CHAPTER II

BIOANALYTICAL METHOD VALIDATION USING LC/MS/MS FOR DETERMINATION OF CURCUMIN IN PLASMA
1.0 INTRODUCTION

Low serum and tissue levels of curcumin are observed irrespective of the route of administration, due to poor solubility and absorption, extensive metabolism (intestinal and hepatic) and rapid elimination (Pan et al., 1999; Sharma et al., 2007). The absorption, metabolism, and tissue distribution of curcumin after oral administration of 400, 80 and 10 mg of $^3$H curcumin in rats (Ravindranath, 1981; Ravindranath and Chandrasekhara, 1980) has been studied. The possible application of this compound in therapy is hampered by its poor bioavailability (BA) resulting in derisory therapeutic levels in vivo.

Even though quite a few methods have been developed for detecting curcumin from biomatrices, but their applicability for in vivo pharmacokinetic determination after administration of formulated curcumin is lacking. In a very recent study in healthy volunteers, a 650 mg/kg dose of solid lipid curcumin particles showed plasma levels of 22.43 ng/ml, while free curcumin (95% curcuminoids extract) at the same dose was undetectable (Gota et al., 2010). To what degree the enhanced BA is a result of increased absorption or due to reduced conversion of free curcumin to conjugates is still not clear, because in this study the samples were not pretreated with glucuronidase. Reports indicate a 2–3-fold increase in curcumin absorption when combined with different types of lipids (Gota et al., 2010).

Low detectable levels of curcumin in human biomatrices and extensive metabolism following oral dosing (Cheng et al., 2001; Garcea et al., 2004) suggest that further clinical development of curcumin would benefit from highly sensitive analytical methods for measurement of curcumin. To the best of our knowledge, the use of liquid chromatographic technique coupled with tandem mass spectroscopy for the determination of curcumin in vivo after oral administration of C-SLNs in rat plasma has not been demonstrated. We therefore developed a liquid chromatographic technique coupled with tandem mass spectroscopy (LC-MS/MS) for the determination of curcumin in plasma and its subsequent application to pharmacokinetics of C-SLNs after oral administration in rats.
2. MATERIALS AND METHODS

2.1 Reagents

Curcumin was a gift sample from Sanat Products Ltd. The sample constituted a mixture of three curcuminoids, namely curcumin (95%), demethoxycurcumin, and bisdemethoxycurcumin (latter two constitute the remaining 5%). Nimesulide was used as the internal standard (IS) and was provided by Panacea Biotec Ltd (Lalru, India). Sulfatase-free β-glucuronidase (type IX-A from *Escherichia coli*) was purchased from Sigma (St. Louis, MO, USA). HPLC grade acetonitrile, acetic acid, diethyl ether and other chemicals used in the preparation of buffer were purchased from Merck KGaA, Darmstadt, Germany.

All other chemicals and reagents were of analytical grade and were used without further purification.

2.2 Mass spectrometry

The liquid chromatograph (Agilent 1100; Agilent Technologies, (Palo Alto, CA)) was coupled to a mass spectrometer with a turbo electrospray ion source (API 4000; LC/MS/MS triple quadruple system, Applied Biosystems, Foster City, CA) and was used in negative ionization mode. Chromolith Performance RP-18e 100 mm x 4.6 mm monolithic column (Merck KGaA, Darmstadt, Germany) kept at ambient temperature was used for the analysis. The mobile phase consisted of acetonitrile and ammonium acetate buffer (10 mM, pH 3.5, adjusted with glacial acetic acid) (80:20, v/v). The flow rate was 0.8 ml/min, and the total run time was 3 min.

The turbo ion-spray interface heater was maintained at 450°C. The GS1 and GS2 was kept at a pressure of 50 p.s.i. The collision-activated dissociation gas pressure was 7 p.s.i., declustering potential was −35 V; entrance potential was −10 V; collision energy was −20 V and collision cell exit potential was −1 V. The multiple reactions monitoring (m/z) as observed for curcumin and nimesulide was 367/217 and 307/229 respectively, with a dwell time of 200 ms. The autoinjector cooler was maintained at 10°C. Analyst software (version 1.42; Applied Biosystems) was used for data registration and calibration.
2.3 Animals

Wistar male rats weighing 200–300 g were used. The animals were kept under standardized conditions (with free access to food and fresh water), with a clean cage being provided twice a week. The animals were acclimatized to laboratory conditions over the week before the experiments. The protocol was duly approved by the Institutional Animal Ethics Committee (IAEC) of Panjab University, Chandigarh, India. To perform the validation study, blood samples (1 ml) were withdrawn from sinus under clavicle from a group of mice (n= 3) and pooled into heparinised vials, centrifuged at 5000 rpm for 10 min and the clear plasma transferred to tightly sealed plastic tubes, which were stored in a frozen form at −20°C until analysis.

