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|  | <p>Journal Homepage: - www.journalijar.com</p> <p>INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)</p> <p>Article DOI: 10.21474/IJAR01/6290 DOI URL: http://dx.doi.org/10.21474/IJAR01/6290</p> |  |
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RESEARCH ARTICLE

A SENSITIVE BIO ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF RUCAPARIB IN HUMAN PLASMA BY LC-ESI-MS/MS.

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Manuscript Info

Manuscript History

Received: 12 November 2017
Final Accepted: 14 December 2017
Published: January 2018

Key words:-

Rucaparib, Mass spectrometry;
Pharmacokinetic study.

Abstract

A simple, sensitive and specific liquid chromatography–tandem mass spectrometry (LC-MS/MS) method was developed for the quantification of Rucaparib (RP) in human plasma using Rucaparib-d3 (RPD3) as an internal standard (IS). Chromatographic separation was performed on Xbridge C18, 50 x 4.6 mm, 5 µm column with an isocratic mobile phase composed of 10mM Ammonium formate and Methanol in the ratio of (20:80 v/v), at a flow-rate of 0.7 mL/min. RP and RPD3 were detected with proton adducts at m/z 323.4 → 170.1 and 328.4 → 170.1 in multiple reaction monitoring (MRM) positive mode respectively. Liquid-Liquid extraction method was used to extract the drug and IS. The method was validated over a linear concentration range of 5.0-5000.0 pg/mL with correlation coefficient (r^2) ≥ 0.9994. This method demonstrated intra and inter-day Precision within 0.7 to 2.0 and 0.7 to 2.7 % and Accuracy within 101.4 to 102.4 and 99.5 to 104.8 %. Rucaparib (RP) was found to be stable throughout freeze-thawing cycles, bench top and postoperative stability studies.

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Introduction:-

Rucaparib is an inhibitor of poly (ADP-ribose) polymerase (PARP) enzymes, including PARP-1, PARP-2, and PARP-3, which play a role in DNA repair. *In vitro* studies have shown that rucaparib-induced cytotoxicity may involve inhibition of PARP enzymatic activity and increased formation of PARP-DNA complexes resulting in DNA damage, apoptosis, and cell death. Increased rucaparib-induced cytotoxicity was observed in tumor cell lines with deficiencies in BRCA1/2 and other DNA repair genes. Rucaparib has been shown to decrease tumor growth in mouse xenograft models of human cancer with or without deficiencies in BRCA^[1-7].

Rucaparib is an inhibitor of the mammalian polyadenosine 5'-diphosphoribose polymerase (PARP) enzyme. The chemical name is 6-fluoro-2-{4-[(methylamino)methyl]phenyl}-3,10-diazatricyclo[6.4.1.0{4,13}]trideca-1,4,6,8(13)-tetraen-9-one. The chemical formula of Rucaparib is C₁₉H₁₈FN₃O and the relative molecular mass is 323.371 g/m^[8-10].

Screening of the literature disclosed few published reports for the quantification of Rucaparib concentration in pharmacokinetics of Rucaparib in rat plasma by LC-MS^[11].

From the literature review it was concluded that the developed methods shows poor sensitivity, long retention time, lack of deuterated internal standard by using HPLC-ESI-M/MS. There are very limited methods were reported for estimation of Rucaparib using deuterated internal standard in biological samples.

Materials and methods:-

2.1. Chemicals and Reagents

Rucaparib (RP) (Cadila Pharmaceuticals, India), Rucaparib-D3 (RPD3) (*ALSACHIM, France*), Ammonium formate and sodium hydroxide (Merck, Mumbai, India), Methanol, ethyl acetate and dichloromethane (J. T. Baker, USA), Ultra pure water (Milli-Q system, Millipore, Bedford, MA, USA), Screened human plasma (navjeevan blood bank, Hyderabad, A.P). The chemicals and solvents were used in this study analytical and HPLC grade.

