The most suitable bio-analytical method based on Liquid-liquid extraction has been developed and validated for quantification of Flecainide in Rat plasma. Flecainide impurity A was used as an internal standard for Flecainide. Zorbax-SB C18 (4.6mm x 75 mm, 3.5 m) column provided chromatographic separation of analyte followed by detection with mass spectrometry. The method involved simple isocratic chromatographic condition and mass spectrometric detection in the positive ionization mode using an API-4000 system. The total run time was 3.5 minutes. The proposed method has been validated with the linear range of 1.01 – 506.04 ng/ml for Flecainide. The intra-run and inter-run precision values were within 2.6 to 6.9% and 5.0 to 5.7% respectively for Flecainide. The overall recovery for Flecainide and Flecainide impurity A was 72.13% and 72.77% respectively. This validated method was successfully applied into the pharmacokinetic study of rat plasma.

1. Introduction
Flecainide is an antiarrhythmic drug available in tablets of 50, 100, or 150 mg for oral administration. Flecainide acetate is benzamide, N-(2-piperidinylmethyl)-2,5-bis (2,2,2-trifluoroethoxy)-monooacetate. The structural formula is given below. [Fig.1]

It has high bioavailability after an oral dose with plasma half-life is about 20 hours with Peak serum concentrations can be seen 1 to 6 hours after ingestion of an oral dose. [1-2]. During oral loading with flecainide, a steady state equilibrium is typically achieved in 3 to 5 days. The majority of flecainide is eliminated by the kidneys, with the remainder metabolized by the cytochrome P450 2D6 isoenzyme in the liver [3]. Therefore, alterations in renal function or urine pH will greatly affect the elimination of flecainide, as more is eliminated by the kidney than by the hepatic route [4]. Because of the dual elimination routes of flecainide and its tendency to decrease myocardial contractility. Flecainide interacts with numerous pharmaceuticals and can potentiate the effects of other myocardial depressants and AV node blocking agents. The molecular formula is C17H20F6N2O3 and the molecular weight is 414.13 [5].

Quantification of such drugs both in Pharmaceutical formulations and as in biological matrices is very important to know the pharmacokinetics of the drug. As of now, to our knowledge, only a few methods were reported for the determination of Flecainide in biological matrices by LC-MS/MS [6-10], GC-MS[11], UPLC-MS[12], Pharmaceutical compounds by HPLC[13-17], Biological matrices especially in humans by HPLC[18-52], in Rabbit by HPLC[53], in Rat by HPLC[54], have been reported. Among all, most sensitive method (6-10) were developed. Still it is required to develop most sensitive method with proper internal standard usage and suitable extraction method for further clinical research. By keep in mind all the requirements, The proposed method is developed with highly sensitive method (1.01-506.04 ng/ml), internal standard usage and LLE method was developed in rat plasma. It was successfully applied to the pharmacokinetic studies of Flecainide after intravenous administration of 23 mg/kg in rat. The purpose of the present investigation is to explore rapid run analysis time (3.5 min), more sensitive method (1.01 ng/ml), rugged and reproducible method with small amount of plasma sample (50µL Plasma utilization during sample processing, specific extraction method (LLE) and analyte comparision with internal standard (Flecainide impurity A).

2. Experimental
Flecainide and Flecainide impurity A were obtained by Hetero drugs Lt.D Hyderabad. LC grade methanol, was purchased from J.T. Baker Inc. (Phillipsburg, NJ, USA). Reagent grade formic acid was procured from Merck (Mumbai, India).

2.1. Instrumentation
HPLC system (1200 series model, Agilent Technologies, Waldbronn, Germany), Mass spectrometry API 4000 triple quadrupole instrument (ABI-SCIEX, Toronto, Canada) multiple reaction monitoring (MRM) with positive ionization mode was used. Data processing was performed on Analyst 1.5.1 software package (SCIEX).

2.2. Detection
The mass spectrometer was operated in the multiple reaction monitoring (MRM) modes. Sample introduction and ionization were electro spray ionization in the positive ion mode. Sources dependent parameters optimized were as follows: nebulizer gas flow: 37 psi; curtain gas flow: 25 psi; ion spray voltage: 5500 V; temperature (TEM): 550°C. The compound dependent parameters such as the declustering potential (DP), focusing potential (FP), entrance potential (EP), collision energy (CE), cell exit potential (CXP) were optimized during tuning as 45, 38, 10, 30, 10 eV for Flecainide and Flecainide impurity A, respectively. The collision activated dissociation (CAD) gas was set at 3 psi using...
nitrogen gas. Quadrupole 1 and quadrupole 3 were both maintained at a unit resolution and dwell time was set at 300 ms for Flecainide and Flecainide impurity A. The mass transitions were selected as m/z 415.1 for Flecainide and m/z 397.1 for Flecainide impurity A. The parent and product ion spectra for Flecainide and Flecainide impurity A are represented in Figs. 2a-2d respectively. The data acquisition was ascertained by Analyst 1.5.1 software.

