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Development of Salt-Assisted Liquid-Liquid Extraction for Simultaneous Quantification of Andrographolide and 14-Deoxy-11,12-Didehydroandrographolide in Plasma Using HPLC-DAD: Method Validation and Pharmacokinetic Assessment Application

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Development of Salt-Assisted Liquid-Liquid Extraction for Simultaneous Quantification of Andrographolide and 14-Deoxy-11,12-Didehydroandrographolide in Plasma Using HPLC-DAD: Method Validation and Pharmacokinetic Assessment Application

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Abstract

A high-performance liquid chromatography method coupled with diode array detection (HPLC-DAD) was developed for the simultaneous quantification of andrographolide (AG) and 14-deoxy-11,12-didehydroandrographolide (DDAG) in rat plasma. A salt-assisted liquid-liquid extraction (SALLE) procedure was optimized, with $MgSO_4$ yielding the highest extraction efficiency (>90% for both AG and DDAG), outperforming conventional solvent extraction, and being comparable to solid-phase extraction. The method exhibited acceptable linearity (125–2000 ng/mL, $r^2 > 0.99$), with low limits of detection and quantification of 60 and 70 ng/mL for AG and 201 and 234 ng/mL for DDAG, respectively, while adhering to the ICH M10 criteria for accuracy, precision, and stability under various storage conditions. Stability testing of the prepared samples demonstrated that > 99% of AG and 95% of DDAG were retained when stored at low temperatures, specifically, below 4 °C. The developed method was successfully applied in a pharmacokinetic study following oral administration of *Andrographis paniculata* extract (containing AG 7.5 mg/kg) to healthy Wistar rats. The SALLE-HPLC-DAD method developed herein enables selective AG quantification without significant matrix interference. In conclusion, this study introduces an alternate sample preparation and analytical method that is fast, cost-effective, and reliable, making it suitable for pharmacokinetic studies of the principal biomarker of *Andrographis paniculata*.

Keywords: *Andrographis paniculata*; Andrographolide; 14-deoxy-11,12-didehydroandrographolide; Diterpene lactones; Method development and validation; Pharmacokinetic study

1. Introduction

Andrographis paniculata (Burm.F.) Nees (AP), commonly known as the king of bitters of the Fah Talai Jone in Thailand, is a medicinal herb native to South and Southeast Asia. It has been employed for centuries in traditional Ayurvedic and Siddha systems, particularly in India and Sri Lanka, primarily to treat fever, respiratory infections, jaundice, and digestive disorders [1]. In Thailand, AP

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is officially listed in the National List of Essential Medicines under the herbal medicine category and is used in formulations such as capsules, tablets, and pills to relieve respiratory symptoms and non-infectious diarrhea [2]. In addition to its long-standing use, numerous pharmacological studies have demonstrated that AP exhibits broad biological activities. The plant extract, particularly its major active constituent "Andrographolide (AG)," has been reported to possess anti-inflammatory [3], antioxidant [4], analgesic [5], antipyretic[6], antimicrobial [7,8], antimalarial [9,10], hepatoprotective [11], immunomodulatory [12,13], and antiviral properties, as well as activity against SARS-CoV-2 [14–17].

Phytochemical investigations of AP have revealed a rich diversity of secondary metabolites. More than 20 diterpenoids and over 10 flavonoid compounds have been identified in various plant parts [10,18,19]. The therapeutic and pharmacological activities of AP have been primarily attributed to its major active compound, andrographolide (AG), followed by 14-deoxy-11,12-didehydroandrographolide (DDAG), which has also been reported to exhibit significant bioactivity [20–24]. Both diterpene lactones have been used as marker compounds in many studies and are quantitatively determined as reference standards in several pharmacopoeias, including the Thai Herbal Pharmacopoeia, United States Pharmacopeia, and British Pharmacopoeia [25–27]. Owing to the extensive pharmacological studies reported, numerous investigations have focused on the pharmacokinetics and development of analytical methods for the determination of these two diterpene lactones. Various analytical techniques have been used to quantify the biological samples. For example, Suo et al. analyzed whole blood samples prepared using liquid-liquid extraction (LLE) and reported a recovery of 66–82%, with quantification performed by HPLC-UV [28]. Songvut et al. investigated plasma and urine samples prepared via protein precipitation (PPT), yielding a recovery range of 78–90 %, and analyzed the samples using LC-MS/MS [29]. In another study, Kovacic et al. examined plasma samples processed by solid-phase extraction and reported a high recovery of 92–99 % with quantification conducted using LC-MS [30]. To date, there are no reports of sample preparation procedures for analyzing AG and DDAG in biological samples that are both economical and straightforward while providing high extraction efficiencies.

Biological samples contain complex mixtures of proteins, lipids, and other endogenous substrates that can interfere with the detection of target analytes. Sample preparation is a critical step in bioanalysis for removing matrix interferences, concentrating analytes, and improving the performance of analytical systems [31]. The procedure used for sample preparation can affect the assay sensitivity. Low recovery limits the ability to achieve the desired limits of detection and quantitation. The effective cleanup and extraction of analytes are crucial for sensitive and selective HPLC assays, particularly as analytes frequently exist at trace levels, and co-components may lead to signal suppression or enhancement if not properly removed. The common sample preparation techniques in LC-based bioanalysis include simple methods, namely PPT and LLE. PPT is widely used because of its simplicity, low cost, and high-throughput compatibility. An organic solvent (such as acetonitrile or methanol) is added to crash out proteins, after which the supernatant can be analyzed. The advantages of PPT include its speed, minimal sample handling, and broad applicability to many analytes [32]. However, PPT only provides crude cleanup, and many residual matrix components remain, leading to significant ion suppression or background interference in HPLC analysis. Thus, while PPT is convenient, it often yields the least clean extract and can be affected by matrix effects [33]. LLE uses an immiscible organic solvent to partition analytes from an aqueous matrix. This method can achieve good cleanup and enrich the analyte in the organic phase [34]. After phase separation, the organic layer (containing the analyte) is collected, evaporated, and reconstituted for HPLC analysis. The advantages of LLE include better removal of proteins and phospholipids than PPT, often resulting in cleaner extracts and reduced matrix interference [33]. LLE is also relatively simple and can be scaled or automated (e.g., in a 96-well format). However, LLE can sometimes form emulsions that complicate phase separation [35]. Moreover, LLE may not efficiently extract highly polar or semipolar analytes, and although LLE yields a clean extract, the recoveries of

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these analytes can be low [33]. Solvent use is also higher, and multiple extractions or solvent optimization may be required to achieve high recovery.

Currently, various advanced sample preparation techniques have been developed to improve extraction efficiency beyond simple methods. These include solid-phase extraction (SPE) [36], supported liquid extraction [37], magnetic separation [38], monolithic spin column extraction [34], and microextraction using a packed sorbent [40]. SPE employs a solid sorbent (packed in cartridges or 96-well plates) to retain analytes, while interfering components are washed away. Compared to PPT and LLE, SPE provides the cleanest extracts and greater selectivity by choosing appropriate sorbent chemistries [33]. For example, using mixed-mode sorbents (combining reverse-phase with ion-exchange) can dramatically reduce residual phospholipids and other matrix elements, thereby minimizing matrix effects [33,37]. The advantages of SPE include excellent cleanup (leading to lower ion suppression or background interference), the ability to concentrate analytes, and flexibility in method design (through different sorbent materials and protocols). SPE is amenable to automation and can improve the robustness of assays. However, the disadvantages of this method include time and cost. SPE involves multiple steps (conditioning, loading, washing, and elution) and often requires method development to optimize recovery and cleanliness [41]. SPE consumables (cartridges or plates) add cost, and evaporation/reconstitution steps are usually needed, which can risk sample loss and increase time [42]. Despite these drawbacks, SPE is often favored when maximum sensitivity and cleanliness are required.

