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A Fast LC-MS/MS Methodology for Estimating Savolitinib in Human Liver Microsomes: Assessment of Metabolic Stability Using In Vitro Metabolic Incubation and In Silico Software Analysis

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Abstract: Savolitinib (Orpathys®), was developed by (HUTCHMED (Shanghai, China) and, AstraZeneca (Gaithersburg, Maryland, USA), is an inhibitor of the c-Met receptor tyrosine kinase that is orally bioavailable. It was designed for the treatment of pillary and clear-cell renal-cell carcinoma (RCC), colorectal cancer, gastric cancer, and metastatic non-small-cell lung cancer (NSCLC). The current work aimed to develop a rapid, specific, green, and sensitive liquid chromatography tandem mass spectrometry (LC-MS/MS) methodology for estimating savolitinib (SVB) in human liver microsomes (HLMs) with application to an in vitro metabolic stability assessment of SVB in HLMs. The validation steps of the current LC-MS/MS methodology in the HLMs were carried out following US FDA bioanalytical method validation guidelines including sensitivity, selectivity, linearity, accuracy, stability, precision, extraction recovery, and matrix effect. SVB and olmutinib (OLM) were chromatographically separated on an Eclipse Plus C8 column using an isocratic mobile phase. SVB parent ions were generated using the positive mode of an electrospray ionization (ESI) source. SVB daughter ions were detected and quantified using the multiple reaction monitoring (MRM) mode of a triple quadrupole mass analyser. The constructed SVB calibration curve showed linearity over the range from 1 to 3000 ng/mL. The interday and intraday accuracy and precision of the developed LC-MS/MS analytical methodology were −6.67–4.11% and −0.51–8.75%, respectively. A lower limit of quantification (LLOQ) of 0.87 ng/mL confirmed the sensitivity of the established method. Furthermore, the eco-scale methodology using the in silico AGREE software was used for the greenness assessment of the current LC-MS/MS method, and the outcomes showed that the established method was very eco-friendly. The intrinsic clearance (Cl_{int}) and in vitro half-life ($t_{1/2}$) of SVB were 33.05 mL/min/kg and 24.54 min, respectively. SVB exhibited a moderate extraction ratio. The current study is the first to establish and validate LC-MS/MS for estimating SVB and assessing the metabolic stability of SVB.

Keywords: savolitinib; intrinsic clearance; in vitro half-life; greenness; metabolic stability; P450 metabolic mode; LC-MS/MS; StarDrop software

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Citation: Attwa, M.W.; AlRabiah, H.; Abdelhameed, A.S.; Kadi, A.A. A Fast LC-MS/MS Methodology for Estimating Savolitinib in Human Liver Microsomes: Assessment of Metabolic Stability Using In Vitro Metabolic Incubation and In Silico Software Analysis. *Separations* **2023**, *10*, 450. https://doi.org/10.3390/separations10080450

Academic Editor: Léon Reubsaet

Received: 7 July 2023
Revised: 3 August 2023
Accepted: 9 August 2023
Published: 14 August 2023

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1. Introduction

Among all cancers, lung cancer now has the uppermost mortality rate in industrialized counties such as Europe and North America. The two main lung cancer subtypes are small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC) [1]. Lung cancer caused more than 1.76 million deaths in 2018 worldwide according to the WHO. Its occurrence continues to grow. In 2018, it was assessed that 2.1 million new lung cancer patietns were diagnosed internationally, accounting for around 11.6% of the global cancer incidence. NSCLC accounts for 90% of all lung cancer cases, with different subtypes caused by a wide range of activated oncogenes [2,3]. The therapeutic methodologies utilized in cancer

management at present depend on the molecular targeting of tumour suppressor genes and oncogenes, which participate in the disease progression in humans [4]. Although the development of new drugs for cancer treatment has been slow, new improvements in molecular targeting approaches have revealed important enhancements in the prognosis of patients [5]. However, pioneering treatments that were presented after the turn of the century, such as immune checkpoint inhibitors and targeted therapies, have significantly extended the survival of patients that suffer from metastatic NSCLC [6,7]. Targeted therapies against ROS1, ALK, EGFR, V600E, NTRK, and BRAF fusions have been permitted by health authorities in numerous countries. Furthermore, therapies targeting aberrant RET, EGFR, exon 20 insertions, HER2, KRAS G12C, and MET are actively being established. The first RET inhibitor, selpercatinib, and the first MET inhibitor, capmatinib, were approved by the US FDA in May 2020.

MET is regarded as a striking therapeutic target for numerous cancer categories, including NSCLC. The MET exon 14 skip mutation was documented as an encouraging biomarker for MET-TKIs that have also been reported as effective in NSCLC cases with MET gene fusions or MET amplification and, depending on these results, various newer MET-TKIs are undergoing active clinical trials at present [8]. Savolitinib (synonyms: Volitinib; HMPL-504; and AZD-6094) is an orally bioavailable inhibitor of the c-Met receptor tyrosine kinase being established for the management of metastatic NSCLC, gastric cancer papillary and clear-cell renal-cell carcinoma (RCC), and colorectal cancer [9,10]. Savolitinib (Figure 1, SVB) is approved under the trade name Orpathys®, which was developed by HUTCHMED (Shanghai, China) and AstraZeneca (Gaithersburg, Maryland, USA). After the outcomes of a pivotal phase-II trial in cases with pulmonary sarcomatoid carcinoma/NSCLC, SVB was recently granted approval in China for the management of metastatic NSCLC with alterations in MET exon 14 skipping in cases where patients are unable to bear platinum-based chemotherapy or have progressed further [11,12].

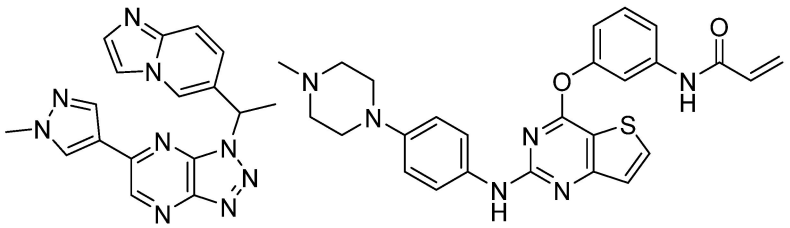

Figure 1. Chemical structure of savolitinib and olmutinib (IS).

Developing a rapid, green, and sensitive analytical LC-MS/MS methodology for the quantification of SVB in different matrices is important. This is because the therapeutic drug monitoring (TDM) of a specific drug (SVB in the current study) is dependent on the accurate analysis of this drug. Moreover, learning the relationship between the activity of SVB and its conc. level is important for its routine and safe use by patients. A literature review revealed that there have been no published articles on the estimation of SVB in various matrices. To date, an assessment of the in vitro metabolic stability of SVB in the metabolic HLM matrix using an LC-MS/MS methodology has not been reported. An evaluation of the metabolic stability of SVB in HLMs is crucial to identify the rate of metabolism and excretion. The metabolic stability of a drug is its susceptibility to metabolism, and is expressed as in vitro half-life ($t_{1/2}$) and clearance intrinsic (Cl_{int}). Half-life ($t_{1/2}$) is described as the time required to metabolize 50% of the parent drug. Intrinsic clearance (Cl_{int}) is the liver's ability to break down the drug in the blood through the metabolic pathway. Thus, the current experiment aimed to establish a rapid, green, sensitive, and specific LC-MS/MS analytical methodology to determine the metabolic stability of SVB in the HLM matrix and to approve the results

in vitro (HLM incubations) and in silico (StarDrop software). Recently, green analytical chemistry (GAC), which aims to remove or decrease dangerous chemicals, lower energy consumption, and decrease waste creation through various analytical steps, has gained increased attention as having crucial scientific value [13,14]. In order to accomplish these objectives, several metric strategies, such as the Analytical Eco-Scale (AES), Red–Green–Blue (RGB), National Environmental Methods Index (NEMI), Analytical Greenness Metric Approach (AGREE), and Green Analytical Procedures Index (GAPI), have been utilised to assess the environmental sustainability of various analytical determinations [13]. Among the mentioned methods, AES, NEMI, GAPI, and RGB relied on specific values of GAC. Notably, the "AGREE" approach, which entails an assessment of the greenness based on the scores of twelve GAC parameters, was employed for the purpose of greenness evaluation.

