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Quantitative Determination of 5-Aminoisoquinoline, a PARP-1 Inhibitor by UPLC-MS/MS: In Silico ADME Profile and In Vitro Metabolic Stability Study

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Abstract: 5-Aminoisoquinoline (5-AIQ) is a water-soluble, potent and selective Poly (ADP-ribose) polymerase 1 (PARP-1) inhibitor, widely used as a biochemical and pharmacological tool to study the inhibitory effect of PARPs enzyme. In this study, a simple, selective and reliable ultra-performance liquid chromatography-tandem mass spectrometry assay has been developed for the quantitative analysis of 5-AIQ in plasma using pantoprazole as an internal standard (IS). Both 5-AIQ and IS were separated on an Acquity CSH_{18} (2.1 × 100 mm; 1.7 µm) column after chromatographic elution of mobile phase comprising of 10 mM ammonium acetate and acetonitrile (35:65; *v*/*v*) at a flow rate of 0.3 mL/min. Electrospray ionization in positive mode was used for sample ionization and precursor to product ion transitions of 145.0 > 91.0; 145.0 > 117.4 for 5-AIQ and 384.0 > 138.1 for IS were used for detection and quantification in multiple reaction monitoring mode. The assay was linear in the concentration range of 1.0 to 666 ng/mL with correlation coefficient of ≥0.995. The precision and bias were within the acceptable limits of ≤12.68% and −8.6 to 5.9%, respectively, with mean recovery of 79.1% from plasma and negligible matrix effects (92.4%). In silico ADME prediction, 5-AIQ showed to be very soluble in water and high gastrointestinal absorption along with blood–brain barrier (BBB) permeability. The validated assay was successfully applied in a metabolic stability study, and 5-AIQ was moderately metabolized by human liver microsomes with an in vitro half-life of 14.5 min and intrinsic clearance of 47.6 µL/min/mg. The validated method can be utilized for future pharmacokinetic and bio-distribution studies.

Keywords: 5-Aminoisoquinoline; UPLC-MS/MS; metabolic stability; microsomes

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1. Introduction

Poly (ADP-ribose) polymerase 1 (PARP-1) is the first and most abundant isoform of the PARP nuclear enzyme family which has a crucial role in the stabilization of DNA replication forks by repairing DNA strands in homeostatic conditions [1,2]. After binding with DNA breaks, the activated PARP cleaves NAD+ into nicotinamide and ADP-ribose which polymerizes the nuclear acceptor proteins including transcription factors and histone. PARP1 initiates and modulates multiple DNA repair pathways and is thus important for maintaining genomic integrity [3]. Nevertheless, its overactivation in several pathological conditions induces a depletion of NAD+ and ATP that triggers cellular death and up-regulation of key inflammatory pathways [4,5]. Therefore, loss of PARP1 activity by its inhibitor is an attractive strategy for treatment of malignancies, both as single agents and in combination to enhance the cytotoxic activity of DNA damaging agents [6]. Beneficial effects of some PARP inhibitors, e.g., nicotinamide and 3-aminobenzamide, have been previously reported

in various pathophysiological conditions [7], but they were not more potent and have poor permeability across cell membranes [8,9]. 5-Aminoisoquinoline (5-AIQ) is a water-soluble, more potent and selective inhibitor of PARP-1 in human cells, being widely used as a biochemical and pharmacological tool to study the effects of inhibition of the PARPs [10–12]. 5-AIQ has also been proven to be useful in producing beneficial effects in various experimental models of ischemia-reperfusion injury for cerebral, cardiac splanchnic, renal and hepatic artery [13–16]. Due to its anti-inflammatory and immunomodulatory properties, it has produced beneficial effects in experimental models of neuroimmune dysfunction, lungs injury, along with remarkable anti-angiogenic activity and anti-metastatic activity [17–19]. In a genotoxicity test, 5-AIQ was found to be non-mutagenic, suggesting that it has no role to play in DNA damage under normal conditions [20]. In addition, 5-AIQ can be a therapeutic alternative in depression, arthritis, asthma, Parkinson's disease, multiple sclerosis and hepatic disease due to its PARP1 antagonist potential [11]. 5-AIQ is also found to be the most active among the TRPV-1 (Transient Receptor Potential Vanilloid-1) antagonists in the class of 1,3-disubstituted urea derivatives and is under development for varying indications including chronic pain [21].