The advanced sample preparation techniques often involve complex procedures and require costly specialized equipment. To overcome these limitations, a method known as salt-assisted liquid-liquid extraction (SALLE) has been developed for the extraction of drugs from biological matrices, such as blood, plasma, and serum [43]. SALLE is a modified LLE technique that leverages the salting-out effect to induce phase separation of a water-miscible organic solvent (such as acetonitrile) from an aqueous sample and enhance extraction efficiency. In a typical SALLE, a high-concentration salt solution is added along with acetonitrile to a plasma/serum sample. The salt causes acetonitrile to separate into a discrete organic layer containing the analytes, effectively combining protein precipitation and extraction in one step [43,44]. This approach offers several advantages: it enables the simultaneous removal of most proteins and endogenous salts while facilitating the efficient transfer of the target analyte from the biological matrix to the organic phase in a single step [44]. SALLE provides a simple protocol, similar to PPT (few steps, easy to automate) [43,44]. SALLE can extract a wide range of compounds, including polar analytes that traditional LLE or SPE might not recover well, because the organic solvent initially mixes with the aqueous sample and then "salts out" the analytes into a separate phase [44,45]. It avoids vigorous shaking and extensive solvent use: no intensive mixing is required, and often the organic layer can be injected directly (or with minimal dilution) into HPLC, eliminating evaporation and reconstitution steps [43,46]. Compared to conventional LLE, SALLE is faster and uses less solvent, and compared to SPE, it is much simpler and more cost-effective (no cartridges or multi-step protocols) [42,47]. Recent reviews have highlighted SALLE's environmental friendliness, high extraction efficiency, and ease of automation relative to PPT and SPE [43,44]. Although the disadvantages of SALLE are few, it requires careful optimization of the salt type/amount and solvent ratio to ensure effective phase separation and recovery. Additionally, SALLE, like PPT, may not remove all very small polar interferents (unless coupled with an additional cleanup) and is not yet as universally adopted as more established methods.

Currently, limited data are available on the SALLE methodology for the determination of diterpene lactones in biological fluids. Therefore, this study aimed to develop a biological sample preparation method for the analysis of AG and DDAG using HPLC-DAD. Chromatographic conditions were developed for the determination of AG and DDAG content. Biological sample preparations using PPT, LLE, and SALLE were compared. Sodium chloride (NaCl), sodium sulfate (Na_2SO_4), and magnesium sulfate ($MgSO_4$) were the salts investigated in the SALLE. Additionally, SALLE in conjunction with HPLC-DAD was used to examine the pharmacokinetics of AG and DDAG

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in AP extract (APE) in Male Wistar rats. SALLE is proposed as a simple, cost-effective, and efficient technique that is compatible with analytical equipment that does not require complicated technology, such as HPLC-DAD, to determine diterpene lactones in biological fluids, such as plasma.

2. Materials and Methods

2.1. Chemicals and Reagents

Andrographolide (AG) reference standard was obtained from the Bureau of Drug and Narcotic Department of Medical Sciences, Ministry of Public Health (Nonthaburi, Thailand). 14-Deoxy-11,12-didehydroandrographolide (DDAG) was purchased from Weifang Wehibest Supply Chain Co., Ltd. (Shandong, China). All HPLC-grade solvents, including acetonitrile (ACN), methanol (MeOH), ethyl acetate (EtOAc), and dichloromethane (DCM), were purchased from Fisher Scientific Korea Ltd. (Seoul, South Korea). Analytical-grade sodium chloride (NaCl, ≥ 99.0%) and anhydrous sodium sulfate (Na_2SO_4, ≥99.0%) were purchased from KemAus (Cherrybrook, Australia). Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$, ≥ 99.5%) of analytical grade was obtained from Loba Chemie PVT. LTD (Mumbai, India). Pharmaceutical-grade absolute ethanol was obtained from the Liquor Distillery Organization (Chachoengsao, Thailand). Blank rat plasma was purchased from the National Laboratory Animal Center, Mahidol University (Nakhon Pathom, Thailand). Thiopental sodium and heparin were obtained from the Faculty of Veterinary Medicine, Khon Kaen University (Khon Kaen, Thailand).

2.2. Plant Materials and AP Extract (APE)

The leaves of *Andrographis paniculata* (Burm.F.) Nees (AP) were harvested from a farm in the Sam Chai District, Kalasin Province, Thailand, in June 2023. The specimens were certified by a botanist, and a herbarium voucher specimen (HB-202-66) was maintained at the Center for Research and Development of Herbal Health Products, Khon Kaen University, Thailand. The APE was processed using a Soxhlet extractor with absolute ethanol as the extraction solvent. The resulting APE contained 24.13 ± 1.11% AG and 4.07 ± 0.90% DDAG, respectively.

2.3. Chromatographic Condition

AG and DDAG were quantified by reverse-phase HPLC using a Shimadzu LC-2050 system (i-Series, Shimadzu, Kyoto, Japan) equipped with an autosampler, column oven, and diode array detector set at 254 nm. Separation was achieved on a ZORBAX Eclipse Plus C18 column (4.6 × 150 mm, 3.5 µm; Agilent Technologies, USA) maintained at 20°C. Gradient elution was performed using mobile phase A, which consisted of 0.1% formic acid in acetonitrile, and mobile phase B, which consisted of 0.1% formic acid in water. The gradient program was as follows: 0–3 min, 30% A (isocratic); 3–6 min, linear increase to 40% A; 6–9 min, 40% A (isocratic); 9–10 min, increase to 80% A; 10–12 min, 80% A (isocratic); 12–13 min, return to 30% A; 13–15 min, 30% A (isocratic). The flow rate was 1.4 mL/min, and the injection volume was 40 µL for each sample. Data acquisition and processing were performed using Shimadzu Class VP software.

2.4. Biological Sample Extraction Procedures

To evaluate the effect of the extraction methods on the chromatographic background signals from the plasma matrices, two sample preparation techniques, PPT and LLE, were compared. MeOH and ACN were the investigated solvents utilized in the PPT technique, whereas EtOAc and DCM were applied for LLE. Briefly, 100 µL of plasma was added with 800 µL of either MeOH or ACN for PPT, or EtOAc or DCM for LLE processing. The mixture was vortexed (VM-500Pro, JOANLAB, Zhejiang, China) for 5 min and then centrifuged at 5000 rpm for 5 min (MC-15Pro, JOANLAB, Zhejiang, China). The resulting supernatant was transferred into a 2 mL centrifuge tube and evaporated in a dry bath at 45°C (DB, JOANLAB, Zhejiang, China). The extraction was repeated once

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under identical conditions, and the supernatants were combined. After complete drying, the residue was reconstituted in 100 µL MeOH and mixed thoroughly. The final solution was filtered through a 0.22 µm syringe filter (Agilent, Santa Clara, USA) before HPLC analysis.