The LC-MS/MS methodology employed an isocratic mobile phase with an elution duration of 2 min, indicating a rapid analytical approach. The flow rate of 0.4 mL (lower ACN content) contributed to the environmentally friendly nature of the developed methodology. Furthermore, this methodology demonstrated linearity throughout a concentration range of from 1 to 3000 ng/mL. The significance of the established LC-MS/MS methodology was validated by an in silico assessment of the metabolic lability of SVB using the in silico P450 software (StarDrop's) prior to the in vitro HLMs incubation [15], with the aim of reducing costs and saving time. The current LC-MS/MS approach was employed to assess the intrinsic clearance (Cl_{int}) and in vitro half-life ($t_{1/2}$) of SVB [16]. These measurements can be used to calculate the in vivo metabolism rate using three models: dispersion, parallel tube, and venous equilibrium [17,18]. The $t_{1/2}$ and Cl_{int} of SVB were determined using an in vitro $t_{1/2}$ approach based on the well-stirred model [17,18], which is commonly employed in drug metabolism research due to its straightforward nature. The rapid metabolism rate of SVB was found to have a low in vivo bioavailability and a short duration of action, as indicated by previous studies [19–21].

2. Materials and Methods

2.1. Materials

The solvents employed in the LC-MS/MS system under consideration were of high-performance liquid chromatography (HPLC) grade. The solid compounds utilised in the study, namely savolitinib and olmutinib, were of analytical grade (AR). The two analytes of interest, namely savolitinib (also known as Volitinib) with a purity of 99.9% and olmutinib (also known as HM61713) with a purity of 99.84%, were procured from MedChem Corporation located in Princeton, New Jersey, United States. Acetonitrile (ACN), ammonium formate (NH4COOH), formic acid (HCOOH), and human liver microsomes (HLMs), received in dry ice as a cooling agent, were purchased from Sigma-Aldrich Company located in St. Louis, MO, USA. The HLMs with a concentration of 20 mg/mL were stored in a refrigerator at a temperature of −78 °C upon receipt until they were ready to be utilised.

2.2. Instruments

The water filtration device utilised to generate HPLC-grade water was the in-house Milli-Q system manufactured by Millipore Company, located in Billerica, Massachusetts, United States. The LC-MS/MS analytical instrument (Waters Acquity, Milford, MA, USA) utilised in this study was the Waters Corporation's Acquity TQD MS (QBB1203) in conjunction with the Acquity UPLC (H10UPH). This instrument was employed for the purpose of conducting mass analysis and estimating the analytical peaks in the target compounds (SVB and OLM) after their extraction from the metabolic HLMs matrix. The LC-MS/MS system was operated using MassLynx (Version 4.1, SCN 805) as the designated operating system. The initial establishment of the vacuum within the triple quadrupole mass analyzer (TQD) was accomplished by employing a vacuum pump manufactured by Sogevac Company located in Murrysville, Pennsylvania, United States. The collected results were analysed and processed utilising the QuanLynx software. The MS parameters for both analytes, SVB and OLM, were optimised using the IntelliStart® module in MassLynx 4.1 software (version 4.1,

SCN 805). Nitrogen gas, produced by a nitrogen generator from Peak Scientific Company located in Scotland, United Kingdom, was employed for the evaporation of droplets within the mobile phase in the ionisation source (ESI). The analyte ions, specifically SVB and OLM, underwent dissociation into their respective daughter ions within the collision cell of the mass analyzer. This dissociation process was facilitated by the use of argon gas (99.999%) as the collision gas.

2.3. In Silico Study of SVB Metabolic Lability

The computational metabolic stability of SVB was evaluated using an in silico P450 model metabolic program (StarDrop's) developed by Optibrium Ltd. (Cambridge, Massachusetts, United States) prior to the in vitro metabolic incubation of SVB with HLMs. The value of executing the in vitro metabolic practical incubations was confirmed by utilising the outcomes and data collected from the StarDrop software application. The findings were further analysed using a composite site lability (CSL) approach, which provided insights into the metabolic stability of SVB. The CSL parameter was employed as a critical factor in the estimation of SVB metabolic stability prior to conducting in vitro metabolic incubation. This was carried out to ascertain the necessity of creating the proposed LC-MS/MS methodology to determine SVB metabolic stability. The SMILES representation (CC(c1ccc2nccn2c1)n3c4c(ncc(n4)c5cnn(c5)C)nn3) was incorporated into the metabolic program to assess SVB metabolic stability (CSL). In order to evaluate the metabolic stability of SVB, the labilities of individual atoms were gathered and utilised to calculate the CSL value, which ultimately provided insight into the metabolic lability of SVB [22]. The CSL was determined using Equation (1).

$$CSL = {}^{k_{total}}/_{(k_{total} + k_w)} \quad (1)$$

where k_w is the rate constant for water formation.

2.4. LC-MS/MS Instrumental Features

The LC-MS/MS analytical parameters were optimised to achieve the optimal sensitivity and effective separation of the SVB and OLM chromatographic peaks, as indicated in Table 1. The analytical characteristics of the HPLC method were improved by considering factors such as pH, composition of the mobile phase, and nature of the stationary phase. This optimisation aimed to achieve the highest possible sensitivity and separation in the peaks corresponding to the target compounds, SVB and OLM, as shown in Table 1. The binary mobile phase system was composed of two lines: line A, which contained an aqueous component consisting of 0.1% formic acid (HCOOH) in water at a pH of 3.2 that represented 45% of mobile phase, and line B, which contained an organic solvent consisting of acetonitrile (ACN) that represented 55% of mobile phase. The mobile phase flowed at a rate of 0.4 mL/min. The utilisation of a 10 mM NH4COOH solution resulted in the generation of SVB analytical peak tailing and an extended elution time when the pH exceeded 3.2. When the proportion of acetonitrile (ACN) exceeds 55%, there is observed overlap between the analytical peaks in SVB and OLM. Conversely, a lower proportion of ACN leads to an increased elution duration. The ESI source operated in the positive mode to facilitate ion creation, as the basic nitrogen atoms present in the SVB and OLM targets were capable of capturing protons, resulting in the formation of positively charged ions.

The MS tuning of SVB ($C_{17}H_{15}N_9$) and OLM ($C_{26}H_{26}N_6O_2S$) was achieved utilising the IntelliStart® program, employing the combined features of SVB and OLM (10 µg/mL) through direct infusion in the mobile phase stream. Multiple reaction monitoring (MRM) was employed as the mass-analyzer detection technique to estimate the concentrations of SVB and OLM, thereby enhancing the specificity and sensitivity of the LC-MS/MS approach that was developed. The dissociation of the SVB and OLM ions into their respective fragment ions within the second quadrupole (collision quadrupole) was achieved using high-purity argon. The duration of the dwell time for the mass change in SVB and OLM,

namely from parent ions to product ions, was 0.025 s. Table 2 displays the various aspects of MRM and mass transition in SVB and OLM (IS).

