A SIMPLE AND RAPID METHOD FOR SIMULTANEOUS QUANTIFICATION OF DOXORUBICIN, EPIRUBICIN, CYCLOPHOSPHAMIDE AND 5-FLUOROURACIL IN HUMAN PLASMA BY LC/MS/MS.

Gopinath Sambasivam*, Deepak Gopal Shewade¹, Biswajit Dubashi², Rajan Sundaram¹

¹Department of Pharmacology, JIPMER, Puducherry, India.
²Department of Medical Oncology, JIPMER, Puducherry, India.

ABSTRACT
A reverse-phase HPLC method with detection by mass spectrometry is described for the simultaneous determination of doxorubicin, epirubicin, cyclophosphamide and 5-fluorouracil in human plasma. The chromatographic separation was carried out on a Waters Xterra® C18 column (3.9mmx150 mm, 5μm particle size) with a mobile phase of 0.1% formic acid in water and methanol (30:70 v/v). The flow rate was kept as 0.4ml/min with total run time of 7 minutes. A tandem quadrupole MS was operated in multiple reaction monitoring (MRM) in both positive and negative electrospray ionization (ESI) mode. Doxorubicin epirubicin and cyclophosphamide were detected in positive mode and 5-fluorouracil was detected well in negative ionisation mode. Protein precipitation method by using 100% methanol was used to extract the drugs. To 200 μL of plasma along with internal standard, 600 μL of 100% methanol was added then vortexed for 1 minute and centrifuged at 13,500 rpm for 5min. After centrifugation 100 μL of the supernatant layer was used for analysis. The retention time of doxorubicin, epirubicin, 5-fluorouracil and cyclophosphamide were 2.32 min, 2.35 min, 2.43 min and 4.64 min respectively. Ifosamide was used as internal standard and its retention time was 4.12 min. The developed method was validated in human plasma with a lowest limit of quantification of 5 ng/mL for all the drugs. The method was accurate with the intra-day and inter-day precision of 15% and the there was no endogenous contamination of any of the analytes. This method was robust, precise and accurate for the
determination of doxorubicin epirubicin cyclophosphamide and 5 fluorouracil in human plasma using LC/MS/MS method.

KEYWORDS: Doxorubicin, Epirubicin, Cyclophosphamide 5-Fluorouracil, LC-MS/MS.

INTRODUCTION

Anthracyclines are one of the most active drugs prescribed for the treatment of breast cancer. Doxorubicin (DOX), epirubicin and daunorubicin are the class of anthracyclines which belongs to the tetracycline class of antibiotics. The anticancer activity of these drugs has been mainly due to strong interactions with DNA in target cells. The cyclophosphamide, an alkylating agent, is often administered with other antineoplastic agents like doxorubicin epirubicin and 5-fluoracil in breast cancers. Anthracyclines and cyclophosphamide combinations are used predominantly in the treatment of breast cancer.[1,2] These combination regimens can be intolerable due to severe and life-threatening toxicities. Therefore maximum clinical assessment is needed for the safer administration in different situations.[3] 5-Flourouracil is an anti-metabolite which is used for the treatment of various cancers such as breast, lung and gastrointestinal tract cancers. Although toxicity is managed by treating the symptoms and reducing the dose of anticancer drugs it results in under-dosing and reduced efficacy. The optimal dose of anticancer drugs should have a maximal antitumor effect with less toxicity. Clinical use of anticancer drugs may require therapeutic drug monitoring which can be possible with analytical evaluation of these agents in biological fluids. A simple, rapid and sensitive analytical method for estimation of these drugs is necessary for better management of cancer patients. Various studies have reported the simultaneous plasma determination of the anthracyclines and metabolites alone or combined with other drugs,[4-7] Some studies have reported the environmental and biological monitoring of anticancer drugs.[8, 9] Few studies described the determination of cyclophosphamide and 5-fluoroacil with its metabolites in human plasma.[10-14] The sample preparation used in various studies mostly involve liquid-liquid extraction (LLE) and solid phase extraction (SPE). Since HPLC methods are labor intensive and have less sensitivity, LCMS method represents the better choice for the analysis of antineoplastic drugs. LCMS methods have desired sensitivity and less run time which enables for getting results within few hours. In the present study, LCMS/MS method was developed to determine four drugs which are prescribed widely in clinical practice. The purpose of this study was to develop a single analytical LC-MS/MS
method for the simultaneous determination of doxorubicin, epirubicin, cyclophosphamide and 5-Flurouracil in human plasma.