2.5. SALLE

Three distinct salt types, NaCl, Na_2SO_4, and $MgSO_4$, at two concentrations (1 and 2 M), were examined to assess their impact on the extraction efficiency of the SALLE process. Briefly, 5 µL of a standard mixture of AG and DDAG was added to 100 µL of plasma and mixed for 30 s. An equal volume of 1–2 M NaCl, Na_2SO_4, or $MgSO_4$ solution was added to the mixture and mixed for 15 s. The mixture was subsequently combined with 800 µL DCM and mixed for 5 min using a vortex mixer (VM-500Pro, JOANLAB, Zhejiang, China). The mixture was then centrifuged at 5000 rpm for 5 min (MC-15Pro, JOANLAB, Zhejiang, China). The resulting supernatant was transferred into a 2 mL centrifuge tube and evaporated using a dry bath at 45°C (DB, JOANLAB, Zhejiang, China). The extraction was repeated once under identical conditions, and the supernatants were combined. After complete drying, the residue was reconstituted in 100 µL MeOH and mixed thoroughly. The final solution was filtered through a 0.22 µm syringe filter (Agilent, Santa Clara, USA) before HPLC analysis.

2.6. SALLE-HPLC-DAD Method Validation

The developed bioanalytical method was validated in compliance with the International Council for Harmonization (ICH) M10 and Q2(R2) [48,49], addressing parameters including selectivity for both AG and DDAG. Validation was carried out across three independent precision–accuracy runs performed on separate days. Each run included duplicate calibration curves and six replicates of quality control (QC) samples at the lower limit of quantification (LLOQ), medium QC (MQC), and upper limit of quantification (ULOQ) to comprehensively assess the intra- and inter-day accuracy and precision. In addition, system suitability was established in accordance with the USP general chapter <chromatography> [50]. Six replicate injections of the LLOQ standard solution were analyzed to determine the capacity factor, resolution between AG and DDAG, tailing factor and column efficiency. The acceptance criteria were as follows: resolution ≥ 2.0; 0.8 ≤ tailing factor ≤ 1.5 for both analytes; N ≥ 2000 for AG and DDAG.

2.6.1. Selectivity and Specificity

To confirm the absence of carry-over or endogenous interference at the retention times of AG and DDAG, six individual blank rat plasma samples were analyzed to evaluate the selectivity and specificity.

2.6.2. Linearity and Range

Primary stock solutions of AG and DDAG were individually prepared by accurately weighing each compound and dissolving it in methanol to a final concentration of 1.0 mg/mL. These stock solutions were serially diluted with methanol to obtain working standard solutions at concentrations of 1.25, 2.5, 5, 10, and 20 µg/mL. Plasma samples were spiked with working standard solutions of AG and DDAG to establish a calibration curve. The final calibration concentrations of AG and DDAG in plasma were 125, 250, 500, 1000, and 2000 ng/mL. This range was selected to provide adequate coverage for quantifying both analytes in pharmacokinetic experiments.

All plasma calibration samples were analyzed under optimized HPLC conditions, with each concentration level tested in six replicates (n = 6). Linearity was evaluated using least-squares regression analysis. The slope (a), y-intercept (b), coefficient of determination (R^2), and relative standard deviation (RSD) were calculated to assess the consistency and precision of calibration curves. The method demonstrated excellent linearity across the selected range, supporting its suitability for the quantitative analysis of AG and DDAG in biological matrices.

2.6.3. Sensitivity

The LLOQ was established according to the ICH M10 guidelines as the lowest concentration that could be quantified with acceptable accuracy (± 20%) and precision (RSD ≤ 20%). The Limit of detection (LOD) and limit of quantification (LOQ) values were calculated using the standard

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deviation of the response (σ) and the slope of the calibration curve (S) using equations (1) and (2) recommended in ICH Q2(R2):

$$LOD = 3.3\ \sigma/S \quad (1)$$

$$LOQ = 10\ \sigma/S. \quad (2)$$

2.6.4. Matrix Effect

To evaluate the matrix effect, the peak area of the analyte in blank plasma after sample preparation was compared with that of a standard solution of equivalent concentration prepared in the solvent. The test was conducted at the LLOQ and ULOQ levels in triplicates for each concentration level. Following ICH M10, the assessment data were accepted as accuracy was within ± 15% and precision (CV%) was ≤ 15%.

2.6.5. Precision and Accuracy

The method precision was evaluated under two conditions: repeatability (intra-day) and intermediate precision (inter-day). Precision was expressed as %RSD. Both intra- and inter-batch precision were assessed by analyzing six replicates of QC samples at three concentrations: 125 ng/mL (LLOQ), 1000 ng/mL (MQC), and 2000 ng/mL (ULOQ) for AG and DDAG. Accuracy was determined by calculating the percentage recovery of each analyte at the LLOQ, MQC, and ULOQ.

2.6.6. Stability

Stability assessments were conducted as part of the method validation process to ensure the reliability of analyte quantification under various storage and handling conditions. The stability of the processed samples was determined by comparing freshly prepared samples (0 h, control) with those re-injected after storage in the autosampler at 4 °C for 24 h. Short-term (benchtop) stability was assessed by leaving six replicates of spiked plasma at room temperature for 4 h prior to analysis. Long-term stability was evaluated by storing spiked rat plasma samples at − 20 °C and analyzing them after 14 days. Additionally, freeze–thaw stability was examined by subjecting the samples to three complete freeze–thaw cycles and comparing their responses to those of freshly prepared control samples.

2.7. Method Application to a Pharmacokinetic Study

2.7.1. Animals

Male Wistar rats weighing 170–200 g were obtained from Nomura Siam International Co., Ltd. (Thailand). Before the pharmacokinetic study, all animals were acclimatized to standardized laboratory conditions for one week. The animal facility was maintained at a controlled temperature of 21–25 °C with relative humidity ranging from 30% to 60%, and a 12-h light/dark cycle was consistently applied throughout the acclimatization and experimental periods. All experimental procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of Khon Kaen University (IACUC-KKU), Thailand. The approved protocol was registered under the certificate number IACUC-KKU-80/67.

2.7.2. Pharmacokinetic Study

Prior to the experiment, all animals were fasted for 12 h with free access to water. A suspension of APE was prepared using 0.3% w/v sodium carboxymethyl cellulose as the suspending agent for the APE. During the study, the test formulation, equivalent to an AG dose of 7.5 mg/kg body weight (BW), was administered orally via a stainless-steel gastric gavage needle. Blood samples (300 µL) were collected from the tail vein at predetermined time points: 0.5, 1, 1.5, 2, 3, 4, 6, and 8 h post-administration. The animals were gently restrained on a customized platform during the blood withdrawal. Each sample was collected in 1.5 mL microcentrifuge tubes preloaded with 30 units of heparin to prevent coagulation.

Immediately after collection, the blood was gently mixed using a vortex mixer and centrifuged at 5,000 rpm to separate the plasma. The obtained plasma was promptly transferred to clean microtubes and stored at –80 °C in a dry ice container until further analysis. At the end of the

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experimental period, all animals were humanely euthanized via intraperitoneal injection of thiopental sodium at a dose of 120 mg/kg BW.

2.8. Data Analysis

The estimated pharmacokinetic parameters were examined using noncompartmental analysis with PKSolver 2.0 USA software. One-way ANOVA was conducted using SPSS software for MS Windows, version 19 (SPSS Co. Ltd., Bangkok, Thailand). Tukey's test was applied to assess the statistical significance of the differences between the means. A 95% confidence level was set for statistical significance, with a *p*-value of less than 0.05.

3. Results and Discussion

3.1. Chromatographic Conditions

The HPLC analytical method developed in this study featured a total run time of 16 min. The retention times of AG and DDAG were observed at 4.2 and 9.7 min, respectively, as shown in Figure 1b. This analytical method was applied to investigate the effects of various factors in the sample preparation process for biological matrices and was also utilized for method validation. Furthermore, it was employed in the pharmacokinetic study of APE in Wistar rats as part of the present research.