Table 1. LC-MS/MS instrumental features.

LC (UPLC)		MS/MS (TQD MS)	
Isocratic mobile phase	0.1% HCOOH in H_2O (45%; pH: 3.2)	ESI	Cone gas: 100 L/H flow rate
	55% ACN		Positive ESI source
	Injection volume: 5.0 µL		The voltage of RF lens: 0.1 (V)
	Flow rate: 0.4 mL/min.		Capillary voltage: 4 KV
Eclipse plus-C8 column	100.0 mm long		The voltage of extractor: 3.0 (V)
	2.1 mm i.d.		Nitrogen (drying gas; 350 °C) at 100 L/hr
	T: 22.0 ± 2.0 °C	Mode	MRM
	3.5 µm particle size	Collision cell	Argon gas at 0.14 mL/min

Table 2. MRM-optimized features for the quantification of the analytes (SVB and OLM).

	Time Segments	Elution Time	MRM Data	
Mass spectra segment	0.0 to 1.0 min	SVB (0.69 min)	First mass transition (m/z)	346.11→ 318.12 CE: 10 and CV: 30
			Second mass transition (m/z)	346.11→ 145.16 CE [a]: 16 and CV [b]: 30
	1.0 to 2.0 min	OLM (IS; 1.16 min)	First mass transition (m/z)	487.15→ 70.13 CE: 40 and CV: 52
			Second mass transition (m/z)	487.15→ 57.98 CE: 44 and CV: 52

[a] Collision energy, [b] cone voltage.

2.5. SVB and OLM Working Dilutions

SVB and OLM exhibited optimum solubility in DMSO at ≥20.83 mg/mL (60.31 mM) and 125 mg/mL (256.89 mM), respectively. Consequently, SVB and OLM stock solutions (1 mg/mL) were dissolved in DMSO. SVB working solutions (WKs) at 100 µg/mL, 10 µg/mL and 1 µg/mL, and OLM at 10 µg/mL were prepared by sequential dilution of the stock SVB and OLM (1 mg/mL) utilising the optimised mobile phase.

2.6. Establishing of SVB Calibration Standards

Prior to conducting the validation procedures for the LC-MS/MS analytical method, HLMs were rendered inactive by introducing an organic solvent (DMSO; 2%) for a duration of 5 min at a temperature of 50 °C. This precautionary measure was implemented to mitigate the potential metabolic impact of HLMs [22–24] on the substances of interest, namely SVB and OLM. To assess the metabolic stability of SVB, a validation matrix for HLMs was prepared. This involved diluting 30 µL of deactivated HLMs (1 mg protein/mL) with 1 mL of metabolic buffer (consisting of 0.1 M sodium phosphate at pH 7.4) containing 3.3 mM MgCl2 and 1 mM NADPH. This approach aimed to replicate the conditions of an actual in vitro metabolic incubation for practical purposes. A series of dilutions of SVB (WK2, and WK3) were performed using the deactivated HLMs matrix to prepare SVB calibration standards (CSs). This resulted in the creation of seven CSs with concentrations of 1, 15, 100, 200, 500, 1500, and 3000 ng/mL. Additionally, four quality controls (QCs) were prepared with concentrations of 1 ng/mL (LLOQ), 3 ng/mL (lower QC, LQC), 900 ng/mL (medium QC; MQC), and 2400 ng/mL (higher QC; HQC). Throughout the dilution process, it was ensured that the concentration of HLMs matrix remained above 90% to minimise the impact of matrix dilution, simulating in vitro incubates. The quality controls

(QCs) were employed as samples with unknown concentrations, and the concentrations were determined by utilising the regression equation derived from the simultaneously injected SVB CSs. A volume of 100 microliters of OLM WK solution with a concentration of 10,000 ng/mL was added to 1 mL of all SVB CSs and QCs as an internal standard.

2.7. Extraction of the Target Analytes (SVB and OLM) from the HLMs Matrix

The SVB and OLM analytes were successfully recovered from the HLMs matrix through the application of the protein precipitation approach, utilising ACN as an organic solvent for quenching and precipitating proteins present in the HLMs matrix. As a result, a volume of 2 mL of ACN was added to each of the SVB CSs and QCs. The mixture was then subjected to continuous shaking for a duration of 5 min to facilitate the extraction of the target analytes (SVB and OLM) from the proteins that had precipitated. Subsequently, the mixture was centrifuged for 12 min at a temperature of 4 °C and a speed of 14,000 rpm. This centrifugation step was performed in order to separate the proteins and clarify the supernatants. In order to verify the appropriateness and integrity of the incubates intended for injection into the LC-MS/MS system, a filtration process was conducted on all samples utilising a syringe filter with a pore size of 0.22 µm. The filtered extracts were transferred into HPLC vials in preparation for injection into an LC-MS/MS equipment. Two control samples were prepared: a negative-control sample consisting of HLMs, and a positive-control sample consisting of HLMs containing OLM. The identical procedures described earlier were followed to confirm that there was no interference from the contents of HLMs during the SVB and OLM processes. The establishment of an SVB calibration curve involved plotting the nominal values of SVB on the x-axis against the peak area ratio of SVB to OLM on the y-axis. The verification of the linearity range of the established SVB CSs was conducted by considering the linear regression equation ($y = ax + b$; r^2) and validation parameters of the LC-MS/MS method.

2.8. Validation Parameters of the Developed LC-MS/MS Methodology

The LC-MS/MS methodology developed in this study was validated according to the recommendations provided by the FDA for the validation of bioanalytical methods. The validation process involved assessing many parameters, including linearity, precision, sensitivity, accuracy, stability, specificity, extraction recovery, and matrix effect [25,26].

2.8.1. Specificity

The determination of the specificity of the designed LC-MS/MS system involved the injection of six sets of blank HLMs matrix samples subsequent to the extraction process. The purified extracts were introduced into an LC-MS/MS system and analysed to identify any intervention peaks that may have been generated by the matrix at the same elution time as the chromatographic peaks (SVB or OLM). These results were then compared to spiked HLMs samples containing the target analytes (SVB and OLM). The MRM mass analyzer mode was employed in order to mitigate the carryover effects of the targets, specifically SVB and OLM, in the quadrupole mass analyzer known as TQD. This was confirmed by analysing the results of the negative control sample HLMs, which were devoid of SVB and OLM.

2.8.2. Sensitivity and Linearity

The sensitivity and linearity of the LC-MS/MS system were assessed by conducting 12 calibration curves (consisting of seven concentration standards) for SVB in the matrix of HLMs on a single day. The resulting calibration curve regression equation was then used to calculate the concentrations of SVB in unknown samples. The limits of detection (LOD) and limits of quantification (LOQ) were determined according to the guidelines outlined in the Pharmacopoeia. These guidelines involved calculating the LOD and the LOQ using

the slope of the linear calibration curve and the standard deviation of the intercept, as described by Equations (2) and (3), respectively.

$$\text{LOD} = 3.3 \times {}^{\text{SD of the intercept}}/_{\text{Slope}} \quad (2)$$

$$\text{LOQ} = 10 \times {}^{\text{SD of the intercept}}/_{\text{Slope}} \quad (3)$$

The linearity of the LC-MS/MS approach was assessed by the utilisation of statistical measures, namely the coefficient of variation (r^2) and the least squared method (y = ax + b).

