INTRODUCTION

Curcumin [1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3, 5-dione] is the major yellow pigment extracted from turmeric, a commonly used spice, derived from the rhizome of the herb Curcuma longa Linn 1. It is a naturally occurring polyphenolic phytochemical currently being examined in preclinical trials for cancer chemoprotective drug development, with pharmacological actions that including antioxidant1,2, anti-inflammatory3,4, and cancer chemopreventive actions5-7. Curcumin, the major yellow-orange pigment extracted from turmeric, may be responsible for much of the bioactive effects. In a recent study, products of curcumin reduction and conjugation had a reduced ability to inhibit cyclooxygenase-2 (COX-2) expression, which correlated to a decrease in the inhibition of prostaglandin biosynthesis when compared to intact curcumin, indicating that the metabolic conversion of curcumin results in pharmacologic deactivation8. Curcumin is also a potent scavenger of various reactive oxygen species (ROS) including superoxide anions2 and hydroxyl radicals2,9. In addition, there have been indications that curcumin may help prevent and treat patients with Alzheimer’s disease by reducing oxidative damage, plaque burden, and suppressing specific inflammatory factors10.

A limitation to the studies cited above was the inability to quantitate low curcumin concentrations and derivatives. Quantitation of curcumin concentrations below 10 ng/ml would allow better characterization and understanding of the disposition and absorption kinetics of this...
compound. Although several methods of detection for curcumin have been published, only one has reported a limit of quantitation below 10 ng/ml\textsuperscript{8}. Of these methods, several involve spectrophotometric\textsuperscript{12}, liquid chromatography-mass spectrophotometric\textsuperscript{13,14}, and radiolabeled determination of curcumin\textsuperscript{15}. HPLC methods have also been developed in order to quantitate curcumin in biological samples\textsuperscript{6,11,16,17}. Ireson et al.\textsuperscript{8} utilized a HPLC gradient system that produced reasonable separation and sensitivity. The retention time for curcumin, however, was greater than 35 min. We therefore focused on developing a rapid and more sensitive HPLC binary method for the estimation of Curcumin.

**EXPERIMENTAL**

**Chemicals and reagents**

Curcumin was obtained from Natural remedies manufacturing company Bangalore. Tetrahydrofuran and citric acid was obtained from Thomas Baker, India. Water was deionised by the Milli-Q Plus system (Millipore).

**Instrumentation**

The HPLC system consists of a Shimadzu SPD-10TVP, Binary pump equipped with a normal sample injector with a 50-microliter loop, SPD-10AVP variable wavelength UV detector and Spincotech station for data analysis.

**Sample preparation**

The stock solutions were prepared by dissolving 5.0 mg of Curcumin was dissolved in 50 ml mobile phase to get a concentration of 1,00,000 ng/ml. Analytical standard solutions for linearity were prepared by diluting the stock solution with tetrahydrofuran and 1% citric acid immediately prior to use. All the preparations were made in borosilicate glass tubes. The standard calibration curve was constructed in the concentration range of 1-100 ng/ml, with concentration on X- axis, peak area on Y-axis and regression equation was calculated.

**Chromatographic conditions**

Chromatographic separations were achieved using a Shimadzu ODS C\textsubscript{18}, 1 cm long Guard column (4.6x250 mm, 5 \textmu m). The mobile phase consisting of tetrahydrofuran: 1\% citric acid 35:65 v/v was passed through a 0.22 mm membrane filter and degassed by ultrasonication under vacuum before use. The flow rate was maintained at 1.2 ml/min and the effluent was monitored for UV absorption at 425 nm. The injection volume was 50 \textmu L. All separations were performed at ambient temperature.

**RESULTS**

**Method development**

The objective of this study to develop method for the determination of Curcumin with short run time, which can also be used for its formulations. The column chosen for this study was 250 mm length, 4.6mm internal diameter and 5-micron particle size. Good sample separation was observed on silica based C\textsubscript{18} Mark column using mobile phase tetrahydrofuran: 1\% citric acid 35:65 v/v. The retention time of Curcumin was found to be 15.892 min. The system suitability results were given in Table 1.

**Method validation**

**Limit of Detection (LOD) and Limit of Quantitation (LOQ)**

The LOD and LOQ were calculated by instrumental and statistical methods. For the instrumental method LOD is determined as the lowest amount to detect and LOQ is the lowest amount to quantify by the detector. For statistical method LOD and LOQ determined by statistical formula.

\[
\text{LOD} = 3.3 \ \text{SD}/S \\
\text{LOQ} = 10 \ \text{SD}/S
\]

where, SD is standard deviation of \text{y} intercept of regression equation and S is slope.

The values for the Limit of Detection and Limit of Quantification for are mentioned in Table 2.