3.2. Biological Sample Preparations: Influences of Extraction Methods

3.2.1. PPT and LLE

The most straightforward approach to preparing biological fluids for quantitative analysis involves the elimination of proteins. Proteins are generally removed by acidification, thermal treatment, or the use of ultrafiltration membranes with specified molecular weight thresholds. PPT with organic solvents preserves the stability of the analyte [51,52]. In this study, two different organic solvents, MeOH and ACN, were used in the PPT process. Their effects on the plasma chromatographic background were investigated, and the results are shown in Figure 1. The HPLC chromatographic background signals of blank plasma prepared via the PPT method with both solvents displayed interference peaks with the MeOH preparation (Figure 1c), showing more pronounced interference peaks than the ACN preparation (Figure 1d). This finding aligns with the prior research conducted by Chamber et al. [33], which indicated that residual phospholipids, specifically phosphatidylcholines, were found at a concentration 40% higher in samples treated with MeOH than in those treated with ACN. This effect primarily arises from the fact that MeOH is a protic solvent capable of donating hydrogen ions. As a result, MeOH tends to preferentially extract more phospholipids into the solvent phase because of hydrogen bonding with the phospholipid head groups [53,54]. Although MeOH is a widely used and effective solvent for PPT in many studies, its use requires caution. This is because residual phospholipids may interfere with the detection process, leading to inaccurate quantification of analytes [55]. In agreement with our study, the interference peaks coincided with the retention times of the target analytes, AG and DDAG, as indicated by the red peaks in Figures 1b-d. Considering the impact of matrix residue extraction, the PPT method is unsuitable for the chromatographic conditions employed in this study.

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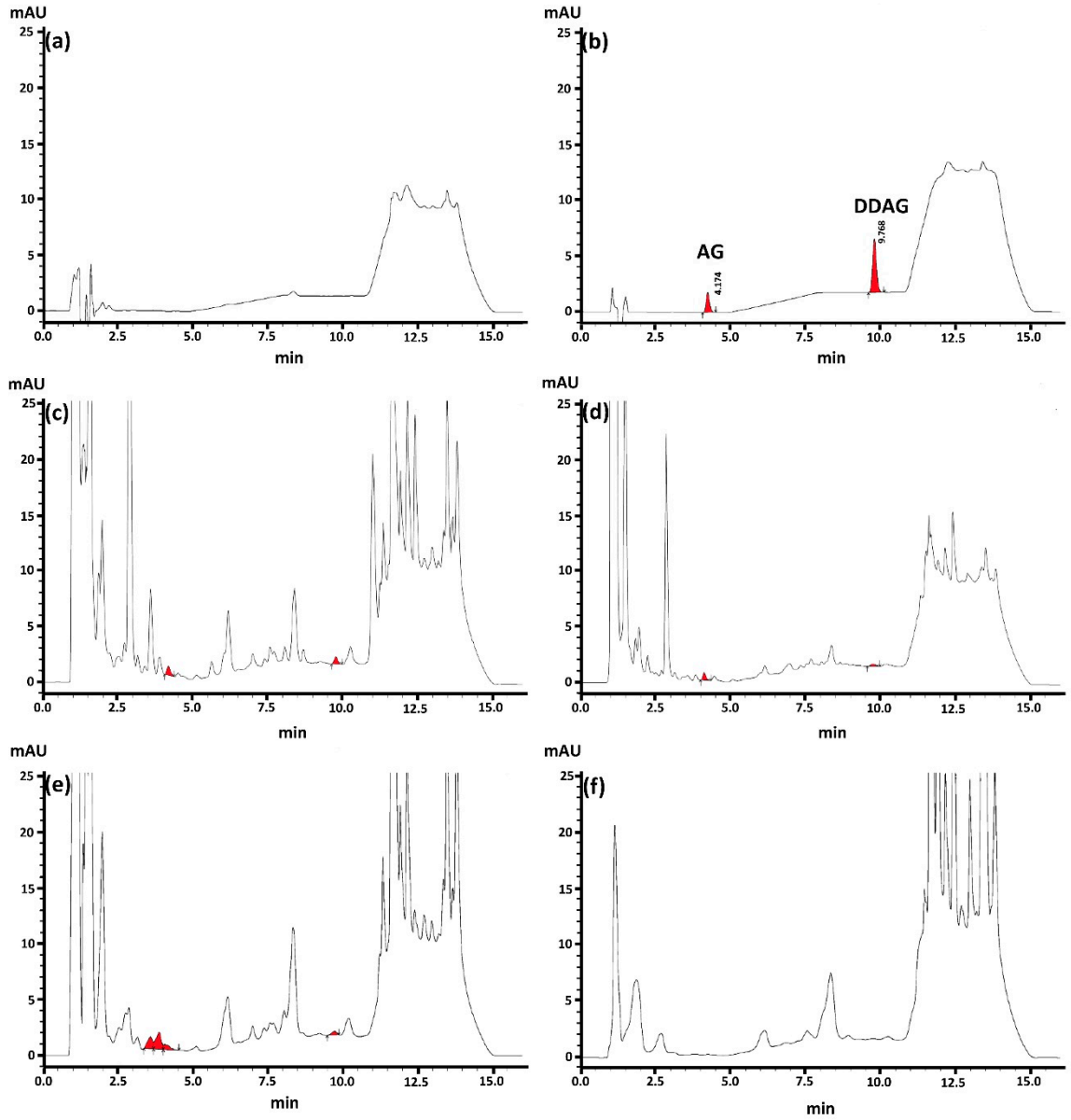

Figure 1. Chromatograms of MeOH (a), standard solution at the MQC level in MeOH (b), PPT-MeOH-blank plasma (c), PPT-ACN-blank plasma (d), LLE-EtOAc-blank plasma (e), and LLE-DCM-blank plasma (f).

The LLE approach utilizing EtOAc and DCM was also examined in this study. EtOAc and DCM are frequently employed solvents for liquid-liquid extraction. LLE is a commonly employed technique for the extraction and preparation of biological products for the analysis of pharmaceutical actives and their metabolites [56,57]. Reports have indicated that the concentration of residual phospholipids in LLE is generally lower than that in PPT. Nevertheless, the choice of extraction solvent is essential for reducing the nonspecific extraction of matrix components. As illustrated in Figures 1e and 1f, the blank plasma extracted using LLE with DCM displayed a reduced number of interference peaks in its chromatographic profile. This was particularly apparent at the retention times associated with the analysis of AG and DDAG, where no interfering peaks were observed for either of the lactone peaks. The results of this study align with those of a prior study by Jiang et al., who assessed the extraction efficacy of supported LLE using different organic solvents. Their findings indicated that DCM and EtOAc resulted in a significant reduction in the phospholipid content in the analytical samples. LLE utilizing DCM can achieve phospholipid removal efficiencies of up to 99.5%, whereas EtOAc exhibits a removal efficiency of approximately 85%. This may be explained by the differences in the solvent polarity and solvation properties. EtOAc, a polar aprotic solvent, can interact with polar lipids, such as phospholipids, resulting in increased phospholipid extraction, which is also a higher risk of interference in chromatographic analysis [58].

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Although LLE demonstrated no interfering peaks at the retention times of AG and DDAG in the LLE-DCM extraction samples, the extraction efficiencies for both compounds were notably low, recorded at 50.66 ± 4.44 % for AG and 56.03 ± 4.32 % for DDAG (Table 1, conventional LLE). The extraction efficiency observed in this study aligns closely with the findings of a previous report that utilized LLE with chloroform as the solvent, achieving an extraction efficiency of the AG compound between 65.7% and 72.6% [28].