The quantitative detection of the active compounds is pivotal to obtain reliable results and to make critical decisions supporting safety and efficacy of compounds [22,23]. In drug development, each compound must undergo metabolic fate and pharmacokinetic studies which enable decisions for optimal route of administration, dosing regimen and its biodistribution. A selective and reliable analytical method is required for determining the quantity of a compound in a biological matrix. In spite of having a broad therapeutic potential, no analytical assay has been reported for the quantitative determination of 5-AIQ in plasma or liver microsomes. Among the available techniques, ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) is considered the "gold standard" method for the determination of target analytes in biological fluids. This article presents the data on the development and validation of a method for quantitative detection of 5-AIQ in plasma using the UPLC-MS/MS method. Furthermore, the developed assay was successfully applied in metabolic stability studies in human liver microsomes. In silico, an absorption, distribution, metabolism and excretion (ADME) profile was also computed to predict the drug-like possibilities of 5-AIQ.

2. Material and Methods

2.1. Chemicals and Materials

The 5-AIQ was obtained from Matrix Scientific (Columbia, SC, USA). Pantoprazole [used as internal standard; (IS)] was obtained from Riyadh Pharma (Riyadh, Saudi Arabia). HPLC grade of acetonitrile (ACN) and methanol were obtained from Merck KGaA (Darmstadt, Germany). The analytical reagent ammonium acetate and phosphate buffers were obtained from Qualikems Fine Chem Pvt. Ltd. (Vadodara, India). Ultrapure deionized water was obtained from the Milli Q purification system, Millipore (Mosheim, France). Blank human plasma from healthy donors was kindly provided from King Khalid University Hospital (Riyadh, Saudi Arabia) and human liver microsomes (HLMs) of 50 mixed-sex donors (HMMCPL; Lot No. PL050D-C) were purchased from GIBCO Thermofisher Scientific (Waltham, MA, USA) and was frozen to -80 ± 5 °C before use. Ethical issue pertaining to use of blank human plasma for scientific experiments differs between countries. Our source was expired plasma which could not be used in treating patients and no identifying information of the donor was retained to seek consent.

2.2. Preparation of Stock, Calibration Standard (CS) and Quality Control (QC) Samples

The stock solution of 5-AIQ and pantoprazole were prepared by dissolving the accurately weighed amount of the neat powders in methanol to achieve a 200 µg/mL solution. The stock solution of 5-AIQ was further serially diluted by 50% ACN to prepare a working standard for CSs 30–2000 ng/mL and for QCs 105–15,000 ng/mL concentration. This working standard solution was further spiked into plasma to achieve CSs in plasma between

1–666 ng/mL. Similarly, the QC samples were prepared by spiking their working standards into plasma to achieve 3.5, 50 and 500 ng/mL concentration and was treated as low, middle and high QC (LQC, MQC and HQC) samples. The IS working solution of 200 ng/mL was prepared by diluting its stock standard with 50% ACN and was used during sample preparation procedure. All of the aqueous solution was stored in a refrigerator maintained at 4 ± 2 °C; however, the spiked plasma CSs and QCs samples were stored in a deep freezer maintained at −80 ± 5 °C.

2.3. UPLC-MS/MS and Chromatographic Conditions

The sample analysis was accomplished by ACQUITY UPLC system coupled to a triple quadrupole detector (TQD) mass spectrometer (Waters Corporation, Milford, MA USA). Chromatographic separation of 5-AIQ and IS were performed on an Acquity CSH_{18} (2.1 × 100 mm; 1.7 µm) column. A mixture of ACN and 10 mM ammonium acetate in ratio of 65:35 was used for isocratic elution of analyte and IS, at flow rate of 0.3 mL/min. The column was heated to 40 ± 5 °C to maintained constant temperature throughout the analysis and the auto-sampler temperature was fixed to 15 ± 5 °C.

