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Original Article

# BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF 2-(4-ETHOXYPHENYL SULPHONAMIDO) PENTANE-DIAMIDE, A NOVEL ANTITUMOR AND ANTIANGIOGENIC AGENT, IN RAT SERUM AND APPLICATION OF THE METHOD IN DETERMINATION OF PHARMACOKINETIC PARAMETERS

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**ABSTRACT**

**Objective:** The present study focuses on the development and validation of a bioanalytical method for the quantification of 2-(4-ethoxyphenyl sulphamido) pentane-diamide, a candidate antitumor and antiangiogenic agent, in rat serum. The developed method was subsequently applied to determine the pharmacokinetic parameters of the compound.

**Methods:** To quantify the compound and determine its pharmacokinetic properties in rats, a liquid chromatography-mass spectrometry (LC-MS) bioanalytical method has been developed and the pharmacokinetic parameters were computed by compartmental model analysis.

**Results:** A linear relationship was detected within the concentration range of 10 to 5000 ng/ml prepared by adding standard solutions of the test compound to the pooled serum of 10 SD rats, which exhibits high levels of precision, accuracy, and reproducibility. An appreciable recovery in the range of 97.20±0.63 to 93.22±1.48 percent was determined, with no noticeable impact from the matrix. The pharmacokinetic parameters, namely oral absorption rate constant (Ka) (5.054±0.238 1/h), elimination rate constant (KE) (2.585±0.357 h), volume of distribution (V) (8.173±0.333 L/kg), and bioavailability of (73.2%), were determined by the utilization of PK-solver software.

**Conclusion:** We developed a simple yet precise and validated LC-MS method to analyze the drug candidate in rat serum. Simple protein precipitation and extraction were cost-effective. This bioanalytical approach was successful due to its good linearity, high recoveries, no matrix influence, and matrix stability. PK solver derived I. V. and oral pharmacokinetics parameters from the best-fit one-compartment model. Because of its high oral absorption, biological half-life, and bioavailability, the compound is suitable for oral administration.

**Keywords:** LC-MS, Validation, Pharmacokinetic study, Bioavailability, PK-solver


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## INTRODUCTION

The compound 2-(4-Ethoxyphenyl sulphonamido) pentanediamide was developed by S. Sen *et al.* in their affiliated laboratory as an antitumor and antiangiogenic drug candidate [1]. The chemical structure of 2-(4-Ethoxyphenyl sulphonamido) pentane-diamide is shown in fig. 1.

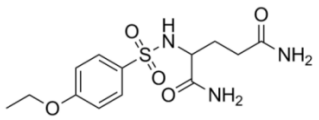

**Fig. 1: Structure of 2-(4-Ethoxyphenyl sulphonamido) pentanediamide**

The cytotoxicity of the molecule was studied in different normal cell lines such as Human Umbilical Vein Epithelial Cells (HUVEC) and Vero cell line (derived from the normal kidney cells of African green monkey) through MTT assay and on a multiple myeloma cell line, RPMI 8226 through MTS assay. The results confirmed that the molecule is a promising antitumor agent against multiple myeloma cancer. The effect of the molecule on the inhibition of phosphorylation of Try-1175 protein on VEGFR-2 also confirmed it as a promising tyrosine kinase inhibitor and an antiangiogenic agent. One of the most interesting results on the normal Vero cell line indicated that the molecule is non-toxic as compared to the standard drug doxorubicin [1].

The studies show that 2-(4-Ethoxyphenyl sulphonamido) pentanediamide is a potential candidate to be a novel anticancer drug and, therefore, requires further investigation for preclinical trial. One of the important and essential steps in preclinical trials is to determine various pharmacokinetics parameters in relevant animal models e. g., rats, rabbits, etc. [2, 3]. The investigation requires the determination of concentrations of the test compound in serum obtained from the animals after administration through a given route. Sensitive and selective analytical methods with accuracy and precision are essential and critical to evaluate the drugs as well as their metabolites quantitively for preclinical studies [4]. To measure the concentration of the test compound in serum, a validated bio-analytical method is required. As 2-(4-Ethoxyphenyl sulphonamido) pentane-diamide is a newly developed molecule, no validated method was developed for its bio-analytical study.

The aim and objective of the present investigation undertaken here are to develop a method using Liquid chromatography hyphenated with Mass spectroscopy (LC-MS) and validate the same for the quantitation of 2-(4-Ethoxyphenyl sulphonamido) pentanediamide in biological fluid and apply the method to determine the concentration of the molecule in serum of the samples obtained for various pharmacokinetic studies following intravenous (I. V.) bolus administration and oral administration. The data were analyzed to determine various pharmacokinetic parameters using compartmental model analysis.