2.8.3. Precision and Accuracy

The precision and accuracy of the LC-MS/MS methodology were assessed by conducting many experiments over a period of three days for inter-day analysis and one day for intra-day analysis. Six sets of SVB quality controls were loaded for inter-day analysis, while twelve sets were loaded for intra-day analysis. The accuracy and precision of the LC-MS/MS analytical method were expressed as the percent error (%E) and percent relative standard deviation (RSD), respectively. These values were estimated using Equations (4) and (5), respectively.

$$\%\ \text{Error} = {}^{(\text{average computed conc.} - \text{supposed conc.})}/_{\text{supposed conc.}} \times 100 \quad (4)$$

$$\%\ \text{RSD} = {}^{\text{SD}}/_{\text{Mean}} \quad (5)$$

2.8.4. Matrix Effect and Extraction Recovery

The impact of HLMs on the ionisation of the target analytes (SVB or OLM) was assessed by preparing two groups of samples. In this study, HLMs samples belonging to group 1 were spiked with the SVB LQC at a concentration of 3 ng/mL, along with OLM. On the other hand, group 2 employed the mobile phase instead of the HLMs. The calculation of the normalised matrix effect (ME) for the IS was performed using Equation (6), while the ME for SVB and OLM were determined using Equation (7).

$$\text{IS normalized ME} = {}^{\text{ME of SVB}}/_{\text{ME of OLM (IS)}} \quad (6)$$

$$\text{ME of SVB or OLM} = \text{mean peak area ratio } {}^{\text{Group 1}}/_{\text{Group 2}} \times 100 \quad (7)$$

The evaluation of the recovery of SVB extraction from the HLMs matrix and the impact of HLMs on the degree of SVB parent ionisation was conducted through the injection of four QCs. The effectiveness of protein precipitation as an extraction method for SVB and OLM was confirmed by injecting six groups of four QCs in a matrix of HLMs (B) and afterwards comparing them with four QCs prepared in the mobile phase (A). The calculation of the extraction recoveries for SVB and OLM involved determining the ratio of B to A, multiplied by 100.

2.8.5. Stability

The stability of SVB in stock preparations and HLMs matrix was assessed under various laboratory conditions, including pre-analysis treatments such as short- and long-term storage, auto sampler storage, and three freeze–thaw cycles.

2.9. In Vitro Assessment of the Metabolic Stability of SVB

The determination of Cl_{int} and in vitro $t_{1/2}$ of SVB involved the estimation of the proportion of SVB that remained following an in vitro metabolic incubation. This incubation was conducted using an active HLMs matrix supplemented with NADPH (a cofactor) and $MgCl_2$. The in vitro experiment was conducted using a four-step procedure. The initial stage involved pre-incubating 1 µL of SVB with metabolic HLMs matrix at a temperature of 37 °C for a duration of 10 min in a water bath that was thermostatically controlled. In the initiation stage, a concentration of 1 mM NADPH was introduced into each sample.

Subsequently, all samples were placed back into a thermostatic shaking water bath set at a temperature of 37 °C. The third stage involved the loading of OLM (100 µL, 1000 ng/mL) prior to the addition of ACN, which served as a quenching agent. This was carried out to maintain a constant concentration of the internal standard (IS) and to eliminate any potential impact of metabolic reactions on the IS concentration. In the fourth step, the termination phase, 2 mL of ACN, was introduced at specific time intervals (0, 2.5, 7.5, 15, 20, 30, 40, 50, 60, and 70 min) to halt the metabolic reaction and induce the precipitation of surplus proteins. This step is regarded as the initial stage in the extraction procedure of the desired analytes, namely SVB and OLM, as outlined in Section 2.7. A negative control incubation of SVB with HLMs was conducted without the presence of NADPH, following the aforementioned technique. This was carried out to ascertain whether the incubation conditions or matrix effects have any influence on the concentration of SVB in the practical in vitro metabolic incubation investigations.

The concentration of the residual SVB was calculated using the regression line equation derived from the simultaneous injection of SVB CSs. The construction of the SVB metabolic stability curve involved charting the chosen time intervals (x-axis) ranging from 0 min to 70 min against the percentage of remaining SVB concentration relative to the initial concentration at time zero (100%) (y-axis). Subsequently, the segment of the metabolic curve spanning from 0 min to 40 min was chosen to construct a logarithmic curve by graphing the natural logarithm (ln) of SVB concentrations versus metabolic time points (0–40 min). The rate constant of SVB metabolic stability can be determined by analysing the slope of the previous curve. This slope was then used to calculate the in vitro $t_{1/2}$ using the formula in vitro $t_{1/2}$ = ln2/ slope. The SVB Cl_{int} (mL/min/Kg) was calculated according to previous studies [27], utilising the liver tissue mass (26 g) per kilogram of body weight and the HLMs matrix mass (45 mg) per gram of liver tissue (Equation (8)) [28].

$$Cl_{int,} = 0693 \times \frac{1}{t_{\frac{1}{2}}(\text{min.})} \times \frac{\text{mL incubation}}{\text{mg protein}} \times \frac{\text{mg microsomal proteins}}{\text{g of liver weight}} \times \frac{\text{g liver}}{\text{Kg b.w.}} \quad (8)$$

3. Results and Discussions

3.1. In Silico Software of Metabolic Lability of SVB

The metabolic landscape of SVB was used for predictions regarding the susceptibility of active sites in SVB's chemical structure to metabolism by the CYP3A4 enzyme. This information was visualised through a pie chart [29–31]. The high metabolic lability of SVB was demonstrated by the composite site lability (CSL) value of 0.9692 (Figure 2). To analyse the metabolic stability of SVB following in vitro incubation with active HLMs, the LC-MS/MS analytical methodology was employed. The metabolic lability was found to be strong at carbon C24 of the N-methyl pyrazole group and carbon C9 of the imidazo [1,2-a] pyridine ring. Additionally, a moderate level of metabolic lability was seen at C23 of the pyrazole ring and carbon C24 of the imidazo [1,2-a] pyridine group. The primary groups identified by CSL as the potential main contributors to SVB metabolic lability were the N-methyl pyrazole and imidazo [1,2-a] pyridine groups (Figure 2; CSL: 0.9692, indicating a high susceptibility to metabolism). These findings were consistent with the results of the subsequent in vitro metabolic experiments, which will be discussed in greater detail later.

3.2. LC-MS/MS Methodology Establishment

Several stationary phases were investigated, including hydrophilic interaction liquid chromatography (HILIC) columns. Both SVB and OLM were not resolved or retained. Despite achieving optimal results with the C8 column as the reversed stationary phase, the utilisation of a C18 column in the LC-MS/MS methodology to separate SVB and OLM demonstrated chromatographic retention of the analytes. However, the targets (SVB and OLM) exhibited poor base peak separation, peak tailing, and a longer elution time. The use of an Eclipse plus-C8 column (with an internal diameter of 2.1 mm, particle size of 3.5 µm, and length of 100 mm) resulted in the achievement of optimal outcomes in terms of

retention time and chromatographic peak shape. In the present LC-MS/MS methodology, the analytes of interest, SVB and OLM, were separated by chromatography employing an isocratic mobile phase at a flow rate of 0.4 mL/min for a duration of 2 min. The SVB calibration curve generated using the established method demonstrated a linear relationship within the concentration range of from 1 to 3000 ng/mL. Table 3 presents a compilation of diverse experiments conducted to optimise and select the most effective features for the separation, extraction, and evaluation of SVB and OLM peaks. These studies aimed to achieve desirable characteristics such as a well-separated chromatographic peak shape and rapid elution time.