Table 1. Extraction efficiency percentage of AG and DDAG via the SALLE procedure as a function of salt type and concentration.

Salt types and concentrations	Extraction Efficiency (%)	
	AG	DDAG
Control (no salt addition)	50.66±4.44	56.03±4.32
NaCl, 1M	81.75±4.81*	69.31±1.64*
NaCl, 2M	86.52±5.59*	93.41±6.33*
Na_2SO_4,1M	0.00±0.00*	7.24±2.41*
Na_2SO_4, 2M	N/A	N/A
$MgsO_4$, 1M	82.64±2.68*	81.70±1.28*
$MgsO_4$, 2M	93.75±0.56*	95.39±2.95*

Data are presented as mean ± SD, n = 3 in all cases. N/A=not applicable. * Significantly different from the control group ($p < 0.05$).

3.2.2. SALLE: Salt Effect

To facilitate sample analysis using the chromatographic conditions developed in this study, SALLE, a modified LLE technique, was utilized. The effects of different salt types (NaCl, Na_2SO_4, and $MgSO_4$) and concentrations (1–2 M) on the extraction efficiencies of AG and DDAG were investigated. During the optimization process, deionized water with a resistivity of 18 MΩ·cm was used to prepare the salt solutions to ensure that the ionic strength originated solely from the salts used in this study. In addition, the extent of protein binding was controlled by defining a fixed incubation period for the mixing of AG and DDAG with plasma. As shown in Table 1, conventional LLE (control, no salt addition) exhibited extraction efficiencies of 50.66% for AG and 56.03% for DDAG, respectively. SALLE with 1 M NaCl significantly improved the extraction efficiency to 81.75% and 69.31% for AG and DDAG, respectively ($p < 0.05$). Notably, SALLE with 1 M $MgSO_4$ yielded the highest efficiencies of 82.64% and 81.70% for AG and DDAG, respectively. Increasing the concentration of either NaCl or $MgSO_4$ to 2 M further improved the extraction efficiencies of both AG and DDAG. Specifically, increasing the NaCl concentration to 2 M resulted in extraction efficiencies of 86.52% and 93.41% for AG and DDAG, respectively. Similarly, a sample prepared with 2 M $MgSO_4$ exhibited extraction efficiencies of 93.75% and 95.39% for AG and DDAG, respectively. In the case of SALLE with Na_2SO_4, the combination with 1 M salt solution resulted in the formation of an emulsion. This limited the complete recovery of the DCM solvent, resulting in markedly reduced extraction efficiencies of AG and DDAG (0.00% and 7.24%, respectively). When the Na_2SO_4 concentration reached 2 M, the resulting emulsion demonstrated stability such that phase separation was unachievable, even after centrifugation, preventing the collection of the organic solvent.

The findings of this study indicate that implementing SALLE in conjunction with an appropriate salt markedly enhances the extraction efficiencies of AG and DDAG. SALLE represents a sample preparation method in which the incorporation of an inorganic salt into the aqueous phase or a water-miscible organic solvent induces phase separation or the establishment of a biphasic system. The salting-out technique serves the dual purpose of facilitating the separation of water-miscible organic solvents while simultaneously improving the extraction efficiency of nonpolar and immiscible organic solvents. In this study, an increase in ionic strength or solvent polarity was found to enhance the transfer of AG into the organic phase, as reflected by the higher extraction efficiency observed with increasing salt concentration. This trend was more pronounced when $MgSO_4$ was used compared to NaCl, which is consistent with its higher ionic strength and stronger salting-out effect in the aqueous phase. These results align with those of a previous study, which suggested that increasing the ionic strength promotes the formation of ion-rich nanodomains and induces stronger solvent-phase structuring, thereby facilitating liquid-liquid phase separation [59]. In addition, the

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introduction of a high concentration of ions binds water molecules into hydration shells, increasing the polarity and surface tension of the aqueous phase. This enhances hydrophobic exclusion, pushing nonpolar organic compounds out of the aqueous phase. Additionally, high-charge-density ions create electrostatic repulsion, which further drives neutral organic molecules into the organic phase [45]. This method has been shown to effectively enhance the extraction efficiency of catechol and hydroquinone from urine samples [60] and improve the sample preparation efficiency for analyzing pyrrolizidine alkaloids [61].

Different salt types influence the performance of SALLE differently. Research indicates that the efficacy of phase separation and precipitation of non-polar compounds aligns with the lyotropic series of cations ($Mg^{2+} > NH_4^+ > Na^+ > K^+$) and anions ($SO_4^{2-} > Cl^-$) [60]. The enhanced separation or precipitation of proteins or non-polar substances, depending on the type of salt employed, is ascribed to the substantial impact of salt solutions on the structure and functional characteristics of hydrophilic colloids or solutions [62]. These effects depend on whether the salt behaves as a chaotrope, which disrupts the structure of water, or as a kosmotrope, which stabilizes the structure of water. Chaotropic ions interfere with the hydrogen bonding network of water [63]. The superior extraction efficiency of Mg_2SO_4 compared to NaCl may be attributed to the pronounced kosmotropic properties of Mg^{2+}, resulting from its high charge density, which effectively organizes water molecules. Despite its small size, Na^+ exhibits weak hydration and is positioned near the medium kosmotropic region within the ion series, exhibiting low salting-out efficacy [52,53]. The presence of salts in solution plays a significant role in surface tension, thereby affecting the energy required to form cavities surrounding nonpolar compounds. Moreover, preferential hydration occurs when ions exhibit stronger interactions with water than with the surfaces of nonpolar compounds. This results in an increase in the surface tension, which can be measured using a parameter referred to as the surface-tension increment. This value correlates with the ability of salts to induce the separation of nonpolar compounds into the organic solvent phase [64]. In the case of Na_2SO_4, emulsion formation and inability to be fully separated may be related to the surface tension modification by Na_2SO_4. Prior research suggests that the presence of Na_2SO_4 in the aqueous phase significantly decreases the interfacial tension between oil and water, resulting in reduced droplet size and enhanced emulsion stability [65].

Regarding extraction efficiency, a 2 M $MgSO_4$ solution was selected to supply the electrolyte to the plasma samples, which permitted acceptable recovery of both AG and DDAG by DCM as the final extraction method.

3.3. SALLE-HPLC-DAD Method Validation

System suitability is an essential assessment for HPLC analysis, guaranteeing that the chromatographic system operates with sufficient efficiency, resolution, and repeatability prior to and throughout the quantitative analysis. It consists of a collection of predetermined parameters that collectively assess the overall performance of the column and analytical system, including the capacity factor, resolution, tailing factor, and column efficiency. The parameters were established in accordance with the USP general chapter <Chromatography> using six replicate injections of the LLOQ standard solution before each analytical run. As summarized in Table 2, the capacity factor values of AG and DDAG were 3.05 ± 0.01 and 8.33 ± 0.02, respectively, which fell within the optimal range of 2–10, indicating sufficient analyte retention without unnecessarily prolonged elution. The resolution between AG and DDAG was exceptionally high, at 16.46 ± 0.41 and 25.49 ± 0.11, respectively, far exceeding the minimum acceptance criterion of 2.0 and confirming complete baseline separation with no evidence of peak overlap. The tailing factors were 1.17 ± 0.06 for AG and 1.23 ± 0.01 for DDAG, both close to unity and within the generally acceptable limit of 2.0, demonstrating excellent peak symmetry and minimal column overloading. The column efficiency reached 6720 ± 147 for AG and 29631 ± 252 for DDAG, indicating high column performance and mass transfer. Collectively, these parameters indicate a stable chromatographic system with strong separation and peak geometry appropriate for the quantitative HPLC-DAD analysis of AG and DDAG.