## MATERIALS AND METHODS

### Chemicals and materials

The test compound, 2-(4-Ethoxyphenyl sulphonamido) pentanediamide was synthesized and purified (99 %) by S. Sen *et al.* in this laboratory [1]. HPLC-grade acetonitrile and distilled water were purchased from 'Merck Life Science Private Limited (Vikhorli, Mumbai)'. Whatman Puradisc 13 syringe filters (PTFE) with pore size of 0.2 µm diameter were used for filtration. Collection of blank serum from Sprague Dawley (SD) rats and pharmacokinetics studies on them were carried out as per the approval given by 'Institutional Animal

Ethics Committee' (IAEC) of College of Pharmaceutical Sciences, Berhampur (CPCSEA, Reg. No. 1170/PO/Re/S/08/CPCSEA).

**Instrumentation and condition for LC-MS method development**

The quantitative analysis of the biological samples was carried out using Liquid Chromatography hyphenated with Mass Spectrometry (LC-MS), which consisted of an HPLC system (Perkin Elmer with LX-50 pump) and Triple Quadrupole Mass Spectrometer (Perkin Elmer, QSight 220). A mobile phase composed of water and acetonitrile in a 70:30 ratio was used for analysis. The HPLC was run in isocratic mode. The flow rate of the solvent system was maintained at 1 ml/min and the injection volume was 10 µl. Separation of molecule was carried out on Waters-Symmetry C18 column (3.9 mm X 150 mm; 5 µm). The temperature of the column oven and autosampler were adjusted at 40 °C and 10 °C, respectively.

The quantitation of the molecule was carried out in multiple reaction monitoring (MRM) mode in the m/z range of 329.104 through 313.031. Negative mode electrospray ionization (ESI-) was used as an ionization source by maintaining ion source temperature at 400 °C and ion spray voltage at-5500V. Nitrogen gas was used to set the temperature of curtain gas, nebulizer gas, and heater gas at 120 °C, 200 °C and 320 °C, respectively. The value of Entrance Potential (EP) and Collision Energy (CE) were optimized at 52 eV and-22 eV, respectively. 'Simplicity 3Q, version 3.0' software was used to analyze the responses.

**Preparation of stock solution, samples for calibration, and quality control**

A stock solution with a concentration of 1 mg/ml was prepared by dissolving 2-(4-Ethoxyphenyl sulphonamido) pentanediamide in HPLC-grade water. The reference standard solutions with different concentrations were prepared from the stock solution, adding HPLC-grade water. Reference standards were used both for preparation of calibration standard (CS) and quality control (QC) samples. Blank blood samples were collected from anesthetized Sprague-Dawley rats (three males and three females) by cardiac puncture. Individual blank serum was obtained from blank blood samples by centrifugation at 3000 rpm (rotor radius 9 cm) for 15 min followed by 7000 rpm (rotor radius 6.5 cm) for 25 min. Serum samples were pooled. Final calibration standards (CS) of 10, 20, 60, 100, 500, 1000, 1500, 2500, 4500, and 5000 ng/ml and quality control (QC) samples of 10, 30, 500, and 4000 ng/ml were prepared in blank serum by adding reference standards for preparation of the individual sample.

**Sample preparation**

Preparation and extraction of CS and QC samples of 2-(4-Ethoxyphenyl sulphonamido) pentanediamide from SD rat serum was carried out by protein precipitation method. 100 µl of blank serum was spiked with 100 µl of specific reference standards for individual samples. The mixtures were vortexed for 2-3 min and set aside for 30 min at room temperature. The individual mixture was mixed with 800 µl of acetonitrile for protein precipitation.

The final mixtures were thoroughly agitated using a vortex mixer for 5 min. Subsequently, the mixtures were centrifuged at 10000 rpm (rotor radius 5 cm) for 15 min. The partial supernatant liquid was collected and passed through a PTFE (hydrophilic filtration) syringe filter with 0.2 µm pore size and 13 mm membrane diameter. The filtrate of every individual sample was collected as final CS and QC samples for LC-MS analysis. As the extraction process involved fewer steps and clean extracts were obtained after filtration, no internal standard (IS) has been used in any steps of sample preparation [5].

**Method validation**

Method validation was performed as per the 'Food and Drug Administration (FDA) guideline on bioanalytical method validation, guidance for industry' [6]. The bioanalytical validation was done in terms of selectivity, sensitivity, calibration curve, and linearity range, the lower limit of quantification (LLOQ), upper limit of quantification (ULOQ), accuracy, precision, matrix effect, recovery, carry-over, stability in the matrix (freeze-thaw stability, bench-top stability, long-term stability) and autosampler stability [7-17].

**Selectivity**

Selectivity was concluded by comparing ~~of~~ the chromatograms of six individual blank sera with sera spiked with reference standard at LLOQ concentration.