MATERIALS AND METHODS

Chemicals and reagents
Pure drugs of doxorubicin, epirubicin ifosfamide, 5-Flourouracil and cyclophosphamide were obtained from Sigma-Aldrich. Methanol (LC-MS chromasolv) and formic acid were also purchased from Sigma-Aldrich (Missouri, USA). Milli-Q water (Millipore Corporation) was used wherever required in this method. Ifosamide was selected as an internal standard as it has similar in its physicochemical properties to that of cyclophosphamide which is one of the drugs of interest for estimation.

LC-MS/MS conditions
An Alliance 2695 HPLC separation module (Waters USA) with PDA detector, cooled autosampler and column oven was used. Chromatographic separation was achieved by using Xterra MS C18 column (3.9mmx150 mm, 5μm; Waters, Milford, MA, USA). Column oven temperature was maintained at 30°C and sample temperature at 20°C. The mobile phases consisted of 0.1% formic acid-water (mobile phase A) and Methanol (mobile phase B). A flow rate was 0.4 ml/min with 30% of Mobile Phase A and 70% of mobile phase B over 7 minutes. Total run time was 7 minutes. A tandem quadrupole MS was operated in multiple reaction monitoring (MRM) in both positive and negative electrospray ionization (ESI) modes for detection of all drugs. Data acquisition and processing were done by using MassLynx 4.1 software. Quan Optimize software was used to optimize individually various MS/MS parameters like cone energy collision energy, and retention time of all the drugs.

Solutions and standards
The stock solutions of doxorubicin, epirubicin, ifosfamide (IS) and cyclophosphamide were prepared in methanol to yield a concentration of 1.0 mg/mL. Preparation of working solutions and calibration standards were done with 50% methanol in water. The working solutions for calibration curve were prepared by serial dilutions of the stock solution. The calibration was done over a range of 10ng/mL to 10μg/mL for doxorubicin and epirubicin, 10ng/mL to 50 μg/mL for cyclophosphamide and 5-flourouracil. Limit of detection (LOD) was observed up to 5ng/mL. The quality control (QC) working solutions were 10 ng/mL, 500 ng/mL, 10 μg/mL and 50 μg/mL of all drugs and internal standard concentration was 100 ng/mL in 50%
methanol. Stock solutions were kept at -80 °C when not in use. Linear regression was used to fit the calibration curves.

Sample preparation
The sample preparation from plasma was carried out using protein precipitation method by using 100% methanol. 50 μL of internal standard working solution (100 ng/mL) was added to 200 μL of plasma and vortexed (30 s), followed by the addition of 600 μL of 100% methanol. The whole solution was then vortexed for 1 minute and centrifuged at 13,500 rpm for 5 min. After centrifugation, 100 μL of the supernatant layer was transferred into vials for analysis and 20 μL were injected into the system.

Method Validation
The bio-analytical method was validated according to the US FDA guidelines. Calibration curves were done by using linear regression analysis of doxorubicin, epirubicin, cyclophosphamide 5-fluorouracil and ifosfamide (IS) versus concentration. The Intra-day and inter-day variation, precision, and accuracy were assessed by analyzing QC samples. The QC samples were also subjected for bench-top stability (27°C, 6 h, 12 h) and freeze/thaw stability (-200°C, 3 freeze/thaw cycles, 48 h) in plasma by comparing samples before and after the stability tests.