2.4 Stock solutions

Concentrated stock solutions of curcumin, and nimesulide (IS, Internal Standard) were prepared by dissolving 10.0 mg of each in 100 ml of methanol to give 100 μg/ml stock solutions (Set I). The second set of stock solutions (Set II) was prepared as a duplicate of Set I.

2.5 Preparation of the calibration curve (CC) and quality controls (QC)

Eight point calibration curve (CC) was prepared by serial dilution of curcumin stock solution (100 μg/ml) in the range of 10– 2000 ng/ml. The concentrations were corrected for potency and the amount weighed. Calibration standards were prepared daily by spiking 0.1 ml of blank plasma with 10 μl of the appropriate working solution resulting in concentrations of 10, 25, 50, 125, 250, 500, 1000 and 2000 ng of curcumin per ml plasma. A plot with the resulting peak area ratios was obtained against the concentrations.

High quality control (HQC:1250 ng/ml); medium quality control (MQC:750 ng/ml); low quality control (LQC: 75 ng/ml) and lowest level of quantitation (LLOQ :10 ng/ml) were prepared by spiking 0.1 ml aliquot of blank plasma with 10 μl of spiking solution of drug as well as the IS. All solutions were stored in a refrigerator at 5.0±3.0°C. The bulk spiked CC and QC samples were stored at -20°C.
2.6 Sample preparation

Drug extraction from the rat plasma was performed using an aliquot of 100 μl of rat plasma in a polypropylene tube followed by addition of 10 μl of internal standard solution. Conversion of any curcumin glucuronide to free curcumin was carried out by addition of 200 μl of β glucuronidase to these samples, trailed by incubation at 37°C for 1 h. Liquid liquid extraction (LLE) was performed using diethylether followed by vortexing (1 min) and cold centrifugation for 5 min at 5000 rpm. The supernatant was decanted in a separate tube and dried using nitrogen gas at 50°C by turbo evaporator. Last step involved the sample extraction in mobile phase and final transfer to auto injector vials which were then subjected to liquid chromatographic technique coupled with tandem mass spectroscopy for analysis.

2.7 Bioanalytical method validation

2.7.1 System suitability

System suitability standards were determined by injecting seven un-extracted MQC before the start of each analytical run. For each system suitability average peak ratio was calculated.

2.7.2 Selectivity and specificity

A specificity exercise was performed for both methanol and plasma. Individual blank plasma samples, LLOQ-QC sample, and methanol (blank) (n=6) were prepared according to the sample preparation procedure described above and screened for interference.

2.7.3 Inter-day and intra-day precision and accuracy

Inter-day and intra-day precision and accuracy were evaluated by spiking known amounts of curcumin and IS in plasma (n = 6). The precisions were expressed as %CV (coefficient of variation) and % accuracy was expressed by using the formula: (measured concentration/nominal concentration) x 100.

Four different concentrations were used, and samples were prepared according to the procedure as mentioned above. Intra-day precision and accuracy were assessed within one batch using replicate (n=6) determinations for each concentration of the
spiked plasma sample, whereas inter-day precision and accuracy were assessed on three separate occasions using replicates (n=6) for each concentration used.

2.7.4 Matrix effect

To study the matrix effect, blank plasma samples were processed and spiked later to obtain MQC and HQC concentrations. The response (area) was compared with directly injected samples at MQC and HQC levels.

2.7.5 Recovery

The LLE efficiency was calculated by comparing the peak areas of extracted plasma standards with areas of reference standards added to blank plasma extract. The reference standards were prepared by extracting rat control plasma and reconstituting the evaporated extracts with stock solutions of curcumin, and internal standard. The concentration of the internal standard was 100 ng/ml. The recovery study for curcumin was accomplished at three concentration levels (75, 750, and 1250 ng/ml) in rat plasma.

3.0 RESULTS

3.1 Bioanalytical method validation

3.1.1 System suitability

The system was found to be suitable for the determination of curcumin under the optimised chromatographic conditions.

3.1.2 Selectivity and specificity

The method was highly selective and specific as observed from the chromatograms. The chromatograms obtained are represented in Figure 1.
Figure 1. Representative chromatograms of A) blank plasma sample B) curcumin sample C) curcumin in plasma
Chromatograms on the left side of the figure represents curcumin whereas those on the right side are indicative of the internal standard peaks.
3.1.3 Linearity

The calibration curve was shown to be linear from 10.0 ng/mL to 2000.0 (Figure 2).

![Calibration Curve](image)

Figure 2. Calibration curve of curcumin from 10.0 ng/ml to 2000.0 ng/ml

3.1.4 Inter-day and intra-day precision and accuracy

The intra-day accuracy of curcumin for rat plasma samples was 102.43–108 QC samples with precision (RSD) less than 3.55%. The inter-day accurcumin for rat plasma samples ranged from 105.7% to 108.5% at QC sample RSD value again less than 3.55% (Table 1).