The TQD equipped with turbo ion source (Z spray) was used for electrospray ionization (ESI) in positive mode and the quantification of the analyte and IS were performed by multiple reaction monitoring (MRM) mode. The transition from precursor to product ion of 145.0 > 117.4 and 145.0 > 91.0 were used as qualifier and quantifier for 5-AIQ while 384.0 > 138.1 was used for IS, respectively. The TQD parameters: capillary voltage (2.41 kV); ion source temperature (150 °C); desolvation temperature (350 °C); desolvation gas (nitrogen) flow (600 L/hr); collision gas (argon) flow (0.15 mL/min) and dwell time (0.160 s) were carefully optimized to produce maximum responses. The molecule related parameters: cone voltage and collision energy were 50 V & 20 V and 22 eV & 30 eV, respectively, for 5-AIQ and IS, respectively (Table 1). The MassLynks and TargetLynks (version 4.1) were used for sample acquisition and processing, respectively.

Table 1. Optimized UPLC-MS/MS parameters for 5-AIQ and IS.

Compound	t_R (min)	Q1 $[M + H]^+$	CV (V)	Q3 $[M + H]^+$	CE (eV)	dt (s)
5-AIQ	0.93	145.0	50	91.0	22	0.160
				117.4 *	20	0.160
IS	0.96	384.02	20	138.05	30	0.160

t_R = retention time; Q1 = precursor ion; CV = cone voltage; dt = dwell time; Q3 = product ion $[M + H]^+$, CE = collision energy), * Qualifier ion.

2.4. Sample Preparation

Protein precipitation was used for plasma sample preparation. An aliquot of 100 µL plasma sample of CSs and QCs was transferred to 1.5 mL of Eppendorf tube. Then, all samples were spiked with 10 µL of IS (200 ng/mL), excluding the blank, and vortexed for proper mixing. The samples were extracted from plasma by using 200 µL of ACN by vortex-mixing gently for 1 min followed by cold centrifugation at 10,000× *g* for 8 min at 4 °C. Then, 100 µL of supernatant samples were transferred to a 150 µL-glass insert. The glass insert was placed in a HPLC vial, and finally, 5 µL was injected to UPLC-MS/MS system for analysis.

2.5. Assay Validation Procedures

The assay validation was performed based on the recommendation described in the recent bioanalytical guideline for method validation [24].

The assay selectivity was determined by comparing the response of 6 different blank plasma samples (obtained from different lots of human plasma) with the response of plasma spiked at lower limit of quantification (LLOQ) and IS concentration. All samples were prepared by following the extraction procedure and were analyzed by developed assay.

For method acceptance, the interference in the blank plasma at the retention time of analyte and IS should be <20% and <5%, respectively.

The assay linearity was determined by preparation of the calibration curve by using eight different concentrations of CSs (1–666 ng/mL) in plasma excluding blank samples. A linear regression model of $1/x^2$ was used to express the area ratio between 5-AIQ/IS against the corresponding 5-AIQ concentration and the assay linearity was confirmed by back calculation of the concentration of each CSs using calibration plots. The value of r^2 (representing the goodness-of-fit) needed to not exceed 0.995; the back-calculated concentration values should be within 15% deviation of the nominal concentration (excluding LLOQ; $\leq$20%) and a minimum of 75% of the nonzero calibration should be within the range of the acceptance criteria.

The intra-day and inter-day precision and bias from nominal value of the developed UPLC-MS/MS method was determined at LLOQ, LQC, MQC, and HQC concentration using five replicates. Precision and bias were expressed by calculating the relative standard deviation (RSD, %) and relative error (RE, %), respectively. The intra-day precision and bias were calculated by analyzing the samples on same day analysis; however, inter-day was calculated by analyzing samples on three different days. For assay acceptance, the precision should not exceed 15% and bias should be within 15%, respectively, for all three QC concentration, except LLOQ which should be within $\leq$20% and $\pm$20%, respectively, for both intra and inter-day studies.