2.3. Chromatography
ZORBAX-SB C18 (4.6mm x 75 mm, 3.5 µm) was selected as the analytical column. Column temperature was set at 40°C. Mobile phase composition was 0.1% formic acid: methanol (40:60, v/v). Source flow rate was 600 µL/min without split with injection volume of 10 µL. Flecainide and Flecainide impurity A were eluted at 2.37 ± 0.2 min, 2.68 ± 0.2 min, with a total run time of 3.5 min for each sample.

2.4. Calibration curve and quality control samples
Two separate stock solutions of Flecainide were prepared for bulk spiking of calibration curve and quality control samples for the method validation exercise as well as the subject sample analysis. The stock solutions of Flecainide and Flecainide impurity A were prepared in milli-Q-water at free base concentration of 100 µg/mL. Primary dilutions and working standard solutions were prepared from stock solutions using water solvent mixture. These working standard solutions were used to prepare the calibration curve and quality control samples. Blank rat plasma was screened prior to spiking to ensure it was free of endogenous interference at retention times of Flecainide and internal standard Flecainide impurity A. Ten point standard curve and four quality control samples were prepared by spiking the blank plasma with an appropriate amount of Flecainide. Calibration samples were made at concentrations of 1.01, 2.02, 5.06, 20.24, 50.60, 101.21, 202.42, 303.62, 404.83, and 506.04 ng/mL and quality control samples were made at concentrations of 1.01, 3.04, 151.91 and 354.46 ng/mL for Flecainide.

2.5. Sample preparation
Prior to analysis, all frozen subject samples, calibration standards and quality control samples were thawed and allowed to equilibrate at room temperature. To an aliquot of 50 L of spiked plasma sample, 50 L internal standard was added and vortexed briefly. To these samples, 3 mL of extraction solvent (methyl tertiary butyl ether: dichloro methane (80:20) v/v) was added, capped and the samples were vortexed for 5 min. Centrifugation of the samples was done at 4000 rpm, for 5 min at 20 °C. Supernatant from each sample was transferred into respective tube and evaporated to dryness under nitrogen at 40 ± 2 °C. The dried samples were reconstituted with 500 µL of methanol: 0.1% formic acid (70:30, v/v). All the tubes containing samples were vortexed briefly and transferred into autosampler vials for injection into the chromatographic system.

2.6. Selectivity
Selectivity was performed by analyzing the six different rat blank plasma samples to test for interference at the retention times of analyte.

2.7. Matrix effect
Matrix effect for Flecainide and IS was evaluated by comparing peak area ratio in post-extracted plasma sample from 6 different drug-free blank plasma samples and aqueous reconstitution samples. Experiments were performed at MQC levels in triplicate with six different plasma lots with the acceptable precision (% CV) of 15%.

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

2.9. Recovery
The extraction efficiencies of Flecainide and Flecainide-impurity A were determined by analysis of six replicates at each quality control concentration level for Flecainide and at one concentration for the internal standard Flecainide-impurity A. The percent recovery was evaluated by comparing the peak concentration of extracted standards to the peak concentration of non extracted standards.

2.10. Stability
Stock solution stability was performed by comparing the area response of analyte and internal standard in the stability sample, with the area response of sample prepared from fresh stock solution. Stability studies in plasma were performed at the LQC and HQC concentration levels using six replicates at each level. Analyte was considered stable if the change is less than 15% per US FDA guidelines [55]. The stability of spiked rat plasma samples stored at room temperature (bench top stability) was evaluated for 26 h. The stability of spiked rat plasma samples stored at 2-8°C in autosampler (autosampler stability) was evaluated for 64 h. The autosampler sample stability was evaluated by comparing the extracted plasma samples that were injected immediately (time 0 h), with the samples that were re-injected after storing in the autosampler at 2-8 °C for 26 h. The freeze-thaw stability was conducted by comparing the stability samples that had been frozen at -30 °C and thawed three times, with freshly spiked quality control samples. Six aliquots each of LQC and HQC concentration levels were used for the freeze-thaw stability evaluation. For long term stability evaluation the concentrations obtained after 41 days were compared with initial concentrations.