Table 1. Intra-day and inter-day precision and accuracy of curcumin plasma (n=6)

<table>
<thead>
<tr>
<th>Nominal concentration (ng/ml)</th>
<th>Observed concentration (ng/ml)</th>
<th>%Precision</th>
<th>%Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-day</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>75</td>
<td>76.78±1.54</td>
<td>2.00</td>
<td>102.4</td>
</tr>
<tr>
<td>750</td>
<td>813.67±28.92</td>
<td>3.55</td>
<td>108.5</td>
</tr>
<tr>
<td>1500</td>
<td>1580.0±43.01</td>
<td>2.72</td>
<td>105.6</td>
</tr>
<tr>
<td>Inter-day</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>75</td>
<td>78.0±2.71</td>
<td>3.55</td>
<td>108.5</td>
</tr>
<tr>
<td>750</td>
<td>789.42±15.93</td>
<td>2.72</td>
<td>105.7</td>
</tr>
<tr>
<td>1500</td>
<td>1571.1±34.08</td>
<td>2.05</td>
<td>106.5</td>
</tr>
</tbody>
</table>
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3.1.5 Matrix effect

There were no matrix effects observed during the process of validation.

3.1.6 Recovery

The overall recovery as determined was found to be 77.15%. The individual recoveries at HQC, MQC and LQC are represented in Table 2.

Table 2. Recovery of curcumin and internal standard in rat plasma

<table>
<thead>
<tr>
<th>QC</th>
<th>Curcumin</th>
<th>Internal standard</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%Recovery</td>
<td>%RSD</td>
</tr>
<tr>
<td>LQC</td>
<td>82.91</td>
<td>1.77</td>
</tr>
<tr>
<td>MQC</td>
<td>76.71</td>
<td>7.09</td>
</tr>
<tr>
<td>HQC</td>
<td>75.13</td>
<td>5.53</td>
</tr>
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</table>

4.0 DISCUSSION

A liquid chromatographic mass spectrometric method for the estimation of curcumin in rat plasma was developed and validated according to the principles of Laboratory Practices.

Optimization trials were carried out using sunfire C18, with mobile phase acetonitrile:methanol (50:50, v/v), acetonitrile:2mM ammonium acetate buffer (80:20, v/v), acetonitrile:10mM ammonium acetate buffer (80:20, v/v), acetonitrile:ammonium acetate buffer (80:20, v/v) with pH adjusted with acetic acid to 3.5 as mobile phase, at flow rate of 0.8 ml/min. The method was validated over a concentration range of 10–2000 ng/ml, and the results obtained were obtained/extrapolated on the calibration curve. Analysis for curcumin in the plasma samples showed a retention time of 1.21±0.3 min, with a total run time of 4.3 min. Representative chromatograms of plasma, control and curcumin in plasma are shown in Figure 1. The chromatograms obtained with one of the precision batches are attached for reference at the end of this chapter, as Appendix.
The lower limit of quantization for curcumin by the developed method was found to be 10.0 ng/ml for curcumin. The between run precision and accuracy for curcumin at 10 ng/ml were 6% and 104%, respectively. The linearity of the method was determined by a weighted least-squares regression analysis of an eight point standard curve. The calibration lines were shown to be linear from 10 to 2000 ng/ml (Figure 2). Best-fit calibration lines of the ratio of curcumin to IS peak area versus the concentration of calibration standards were determined by least-squares regression analysis with weighting factors of $1/x^2$.

The $r^2$ values were consistently 0.999 during the entire course of validation. Table 1 shows a summary of intra- and inter-day precision and accuracy for curcumin in rat plasma. The absolute recovery of curcumin and IS was calculated for replicate spiked QC samples (LQC, MQC and HQC) (Table 2). Extraction recovery is calculated by comparing the peak area ratios of curcumin and IS respectively, with the peak area of curcumin and IS added to blank plasma at concentrations ranging from 75 to 1500 ng/ml. Results show an overall mean percent recovery of 77.15% for curcumin and 67.95% for IS added to blank plasma extract. It may be noted that as per the US FDA guidelines of May 2001, recovery pertains to the extraction efficiency of an analytical method within the limits of variability. It is stated that recovery of the analyte need not be 100%, but the extent of recovery of an analyte and of the internal standard should be consistent, precise, and reproducible. Hence the method developed and reported presently though showing a recovery of 77.15% (for curcumin) may be considered suitable taking into account the consistency and reproducibility of the results obtained upon repetitive evaluation.

The validated method was successfully applied to measure curcumin in rat plasma and was also used to study its pharmacokinetics when administered orally in free form and as C-SLN to rats.

5.0 CONCLUSION

A highly validated, sensitive and specific liquid chromatographic technique coupled with tandem mass spectroscopy method for the quantization of curcumin as such and from its prepared SLNs was developed and validated with a routine sensitivity limit of 10.0 ng/ml in 0.1 ml rat plasma.