Recovery and matrix effects (ME) of the assay was determined at LQC, MQC and HQC concentration level by spiking them in blank plasma samples. The percentage recoveries were determined by comparing the mean peak response of 5-AIQ between pre- and post-extraction samples. The % ME were determined by comparing the peak response of 5-AIQ between post extracted and aqueous samples. A similar procedure was followed for the determination of ME for IS samples. For assay acceptance, the % ME should be $\leq$15%.

The stability of 5-AIQ in plasma at different anticipated conditions of storage, handling, preparation and analysis were examined by analyzing the recovery of LQC and HQC samples in five replicates. The evaluation parameters include short-term storage, freeze-thaw cycles, auto-sampler and long-term storage stability of 5-AIQ prior to analysis. The short-term stability was determined by keeping the samples at benchtop at least for 8 h while long-term stability was determined by storing the sample at -70 °C for two months before analysis. The freeze-thaw cycles stability was determined by exposing samples for three cycles of freeze (-70 °C) and thaw (room temperature) before analysis. The auto-sampler stability was determined by placing the processed sample in an instrument auto-sampler for 24 h at 15 °C before analysis. The assay was deemed to be stable if the calculated concentration of 5-AIQ were within $\pm$15% of the nominal values after exposing all above anticipated conditions.

2.6. In Silico ADME Analysis

To predict the expected ADME properties of 5-AIQ, SwissADME software, version.1. (Swiss Institute of Bioinformatics Lausanne Switzerland), freely available online at http://www.swissadme.ch/ (accessed on 25 March 2022) was used.

2.7. In Vitro Metabolic Stability Study Using Liver Microsomes

In vitro metabolic stability study was performed to predict the in vivo metabolism of the drug [25]. Pooled HLMs are a suitable source of enzymes for examining in vitro human metabolism as they contain the major drug metabolizing enzymes was used to measure the metabolic activity of 5-AIQ. Microsomal incubation was performed in 0.1 M phosphate buffer solution (PBS) placed in a shaking water bath as described in our previous method [26,27]. Briefly, 5 µL of 5-AIQ (50 µg/mL) solution was transferred to 1.5 mL Eppendorf tube. Then, 460 µL of PBS (pH 7.6) was added. For NADPH generating process, 25 µL of freshly prepared 20 mM NADPH was added into mixture. All the samples were placed for incubation in a shaking water bath maintained at 37 °C. After 5 min, 10 µL of

microsomes (0.5 mg/mL) was transferred into each tube for reaction initiation and the incubation was continued for up to 45 min. Reaction was terminated at different time intervals (0, 2.5, 5, 10, 20, 30 and 45 min) by addition of 250 µL of cold ACN containing IS (500 µg/mL). After incubation termination, samples were properly vortex-mixed followed by cold centrifugation at 8500× *g* for 8 min and 5 µL was injected for UPLC-MS/MS analysis. The 5 µL of the sample was transferred to HPLC vials for analysis by using the developed assay. A graph was plotted between the percentage of the 5-AIQ percent remaining versus time. From the plot, time points in the linear range were chosen to plot the natural logarithm of percent parent compound remaining versus time. The slope of the linear part gives the rate constant for the disappearance of the 5-AIQ that was used for in vitro half-life ($T^1/_2$) and intrinsic clearance (Clint) calculation.

3. Result and Discussion

3.1. Optimization of Mass Spectrometry and Chromatographic Conditions

During the method development process, mass spectrometry condition and its compound specific parameters were carefully optimized to achieve best possible response. The sample tuning of 5-AIQ was performed by direct infusion of standard solution (500 ng/mL) in mass spectrometry using IntelliStart software. The compound 5-AIQ was given single peak of precursor ion $[M + H]^+$ at m/z of 145.0 with strong intensity in ESI positive mode. After the mass fragmentation by MRM mode, the precursor ion produced four abundant product ions $[M + H]^+$ at m/z of 91.0, 117.4, 101.3 and 128.3 (Figure 1). Finally, MRM precursor to product ion transition of 145.0 > 91.0 and 145.0 > 117.4 were used for identification and quantification of 5-AIQ. Similarly, the IS (pantoprazole) ionization was also optimized to achieve best possible transitions at m/z of 384 > 138.1. Finally, the compound dependent parameters: cone voltage and collision energy were optimized to achieve high intensity response.