**Sensitivity and calibration curve**

The sensitivity of the instrument towards the 2-(4-Ethoxyphenyl sulphonamido) pentanediamide molecule was evaluated by measuring the LOD and LLOQ. The LOD and LLOQ values were established by considering signal-to-noise ratio (S/N) 3:1 and 10:1, respectively [18].

The calibration range was established by measuring peak area vs concentration linearity. A total of ten different concentrations were taken in the range of 10 to 5000 ng/ml to make a calibration curve. Least-square linear regression of the areas under the peaks of the analyte plotted along the Y-axis and nominal concentrations plotted along the X-axis with $1/X^2$ as a weighting factor was used to get the calibration curve.

**Accuracy and precision**

Four QC samples (10, 30, 500 and 4000 ng/ml) were analyzed, repeating six times on a given day and on three different days to determine accuracy, precision, and reproducibility for intra-day and inter-day analysis, respectively. Accuracy was calculated in terms of percentage relative error (% RE) from nominal concentration and precision was calculated in terms of % coefficient of variation (% CV) [19]. If the value of accuracy and precision are within±15% of the nominal concentration, results will be accepted.

**Matrix effect**

The unidentified components present in rat serum may interfere with the ionization of the analyte during analysis. The matrix effect evaluation is necessary during method validation. Four QC samples (10, 30, 500, 4000ng/ml) in sextuplicate were prepared in blank rat serum (n=6) and samples were extracted by protein precipitation. Solutions of the same concentrations as QC samples were prepared in sextuplicate in neat solvent (double distilled water). A similar extraction process was used for the preparation of samples in a neat solution to avoid process-related interference. The matrix effect was calculated as follows [20-23]:

$$\text{Matrix effect} = \frac{\text{Peak area of analyte in neat solvent} - \text{Peak area of analyte in serum}}{\text{Peak area of analyte in neat solvent}} \times 100$$

**Recovery**

Four QC samples were prepared by adding reference standard solutions into blank serum followed by extraction using the protein precipitation method. Another four samples of similar concentrations of QC samples were prepared by adding reference standard solutions directly to the extracted serum i.e., after protein precipitation. A comparison of the peak areas was made to calculate the recovery of the analyte.

**Carry-over**

After consecutive three samples run at a concentration of the upper limit of quantification (ULOQ) i.e., 5000 ng/ml, blank samples in triplicate are run thereafter. If blank samples' responses are not more than 20 % of the LLOQ response, carry-over concentration does not affect the analysis.

**Stability**

According to the FDA guideline, stability studies were conducted by considering different conditions to which the samples were supposed to be exposed during experimental work and storage. Stock solution stability of samples was evaluated by comparing the chromatogram of QC samples prepared two months ago (stored at 25 °C) to the freshly prepared QC samples. Benchtop matrix stability study was conducted using QC samples present in serum for 6 h at room temperature and then extracted. The above samples were compared to the freshly prepared QC samples in serum, followed by extraction. Extracted or postpreparative stability was evaluated by comparing the extracted QC samples in an autosampler for 12 h to

the freshly prepared extracted QC samples. QC samples in serum were frozen at-20 °C for 12 h. Subsequently, the samples were thawed at room temperature. Every QC sample was frozen and thawed thrice. The above samples' chromatograms were compared with the chromatograms of the freshly prepared QC samples and freeze-thaw stability was evaluated. For a long-term matrix stability study, The QC samples in serum were stored at-20 °C for 30 d. These samples were compared with the freshly prepared QC samples in serum. For all the stability studies, four QC samples with different concentrations in triplicate were used. The test molecule will be considered stable when accuracy at each level±15 %.

**Pharmacokinetic study**

**Experimental animals and laboratory conditions**

Two to three months old healthy SD rats weighing 200-250 g were used as experimental animals. Animals were purchased from West Bengal Livestock Development Corporation Limited, Buddha Park, Kalyani, Nadia (RegNo. 2109//GO/ReRcBiBt/S/20/CPCSEA). Before administration of the test compound, animals were kept in the laboratory for seven days to acclimatize with laboratory conditions. A proper diet and unlimited water were provided to the animals by maintaining standard laboratory conditions of 12 h dark-light cycle, 25±2 °C temperature, and 50±20 % humidity [24-26]. Pharmacokinetic studies were conducted as per protocol for experiments on animals approved by the Institutional Animals Ethics Committee (IAEC).