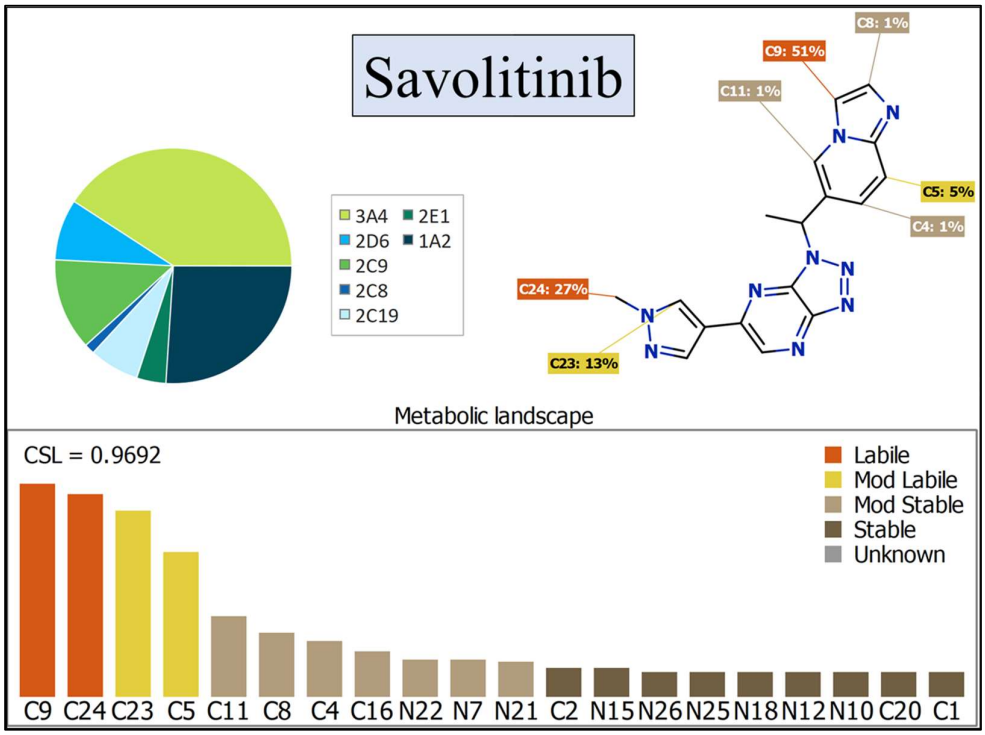

Figure 2. Composite site lability (CSL, 0.9692) revealing the high lability of SVB to metabolism. The outcomes were assessed utilizing WhichP450 (StarDrop's software).

Table 3. Optimized features of the current LC-MS/MS system for SVB and OLM.

Analytes	Mobile Phase		Extraction Method Recovery		Stationary Phase	
	ACN	Methanol	Protein Precipitation Using ACN	Solid Phase Extraction	C8 Column	C18 Column
SVB	0.69 min Good peak	1.0 min Tailed	High (100.88 ± 2.31%) Precise (RSD < 2.29%)	Low (80.54%) Not precise	0.69 min Perfect shape	1.45 min Tailed peaks
OLM	1.16 min Good peak shape	1.15 min Overlapped	High (99.61 ± 2.82%) Precise (RSD < 2.17%)	Low (77.89%) Not precise	1.16 min Perfect shape	1.84 min Perfect shape

In order to improve the sensitivity of the LC-MS/MS analytical system, the MRM mass analyzer mode was employed for the purpose of detecting and estimating SVB and OLM. This aimed to eliminate any interference caused by the matrix components present in the HLMs (Figure 3).

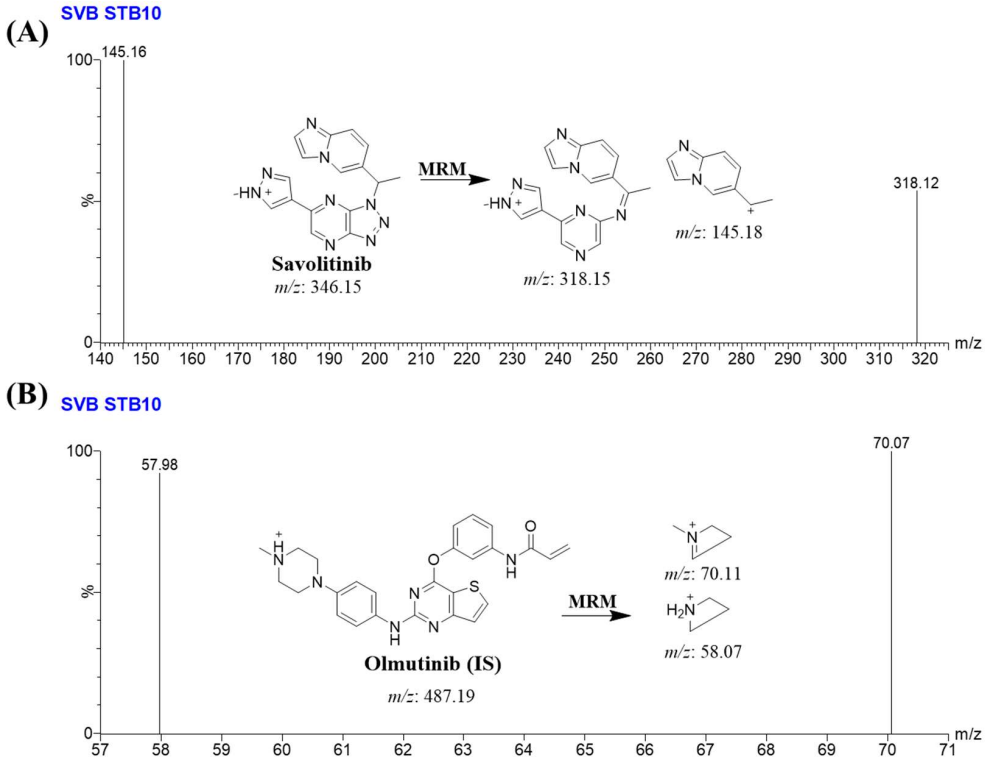

Figure 3. MRM mass spectrum of SVB $[M+H]^+$ (**A**) and OLM (**B**) $[M+H]^+$. The proposed dissociation patterns are exhibited.

The utilisation of OLM as an IS in the estimation of SVB was conducted in the current well-established analytical LC-MS/MS methodology, owing to three underlying factors. Initially, it is notable that the protein precipitation extraction approach can be effectively employed for both SVB and OLM targets, resulting in a substantial yield of 100.88 ± 2.31% (RSD: 2.29) and 99.61 ± 2.82% (RSD: 2.17%), respectively. Furthermore, the eluted peaks in the target compounds SVB (0.69 min) and OLM (1.16 min) were successfully obtained within a two-minute timeframe, demonstrating the effectiveness of the created LC-MS/MS approach as a rapid analytical technique. This method not only reduces the overall run time but also utilises a smaller amount of ACN, aligning with the principles of green chemistry. Furthermore, it is important to note that the patients did not consume both analytes (SVB and OLM) simultaneously in a certain medical case. Thus, the present LC-MS/MS analytical technique can be employed for therapeutic drug-monitoring (TDM) and pharmacokinetic investigations of SVB. There was no observed carry-over influence in the MRM mass chromatograms of HLMs negative controls (Figure 4A) and positive controls (Figure 4B) for SVB. Figure 4C displays the superimposed MRM mass chromatograms of SVB CSs and OLM at concentrations of 1–3000 ng/mL and 1000 ng/mL, respectively.