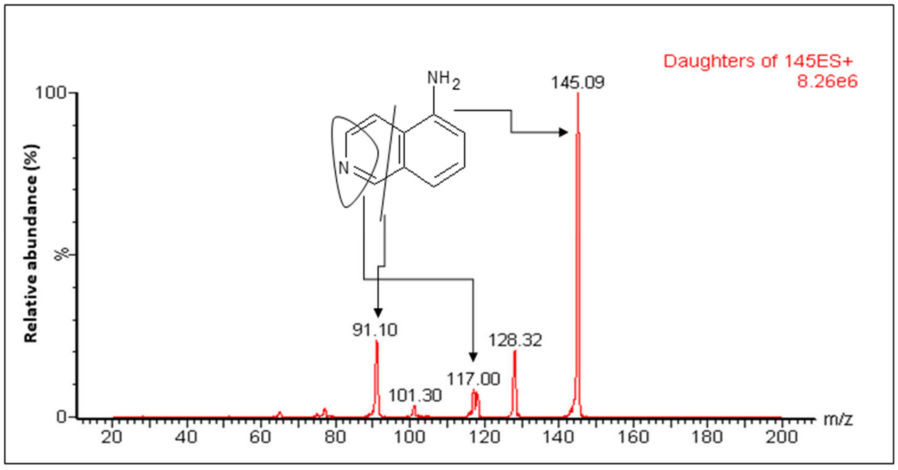

Figure 1. Representative precursor to product ion (m/z) transition of 5-AIQ in ESI positive mode.

An Acquity HILIC column was initially tried for separation of 5-AIQ due to its water solubility properties, but the result was not satisfactory and thus was switched to a C_{18} column. Compared to normal Acquity BEH C18 column, Acquity CSH C18 column showed better peak shape and high sensitivity and was selected for stationary phase separation. A mixture of aqueous and organic mobile phase composition: 10 mM ammonium acetate, 0.1% formic acid and acetic acid in waters and methanol, ACN were tried in different ratio and flow rate to achieve good peak shape with satisfactory peak resolution. The presence of ammonium ions improved the response of protonated ions and finally, the composition of mobile phase containing 10 mM ammonium acetate and acetonitrile in ratio of 35:65 (v/v) at flow rate of 0.3 mL/min was used for chromatographic elution of 5-AIQ and IS, and their retention time were 0.93 and 0.96 min, respectively.

3.2. Assay Validation

3.2.1. Selectivity and Specificity

The chromatogram of blank plasma for 5-AIQ and IS did not show any significant interfering peaks of endogenous substances at their retention time, indicating good assay selectivity (Figure 2A). This result confirmed that the developed assay is selective and specific to the analyte of interest.