2.11. Application of method
The validated method has been successfully used to analyze Flecainide concentrations in rat plasma. The study was conducted according to current GCP guidelines. Before conducting the study it was also approved by an authorized animal ethics committee. There were a total of 8 blood collection timepoints including the predose sample. The blood samples were collected in separate vacutainers containing K2EDTA as anticoagulant. The plasma from these samples was separated by centrifugation at 3000 rpm within the range of 2-8 °C. The plasma samples thus obtained were stored at ~30 °C till analysis. Post analysis the pharmacokinetic parameters were computed using WinNonlin® software version 5.2 and 90% confidence interval was computed using SAS® software version 9.2.

3. 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 10 µL/min. Flecainide gave more response in positive ion mode as compared to the negative ion mode. The predominant peaks in the primary ESI spectra of Flecainide and Flecainide-impurity A correspond to the MH+ ions at m/z 415.1 and 397.1 respectively. [Fig. 2a, Fig.2c]. Product ions of Flecainide and Flecainide-impurity A scanned in quadrupole 3 after a collision with nitrogen in quadrupole 2 had an m/z of 398.1 and 231.2 respectively. [Fig. 2b, Fig.2d].
Fig. 2a. Mass spectrum of Flecainide Parent ion.

Fig. 2b. Mass spectrum of Flecainide Product ion.

Fig. 2c. Mass spectrum of Flecainide impurity A Parent ion.

Fig. 2d. Mass spectrum of Flecainide impurity A Product ion.
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 acetic acid: acetonitrile (30:70, v/v) and acetic acid: methanol (30:70, v/v) gave 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 was tried. Using a mobile phase containing 0.1% formic acid in water in combination with methanol (40:60, v/v), the best signal along with a marked improvement in the peak shape was observed for Flecainide and Flecainide impurity A. Short length columns, such as Symmetry Shield RPLC (50mm x 2.1 mm, 3.5 µm), Inertsil ODS-2V (50mm x 4.6 mm, 5µm), Zorbax SB- C18 (75mm x 4.6 mm, 3.5 µm) and Hypurpurp Advance (50mm x 4.0 mm, 5 µm) YMC basic (50mm x2 mm, 5µm), Zorbax Eclipse Plus C18, (2.1mm x 50 mm, 3.5 µl) were tried during the method development. The best signal and good peak shape was obtained using the Zorbax SB- C18 (75mm x 4.6 mm, 3.5 µm), column. It gave satisfactory peak shapes for both Flecainide and Flecainide impurity A. Flow rate of 0.6mL/min without splitter was used and reduced the run time to 3.5 min. Both drug and internal standard were eluted with shorter time at 2.37 and 2.69 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, Flecainide impurity A 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 10µL 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 Protein precipitation (PPT), Liquid-liquid extraction(LLE) and Liquid-liquid precipitation(SPE). 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. Several compounds were investigated to find a suitable IS, and finally Flecainide IMPURITY A was found to be the most appropriate internal standard for the present purpose. There was no significant effect of IS on analyte recovery, sensitivity or ion suppression. 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 Flecainide in rat plasma.

3.2. Method validation
A thorough and complete method validation of Flecainide in rat plasma was done following US FDA guidelines [53]. The method was validated for selectivity, sensitivity, matrix effect, linearity, precision and accuracy, recovery, reinjection reproducibility and stability.

3.2.1. Selectivity and sensitivity
Representative chromatograms obtained from blank plasma and plasma spiked with a lower limit of quantification (LOQ) sample are shown in Fig. 3 and Fig. 4., for Flecainide and Flecainide impurity A. The mean % interference observed at the retention time of analytes between six different lots of rat plasma, containing K2EDTA as an anti-coagulant calculated for Flecainide and Flecainide impurity A respectively, which was within acceptance criteria. Six replicates of extracted samples at the LLOQ level in one of the plasma sample having least interference at the retention time of Flecainide were prepared and analyzed. The % CV of the area ratios of these six replicates of samples was 2.1% for Flecainide, confirming that interference does not affect the quantification at LLOQ level. The LLOQ for Flecainide was 1.01 ng/mL. All the values obtained below 1.01 ng/mL for Flecainide were excluded from statistical analysis as they were below the LLOQ values validated for Flecainide.

3.2.2. Matrix Effect
The % CV of ion suppression/enhancement in the signal was found to be 0.64% at LQC and 0.74 at HQC level for Flecainide, indicating that the matrix effect on the ionization of analyte is within the acceptable range under these conditions.