3.3. Validation of the Developed LC-MS/MS Analytical Methodology

3.3.1. Specificity

The validity of the LC-MS/MS methodology was confirmed through the distinct separation of chromatographic peaks corresponding to SVB and OLM, as illustrated in Figure 4. In addition, it was observed that the chromatographic peaks in the target analytes (SVB and OLM) did not exhibit any significant interference from the matrix components present in the HLMs. The control MRM mass chromatograms did not exhibit any observable carry-over influence from SVB.

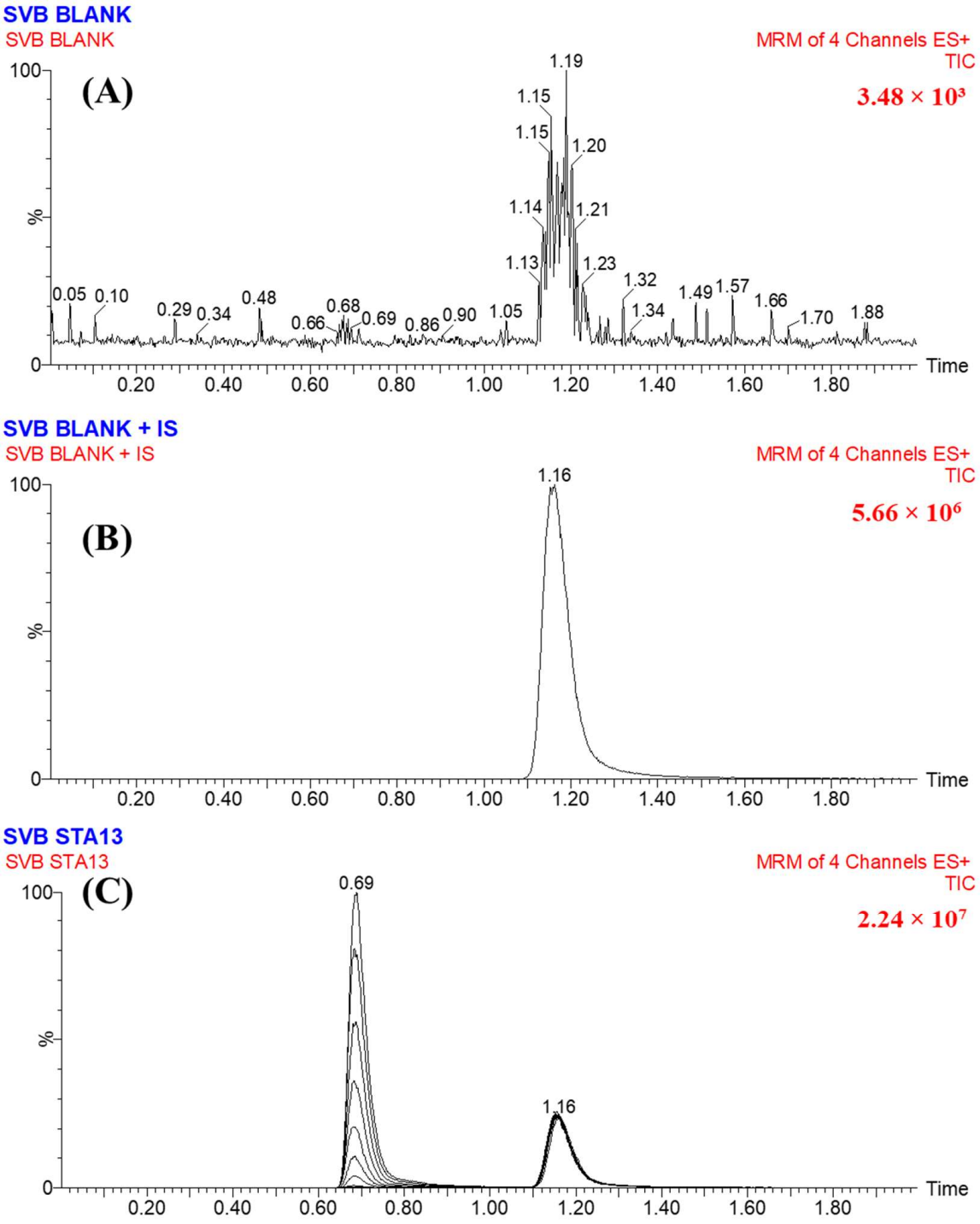

Figure 4. (**A**) Negative-control HLMs exhibiting no interfering chromatographic peaks at savolitinib (SVB) and olmutinib (OLM) elution times; (**B**) MRM mass chromatogram of Blank HLMs plus OLM (1000 ng/mL); (**C**) superimposed MRM mass chromatograms of the seven SVB CSs (1, 15, 100, 200, 500, 1500, and 3000 ng/mL) and the three QCs (3, 900, and 2400 ng/mL) (revealing the SVB peaks (0.69 min) and OLM peaks (1000 ng/mL; 1.16 min).

3.3.2. Linearity and Sensitivity

The statistical confirmation of linearity for the established LC-MS/MS analytical approach was conducted within the concentration range of 1–3000 ng/mL. This was achieved by loading SVB CSs into the HLMs matrix and subsequently back-computing the results as unknowns. The linearity was determined to be $y = 1.244x + 3.314$ with an R^2 value

of 0.998. The utilisation of a weighting factor of (1/x) was employed in the construction of the SVB calibration curve in response to the extensive range of the CSs. The RSD of the six replicates, including both CSs and QCs, was found to be less than 7.9% according to the data presented in Table 4. The LOD and LOQ were determined to be 0.29 ng/mL and 0.87 ng/mL, respectively.

Table 4. Back-calculation outcomes of six replicates (CSs) of savolitinib (SVB).

SVB (ng/mL)	Mean	SD	RSD (%)	Accuracy (%)	Recovery
1.0	0.97	0.08	7.90	−3.33	96.67
15.0	15.12	0.30	1.98	0.80	100.80
100.0	103.80	2.69	2.59	3.80	103.80
200.0	202.79	3.61	1.78	1.39	101.39
500.0	509.41	3.01	0.59	1.88	101.88
1500.0	1534.13	9.64	0.63	2.28	102.28
3000.0	2980.00	36.13	1.21	−0.67	99.33
% Recovery					100.88 ± 2.31

3.3.3. Accuracy and Precision

The precision and accuracy of the LC-MS/MS methodology were assessed by 12 sets (consisting of four QCs) in a single day, followed by six sets (also consisting of four QCs) during the subsequent three days. The results fell within the specified acceptable range, as outlined in the validation requirements set forth by the Food and Drug Administration (FDA) [32]. The inter-day and intra-day accuracy and precision of the LC-MS/MS methodology that was established exhibited a range of from −6.67 to 4.11% and −0.51% to 8.75%, respectively, as shown in Table 5.

Table 5. Precision and accuracy validation features of the current LC-MS/MS methodology.