2.2. Instrumentation

The 1200 Series HPLC system (Agilent Technologies, Germany). Mass spectrometric detection was performed on an API 4000 triple quadrupole instrument (ABI-SCIEX, Toronto, Canada) using MRM. Data processing was performed on Analyst 1.4.1 software package (SCIEX).

2.3. Detection

Detection was performed by Turbo ion spray (API) positive mode with Unit Resolution using MRM positive ion mode with mass transitions of m/z (amu) 323.4 \rightarrow 170.1 and 328.4 \rightarrow 170.1 for RP and RPD3. The mass spectras of parent and product ions of RP and RPD3 shown in fig- 2 and 3.

2.4. Chromatographic conditions

Chromatographic separation was performed by Xbridge C18, 50 X 4.6 mm, 5 μ m analytical column at 40°C. 10mM ammonium formate and methanol in the ratio of (20:80 v/v) was used as mobile phase at a flow rate of 0.7 mL/min. Deuterated internal standard Rucaparib-d4 was used as appropriate internal standard in terms of chromatography and extractability. Rucaparib and Rucaparib -D3 was eluted at 0.9 ± 0.2 min approximately with a total run time of 3 min for each sample.

2.5. Preparation of standards and quality control (QC) Samples

Standard stock solutions of Rucaparib (100.0 μ g/mL) and Rucaparib-D3 (IS) (100.0 μ g/mL) was prepared in methanol. The IS spiking solutions (10.0 ng/mL) were prepared in reconstitution solution (10mM ammonium formate and methanol in the ratio of (20:80 v/v)) from IS stock solution. Standard stock solutions and IS spiking solutions stored in refrigerator conditions 2-8 °C until analysis. Standard stock solutions of Rucaparib (100.0 μ g/mL) was added to drug-free screened human plasma to obtain concentration levels of 5.0, 10.0, 200.0, 800.0, 1400.0, 2000.0, 3000.0, 4000.0 and 5000.0 pg/mL for analytical standards and 5.0, 15.0, 2500.0 and 3500.0 pg/mL for Quality control standards and stored in the freezer at -30°C until analysis. The Aqueous standards were prepared in reconstitution solution (10mM ammonium formate and Methanol in the ratio of (20:80 v/v) for validation exercises and stored in the refrigerator at 2-8°C until analysis.

2.6. Sample preparation

Liquid-Liquid extraction method was used to isolate Rucaparib and Rucaparib-D3 from human plasma. For this, 100 μ L of Rucaparib-D3 (10 ng/mL) and 400 μ L of plasma sample (respective concentration) were added into labeled polypropylene tubes and vortexed briefly about 5 minutes. Followed by, 100 μ L of 0.1N NaOH solution and 3 mL of extraction solvent (ethyl acetate: dichloromethane 80:20v/v) were added and vortexed about 10 minutes. Then the samples were centrifuge at 4000 rpm for approximately 5min at an ambient temperature. From this, supernatant sample was transferred into labeled polypropylene tubes and evaporate to dryness at 40°C briefly, and then reconstituted with reconstitute solution (10mM ammonium formate and Methanol in the ratio of (20:80 v/v) , vortexed and transferred the sample into auto sampler vials for injection.

2.7. Selectivity and Sensitivity

Selectivity was performed by analyzing the human blank plasma samples from six different sources (donors) with an additional hemolysed group and lipidemic group to test for interference at the retention times of analytes. The sensitivity was compared with LLOQ of the analyte with its blank plasma sample. The peak area of blank samples should not be more than 20% of the mean peak area of LOQ of Rucaparib and 5% of the mean peak area of Rucaparib-D3.

2.8. Precision and Accuracy

It was determined by replicate analysis of quality control samples ($n = 6$) at LLOQ (lower limit of quantification), LQC (low quality control), MQC (medium quality control), HQC (high quality control) and ULOQ (upper limit of quantification) levels. The % CV should be less than 15%, and accuracy should be within 15% except LLOQ where it should be within 20%.

2.9. Matrix effect

The matrix effect due to the plasma matrix was used to evaluate the ion suppression/enhancement in a signal when comparing the absolute response of QC samples after pretreatment (LLE) with the reconstitution samples extracted blank plasma sample spiking with analyte. Experiments were performed at MQC levels in triplicate with six different plasma lots with the acceptable precision (%CV) of $\leq 15\%$.