RESULTS
The separation of four anticancer drugs by LC-MS/MS using ifosamide as an internal standard was done. The mobile phases consisted of 0.1% formic acid-water (mobile phase A) and methanol (mobile phase B). A flow rate of 0.4 ml/min with 30% of mobile. Phase A and 70% of mobile phase B with runtime of 7 minutes. The retention time of doxorubicin, epirubicin, cyclophosphamide and ifosfamide (IS) in positive ion mode were 2.32 min, 2.35 min, 4.64 and 4.12 min respectively. 5-fluorouracil retention time in negative ion mode was 2.43 min. A runtime of 7 min was set to avoid carry-over effect of biological matrices. The MRM transitions, cone voltage and collision energy of the analytes and IS are presented in Table 1. A representative chromatograms of 100 ng of all four compounds after extraction in plasma are given in Fig.1.
Table 1: Multiple reaction monitoring of various parameters of drugs

<table>
<thead>
<tr>
<th>Compound</th>
<th>Formula/Mass</th>
<th>Parent Ion m/z</th>
<th>Cone Voltage (Volt)</th>
<th>Daughter Ion m/z</th>
<th>Collision Energy</th>
<th>Ion Mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin</td>
<td>543.17</td>
<td>544.27</td>
<td>16</td>
<td>397.05</td>
<td>12</td>
<td>ES+</td>
</tr>
<tr>
<td>Epirubicin</td>
<td>543.17</td>
<td>544.20</td>
<td>22</td>
<td>396.98</td>
<td>14</td>
<td>ES+</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>260.02</td>
<td>283.05</td>
<td>22</td>
<td>225.01</td>
<td>16</td>
<td>ES+</td>
</tr>
<tr>
<td>Ifosfamide</td>
<td>260.02</td>
<td>261.05</td>
<td>28</td>
<td>92.03</td>
<td>26</td>
<td>ES+</td>
</tr>
<tr>
<td>5-Flurouracil</td>
<td>130.01</td>
<td>128.98</td>
<td>24</td>
<td>42.10</td>
<td>12</td>
<td>ES-</td>
</tr>
</tbody>
</table>

Fig.1: Chromatogram of 100ng pure drug extracted in human plasma

Method validation

Linearity and Sensitivity

The calibration curves were plotted by spiking blank human plasma over a wide range of 10ng/mL to 10μg/mL for doxorubicin and epirubicin, 10ng/mL to 50μg/mL for cyclophosphamide and 5-flurouracil. The r2 value was >0.99 for all the analytes over this
wide range. The lower limit of quantification (LLOQ) was 5 ng/mL for all the drugs which defined the sensitivity of the method.

**Precision and accuracy**
The accuracy and precision were done for the QC samples at 10 ng/mL, 500 ng/mL, 10μg/mL and 50 μg/mL for doxorubicin, epirubicin, cyclophosphamide, and 5-flourouracil. The accuracy of the present method was close to 100% over this range. The intra-day and inter-day precision were within the limit of 15%.

**Specificity and stability**
The analytical run of blank samples with IS and without IS in plasma indicates there is no cross interference of the drugs. The stability test was performed using four sets of QC samples for benchtop stability and freeze-thaw stability. The first set of QC samples were estimated immediately after preparation. The second set was kept at 27°C and was analyzed after 6 hrs and 12 hrs for measuring bench top stability. The freeze-thaw stability was done by keeping the fourth set of QC samples at -200°C and was analyzed at 24th hr and 48th hr. The response deviation of the second set, third set and fourth set of samples were less than 10% from the first set of QC samples which indicated the stability of all these drugs in human plasma.