Table 2. System suitability parameters for HPLC analysis of AG and DDAG.

Parameters	AG	DDAG
Capacity factor	3.05 ± 0.01	8.33 ± 0.02

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Resolution	16.46 ± 0.41	25.49 ± 0.11
Tailing factor	1.17 ± 0.06	1.23 ± 0.01
Column efficiency	6702 ± 147	29631 ± 252

Data are presented as mean ± SD, n = 6.

The guidelines outlined in the ICH Bioanalytical Method Validation section were followed to evaluate the analytical method used for plasma sample analysis [48]. The assessed parameters included linearity, LOD, LOQ, precision, accuracy, and analyte stability in the sample matrix.

3.3.1. Matrix Effect, Separation, and Specificity

Rat plasma from the same strain intended for use in the pharmacokinetic study was employed to evaluate the matrix effect. The assessment was performed at analytical concentration levels of LLOQ and ULOQ. The precision was expressed as the coefficient of variation (%CV), which was 4.34% and 0.65% for AG at the LLOQ and ULOQ levels, respectively, while DDAG showed values of 12.34% and 12.68%, respectively. The accuracy of the matrix effect for AG ranged from 92.9% to 101.1% and from 92.9% to 94.0% at the LLOQ and ULOQ levels, respectively. For DDAG, the accuracy of the matrix effect ranged from 86.5% to 107.1% and 86.7% to 111.0%, respectively. The precision and accuracy results met the ICH M10 acceptance criteria (accuracy ± 15% and precision ≤ 15%), confirming the reliability of the analytical method, without significant matrix interference. The specificity was determined by analyzing blank plasma samples extracted using the SALLE method. No interference peaks were observed in any of the chromatograms obtained from the replicate blank plasma samples analyzed using HPLC-DAD (Figure 2a). At the retention times of AG (4.2 min) and DDAG (9.7 min) (Figure 2b), clear and distinct peaks were detected in the chromatogram of the LLOQ concentration sample, with signal-to-noise ratios of 11.6 and 32.0 for AG and DDAG, respectively. Generally, the response of any co-eluting peak at the analyte retention time should be less than 20% of the analyte response at the LLOQ.

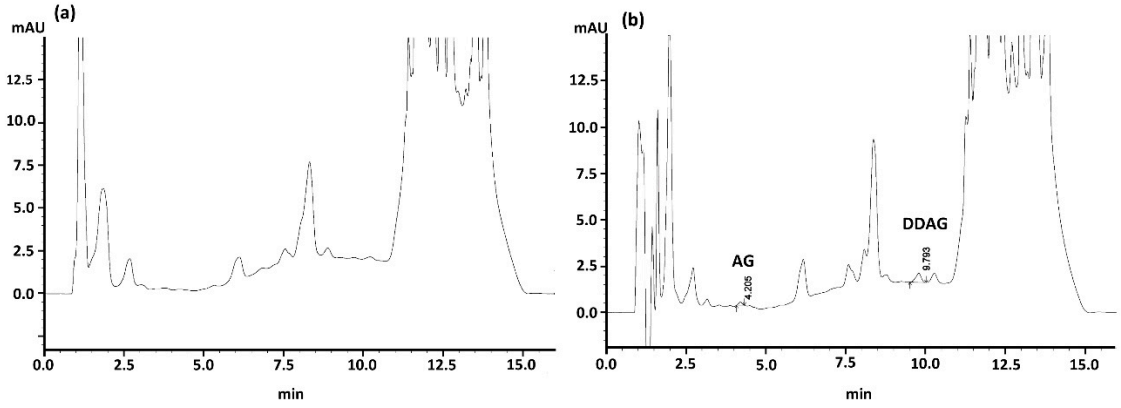

Figure 2. Representative chromatograms of blank plasma (a) and plasma spiked with AG and DDAG at the LLOQ level (125 ng/mL) (b).

3.3.2. Range and Linearity of Calibration Curve

A five-point calibration curve was constructed for AG and DDAG at concentrations of 125, 250, 500, 1000, and 2000 ng/mL. The results demonstrated that the standard curves of AG and DDAG exhibited good linearity, with R^2 values of 0.9973 and 0.9974, respectively, both exceeding 0.99, indicating strong linearity (Table 2). The resulting regression equations were $y = 10.9x - 400.9$ for AG and $y = 32.3x + 168.3$ for DDAG. The established SALLE-HPLC-DAD technique for quantifying AG and DDAG was assessed and compared with previously documented methods. As summarized in Table 3, the approach used in this study showed superior extraction efficiency compared to traditional LLE and PPT, while demonstrating comparable performance to SPE for both AG and DDAG. In terms of the analytical range, the linearity limits were comparable to those of other studies that employed the same type of detector. The LOD and LOQ values for AG were similar to those reported in the literature, whereas for DDAG, the sensitivity was slightly lower than that in some previous reports.

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Table 3. Summary of SALLE-HPLC-DAD quantitative results obtained from plasma samples compared with the established measurement method for AG and DDAG utilizing various sample preparation techniques.

Analytical method	Actives	Linearity range (ng/mL)	R^2	LOD (ng/mL)	LOQ (ng/mL)	EE (%)	Refs.
SALLE-HPLC-DAD *	AG	125 – 2000	0.9973 ± 0.0029	60.3 ± 13.6	70.3 ± 28.9	93 – 94	-
	DDAG	125 – 2000	0.9974 ± 0.0023	201.1 ± 45.5	234.3 ± 96.3	92 – 98	
LLE-HPLC-DAD	AG	53 – 530000	0.996	15	53	65 – 72	[28]
	DDAG	-	-	-	-	-	
PPT-HPLC-MS/MS	AG	0.98 – 1000	>0.99	-	0.98	78 – 81	[29]
	DDAG	0.98 – 1000	>0.99	-	0.98	81 – 89	
SPE-HPLC-MS/MS	AG	4000 – 12000	0.9989	40	150	97 – 99	[30]
	DDAG	4000 – 12000	0.9987	20	60	92 – 95	

* Data are presented as mean ± SD, n = 6 for all cases. EE denotes the extraction efficiency (%).

The relatively high LOD and LOQ values of DDAG observed in this study may represent a limitation for pharmacokinetic investigations involving low-dose administration. This could primarily be attributed to the limited sensitivity of the DAD detector. Combining the optimized SALLE-based sample preparation, which achieved extraction efficiency exceeding 90%, with a more sensitive detection system, such as mass spectrometry, has been reported to provide higher sensitivity than that of DAD [66]. This may help overcome the analytical limitations. Overall, the sample preparation procedure developed in this study can be considered a potential alternative to previously reported methods owing to its simplicity, cost-effectiveness, and high extraction efficiency.

3.3.3. Accuracy, Inter- and Intra-Day Precision

In this study, sample preparation using SALLE with $MgSO_4$ involved a phase separation time of 5 min and an organic-to-aqueous ratio of 1:4. The batch-to-batch reproducibility tested for sample preparation inter- and intra-day met the repeatability criteria specified in the guidelines. As presented in Table 4, both inter-day and intra-day precision for AG and DDAG were within the acceptable criteria, with RSD ranging from 2.0% to 9.9%, not exceeding the guideline threshold of 15% for MQC and ULOQ. Similarly, the accuracy of the method met the acceptance criteria, with recovery values ranging from 85.6% to 119.0%.

Table 4. Intra-day, inter-day precision, and accuracy of AG and DDAG in rat plasma.