2.10. Recovery

The extraction recovery of Analyte and IS from human plasma was determined by analyzing quality control samples. Recovery at three concentrations (15.0, 2500.0, and 3500.0 pg/mL) was determined by comparing peak areas obtained from the plasma sample, and the standard solution spiked with the blank plasma residue. A recovery of more than 50 % was considered adequate to obtain required sensitivity.

2.11. Stability (Freeze - thaw, Auto sampler, Bench top, Long term)

Low quality control and high quality control samples ($n=6$) were retrieved from the deep freezer after three freeze-thaw cycles according to the clinical protocol. Samples were stored at -30°C in three cycles of 24, 36 and 48 h. In addition, the long-term stability of DL in quality control samples was also evaluated by analysis after 105 days of storage at -30°C . Autosampler stability was studied following 53 hr storage period in the autosampler tray with control concentrations. Room temperature stability was studied for 24.5 hr period with control concentrations. Stability samples were processed and extracted along with the freshly spiked calibration curve standards. The precision and accuracy for the stability samples must be within ≤ 15 and ± 15 % respectively of their nominal concentrations.

Results and discussion:-

3.1. Method development

During method development, different options were evaluated to optimize mass spectrometry detection parameters, chromatography and sample extraction.

3.1.1. Mass spectrometry detection parameters optimization

Electro spray ionization (ESI) provided a maximum response over atmospheric pressure chemical ionization (APCI) mode, and was chosen for this method. The instrument was optimized to obtain sensitivity and signal stability during infusion of the analyte in the continuous flow of mobile phase to electrospray ion source operated at both polarities at a flow rate of 5 $\mu\text{L}/\text{min}$. Rucaparib gave more response in positive ion mode as compare to the negative ion mode. The predominant peaks in the primary ESI spectra of Rucaparib and Rucaparib-D3 corresponds to the $[\text{M}+\text{H}]^{+}$ ions at m/z 323.4 and 328.4 respectively [Fig.2 and 3]. Product ions of Rucaparib and Rucaparib-D3 scanned in quadrupole-3 after a collision with nitrogen in quadrupole-2 had an m/z of 170.1 and 170.1 respectively [Fig.2 and 3]. Mass parameters were optimised as Source temperature 500°C , Heater gas 45 (nitrogen) psi, nebulizer gas 30 (nitrogen) psi, Curtain gas 20 (nitrogen) psi, CAD gas 5 (nitrogen) psi, Ion Spray (IS) voltage 5500 volts, Source flow rate 600 $\mu\text{L}/\text{min}$ without split, Entrance potential 10 V, Declustering potential 70 V, Collision energy 30 V, Collision cell exit potential 15 V for both Analyte and I.S.

3.1.2. Chromatography optimization

Initially, a mobile phase consisting of ammonium acetate and acetonitrile in varying combinations was tried, but a low response was observed. The mobile phase containing 5mM ammonium formate: acetonitrile (20:80 v/v) and 5mM ammonium formate: methanol (20:80 v/v) gives the better response, but poor peak shape was observed. A mobile phase of 0.1% formic acid in water in combination with methanol and acetonitrile with varying combinations were tried. Using a mobile phase containing 5mM ammonium formate: acetonitrile (20:80 v/v) gave the best signal along with a marked improvement in the peak shape was observed for Rucaparib and Rucaparib-D3. Short length columns, such as Symmetry Shield RP18 (50 x 2.1 mm, 3.5 μm), Inertsil ODS-2V (50 x 4.6 mm, 5 μm), Hypurity C18 (50 x 4.6 mm, 5 μm) and Hypurity Advance (50 x 4.0 mm, 5 μm), Xbridge C18, 50x4.6 mm 5 μm were tried during the method development. Xbridge C18, 50x4.6 mm 5 μm column gave a relatively good peak shape with the

best signal was obtained. It gave satisfactory peak shapes for both Rucaparib and Rucaparib-D3. Flow rate of 0.7mL/min without splitter was utilized and reduced the run time to 2.5 min both Drug and IS were eluted with shorter time at 0.9 ± 0.2 min. For an LC-MS/MS analysis, utilization of stable isotope-labeled or suitable analog drugs as an internal standard proves helpful when a significant matrix effect is possible. In our case, Rucaparib-D3 was found to be best for the present purpose. The column oven temperature was kept at a constant temperature of about 40 °C. Injection volume of 5µL sample is adjusted for better ionization and chromatography.