**Single-dose intravenous (I. V.) bolus administration**

Rats were divided into nine groups (n=3 for each group) for a pharmacokinetic study of single-dose I. V. administration. Rats were placed in rat restrainers for dose administration. After proper disinfection of the distal region of the tail with 70 % alcohol and dilation of the vein using a heating pad or warm water (approximate temperature 45 °C), a 50 mg/KgBW dose (prepared in sterile water for injection) was administered using a 24G needle through left or right lateral vein [27]. Rats were anesthetized using isoflurane. The blood samples were collected from the heart by cardiac puncture at predetermined time intervals such as 5 min, 10 min, 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, and 12 h. Immediately after collection, the blood samples were centrifuged at 3000 rpm (rotor radius 9 cm) for 15 min, followed by 7000 rpm (rotor radius 6.5 cm) for 25 min to avoid hemolysis as well as to get clear serum. Every individual serum sample was prepared for analysis by the protein precipitation method. The serum samples were precipitated by mixing acetonitrile in two different ways. In method-1, 900 µl of acetonitrile was directly added to 100 µl of serum sample and the mixture was vortexed for 5 min. In method-2, 100 µl of serum sample was diluted with 100 µl of HPLC-grade water and vortexed for 2 min. 800 µl of acetonitrile was added to the serum-water mixture and mixed thoroughly for 5 min using a vortex mixer. In method-2, the ratio of serum: water: acetonitrile was 1:1:8. In both methods, after vortexing with acetonitrile, the mixtures were centrifuged at 10,000 rpm (rotor radius 5 cm) for 15 min. The supernatant liquid was collected partially and passed through a PTFE (hydrophilic filtration) syringe filter with 0.2 µm pore size and 13 mm membrane diameter. The filtrate of every individual sample was collected as the final sample for LC-MS analysis.

**Single-dose oral administration**

In the pharmacokinetic study for single-dose oral administration, rats were divided into nine groups (n=3 for each group). A dose of 125 mg/kgBW (prepared in distilled water) was administered orally to every rat using an 18G gavage needle. Rats were anesthetized using isoflurane. The blood samples were collected from the heart using an 18G needle at predetermined time intervals such as 15 min, 30 min, 1 h, 2 h, 3 h, 4 h, 8 h, 12 h, and 24 h. Blood samples were processed in a similar manner as that of blood samples collected for pharmacokinetic study following I. V. administration of the test compound. The final samples were analyzed in LC-MS.

**Pharmacokinetics data analysis**

Both for I. V. and oral pharmacokinetic study, the serum concentration-time data of 2-(4-Ethoxyphenyl sulphonamido) pentanediamide were analyzed to get pharmacokinetic parameters using PK-Solver, an add-in program in Microsoft Excel [28].

**RESULTS AND DISCUSSION**

**Validation**

Selectivity of this method for 2-(4-Ethoxyphenyl sulphonamido) pentanediamide was confirmed observing the reproducibility of the retention time ($R_t$) at 3.1 min of the peak of the test compound. It is also confirmed that there was no interference of any peak of the 'blank' serum extract at the same retention time (3.1 min) (fig. 2).

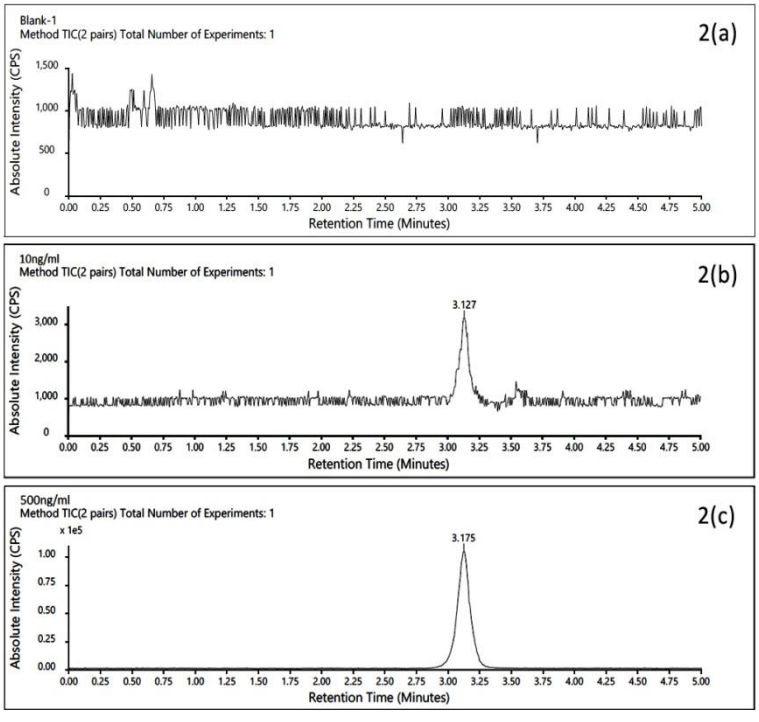


**Fig. 2: Typical LC-MS chromatogram of 2-(4-Ethoxyphenyl sulphonamido) pentanediamide in serum (a) Blank serum (b) at LLOQ (10 ng/ml) (c) at 500ng/ml**

The calibration curve was plotted taking each concentration and corresponding peak area in six replicates. The linearity range was found in the concentration range of 10 through 5000 ng/ml, with a correlation coefficient ($r^2$) = 0.9999. The linear equation of the best-fit calibration curve was y = 797.96x-3298.1 (fig. 3)

LOD and LLOQ were found to be 1 ng/ml and 10 ng/ml respectively. The S/N>3 for LOD and S/N>10 for LLOQ, indicated high sensitivity of the developed method.