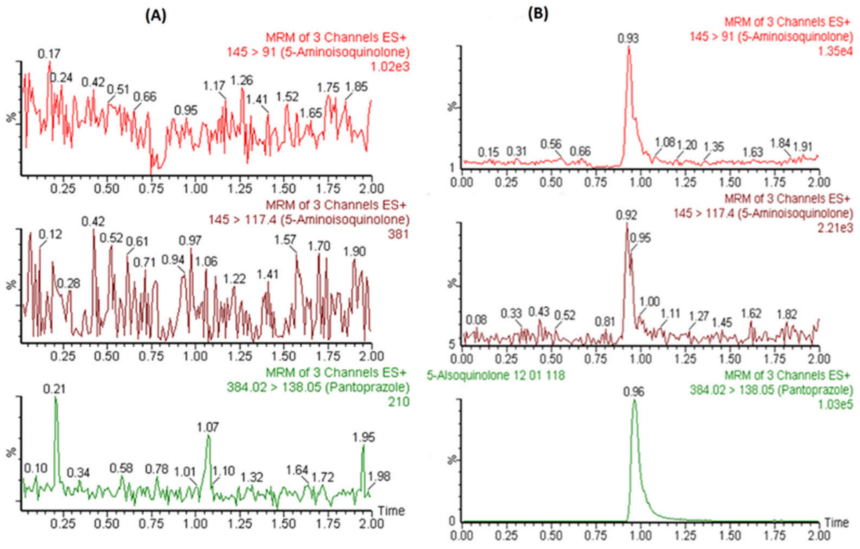

Figure 2. Representative MRM chromatograms of 5-AIQ and IS for blank plasma (**A**) and plasma spiked at LLOQ (1.0 ng/mL) concentration (**B**).

3.2.2. Calibration Curves and Linearity

The developed analytical method exhibited a good linearity over the concentration range of 1.0–666 ng/mL with excellent fit ($r^2 \geq 0.995$). The mean calibration curve linear regression equation of y = 0.102579x + 0.0376 was obtained by using weighing factor $1/\chi^2$. The lowest concentration of CS, 1.0 ng/mL was considered as LLOQ of the method which was quantified with acceptable limit of precision and accuracy (±20%) and having an acceptable signal-to-noise (S/N) ratio, revealing the good sensitivity of the UPLC-MS/MS assay (Figure 2B). The back calculated concentration of remaining calibration standard was also within the acceptable limits of precision and accuracy (±15%).

3.2.3. Precision and Bias

The precision and bias data for all QC samples are presented in Table 2. The intra-day and inter-day precision of the developed assay was ≤7.3 and ≤12.7 (RSD, %), respectively, while the bias value in comparison with nominal value was in the range of −8.6 to 59 and −4.3 to 1.8 (% RE), respectively. These values confirmed that the method is accurate and reliable to use for future pharmacokinetic studies.

3.2.4. Extraction Recovery and ME

The recovery percentage and the ME for 5-AIQ with plasma as a sample matrix was evaluated and presented in Table 3. The overall mean % recovery of 5-AIQ between LQC, MQC and HQC were 79.1% (% RSD = 4.63). This indicates that the recovery of analyte from the proposed extraction procedure was consistent and concentration independent. The mean value of matrix effects for 5-AIQ was 92.4% (RSD, 3.18%) which reflect the ion suppression effects of endogenous phospholipid on analyte response. Since these effects were within the acceptable limit of ±15% with % RSD of ≤5%, it indicates that the developed assay will produce a negligible impact on the quantification of 5-AIQ in actual sample analysis.

Table 2. Intra- and inter-day precision and bias of 5-AIQ in plasma at LLOQ, LQC, MQC and HQC concentration).

Nominal Conc. (ng/mL)	Precision (RSD, %)	Bias (%)
	Intra-day (n = 5)	
1.0	5.6	−8.6
3.5	4.2	5.9
50	7.3	2.3
500	1.9	−6.6
	Inter-day (n = 15)	
1.0	12.7	−4.3
3.5	10.3	−2.6
50	8.1	1.8
500	5.3	−3.4

Table 3. The % recovery and % ME of 5-AIQ and IS in human plasma at LQC, MQC and HQC concentration (n = 5).

Compound	Nominal QC (ng/mL)	% Recovery		% Matrix Effects	
		Mean	%, RSD	Mean	%, RSD
5-AIQ	3.5	76.9	7.2	92.5	5.5
	50	83.3	5.5	95.3	3.4
	500	77.0	9.5	89.4	3.9
Overall		79.1	4.6	92.4	3.2
IS	20	81.9	10.7	91.7	6.1

3.2.5. Stability

The stability study results, which were tested under a variety of conditions using both LQC and HQC concentration, are presented in Table S1. The 5-AIQ was found to be stable under the tested conditions as precision and bias for benchtop stability, freeze thaw stability, in injector stability and long-term stability were within the acceptable limits of $\leq$15% (RSD, %) and of $\pm$15% (relative error; RE, %), respectively. This confirmed that the samples will remain stable at different anticipated conditions during analysis of 5-AIQ.