3.1.3. Extraction optimization

Prior to load the sample for LC injection, the co-extracted proteins should be removed from the prepared solution. For this purpose, initially we tested with different extraction procedures like PPT (Protein Precipitation), LLE (Liquid Liquid extraction), and SPE (Solid Phase extraction). We found ion suppression effect in protein precipitation method for drug and internal standard. Further, we tried with SPE and LLE. Out of all we observed LLE is suitable for extraction of drug and IS. We tried with several organic solvents (ethyl acetate, chloroform, n-hexane, dichloro methane and methyl tertiary butyl ether) individually as well with combinations in LLE to extract analyte from the plasma sample. In our case ethyl acetate: dichloromethane (80:20) combination served as good extraction solvent. Auto sampler wash is optimized as 80% methanol. High recovery and selectivity was observed in the Liquid-Liquid extraction method. These optimized detection parameters, chromatographic conditions and extraction procedure resulted in reduced analysis time with accurate and precise detection of Rucaparib in human plasma.

3.2. Method validation

A thorough and complete method validation of Rucaparib in human plasma was done following US FDA guidelines^[12]. The method was validated for selectivity, sensitivity, matrix effect, linearity, precision and accuracy, recovery and stability.

3.2.1. Selectivity and specificity

The analysis of RP and RPD3 using MRM function was highly selective with no interfering compounds. (Fig: 4). Specificity was performed by using six different lots of human plasma. Here showing only one blank plasma interference. Chromatograms obtained from plasma spiked with RP (5.0 pg/mL) and RPD3 (10.0 ng/mL) are shown in (Fig. 5).

3.2.2. Matrix effect

The overall precision of the matrix factor is expressed as Coefficient of Variation (CV%) and was determined to be 1.34 for RP and 1.41 for RPD3.

3.2.3. Linearity

Calibration curves were plotted as the peak area ratio (RP/RPD3) versus (RP) concentration. Calibration was found to be linear over the concentration range of 5.0-5000.0 Pg/mL. The % CV was less than 3.21%, and the accuracy ranged from 97.6 to 102.2 %. The determination coefficients (r^2) were greater than 0.9994 for all curves (Table 1).

3.2.4. Precision and Accuracy

Precision and accuracy for this method were controlled by calculating the intra and inter-batch variations at four concentrations (5.0, 15.0, 2500.0 and 3500.0 pg/mL) of QC samples in six replicates. As shown in (Table 2), the intra-batch % CV was less than 2.0 % and the accuracy ranged from 101.4 to 102.4 %. Inter-batch % CV was less than 2.7 % and the accuracy ranged from 99.5 to 104.8 %. These results indicate the adequate reliability and reproducibility of this method within the analytical range.

3.2.5. Recovery

The recovery following the sample preparation using Liquid-Liquid extraction method was calculated by comparing the peak area ratios of RP in plasma samples with the peak area ratios of solvent samples and was estimated at control levels of RP. The recovery of RP was determined at three different concentrations 15.0, 2500.0 and 3500.0 ng/mL, were found to be 89.6, 90.2 and 91.2 %, respectively. The overall average recovery of RP and RPD3 was found to be 90.3 and 92.5% respectively.

3.2.6. Limit of quantification (LOQ) and Limit of Detection (LOD)

The limit of quantification was determined at 5 pg/ml. The limit of detection was determined at 50 fg /10 μ L injection volume.