Accuracy (% RE) and precision (% CV) were evaluated for intra-day as well as for inter-day using four QC samples in sextuplicate. The result is presented in table 1. The accuracy and precision were in the range of-1.60 to 3.5 and 1.67 to 5.85, indicated within the acceptance limit.

The matrix effects and extraction recovery are presented in table 2. The matrix effects on all four QC samples were less than 15 %, indicating that there is negligible effect of matrix on suppression or enhancement of analyte ions. The recovery range was 97.20±0.63 to 93.22±1.48 percent, confirming proper protein precipitation and extraction process for the analyte.

In the carry-over study, the concentrations in blank samples were below the detectable limit.

The stability of samples at different conditions in which samples were supposed to be exposed, was evaluated by comparing them with freshly prepared samples. All stability conditions with their study reports are illustrated in table 3. The accuracy of each level of QC samples at different stability study conditions was within±15 %, indicating great stability of the molecule.

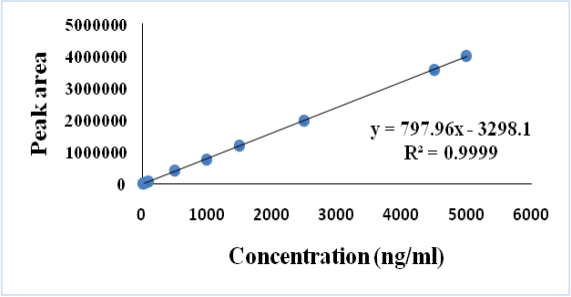


**Fig. 3: Calibration curve of 2-(4-Ethoxyphenyl sulphonamido) pentanediamide**

**Table 1: Accuracy and precision assay**

| Biological sample | Nominal concentration (ng/ml) | Observed concentration (ng/ml) | Accuracy (RE %) | Precision (CV %) |
|---|---|---|---|---|
| Rat Serum | *Intra-day | | | |
| | 10 | 10.35±0.58 | 3.5 | 5.67 |
| | 30 | 29.75±1.66 | -0.83 | 5.59 |
| | 500 | 501.00±9.62 | 0.20 | 1.92 |
| | 4000 | 3935.66±73.29 | -1.60 | 1.86 |
| | **Inter-day | | | |
| | 10 | 10.18±0.59 | 1.82 | 5.85 |
| | 30 | 30.06±1.44 | 0.22 | 4.82 |
| | 500 | 498.57±11.23 | -0.28 | 2.25 |
| | 4000 | 3939.45±66.17 | -1.51 | 1.67 |

*Data expressed as mean±SD; n=6, **Data expressed as mean±SD; n=18

**Table 2: Matrix effect and recovery**

| Biological sample | Nominal concentration (ng/ml) | Matrix effect (%) | Recovery (%) |
|---|---|---|---|
| Rat serum | 10 | 2.79±0.63 | 97.20±0.63 |
| | 30 | 3.59±0.34 | 96.40±0.003 |
| | 500 | 5.69±0.87 | 94.30±0.87 |
| | 4000 | 6.77±1.48 | 93.22±1.48 |

Data expressed as mean±SD; n=6

**Table 3: Stock solution and matrix stability study**

| Biological sample | Stability test/condition | Nominal concentration (ng/ml)* | Observed concentration (ng/ml)* | Accuracy (RE %) |
|---|---|---|---|---|
| Rat serum | Stock solution stability | 10 | 10.19±0.71 | 1.96 |
| | (at 25 °C for 2 mo) | 30 | 29.48±1.64 | -1.72 |
| | | 500 | 485.00±22.24 | -2.99 |
| | | 4000 | 3918.71±60.31 | -2.03 |
| | Bench-top matrix | 10 | 10.03±0.66 | 0.35 |
| | stability | 30 | 30.08±1.20 | 0.22 |
| | (at RT for 6 h) | 500 | 500.91±10.63 | 0.18 |
| | | 4000 | 3931.96±76.05 | -1.70 |
| | Auto sampler stability | 10 | 10.006±0.71 | 0.06 |
| | (at 10 °C for 12 h) | 30 | 29.57±1.61 | -1.41 |
| | | 500 | 493.69±13.35 | -1.26 |
| | | 4000 | 3961.01±45.03 | -0.97 |
| | Freeze-thaw stability | 10 | 9.68±0.92 | -3.125 |
| | (at-20 °C and RT, 12 h | 30 | 29.42±0.64 | -1.93 |
| | cycle) | 500 | 488.49±23.10 | -2.30 |
| | | 4000 | 3908.67±127.70 | -2.28 |
| | Long-term matrix | 10 | 9.69±0.93 | -3.08 |
| | stability | 30 | 28.91±0.92 | -3.62 |
| | (at-20 °C for 30 d) | 500 | 471.94±22.38 | -5.61 |
| | | 4000 | 3841.68±93.80 | -3.95 |

