision (% CV) observed for the calibration curve standards varied from 93.5 to 98.1% and 1.00 to 5.72 respectively. The limit of detection (LOD) and LLOQ of the method were 1.50 and 5.00 ng/mL for etoposide at a signal-to-noise ratio of 10 and 42 respectively. The intra-batch and inter-batch precision and accuracy results across four QC levels for etoposide are shown in Table 1. The intra-batch precision (% CV) ranged from 0.59 % to 2.44 % and the accuracy was within 95.74 - 98.09 %. Similarly for inter-batch experiments, the precision varied from 1.00 to 5.72 % and the accuracy was within 93.09 - 98.19 %.

<table>
<thead>
<tr>
<th>QC level</th>
<th>Intra-batch (n = 6; single batch)</th>
<th>Inter-batch (n = 30; 6 from each batch)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nominal Conc. (ng/mL)</td>
<td>Mean conc. found (ng/mL)</td>
<td>% CV</td>
</tr>
<tr>
<td>HQC (7000)</td>
<td>6702</td>
<td>0.93</td>
</tr>
<tr>
<td>MQC-1 (4000)</td>
<td>3882</td>
<td>0.86</td>
</tr>
<tr>
<td>MQC-2 (800)</td>
<td>785</td>
<td>0.59</td>
</tr>
<tr>
<td>LQC (15.0)</td>
<td>14.6</td>
<td>1.64</td>
</tr>
<tr>
<td>LLOQ QC (5.00)</td>
<td>4.85</td>
<td>2.44</td>
</tr>
</tbody>
</table>

CV: Coefficient of variation; LLOQ: lower limit of quantitation; LQC: low quality control; MQC: medium quality control; HQC: high quality control

**Extraction recovery and relative matrix effect**

The extraction recovery and matrix effect data for etoposide is presented in Table 2. The mean extraction recovery for etoposide and IS ranged from 92.3 to 96.5 % and 90.8 to 94.3 % respectively across QC levels.

The coefficient of variation (% CV) of the slopes of calibration lines for relative matrix effect in eight different plasma lots was 2.88, which is within the acceptance criteria of < 3-4 % [22] as shown in Table 3. This proves the absence of matrix effect in the present method.
Table 3. Relative matrix effect in 8 different lots of human plasma

<table>
<thead>
<tr>
<th>Plasma lot</th>
<th>Slope of calibration curve</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lot-1</td>
<td>0.00239</td>
</tr>
<tr>
<td>Lot-2</td>
<td>0.00225</td>
</tr>
<tr>
<td>Lot-3</td>
<td>0.00238</td>
</tr>
<tr>
<td>Lot-4</td>
<td>0.00228</td>
</tr>
<tr>
<td>Lot-5</td>
<td>0.00235</td>
</tr>
<tr>
<td>Lot-6 (heparinized)</td>
<td>0.00240</td>
</tr>
<tr>
<td>Lot-7 (haemolysed)</td>
<td>0.00239</td>
</tr>
<tr>
<td>Lot-8 (lipemic)</td>
<td>0.00224</td>
</tr>
<tr>
<td>Mean</td>
<td>0.00234</td>
</tr>
<tr>
<td>± Standard deviation</td>
<td>0.0000674</td>
</tr>
<tr>
<td>% Coefficient of variation</td>
<td>2.88</td>
</tr>
</tbody>
</table>

Stability under various conditions

The stability of the etoposide and IS in human plasma and stock solutions was examined under different storage conditions. Samples for short-term and long-term stock solution stability of etoposide and IS remained unchanged up to 14 h
and 7 days respectively. Bench top stability of etoposide in plasma was established up to 14 h and for minimum of five freeze and thaw cycles at –20 °C and –70 °C. Auto sampler stability (wet extract) of the spiked quality control samples was determined up to 44 h and dry extract up to 43 h without significant loss of analyte. Spiked plasma samples stored at –20 °C and –70 °C for long term stability experiment were found stable for a minimum period of 92 days. The detailed results for stability study are presented in Table 4.