3.2.7. Stability (Freeze-thaw, Auto sampler, Bench top, Long term)

Quantification of the RP in plasma subjected to 3 freeze-thaw (-30°C to room temperature) cycles showed the stability of the analyte. The concentrations ranged from 99.3 to 98.5% of the theoretical values. No significant degradation of the Rucaparib was observed even after 53 h storage period in the autosampler tray, and the final concentration of RP was between 97.3 to 103.2 % of the theoretical values. Room temperature stability at 24.5h was between 98.0 to 99.4 % of the theoretical values. In addition, the long-term stability of RP in QC samples after 105 days of storage at -30°C was also evaluated. The concentrations ranged from 94.6 to 101.8% of the theoretical values. These results confirmed the stability of RP in human plasma for at least 105 days at -30°C. (Table 3)

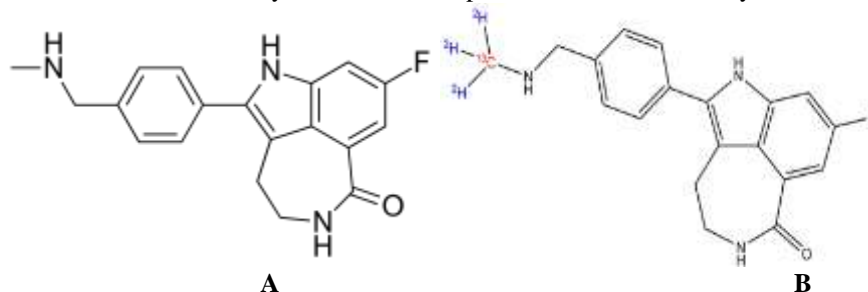


Fig. 1:- Chemical structures of A) Rucaparib (RP) B) Rucaparib-D3 (RPD3)