**Precision**

Precision of the procedure was determined by repeatability method. A solution of Curcumin containing 10ng and 5000ng/ml respectively was injected into the system repeatedly six times. The percentage RSD of injection repeatability and analysis repeatability for Curcumin was found to be
Table 1: Validation data of Curcumin

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Parameters</th>
<th>Observations</th>
<th>Acceptance Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>LOD (mcg/ml)</td>
<td>Visualization 0.2</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Statistical 1.33</td>
<td>-</td>
</tr>
<tr>
<td>2.</td>
<td>LOQ (mcg/ml)</td>
<td>Visualization 0.4</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Statistical 4.03</td>
<td>-</td>
</tr>
<tr>
<td>3.</td>
<td>Linearity</td>
<td>Range (mcg/ml) 1 - 40</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Regression eq^n 178913x - 37010</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>R^2</td>
<td>0.9997</td>
<td>-</td>
</tr>
<tr>
<td>4.</td>
<td>Accuracy(%Recovery)</td>
<td>Level I (80%) 97.32</td>
<td>% Recovery within 90 to 120%.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Level II (100%) 104.50</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Level III (120%) 95.56</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>Precision(%RSD)</td>
<td>Method 0.4324</td>
<td>% RSD should be less than 2%.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>System 0.797</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Interday 0.905</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Intraday 1.2917</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>Ruggedness (%Assay)</td>
<td>Analyst 1 99.51</td>
<td>% Assay should be within 95-102%.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Analyst 2 99.34</td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>Robustness (Flow rate (ml/min))</td>
<td>0.8 95.53</td>
<td>should be within 95-102%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tetrahydrofuran: 1.2 99.76</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1% citric acid 35:65 101.48</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wavelength 40:60 98.54</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>425 95.02</td>
<td></td>
</tr>
</tbody>
</table>

Table 1: System-suitability report

<table>
<thead>
<tr>
<th>Compound (n=3)</th>
<th>Asymmetry/ Tailing factor</th>
<th>Capacity factor</th>
<th>Efficiency (N) (No. of theoretical plates)</th>
<th>Eff/l [t.p/m] (Relative efficiency in plates per meter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcumin</td>
<td>1.023</td>
<td>5.92</td>
<td>4886.77</td>
<td>37538</td>
</tr>
</tbody>
</table>

n = Number of determinations

0.812 %and 0. 856% % respectively. The results obtained confirm good precision of the method developed.

Table 2: Recovery results of Curcumin in sample

<table>
<thead>
<tr>
<th>Added (ng)(n=3)</th>
<th>Recovered (ng)(n=6)</th>
<th>%Recovery</th>
<th>% RSD (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>200 ng</td>
<td>197.49 ng</td>
<td>98.74</td>
<td>1.274</td>
</tr>
<tr>
<td>500 ng</td>
<td>497.92 ng</td>
<td>99.58</td>
<td>1.316</td>
</tr>
</tbody>
</table>

n = Number of determinations

Linearity

The linearity of the method for Curcumin was checked at ten concentration levels over the concentration range of 50-5000 ng/ml. The typical equation describing the calibration curve is y=0.830x where y is the peak area of Curcumin and x is the concentration of Curcumin, with a mean correlation coefficient (R^2) of 0.9997. Linearity is presented in Fig. 2.
Quantification of Curcumin in natural samples
The standard addition and recovery experiments were conducted to determine the accuracy of the present method for the quantification of Curcumin samples.

The recovery of Curcumin was calculated from the slope and the intercept of the calibration curve drawn in the concentration range of 50-100,000 ng/ml. The percentage recovery of Curcumin was ranged from 98.6 % to 99.4 % in samples of Curcumin. The results were shown in Table 2. HPLC chromatograph of Curcumin in samples was shown in Figure 1.

Limit of detection
The limit of detection represents the concentration of analyte that would yield a signal to noise ratio equal to $3\sigma (DL = 3\sigma / S)^5$. The limit of detection for Curcumin was found to be 3.68 to 8.125 ng/ml for 50 µL injection volume. The limit of quantification represents the concentration of analyte that would yield a signal to noise ratio equal to $10\sigma (DQ = 10\sigma / S)^5$. Limit of quantification for Curcumin was 8.125 ng/ml for 50 µL injection volume.

Solution stability
Solution stability of Curcumin was studied

Fig. 1: Chromatograph of Curcumin

Fig. 2: Linearity graph of curcumin
by leaving the solution (10 and 5000ng/ml prepared in diluent) in tightly capped ambered colour volumetric flasks at room temperature for three days. Content of Curcumin was checked for 12 hours interval and compared with freshly prepared solutions. No variation was observed in the content of Curcumin for the study period, which indicates that the Curcumin sample solutions prepared in the said diluents are stable for at least 3 days.

Ruggedness
The ruggedness was established by carrying out the assay of curcumin using the same chromatographic system and the same column by two analysts on a different day. The assay results were found within the acceptance criteria of 95 to 102% w/w, hence the proposed method was said to be rugged.

Robustness
It is the measure of capacity of an assay to remain unaffected by small but deliberate variations in method parameters and provide an indication of its reliability in normal usage. For the robustness study small variations in columns, mobile phase, detection wavelength and flow rate have been performed and percentage assay of curcumin was calculated. The percentage assay in all varied chromatographic conditions was found within the acceptance criteria.

DISCUSSION
A simple and sensitive HPLC method was developed for Curcumin. This assay method provided excellent sensitivity, accuracy and precision, with relatively short retention time for Curcumin. This HPLC method can therefore be applied to both in vitro studies of Curcumin formulations as well as drug estimation in biological samples.

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REFERENCES