3.3. In Silico ADME Profile

The drug likeliness of 5-AIQ was evaluated using an ADME properties analysis study. Based on the predicted log *p* value from SwissADME software, the 5-AIQ showed to be very soluble in water (Log S = −2.14). Moreover, the predicted pharmacokinetic value for gastrointestinal absorption is high along with BBB permeability. The bioavailability score is 0.55. The 5-AIQ is predicted to be an inhibitor of cytochrome P450 1A2 (CYP1A2) but not P-glycoprotein (P-gp) substrate. The skin permeability prediction Log Kp value is = −6.36 cm/s, and for drug likeness, it follows the Lipinski rule with violation of molecular weight (MW < 160). The ADME radar chart of 5-AIQ is presented in Figure 3, and data are presented in Table S2.

3.4. Assay Application into Metabolic Stability Study

In the metabolic stability study, the concentration of 5-AIQ in post incubated samples (various time intervals) were quantified against prepared calibration curves between an area ratio of 5-AIQ/IS versus nominal concentration of 5-AIQ. The % remaining concentration of 5-AIQ at different time intervals in incubated reaction mixtures was measured. Figure 4A represents the % remaining of 5-AIQ versus the incubation time point interval. A biphasic pattern of curve indicates rapid metabolic degradation of 5-AIQ for the first 10 min followed by slow degradation up to 45 min. Around 40% of the compound 5-AIQ remained in the mixture after 45 min of the incubation.

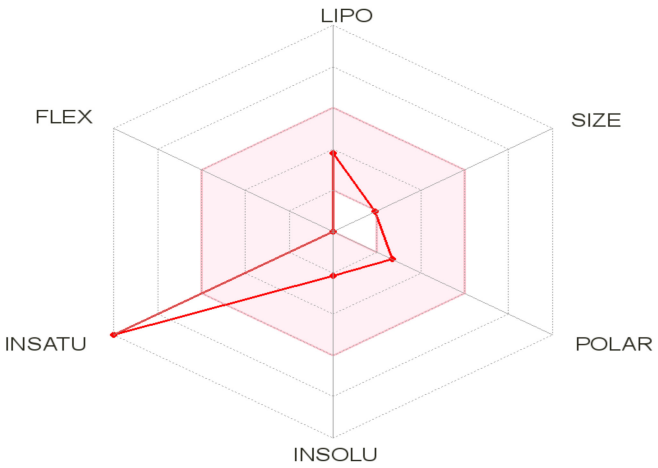

Figure 3. The ADME radar chart of 5-AIQ obtained from SwissADME software.