*Data expressed as mean±SD; n=6

**Pharmacokinetic studies**

Above mentioned validated LC-MS method was applied to pharmacokinetic studies to determine the concentration of 2-(4-Ethoxyphenyl sulphonamido) pentanediamide in serum. The extraction of the test compound from the serum sample is one of the most critical steps in bioanalytical study, to be performed scrupulously. Not only the proper selection of extraction method is sufficient, but also proper utilization of that method is important to get better recovery. Elizaveta N. Fisher *et al.*, found that the direct addition of acetonitrile to serum sample in the protein precipitation step led to poor and incomplete recovery. They used acetonitrile along with 10% trichloroacetic acid in the protein precipitation step and got better recovery [9]. In our study, we utilized two different ways to add acetonitrile to the serum sample in the protein precipitation step. In method-1, it was observed that the direct addition of acetonitrile formed a lump of precipitated proteins which may lead to the entrapment of molecules of the test compound in it. In method-2, proteins were precipitated as fine particles without forming any lump. The final samples were analyzed in LC-MS and concentrations of the test compound were calculated (table 4 and 5). The concentrations of all samples were much higher for method-2, where serum was diluted with water, as compared to method-1, where acetonitrile was directly added to serum. It is quite evident that dilution of serum with water before the addition of acetonitrile prevents lump formation and thereby reduces the entrapment of test compound in that lump of proteins and increases recovery.

The representative LC-MS chromatograms sampled after I. V. and oral administration of the test compound are shown in fig. 4. After sampling at each point, the concentrations were calculated with the help of the calibration curve. The concentration versus time curves for the samples obtained after I. V. and oral administration are presented in fig. 5.