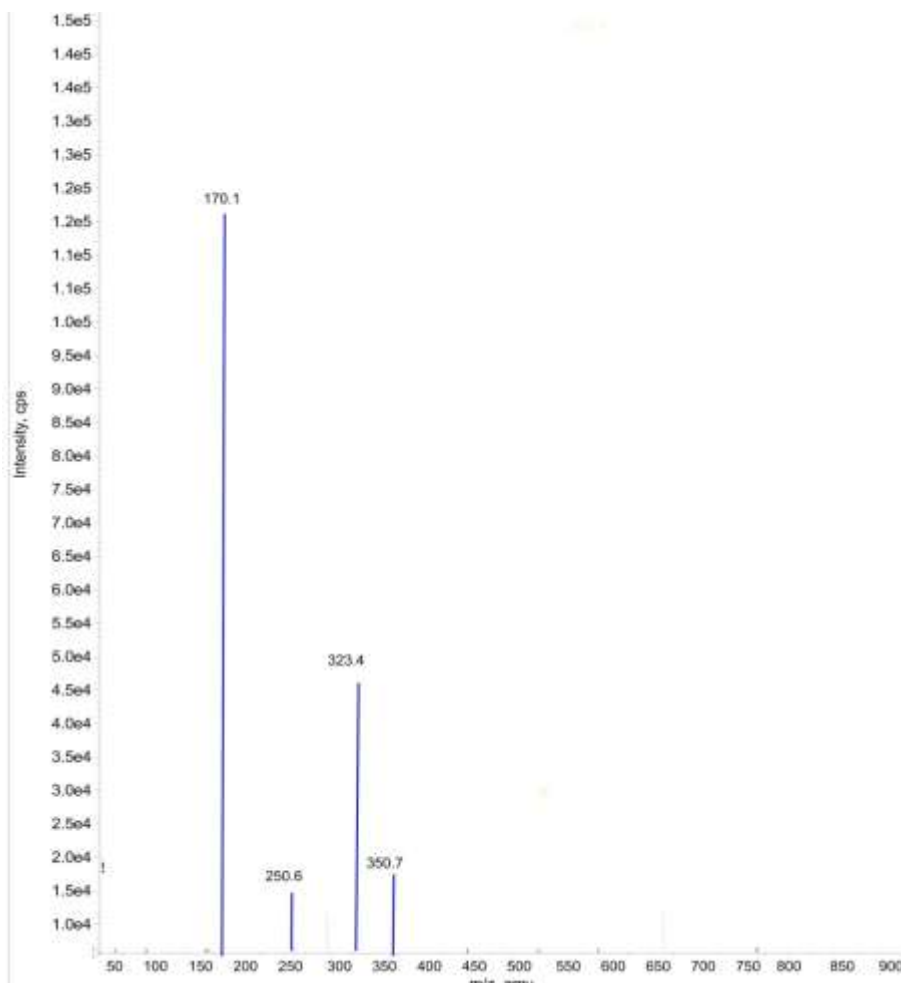


Fig. 2:- Parent and product ion mass spectra of Rucaparib

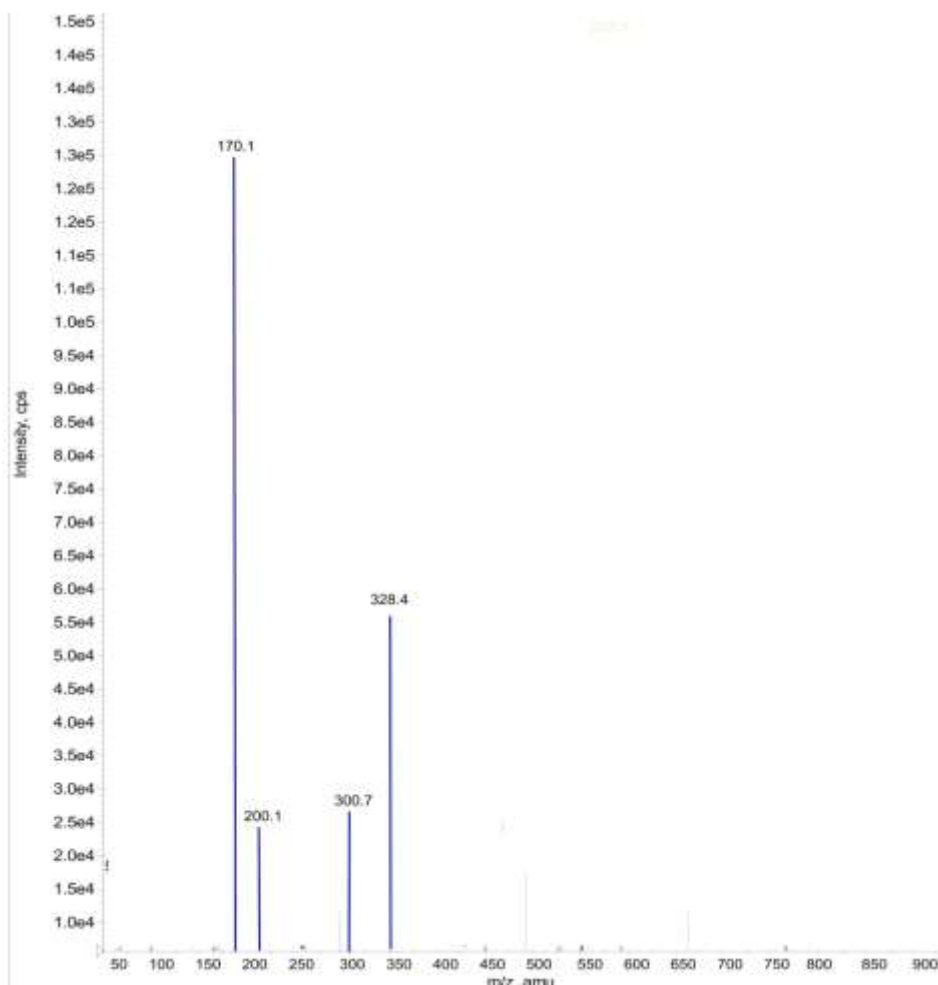


Fig. 3:- Parent and product ion mass spectra of Rucaparib-D3

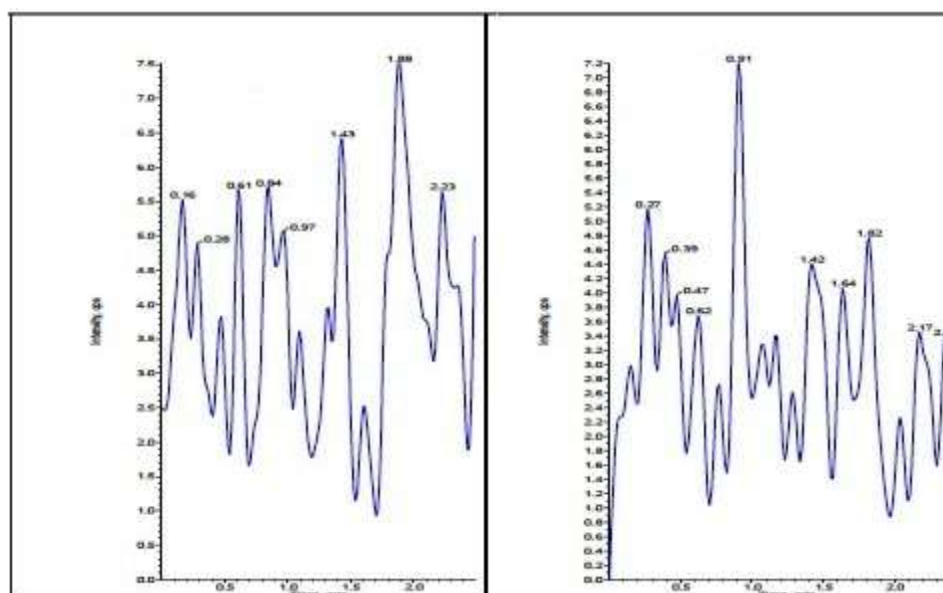


Fig. 4:- Chromatogram of Blank human plasma

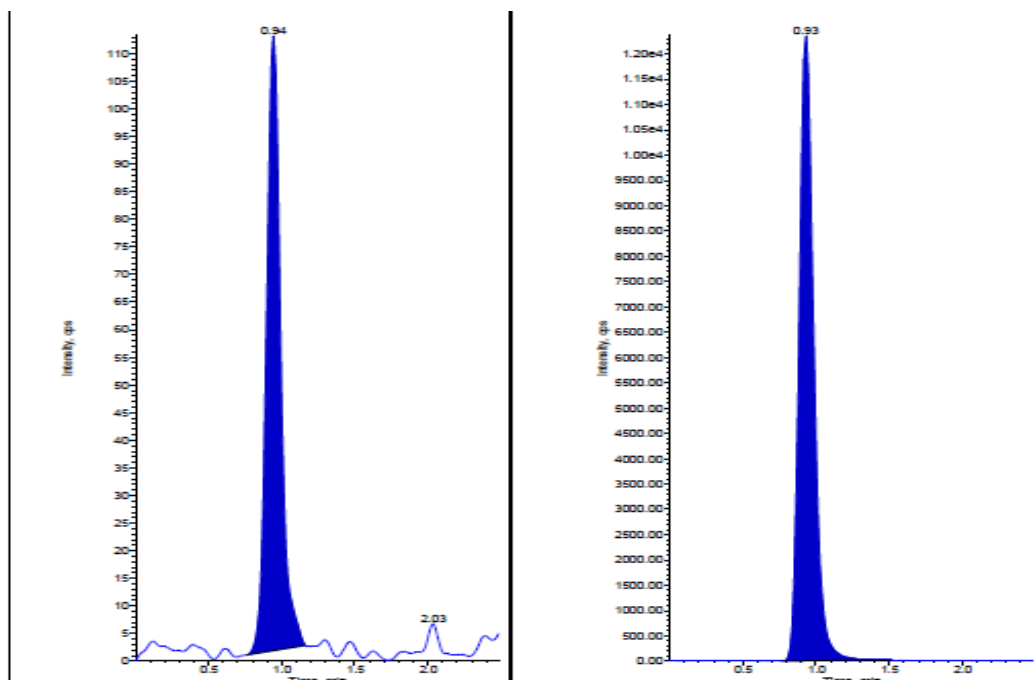


Fig. 5:- Chromatogram of Rucaparib and Rucaparib-D3 at LOQ level

Table 1:- Calibration curve

| Spiked plasma concentration (pg/mL) | Concentration measured(mean) (pg/mL) | SD | (%) CV (n = 5) | Accuracy % |
|-------------------------------------|--------------------------------------|------|----------------|------------|
| 5.0 | 5.1 | 0.1 | 1.9 | 102.0 |
| 10.0 | 9.76 | 0.3 | 3.0 | 97.6 |
| 200.0 | 199.6 | 6.4 | 3.2 | 99.8 |
| 800.0 | 817.2 | 15.2 | 1.8 | 102.2 |
| 1400.0 | 1389.5 | 19.5 | 1.4 | 99.3 |
| 2000.0 | 2006.6 | 23.1 | 1.1 | 100.3 |
| 3000.0 | 2999.7 | 19.6 | 0.6 | 100.0 |
| 4000.0 | 3968.9 | 75.1 | 1.8 | 99.2 |
| 5000.0 | 5026.8 | 91.6 | 1.8 | 100.5 |

Table 2:- Precision and accuracy

| Spiked plasma concentration (pg/mL) | Within-run | | | Between-run | | |