Concentration (ng/mL)	AG			DDAG		
	Precision (RSD, %)		Accuracy* (% Recovery)	Precision (RSD, %)		Accuracy* (% Recovery)
	Inter-day	Intra-day		Inter-day	Intra-day	
125	9.5	5.1	91.8 ± 4.7	9.9	2.0	103.1 ± 10.7
1000	5.6	2.0	99.2 ± 2.0	8.6	9.6	99.3 ± 8.6
2000	8.6	2.6	91.4 ± 2.4	5.2	4.7	102.3 ± 5.0

*Data are presented as mean ± SD, n = 6 for all cases.

3.4. Sensitivity (LLOQ)

According to ICH M10, the LLOQ was defined as the lowest concentration that could be quantified with an accuracy within ± 20% and a precision not exceeding 20% RSD. The assay achieved LLOQ of 125 ng/mL for both AG and DDAG. At this level, intra- and inter-day precision in terms of RSD were 5.1% and 9.5%, respectively, and accuracy in terms of % recovery ranged from 85.9 % to 98.8 %. These results met the acceptance criteria of the guidelines [48].

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Table 5. Short-term stability (4 h under ambient conditions and 24h in an auto-sampler), three freeze-thaw cycles, and long-term storage (14 days at -20 °C) of AG and DDAG in processed plasma.

Storage time and condition	Percentage remaining (%)	
	AG	DDAG
Control (0 h)	102.83 ± 6.70	95.48 ± 3.40
4 h Bench-top	67.44 ± 6.72	88.20 ± 9.50
24 h auto-sampler	99.94 ± 6.70	96.70 ± 4.73
Freeze-thaw (3 cycles)	90.64 ± 5.81	83.99 ± 2.01
14 days at -20 °C	106.74 ± 6.83	94.57 ± 6.58

Data are presented as mean ± SD, n = 6 for all cases.

3.5. Sample Stability

To maintain the active compound at a level exceeding ± 15% of the initial concentration, the sample preparation and analysis should be performed immediately. Table 5 lists the percentages of remaining AG and DDAG in spiked plasma under different conditions. The data indicated that storage at room temperature, with a 4-hour interval at the bench-top, led to a notable reduction in the concentrations of both AG and DDAG, with the remaining concentration of AG decreasing to 67.44%, whereas DDAG exhibited relative stability of 88.20%. However, the analytes remained stable in the autosampler for up to 24 h, with the percentages of AG and DDAG remaining between 95.48% and 102.83%. This stability was attributed to the autosampler of the Shimadzu instrument, which maintained the sample compartment at 4 °C. Additionally, long-term stability was evaluated by storing the samples at -20 °C for 14 days and by assessing the effects of freeze-thaw cycles. These conditions did not significantly affect the concentration of AG and DDAG, with remaining levels ranging from 94.57% to 106.74% for the -20 °C storage and from 83.99% to 90.64 % for the freeze-thaw cycles.

The stability of both AG and DDAG in spiked plasma was directly affected by the storage temperature. These findings are consistent with those of an earlier report by Lomlim et al. [67], which showed that crystalline AG remains highly stable at elevated temperatures, whereas its amorphous forms degrade rapidly even when stored at room temperature, with DDAG identified as the major degradation product. Similarly, in our investigation, a significant reduction in AG content was observed, whereas the concentration of DDAG exhibited a modest decrease.

The lower stability of AG relative to its dehydro-analogue (DDAG) may be attributed to the higher susceptibility of its lactone and C-14 allylic hydroxyl groups to hydrolysis and dehydration under aqueous conditions, consistent with previous reports describing the spontaneous transformation of AG into DDAG [68,69]. Furthermore, previous reports have shown that AG possesses greater aqueous solubility than DDAG [70]. This property may partly account for its increased exposure to plasma enzymes and, consequently, its reduced stability. In addition, the dehydro analog of andrographolide exhibits a higher extent of protein binding, approximately 82.4 % in rat plasma [71], whereas AG has been reported to display protein binding in the range of 55 – 60% [72]. The lower degree of protein binding of AG may represent another possible mechanism underlying its stability. As described in a previous study, protein binding can exert dual effects on the stability of lactone-containing compounds, either enhancing or accelerating their degradation, depending on the binding pattern with plasma proteins [73]. Further studies on the protein interactions affecting the stability of AG and DDAG should be elucidated.

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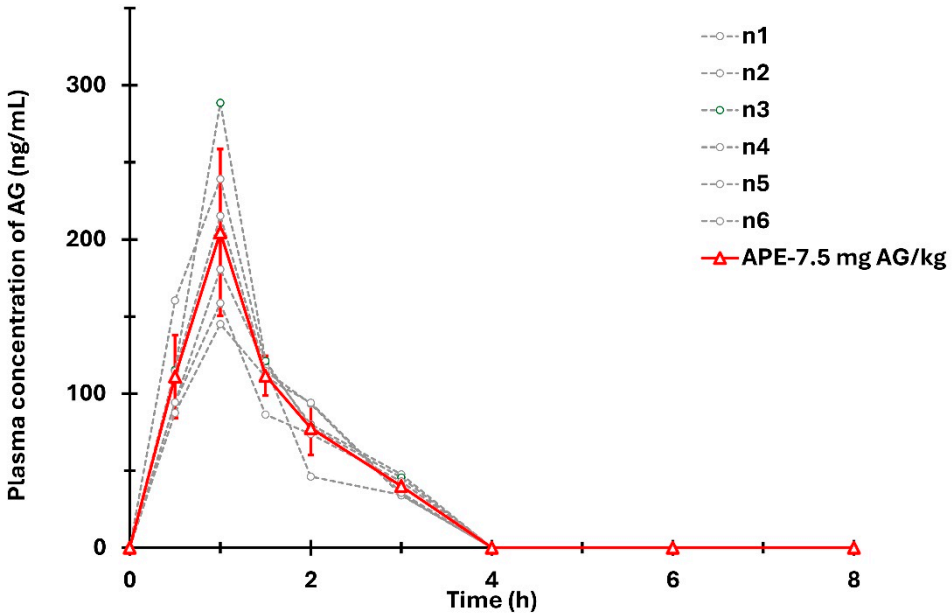

Figure 3. Plasma concentration-time profiles of AG following oral administration of APE at a single dose equivalent to 7.5 mg AG/kg BW in rats. Dashed lines represent individual animal data (n1–n6), while the solid red line indicates the mean concentration with SD (n=6).

3.6. Application to Pharmacokinetic Studies

To evaluate the application of the developed SALLE-HPLC-DAD method for the quantification of AG and DDAG in blood samples, a pharmacokinetic study was conducted following APE administration, corresponding to 7.5 mg/kg of AG, in Wistar rats. In the present study, 0.1 mL of plasma was used for the analysis. The sensitivity of the method was sufficient to detect AG up to 8 h post-administration; however, DDAG was not detected in any of the collected samples. This may be attributed to the lower proportion of DDAG in the investigated APE, which contained only 4% DDAG compared to 24% AG. This corresponded to a DDAG:AG ratio of approximately 1:6, resulting in a DDAG dose of approximately 1.25 mg/kg. Moreover, several studies have indicated that DDAG exhibits low oral absorption owing to physicochemical and metabolic limitations. In rats, oral administration resulted in a very low bioavailability (~3.4%), suggesting poor permeability and extensive first-pass metabolism [74]. Another study similarly showed that the major parent diterpenes are rarely detected in plasma, whereas glucuronide and sulfate conjugates predominate, confirming rapid phase II metabolism after absorption [29]. Additionally, DDAG displays limited aqueous solubility compared to AG and is recognized as a P-glycoprotein substrate [70]. It has been reported that DDAG possesses poor water solubility [75]. These factors likely explain why DDAG could not be quantified in the present pharmacokinetic study.