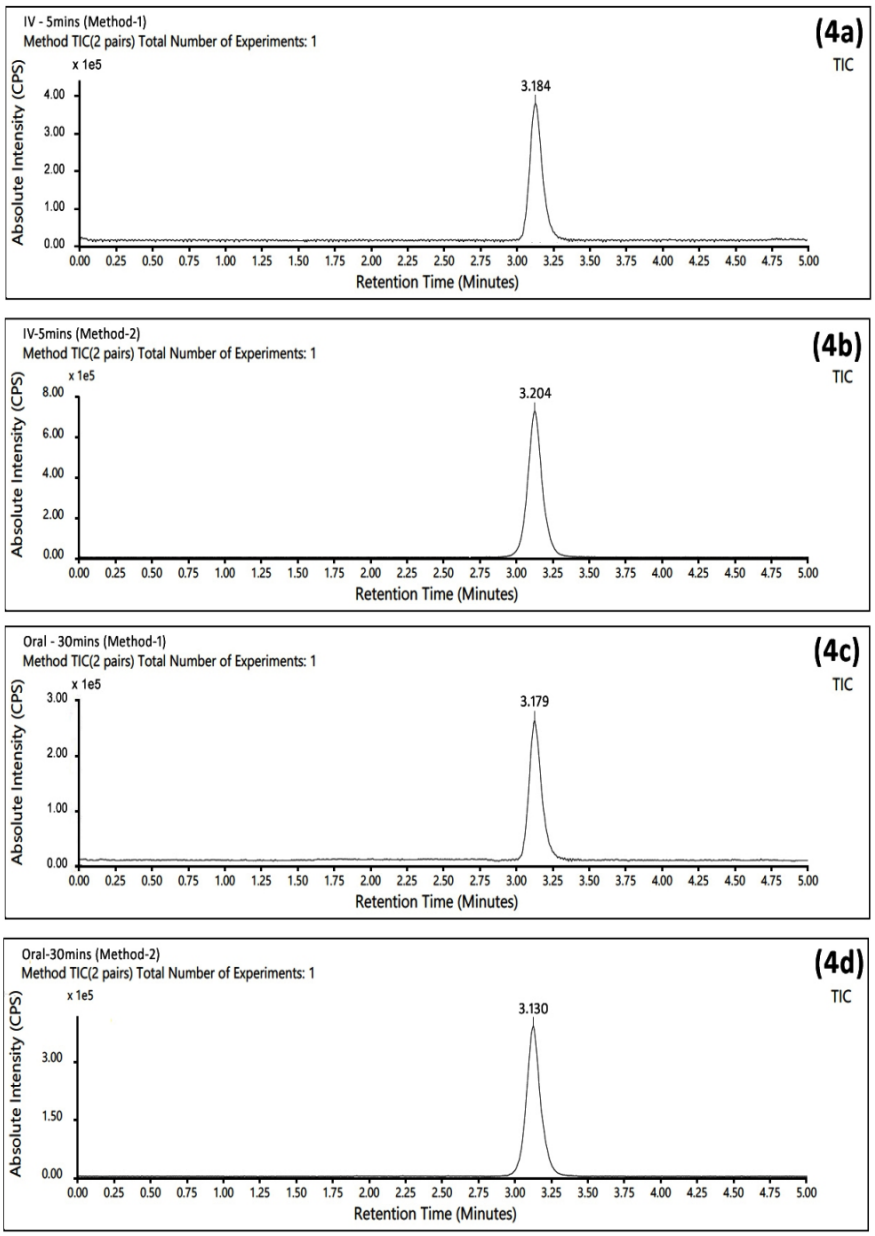


**Fig. 4: Representative LC-MS chromatogram obtained in pharmacokinetic studies. (a) Serum sample collected after 5 min of I. V. administration where protein precipitation was done by method-1, (a) Serum sample collected after 5 min of I. V. administration where protein precipitation was carried out following method-2, (c) Serum sample collected after 30 min of oral administration where protein precipitation was done following method-1, (d) Serum sample collected after 30 min of oral administration where protein precipitation was done by method-2**

**Table 4: The concentrations of the test compound obtained from method-1 and method-2 protein precipitation processes at different time points after single intravenous bolus administration in SD rats**

| Time (h) | Concentrations of the compound in serum (µg/ml) after a single intravenous bolus administration (mean±SD) | |
|---|---|---|
| | Protein precipitation method-1 | Protein precipitation method-2 |
| 0.0833 | 21.016±1.03 | 30.912±3.17 |
| 0.1666 | 20.658±1.13 | 28.750±3.30 |
| 0.25 | 19.344±0.65 | 28.038±3.76 |
| 0.5 | 11.118±0.90 | 16.928±1.78 |
| 1 | 7.346±1.08 | 13.620±2.04 |
| 2 | 4.120±0.65 | 8.504±0.87 |
| 4 | 3.186±0.33 | 5.734±0.44 |
| 8 | 2.319±0.57 | 3.167±0.59 |
| 12 | 1.769±0.17 | 2.490±0.36 |

Single intravenous bolus dose= 50 mg/kgBW; Data expressed as mean±SD; n=3

**Table 5: The concentrations of the test compound obtained from method-1 and method-2 protein precipitation processes at different time points after single-oral administration in SD rats**

| Time (h) | Concentrations of the compound in serum (µg/ml) after single-oral dose administration (mean±SD) | |
|---|---|---|
| | Protein precipitation method-1 | Protein precipitation method-2 |
| 0 | 0 | 0 |
| 0.25 | 7.195±1.05 | 12.715±1.11 |
| 0.5 | 14.126±0.54 | 20.613±2.30 |
| 1 | 8.347±0.70 | 14.771±1.06 |
| 2 | 7.961±0.82 | 14.252±1.62 |
| 3 | 3.970±0.71 | 8.321±2.52 |
| 4 | 4.798±0.08 | 7.304±0.91 |
| 8 | 1.423±0.06 | 3.925±1.39 |
| 12 | 0.837±0.106 | 0.861±0.31 |
| 24 | 0.243±0.04 | 0.638±0.22 |

Single-oral dose= 125 mg/kg BW; Data expressed as mean±SD; n=3

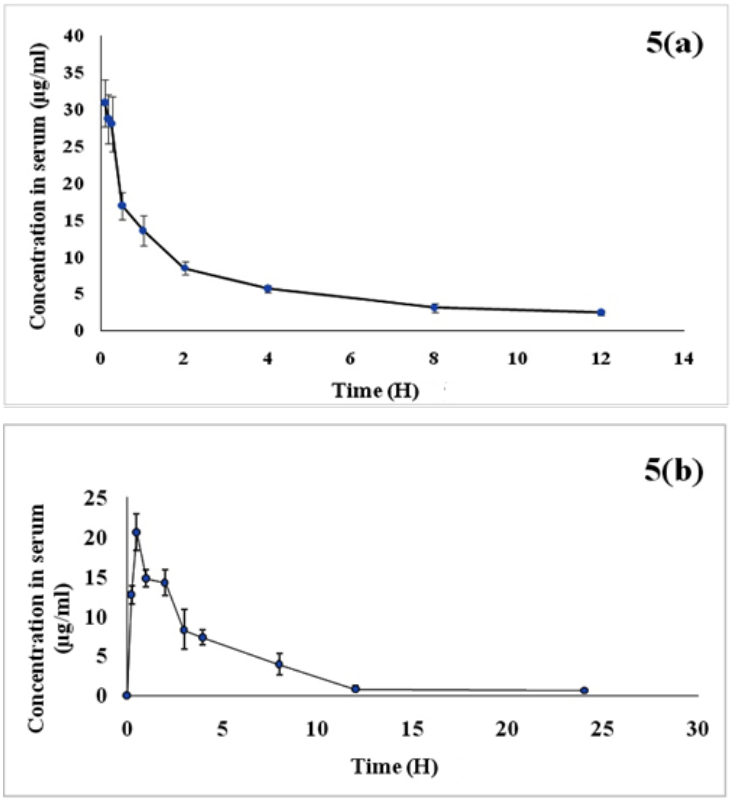


**Fig. 5: Serum concentration vs time curves (a) I. V. bolus administration (b) Oral administration. *Data expressed as mean±SD; n=3**