3.2.3. Linearity
The peak area ratios of calibration standards were proportional to the concentration of Flecainide in each assay over the nominal concentration range of 1.01-506.04 ng/mL. The calibration curves appeared linear and were well described by least-squares linear regression lines. As compared to the 1/x weighing factor, a weighing factor of 1/x2 properly achieved the best result and was chosen to achieve homogeneity of variance. The correlation coefficient was 0.9850 for Flecainide. The observed mean back-calculated concentration with accuracy and precision (% CV) of five linearity’s analyzed during method validation is given in Table 1. The deviations of the back calculated values from the nominal standard concentrations were less than 15%. This validated linearity range justifies the concentration observed during real sample analysis.

Table 1: Precision and accuracy data of back calculated concentrations of calibration samples of Flecainide in Rat plasma

<table>
<thead>
<tr>
<th>Spiked plasma concentration (ng/mL)</th>
<th>Concentration measured (mean) (ng/mL)</th>
<th>SD</th>
<th>(%) CV (n = 5)</th>
<th>Accuracy %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.01</td>
<td>1.01</td>
<td>0.02</td>
<td>1.63</td>
<td>99.80</td>
</tr>
<tr>
<td>2.02</td>
<td>2.05</td>
<td>0.06</td>
<td>2.73</td>
<td>101.29</td>
</tr>
<tr>
<td>5.06</td>
<td>5.01</td>
<td>0.11</td>
<td>2.10</td>
<td>99.05</td>
</tr>
<tr>
<td>20.24</td>
<td>19.87</td>
<td>0.12</td>
<td>0.62</td>
<td>98.16</td>
</tr>
<tr>
<td>50.60</td>
<td>51.23</td>
<td>0.82</td>
<td>1.60</td>
<td>101.25</td>
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<tr>
<td>101.21</td>
<td>102.38</td>
<td>3.38</td>
<td>3.31</td>
<td>101.16</td>
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<tr>
<td>202.42</td>
<td>201.33</td>
<td>7.00</td>
<td>3.48</td>
<td>99.46</td>
</tr>
<tr>
<td>303.62</td>
<td>302.51</td>
<td>4.17</td>
<td>1.38</td>
<td>99.63</td>
</tr>
</tbody>
</table>
3.2.4. Precision and accuracy
The inter-run precision and accuracy were determined by pooling all individual assay results of replicate (n = 6) quality control over five separate batch runs analyzed on four different days. The inter-run, intra-run precision (% CV) was 5% and inter-run, intra-run accuracy was in between 85-115% for Flecainide. All these data presented in Table 2 indicate that the method is precise and accurate.

Table 2: Precision and accuracy (analysis with spiked plasma samples at four different concentrations)

<table>
<thead>
<tr>
<th>Spiked plasma concentration (ng/mL)</th>
<th>Within-run</th>
<th>Between-run</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration measured (n=6) (ng/mL) (mean ± S.D.)</td>
<td>Concentration measured (n=30) (ng/mL) (Mean ± S.D.)</td>
</tr>
<tr>
<td></td>
<td>1.01</td>
<td>1.05 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>3.04</td>
<td>2.95 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>151.91</td>
<td>148.65 ± 9.49</td>
</tr>
<tr>
<td></td>
<td>354.46</td>
<td>347.44 ± 18.26</td>
</tr>
</tbody>
</table>

3.2.5. Recovery
Six aqueous replicates (samples spiked in reconstitution solution) at low, medium and high quality control concentration levels for Flecainide were prepared for recovery determination, and the areas obtained were compared versus the areas obtained for extracted samples of the same concentration levels from a precision and accuracy batch run on the same day. The mean recovery for Flecainide was 72.13% with a precision of 1.1%, and the mean recovery for Flecainide impurity A was 72.77% with a precision of 1.3%. This indicates that the extraction efficiency for the Flecainide as well as Flecainide impurity A was consistent and reproducible.

3.2.6. Readministration reproducibility
Readministration reproducibility exercise was performed to check whether the instrument performance remains unchanged after hardware deactivation due to any instrument failure during real subject sample analysis. The change was less than 1.5% for LQC and HQC level concentration; hence batch can be re-administered in the case of instrument failure during real subject sample analysis. Furthermore, samples were prepared to be re-administered after 25 hours, which shows % change less than 1.8% for LQC and HQC level concentration; hence batch can be re-administered after 25 hours in the case of instrument failure during real subject sample analysis.