Table 6. Pharmacokinetic parameters of AG following oral administration of APE at a single dose equivalent to 7.5 mg AG/kg BW.

Parameters	Values
T_{max} (h)	1.0 ± 0.0
C_{max} (ng/mL)	219.0 ± 46.5
AUC (ng·h/mL)	309.5 ± 4.8
$T_{1/2}$ (h)	0.8 ± 0.1

Data are presented as mean ± SD, n = 6.

The plasma concentration-time profile of AG following a single oral dose of APE is shown in Figure 3, and the calculated pharmacokinetic parameters are summarized in Table 6. As seen, the maximum plasma concentration (C_{max}) of AG was 219.0 ± 46.5 ng/mL, observed at 1 h after oral administration, with a half-life of 0.8 ± 0.1 h, while the total exposure (AUC) was 309.5 ± 4.8 ng·h/mL. In comparison, Chellampilai et al. reported that administration of pure AG at a dose of 10 mg/kg to

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experimental animals yielded a higher maximum plasma concentration of 830 ng/mL, whereas the T_{max} was identical to that observed in the present study [76]. A recent investigation on the pharmacokinetic profile of AG compounds isolated from APE in rats, utilizing a validated LC-MS/MS methodology, revealed an apparent C_{max} of 115.8 ± 17.6 ng/mL and a T_{max} of 0.8 ± 0.3 h after oral administration at 30 mg/kg of AG [77]. As is known, the variations in pharmacokinetic parameters are influenced by various factors, including the form of the compound, the dosage administered, and the species of the animal involved.

It should be noted that in the pharmacokinetic analysis of this study, plasma concentrations assessed at 0.5, 1.5, 2, and 3 hours post-administration were observed to be below the LLOQ, with the 3-hour time point also registering below the LOQ, as depicted in Figure 3. However, in accordance with the Bioanalytical Method Validation: Guidance for Industry (U.S.FDA), values below the limit of quantitation (BLQ) may be reported if clearly indicated as such. The BLQ values fall below the calibration range defined during the validation process, and there is inadequate evidence to confirm the reliability of these values. The removal of BLQ values in both the absorption and, notably, the elimination phase significantly impacts the estimation of the absorption rate, restricts the lower end of the drug's concentration-time profile, and influences other related parameters. Neglecting BLQ data may lead to bias and imprecision in pharmacokinetic analyses based on models. Prior reports have indicated that the use of BLQ data would be advantageous. Incorporating BQL values in pharmacokinetic evaluation may more accurately reflect the actual data trend, offering a more precise representation of drug disposition compared to excluding the points or assigning them a value of zero [78–80].

The pharmacokinetic behavior of AG can be elucidated by considering its physicochemical and biopharmaceutical properties. The relatively low C_{max} observed following oral administration can be attributed to the poor aqueous solubility and moderate lipophilicity (log P ≈ 2.6) of the compound [70]. These properties limit its dissolution and passive diffusion across the intestinal membranes. Furthermore, AG has been identified as a P-glycoprotein substrate [81,82]. The active efflux of AG from enterocytes likely contributes to limited systemic absorption, leading to a low C_{max} despite sufficient dosage. The short T_{max} observed in this study reflects a rapid but transient absorption phase, which may result from the swift distribution and elimination processes discussed in earlier studies [83]. This is consistent with the short $T_{1/2}$ observed, possibly because AG is rapidly metabolized and eliminated from the body. This finding aligns with previous reports indicating that AG is converted into its conjugated metabolites, which are the predominant forms detected in the blood [29].

In summary, these mechanistic explanations indicate that the low C_{max}, short T_{max}, limited AUC, and rapid half-life ($T_{½}$) of AG are pharmacokinetic consequences of its physicochemical constraints and high metabolic susceptibility. Although both AG and APE have been reported to exhibit a wide range of pharmacological activities, including anti-inflammatory, antioxidant, analgesic, antipyretic, antimicrobial, antimalarial, hepatoprotective, immunomodulatory, and antiviral properties, including activity against SARS-CoV-2 [3–14], to achieve clinical efficacy would probably necessitate superior pharmacokinetic parameters. Therefore, formulation optimization, the use of a drug delivery system, or modulation of transporter and metabolic pathways may be necessary to enhance the systemic exposure and improve the therapeutic performance of AG [84–86].

Currently, the bioanalysis of AG lacks a rapid, low-cost, and greener LC workflow that supports high-throughput plasma analysis without relying on mass spectrometry. Previous liquid chromatography methods often required labor-intensive liquid-liquid extraction, had matrix carry-over post-protein precipitation, or utilized excessive solvents, all of which hinder scalability and sustainability. This study integrated HPLC-DAD with SALLE to address these deficiencies. SALLE induces phase separation, simultaneously removing proteins and reducing organic solvent usage, delivering cleaner extracts, and enabling faster sample preparation suitable for large pharmacokinetic study batches [60,61]. In particular, SALLE demonstrates a lower overall cost than SPE [42]. Leveraging DAD detection makes the platform cost-effective and accessible to laboratories without LC-MS, while still providing robust quantification when extraction and chromatographic conditions are optimized.

4. Conclusions

A bioanalytical method combining HPLC with optimized sample preparation was successfully developed to determine AG and DDAG in blood samples. To the best of our knowledge, this is the

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first report describing the preparation of biological samples for AG and DDAG analyses using the SALLE method. The developed SALLE demonstrated a higher extraction efficiency than conventional solvent-based preparation methods and comparable performance to advanced techniques such as SPE. An HPLC method was developed to quantify AG and DDAG in SALLE-prepared samples, demonstrating that this approach effectively minimized carryover interference from plasma samples. Furthermore, the analytical method developed in this study was successfully applied to determine AG levels in biological samples obtained from pharmacokinetic studies in healthy Wistar rats. This alternative sample preparation and analytical approach has significant utility in routine sample preparation, notably for pharmacokinetic studies of AG, a key biomarker of APE, in experimental rat samples. The potential utilization of this established HPLC analytical methodology in tissue distribution analysis, as well as in formulation development and quality control of AP formulations, should be examined. Furthermore, a comparative pharmacokinetic study of pure AG and DDAG compounds, in relation to the crude APE, presents an intriguing opportunity to investigate whether the pharmacokinetic behavior observed in AG and DDAG is attributable to their inherent properties or the intricate interactions with other components present in the extract. A further investigation of this comparison shall be conducted.

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

Abbreviations

The following abbreviations are used in this manuscript.

ACN	Acetonitrile
AG	Andrographolide
AP	*Andrographis paniculata*
APE	AP extract
AUC	Area under the curve
BW	Body weight
C_{max}	Maximum plasma concentration
DCM	Dichloromethane
DDAG	14-deoxy-11,12-didehydroandrographolide
EtOAc	Ethyl acetate
HPLC-DAD	high-performance liquid chromatography method coupled with diode array detection
LLE	liquid-liquid extraction
LLOQ	Lower limit of quantification
LOD	Limit of detection
LOQ	Limit of quantification
MeOH	Methanol
MgSO4	magnesium sulfate
MQC	Medium QC
Na_2SO_4	sodium sulfate
NaCl	Sodium chloride
PPT	protein precipitation
QC	Quality control
RSD	Relative standard deviation
SALLE	salt-assisted liquid-liquid extraction
SPE	solid-phase extraction

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$T_{1/2}$	Half-life
T_{max}	Time to maximum concentration
ULOQ	Upper limit of quantification

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