### Table 4. Stability of etoposide in human plasma under different conditions (n = 6)

<table>
<thead>
<tr>
<th>Storage conditions</th>
<th>Nominal conc. (ng/mL)</th>
<th>Mean stability sample (ng/mL) ± SD</th>
<th>% Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bench top stability at room temperature, 14 h</td>
<td>7000</td>
<td>6537 ± 93</td>
<td>-6.62</td>
</tr>
<tr>
<td></td>
<td>15.0</td>
<td>15.2 ± 0.12</td>
<td>1.50</td>
</tr>
<tr>
<td>Freeze-thaw stability after 5th cycle at -20 °C</td>
<td>7000</td>
<td>6722 ± 55</td>
<td>-3.97</td>
</tr>
<tr>
<td></td>
<td>15.0</td>
<td>15.3 ± 0.14</td>
<td>2.17</td>
</tr>
<tr>
<td>Freeze-thaw stability after 5th cycle at -70 °C</td>
<td>7000</td>
<td>6742 ± 85</td>
<td>-3.68</td>
</tr>
<tr>
<td></td>
<td>15.0</td>
<td>15.7 ± 0.09</td>
<td>4.83</td>
</tr>
<tr>
<td>Wet extract stability at 5°C, 44 h</td>
<td>7000</td>
<td>6763 ± 69</td>
<td>-3.38</td>
</tr>
<tr>
<td></td>
<td>15.0</td>
<td>14.9 ± 0.10</td>
<td>-0.67</td>
</tr>
<tr>
<td>Dry extract stability at 5°C, 43 h</td>
<td>7000</td>
<td>6794 ± 105</td>
<td>-2.94</td>
</tr>
<tr>
<td></td>
<td>15.0</td>
<td>15.2 ± 0.12</td>
<td>1.00</td>
</tr>
<tr>
<td>Long term stability at -20 °C, 92 days</td>
<td>7000</td>
<td>7057 ± 45</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td>15.0</td>
<td>14.1 ± 0.13</td>
<td>-5.88</td>
</tr>
<tr>
<td>Long term stability at -70 °C, 92 days</td>
<td>7000</td>
<td>7107 ± 23</td>
<td>1.53</td>
</tr>
<tr>
<td></td>
<td>15.0</td>
<td>14.3 ± 0.15</td>
<td>-4.67</td>
</tr>
</tbody>
</table>

SD: Standard deviation, CV: Coefficient of variation n: Number of replicates

%Change = \( \frac{\text{Mean stability samples} - \text{Mean comparisons samples}}{\text{Mean comparisons samples}} \times 100 \)

**Dilution reliability and method ruggedness**

The precision (% CV) for dilution reliability of 1/5<sup>th</sup> and 1/10<sup>th</sup> were between 1.17 and 1.48 %, while the accuracy results were within 99.31-106.89 % respectively, which is within the acceptance limit of 15 % for precision (% CV) and
85 % to 115 % for accuracy. The precision (% CV) and accuracy for method ruggedness with different columns and analysts ranged from 2.57 % to 3.15 % and 96.01 % to 99.63 % respectively at four QC levels.

4.5 Conclusion

To summarize, the UPLC-MS/MS method for the quantitation of etoposide in human plasma was developed and fully validated as per USFDA guidelines. To the best of our knowledge this is the first report on the use of UPLC-MS/MS for determination of etoposide in human plasma. The method offers significant advantages over those previously reported [7-18], in terms of simplicity of extraction procedure, sensitivity and overall analysis time. The procedure employed small plasma volume (200 µL) for processing, which is lower compared to existing methods. The efficiency of liquid-liquid extraction and a chromatographic run time of 2.0 min per sample make it an attractive procedure in high-throughput bioanalysis of etoposide. With dilution reliability up to 10-folds, it is possible to extend the upper limit of quantification to 16000 ng/mL. In addition, the carry-over test and post column infusion study is also studied in the present work. The current method has shown adequate sensitivity and selectivity for the quantification of etoposide in human plasma and can be highly useful in clinical studies.

4.6 References

Evaluation and Research (CDER), Centre for Veterinary Medicine (CVM), May 2001.