3.2.7. Stabilities
Stock solution stability was performed to check stability of Flecainide and Flecainide impurity A in stock solutions prepared in methanol and stored at 2-8 °C in a refrigerator. The freshly prepared stock solutions was compared with stock solutions prepared before 41 days. The % change for Flecainide and Flecainide impurity A were -0.02% and 0.03% respectively indicate that stock solutions were stable at least for 41 days. Bench top and autosampler stability for Flecainide was investigated at LQC and HQC levels. The results revealed that Flecainide was stable in plasma for at least 24 h at room temperature, and 36 h in an auto sampler at 20 °C. It was confirmed that repeated freezing and thawing (three cycles) of plasma samples spiked with Flecainide at LQC and HQC levels did not affect their stability. The long-term stability results also indicated that Flecainide was stable in a matrix up to 60 days at a storage temperature of -30 °C. The results obtained from all these stability studies are tabulated in Table 3.

Table 3: Stability of the samples

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Room temperature stability</th>
<th>Autosampler stability</th>
<th>Long term stability</th>
<th>Freeze and thaw stability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration measured (ng/mL) (mean ± S.D.) (n=6)</td>
<td>Concentration measured (ng/mL) (mean ± S.D.) (n=6)</td>
<td>%CV</td>
<td>% Accuracy</td>
<td>%CV</td>
</tr>
<tr>
<td>24.0 h</td>
<td>36 h</td>
<td>41 days</td>
<td>Cycle 3 (48 h)</td>
<td></td>
</tr>
<tr>
<td>3.04</td>
<td>3.08 ± 0.15</td>
<td>4.86</td>
<td>3.24 ± 0.10</td>
<td>3.02</td>
</tr>
<tr>
<td>354.46</td>
<td>373.63 ± 11.83</td>
<td>3.17</td>
<td>364.27 ± 5.47</td>
<td>1.50</td>
</tr>
</tbody>
</table>

3.3. Application
The validated method has been successfully applied to quantify Flecainide concentrations in to a single dose (23mg/1kg) in rats. Male Sprague-Dawley rats were obtained from Bioneeds, Bangalore. After i.v administration of drug via left femoral vein 0.2 ml of blood samples for analytical determinations were collected via the right femoral vein at specific time intervals for 30 h. Plasma samples were stored at 30 °C until analysis. The study was carried out after approval from an independent animal ethics committee. The pharmacokinetic parameters evaluated were Cmax (maximum observed drug concentration during the study), AUC0-30 (area under the plasma concentration–time curve measured 30 hours, using the trapezoidal rule), T1/2 (terminal half-life as determined by quotient 0.693/Kel). Pharmacokinetic details were shown in Table 4, Fig.5

Table 4: Pharmacokinetic Parameters of Flecainide in Rat Plasma

<table>
<thead>
<tr>
<th>Pharmacokinetic Parameter</th>
<th>values</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC0-1 (ng · h/mL)</td>
<td>2218</td>
</tr>
<tr>
<td>Cmax (ng/mL)</td>
<td>145</td>
</tr>
<tr>
<td>AUC0- (ng · h/mL)</td>
<td>2258</td>
</tr>
<tr>
<td>T1/2 (h)</td>
<td>3.42</td>
</tr>
<tr>
<td>T1/2</td>
<td>11.1</td>
</tr>
</tbody>
</table>

AUC0—∞: area under the curve extrapolated to infinity; AUC0—t: area under the curve up to the last sampling time; Cmax: the maximum plasma concentration; T1/2: the time to reach peak concentration.
4. Conclusion
The proposed bio-analytical method is most specific, highly sensitive, rugged and reproducible. The major advantage of this method is its rapidity, simplicity, less plasma volume (0.1 ml), usage for analysis, suitable internal standard usage. This method was successfully applied in Pharmacokinetic study to evaluate the plasma concentrations of Flecainide in healthy male rats.

Acknowledgments
The authors are grateful to the Indian Institute of technology, Hyderabad for Literature survey and YONTUS Life sciences Pvt.LTD, India for their Lab facility of this research work.

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Fig. 2b. Mass spectrum of Flecainide Product ion.
Fig. 2c. Mass spectrum of Flecainide impurity A Parent ion.
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Fig. 3. Blank plasma chromatogram of Flecainide and Flecainide impurity A in
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Fig. 5: Mean plasma concentrations vs. time graph of Flecainide after intravenous administration of 23 mg/kg in male rat

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Table 2. Precision and accuracy (analyzed with spiked plasma samples at four different concentrations)
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Table 4. Mean Pharmacokinetic Parameters of Flecainide in Rat plasma after intravenous administration of 23 mg/kg in male rat

References
5. https://www.drugbank.ca/drugs/DB01195