**DISCUSSION**
The use of analytical methods for the simultaneous estimation of various drugs considerably reduced the cost and time required for the analysis of drugs. HPLC methods with different detectors were used for the estimation of doxorubicin, epirubicin cyclophosphamide and 5-flurouracil. Most of the methods focused on individual drug estimation of these commonly prescribed drugs. Sanson described the Simultaneous estimation methods of five anticancer drugs using HPLC and liquid-liquid extraction method. Ahmad described the simultaneous determination of 5-flourouracil doxorubicin and cyclophosphamide by HPLC with PDA detector. Nevertheless, the mass spectrometry is an advanced detection method which offers a lot of advantages over HPLC and more accessible to analysts as its cost decreases. The detector is more superior in MS than UV because of its specificity and range, and its detection limits are greater. Various studies have been carried out for the determination of anthracyclines in LCMS. Author wall described the method for the estimation of epirubicin in LCMS by using the serum. A mixture of acetonitrile/ water/formic acid (72:28:0.1 v/v/v) at 0.2 mL/min was used for separation. Lachatre developed a
method for the simultaneous determination of four anthracyclines and their metabolites by LC-MS. The mobile phase was composed of 5 mM ammonium formate /acetonitrile (70:30 v/v) and the flow rate was 50 μL/min.\textsuperscript{[24]} Mahnik developed the method for the estimation of anthracyclines in hospital effluents. The mobile phases were water/acetonitrile adjusted to pH 2.0. The flow rate was kept as 0.6 mL/min. Doxorubicin and Daunorubicin retention times were 13.0 and 16.0 min.\textsuperscript{[25]} In the present study, the mobile phases were 0.1% formic acid in water and methanol (30:70) and flow rate was 0.4 mL/min. The retention time is 2.32 min and 2.35 min for doxorubicin and epirubicin which is very much less than the other reported studies. It offers advantages like faster estimation and the Limit of detection is 5 ng/ml which shows the good sensitivity. Di Francesco described a method for the simultaneous estimation of cyclophosphamide, doxorubicin, and doxorubicinol.\textsuperscript{[26]} Solid-phase extraction was used for sample extraction. Detection was achieved in positive, mixed reaction monitoring mode on a triple quadrupole mass spectrometer. In the present study detection of cyclophosphamide doxorubicin and epirubicin was achieved by positive MRM mode which is like in other studies. In our study protein, precipitation method was used. It is a very convenient method and less cumbersome than solid phase extraction. Few studies described the quantification and validation of 5-fluorouracil and its metabolites in dog and human plasma.\textsuperscript{[27-29]} Author usawanuwat described the monitoring of 5-fluorouracil cyclophosphamide and hydroxyl urea in water samples.\textsuperscript{[30]} Most of the studies described the estimation of 5-fluorouracil was carried out in negative ionization mode in multiple reaction monitoring which is in agreement with our study. The mobile phase of formic acid in water and methanol was suitable for separation of the cyclophosphamide hydroxyl urea and 5 fluorouracil in waters sample.\textsuperscript{[30]} In the present study, the mobile phase of 0.1 formic acid in water and methanol was sufficient enough for the separation of 5-fluorouracil cyclophosphamide as well as doxorubicin and epirubicin. Calibration standard in our study was 10ng/mL to 10μg/mL for doxorubicin and epirubicin. 10ng/mL to 50 μg/mL for cyclophosphamide and 5-fluorouracil. The present method was accurate with the intra-day and inter-day precision of 15% which is the accepted criteria. The analytical run of blank samples with IS and without IS shown that there is no cross interference of the drugs. The good background in the analytical run indicates that there was no endogenous contamination by any of the analytes. It was indicative of the excellent specificity of this current method for quantification of all four drugs used over a wide range. Our method is robust precise and accurate for the estimation of doxorubicin epirubicin cyclophosphamide and 5-fluorouracil in human plasma.
CONCLUSION
In the current study, an LC/MS/MS method for the simultaneous estimation of three chemically and structurally different anticancer drugs was developed. Protein precipitation was used for the extraction of all drugs. This method is simple, fast and validated for the estimation of doxorubicin, epirubicin, 5-flourouracil and cyclophosphamide in human plasma.

ACKNOWLEDGEMENT
We acknowledge the JIPMER and ICMR for funding the study.

CONFLICT OF INTEREST
There is no conflict of interest.

REFERENCES