The data were analyzed using PK solver software. Compartment models were used to analyze the data. In this study, one compartment model is found to be the best-fit model for both I. V. and oral administration. The pharmacokinetics parameters are shown in table 6.

**Table 6: The pharmacokinetic parameters obtained by compartmental model analysis**

| Parameter | Unit | Route of administration | |
|---|---|---|---|
| | | Intravenous bolus (50 mg/KgBW) | Oral (125 mg/KgBW) |
| $C_0$ | µg/ml | 32.080±5.116 | ----- |
| A | µg/ml | ----- | 22.175±1.096 |
| Ka | 1/h | ----- | 5.054±0.238 |
| $K_E$ | 1/h | 0.789±0.263 | 0.271±0.034 |
| $t_{1/2\ a}$ | h | ----- | 0.137±0.006 |
| $t_{1/2\ E}$ | h | 0.772±0.165 | 2.585±0.357 |
| V | L/kg | 1.492±0.245 | 8.173±0.335 |
| CL | ml/min | 20.178±4.019 | 36.813±3.329 |
| Tmax | h | ----- | 0.612±0.002 |
| Cmax | µg/ml | ----- | 17.755±0.351 |
| AUC 0-t | µg/mlh | 42.497±9.361 | 77.775±7.275 |
| AUC 0-inf | µg/mlh | 42.529±9.412 | 77.940±7.437 |
| AUMC | $\mu g/mlh^2$ | 61.141±34.720 | 308.684±70.904 |
| MRT | h | 1.369±0.473 | 3.928±0.506 |
| F% | % | ----- | 73.2 |

Data expressed as mean±SD, n=3

The absorption rate constant (Ka) was high i.e., 5.054±0.238 1/h, indicating faster oral absorption and maximum concentration ($C_{max}$) 17.755±0.351 µg/ml was reached at 0.612±0.002 h ($T_{max}$). Oral biological half-life ($t_{1/2\ E}$) was 2.585±0.357 h, indicating 90 % of the administered dose was removed from the body within 8 to 10 h of administration. The volume of distribution (V) was 8.173±0.333 L/kg for oral administration, which indicates the perfusion of the candidate drug in different tissues, and organs is significantly high. The total clearance (CL) was high i.e., 36.813±3.329 ml/min, indicating less chance of accumulation in the body. The percentage bioavailability (F %) was 73.2 %, indicating that 2-(4-Ethoxyphenyl sulphonamido) pentanediamide is suitable for oral administration.

## CONCLUSION

A simple, specific LC-MS method was developed and validated to study a novel antitumor and antiangiogenic agent, 2-(4-Ethoxyphenyl sulphonamido) pentanediamide, in rat serum. Simple protein precipitation and extraction process made the method simple and cost-effective. Owing to good linearity, high recoveries, no significant matrix effect, and great stability in the matrix, this bioanalytical method was successfully applied to pharmacokinetic studies of 2-(4-Ethoxyphenyl sulphonamido) pentanediamide. In pharmacokinetic studies, the recovery of the test compound was better in method 2, where serum was diluted with water, before the addition of acetonitrile in the protein precipitation process, as compared to method 1, where acetonitrile was directly added to the serum. So, this is one of a new and simple way to enhance recovery of the test compounds in bioanalytical study. Pharmacokinetics parameters both for I. V. and oral administration were successfully calculated from the best fit one-compartment model using PK solver. To sum up, by considering, high oral absorption rate, oral biological half-life (2.585±0.357 h), and high oral bioavailability (73.2 %), 2-(4-Ethoxyphenyl sulphonamido) pentanediamide is a suitable candidate for oral administration.

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## AUTHORS CONTRIBUTIONS

Study conception and design were prepared by Nilufa Yeasmin, PhD scholar of Biju Patnaik University of Technology, Rourkela, Odisha-769015, and Subrata Sen. Data Collection, Analysis, and their interpretations were made by all four authors. Draft manuscript was prepared by Nilufa Yeasmin. The final manuscript was reviewed by all the authors and approved for submission..

## CONFLICTS OF INTERESTS

The authors declare that there are no conflicts of interest.

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