|-------------------------------------|--|-------|------------|---|-------|------------|
| | Concentration measured (n=6) (Pg/mL) (mean \pm S.D.) | (%)CV | Accuracy % | Concentration measured (n=30) (Pg/mL) (mean \pm S.D.) | (%)CV | Accuracy % |
| 5.0 | 5.12 \pm 0.1 | 2.0 | 102.4 | 5.24 \pm 0.1 | 1.9 | 104.8 |
| 15.0 | 15.28 \pm 0.3 | 2.0 | 101.9 | 14.92 \pm 0.3 | 2.0 | 99.5 |
| 2500.0 | 2535 \pm 21.4 | 0.8 | 101.4 | 2495.2 \pm 25.1 | 1.0 | 99.8 |
| 3500.0 | 3577.1 \pm 25.2 | 0.7 | 102.2 | 3550.7 \pm 25.5 | 0.7 | 101.4 |

Table 3:- Stability of the samples

| Spiked plasma concentration (Pg/mL) | Room temperature stability | | Autosampler sample stability | | Long term stability | | Freeze and thaw stability | |
|-------------------------------------|----------------------------|------|------------------------------|------|------------------------|------|---------------------------|--------|
| | 24.5 h | | 53 h | | 105 days | | Cycle 3 (48 h) | |
| | Concentration measured | (%)C | Concentration measured | (%)C | Concentration measured | (%)C | Concentration measured | (%)C V |

| | (n=6) (Pg/mL) (mean±S.D) | V (n=6) | (n=6) (Pg/mL) (mean±S.D) | V (n=6) | (n=6) (Pg/mL) (mean±S.D) | V (n=6) | (n=6) (Pg/mL) (mean±S.D) | (n=6) |
|--------|--------------------------------|------------|--------------------------------|------------|--------------------------------|------------|--------------------------------|-------|
| 15.0 | 14.7± 0.5 | 3.4 | 14.6±0.5 | 3.4 | 14.2± 0.5 | 3.5 | 14.9± .2 | 1.3 |
| 3500.0 | 3480.3± 43.1 | 1.2 | 3614.4± 256.6 | 7.1 | 3563.7± 63.4 | 1.8 | 3450.2±39.1 | 1.1 |

Conclusions:-

The proposed method was five folds higher sensitive than the reported method and analyte was compared with deuterated internal standard. The method described here is fast (requires less than 2.5 min of analysis time), rugged, reproducible bioanalytical method. The developed method is simple and efficient and can be used in pharmacokinetics studies as well as in the monitoring of the investigated analyte in body fluids.

Acknowledgements:-

Authors wish to thank the support received (for providing Literature survey) from IICT (Indian institute of chemical technology) Hyderabad India., APL Research Centre Pvt. Ltd (Hyderabad) India to carry out this Research work.

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