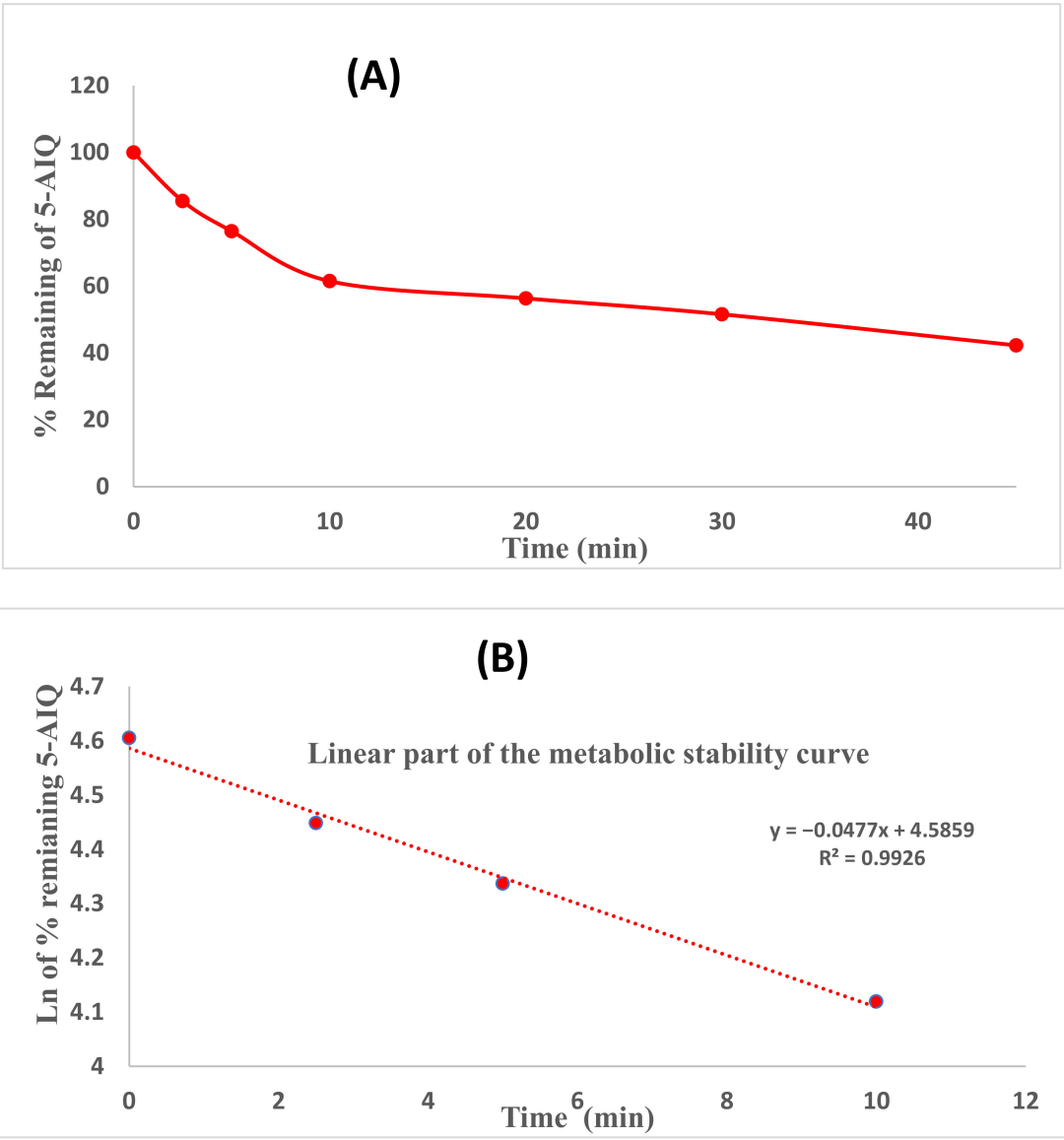

Figure 4. The metabolic stability curve of 5−AIQ in HLMs (**A**) and the regression equation of the linear part of the curve (**B**).

From the constructed curve, the concentrations between the time duration between 0 to 10 min were selected to plot another curve of time versus logarithm (ln) % of remaining exhibited linearity (0–10 min) shown in Figure 4B. The slope of the line (0.0477) described the rate constant of elimination for 5-AIQ. The linear curve regression equation was $y = -0.0477x + 4.55859$ with $r^2 = 0.9926$, which was used for the determination of in vitro $T_{1/2}$ [26]. The slope was 0.0477, so the in vitro $T_{1/2}$ was 14.5 min. The CLint was also calculated based on the in vitro $T_{1/2}$ and was 47.6 µL/min/mg.

4. Conclusions

An accurate, specific, and precise UPLC–MS/MS method was developed and validated for determination of 5-AIQ in plasma sample. The calibration curve was linear between the concentration range of 1.0–666 ng/mL and the stability of the analyte was not impacted by the expected sample handling, storage, preparation, and analysis conditions. The 5-AIQ showed to be very soluble in water and high gastrointestinal absorption along with BBB permeability. 5-AIQ was moderately metabolized by human liver microsomes with in vitro $T_{1/2}$ of 14.5 min and intrinsic clearance of 47.6 µL/min/mg in a metabolic stability study. The current UPLC–MS/MS method was validated and can be used for future applications in pharmacokinetic studies of 5-AIQ.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app12125998/s1, Table S1: Stability data of 5-AIQ in human plasma (n = 5). Table S2: ADME properties of 5-AIQ screened by SwissADME software.

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