SVB (ng/mL)	Intra-Day (Twelve Sets in One Day)				Inter-Day (Six Sets in Three Days)			
QCs	1.0	3.0	900.0	2400.0	1.0	3.0	900.0	2400.0
Mean	1.04	3.00	895.43	2389.58	0.93	3.12	887.65	2396.13
SD	0.09	0.13	7.37	20.14	0.02	0.05	6.13	18.12
Precision (%RSD)	8.75	4.36	0.82	0.84	1.64	1.44	0.69	0.76
% Accuracy	3.67	0.00	−0.51	−0.43	−6.67	4.11	−1.37	−0.16
Recovery (%)	103.67	100.00	99.49	99.57	93.33	104.11	98.63	99.84

3.3.4. HLMs Matrix Does Not Affect the Recovery and Extraction of SVB in the Current LC-MS/MS System

The efficacy of the selected LC-MS/MS analytical system for the protein precipitation extraction methodology of SVB and OLM was determined by estimating six loaded repeats (four QCs) in the HLMs matrix and then comparing the same with QCs made in the mobile phase. The data showed a high rate for SVB extraction recovery (100.88 ± 2.31% and RSD < 2.29%) and OLM (99.61 ± 2.82% and RSD: <2.17%). The analysis of two injected sample groups of HLMs matrix verified its absence of effect on the extent of ion (SVB or OLM) formation. Group set 1 was loaded with SVB (LQC, 3 ng/mL) and OLM (LQC, 1000 ng/mL), while sample group 2 was made by substituting the HLMs matrix with the mobile phase. HLMs involving SVB and OLM revealed an ME of 100.23 ± 2.67% and 98.4 ± 2.5%, respectively. The IS-normalised ME was found to be 1.02, which was within the adequate limit according to the FDA guidelines. The outcomes verified that the HLMs matrix does not affect the degree of OLM or SVB parent ionisation.

The effectiveness of the chosen extraction method (protein precipitation) for SVB and OLM in the LC-MS/MS analytical system was evaluated by conducting six replicates (including four QCs) in the HLMs matrix. These results were then compared to the QCs

prepared in the mobile phase. The data presented in the study demonstrated a notable rate of recovery for SVB extraction (100.88 ± 2.31% with a relative standard deviation (RSD) below 2.29%) and OLM (99.61 ± 2.82% with an RSD below 2.17%). The absence of any effect of the HLMs matrix on the creation of ion (specifically SVB or OLM) was confirmed through an analysis of two groups of HLMs samples. Group set 1 was prepared by incorporating SVB (LQC, 3 ng/mL) and OLM (1000 ng/mL), whereas sample group 2 was generated by replacing the HLMs matrix with the mobile phase. The HLMs matrix that incorporated SVB and OLM demonstrated a matrix effect (ME) of 100.23 ± 2.67% for SVB and 98.4 ± 2.5% for OLM, respectively. The normalised ME of the IS was determined to be 1.02, falling within the acceptable range as outlined by the rules set forth by the Food and Drug Administration (FDA). The results demonstrated that the HLMs matrix does not have a significant impact on the level of parent ionisation for both OLM and SVB.

3.3.5. SVB Was Stable in the HLMs Matrix and DMSO

The evaluation of the stability of SVB in the stock solution (DMSO) and the matrix (HLMs) demonstrated that the most favourable stability was achieved by storage in DMSO at a temperature of −80 °C for a duration of 28 days. The precision (RSD%) of all SVB samples was found to be less than 4.29% across different storage features, as indicated in Table 6. No significant decrease in SVB concentration was observed following auto-sampler storage, short-term storage, three freeze–thaw cycles, and long-term storage. The results confirmed the highest level of stability of SVB.

Table 6. SVB stability analysis.

Stability Features	3.0	2400.0	3.0	2400.0	3.0	2400.0	3.0	2400.0
	Mean		SD		RSD (%)		Accuracy (%)	
Long-Term Stability (−80 °C for 28 d)	2.98	2389.38	0.07	22.57	2.18	0.94	−0.56	−0.44
Auto-Sampler Stability (24 h at 15 °C)	2.96	2387.65	0.05	11.00	1.70	0.46	−1.44	−0.51
Freeze–Thaw Stability (three cycles at −80 °C)	2.89	2356.83	0.02	15.13	0.69	0.64	−3.67	−1.80
Short-Term Stability (4 h at room temperature)	2.97	2404.27	0.13	9.53	4.29	0.40	−0.89	0.18

3.4. Evaluation of the Greenness of the Established LC-MS/MS Methodology Using AGREE Software

The evaluation of the environmental sustainability (greenness) of the proposed LC-MS/MS approach was conducted through the utilisation of the in silico software (AGREE), which encompasses all 12 GAC elements [13]. The software assigns weights ranging from 0.0 to 1.0 to the various parameters of the GAC system, thereby establishing analytical eco-friendly scales. The findings are visually presented in the form of a circular diagram, encompassing a diverse spectrum of colours ranging from red to dark green, symbolising twelve distinct attributes. Figure 5 presents a profile of the eco-friendly scale for the LC-MS/MS technology that is currently employed. The scores for all 12 attributes were documented in Table 7. The obtained score of 0.76 was determined based on the different characteristics of the current approach, indicating the level of environmental sustainability of the LC-MS/MS methodology (where a higher value closer to 1.0 indicates a more environmentally friendly analytical process). Eco-scale values ranging from 0.75 to 1.00 indicate a high level of eco-friendliness for the current LC-MS/MS approach.

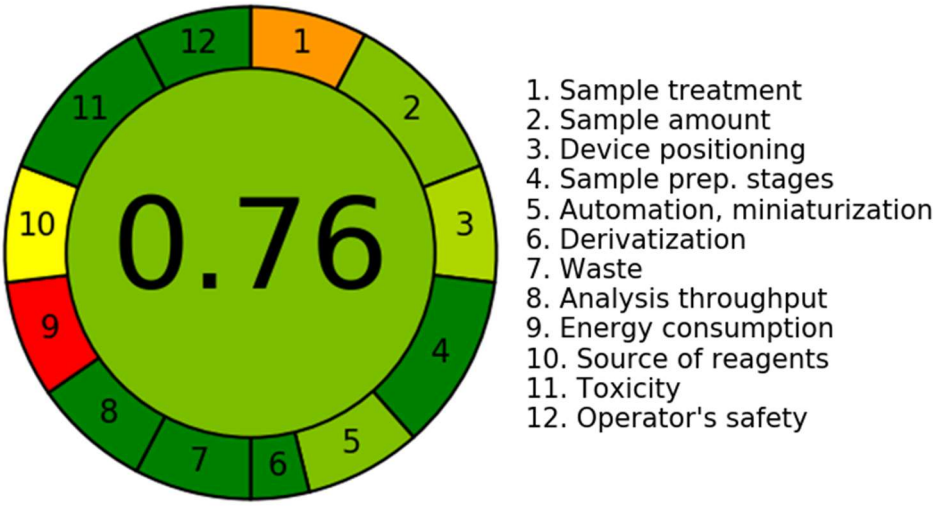

Figure 5. The eco-friendly scale profile of the established LC-MS/MS methodology was exhibited utilizing AGREE software. The results are exhibited as a circle a wide range of colors, from red (not greenness method) to dark green (the most greenness), representing 12 features as exhibited in the figure.

Table 7. The greenness report sheet of the established LC-MS/MS method involving individual score according to GAC standards. The score are presented as a circle a wide range of colors, from red (0.0: not greenness method) to dark green (1.0: the most greenness), representing 12 features that are listed in the table.

Criteria	Score	Weight
1. Direct analytical techniques should be applied to avoid sample treatment.	0.3	2
2. Minimal sample size and minimal number of samples are goals.	0.75	3
3. If possible, measurements should be performed in situ.	0.66	
4. Integration of analytical processes and operations saves energy and reduces the use of reagents.	1.0	2
5. Automated and miniaturized methods should be selected.	0.75	3
6. Derivatization should be avoided.	1.0	2
7. Generation of a large volume of analytical waste should be avoided, and proper management of analytical waste should be provided.	1.0	1
8. Multi-analyte or multi-parameter methods are preferred versus methods using one analyte at a time.	1.0	2
9. The use of energy should be minimized.	0.0	2
10. Reagents obtained from renewable sources should be preferred.	0.5	2
11. Toxic reagents should be eliminated or replaced.	1.0	3
12. Operator's safety should be increased.	1.0	3

3.5. In Vitro Metabolic Incubations of SVB with HLMs Matrix

No significant reduction in the concentration of SVB was seen in the negative-control sample. In order to evaluate the metabolic stability of SVB, a concentration of 1 μM/mL was employed in the in vitro metabolic incubation tests using HLMs. This concentration was chosen to ensure that it remained below the Michaelis–Menten constant, hence maintaining a linear relationship between the rate of SVB metabolism and the duration of the in vitro metabolic incubation. A concentration of 1 mg/mL of HLMs protein was employed to mitigate nonspecific protein-binding. The initial SVB metabolic stability curve was constructed by graphing specific time intervals for quenching (ranging from 0 to 70 min) on the x-axis, while representing the remaining ratio of SVB on the y-axis (Figure 6A). The linear segment of the preceding curve was selected within the time range of 0–40 min in order to construct a subsequent curve depicting metabolic incubation time points (0–40 min) versus the natural logarithm of the remaining ratio of SVB (Figure 6B). The study determined

that the SVB metabolic rate (slope) had a value of 0.02825, as indicated by the equation y = −0.02825x + 4.654 and a coefficient of variation (r^2) of 0.9904 (Table 8). The in vitro half-life can be calculated using the formula ln2/slope. In this case, the calculated in vitro $t_{1/2}$ was determined to be 24.54 min. The SVB Clint was measured to be 33.05 mL/min/kg. Based on the scoring system developed by McNaney et al. [27], SVB is classified as an intermediate clearance medicine that is suggested to be administered without the fear of dose accumulation within the human body. Additional physiological factors, such as the in vivo pharmacokinetics of SVB, might be suggested by the employment of various software tools, such as Cloe PK and simulation software [33].

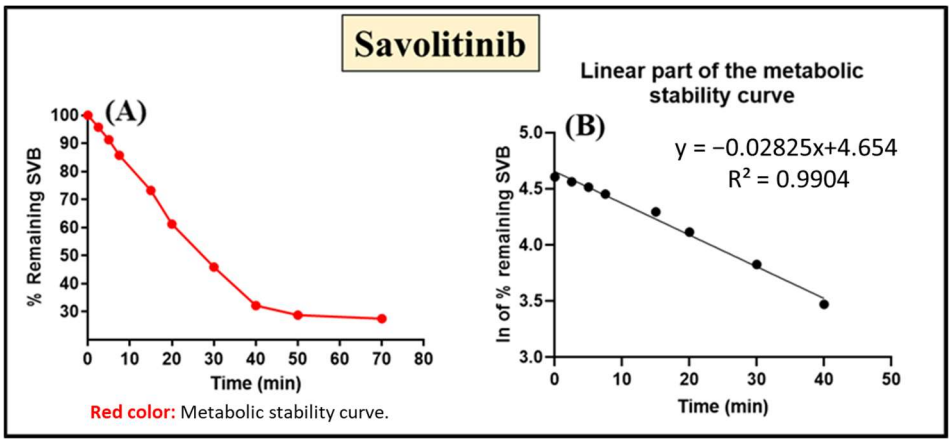

Figure 6. (**A**) SVB metabolic stability curve (red color) in the incubation matrix (HLMs); (**B**) linear segment of the logarithm (ln) calibration curve showing the linear regression equation that revealed the rate of SVB metabolism (black color).

Table 8. Metabolic stability of savolitinib (SVB).

Time Points (min.)	Mean [a] (ng/mL)	X [b]	LN X	Linearity Features
0.00	872.95	100.00	4.61	Regression line equation: y = −0.02825x + 4.654
2.50	815.77	93.45	4.54	
5.00	754.67	86.45	4.46	R^2 = 0.9904
7.50	667.63	76.48	4.34	
15.00	478.20	54.78	4.00	Slope: −0.02825
20.00	347.26	39.78	3.68	
30.00	277.42	31.78	3.46	$t_{1/2}$: 24.54 min and
40.00	243.29	27.87	3.33	Cl_{int}: 33.05 mL/min/kg
50.00	233.78	26.78	3.29	
70.00	216.32	24.78	3.21	

[a] Mean of three replicates, [b] X: Mean of the % remaining of SVB in three replicates.

4. Conclusions

The present study involved the development and evaluation of an LC-MS/MS methodology for the quantification of SVB in the metabolic incubation matrix (HLMs). Subsequently, this technique was employed to assess the metabolic stability of SVB. The LC-MS/MS approach demonstrated optimal sensitivity, environmental friendliness, selectivity, and the excellent recovery of SVB and OLM from the HLMs matrix by utilising protein precipitation as the extraction process. The utilisation of a reduced flow rate (0.4 mL/min), a diminished quantity of organic solvent (ACN; 55%), and a small duration of analysis (2 min) contributed to the eco-friendly nature of the LC-MS/MS approach that was devised. Based on an evaluation of environmental sustainability using the AGREE software, it can be concluded that the current LC-MS/MS technology is environmentally friendly and may be better-suited to the regular analysis of SVB without adverse effects on the surrounding ecosystem. The results obtained from the in silico metabolic P450 analysis using StarDrop's

software were validated using in vitro metabolic incubation tests conducted with HLMs. The observed results pertaining to metabolic stability, specifically a half-life ($t_{1/2}$) of 24.54 min and a moderate clearance (Cl_{int}) of 33.05 mL/min/kg, indicate that the medication SVB exhibits characteristics of a moderate extraction ratio drug. Therefore, it is hypothesised that SVB administration may be feasible for patients without the risk of dose buildup within the human body. Potential future research could involve the utilisation of in silico software tools and in vitro metabolic incubations, which are essential for the development of new medications with enhanced metabolic stability. The comparison of outcomes between in vitro incubation assays and in silico software analysis of SVB demonstrates the efficacy of in silico software in conserving both effort and resources.

Author Contributions: M.W.A., A.S.A. and A.A.K. establish the experimental steps. M.W.A. and H.A. made the practical experiments and wrote the first version of the manuscript. A.S.A. and A.A.K. participated in software applications and designing the current LC-MS/MS system. All data and outcomes were performed in-house and no paper mill was utilized in the current research. All authors have read and agreed to the published version of the manuscript.

Funding: The authors extend their appreciation to the Deputyship for Research & Innovation, Ministry of Education in Saudi Arabia for funding this research work through the project no. (IFKSUOR3-252-2).

Institutional Review Board Statement: The utilization of HLMs that were purchased from Sigma company excepts it from the requirement of ethical approval.

Data Availability Statement: All data are available within the manuscript.

Acknowledgments: The authors extend their appreciation to the Deputyship for Research and Innovation, "Ministry of Education" in Saudi Arabia for funding this research (IFKSUOR3-252-2).

Conflicts of Interest: The authors declare no conflict of interest.

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