Journal of Research in Pharmacy

Research Article

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An LC-MS/MS method development for dapagliflozin-loaded nanostructured lipid carrier formulation in rabbit plasma

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Received: 30 November 2023 / Revised: 04 January 2024 / Accepted: 04 January 2024

ABSTRACT: The primary goal of this study was to develop and validate an LC-MS/MS method for the detection of dapagliflozin in nanostructured lipid carriers (NLC) using ion-interaction chromatography. The reversed-phase InfinityLab Poroshell 120 (150 × 4.6 mm, 4 µm) column, using a mobile phase of acetonitrile-25 mM ammonium acetate solution with pH 4.1 (35:65, v/v), effectively separated the analytes and their internal standards. This method has been thoroughly tested and validated to ensure accurate and reliable results. To improve sensitivity and selectivity, mass spectrometry was used in polarity switching mode. In order to study ion transitions for dapagliflozin in both positive and negative mode, multiple reaction monitoring mode was utilized, with the ion transitions being m/z 467.1 [M+CH3COO]- /329.1. The assay's linear calibration range for dapagliflozin was established from 0.05-150 ng/mL to improve drug pharmacokinetics assessment. The analyte's limit of detection (LOD) and limit of quantitation (LOQ) were 0.07 and 0.35 ng/mL, respectively. After testing, no interference was observed in plasma matrices from different sources, including haemolysed and lipemic plasma. The impact of Dapagliflozin-loaded NLC on plasma levels were investigated using this method.

KEYWORDS: Nanostructured lipid carriers; quantitative method; SGLT-2 inhibitors; dapagliflozin; plasma extraction

1. INTRODUCTION

It is imperative to maintain continuous glycemic control when dealing with type 2 diabetes mellitus (T2DM), a chronic disease that progresses over time. This control is necessary to minimize long-term microvascular and macrovascular complications [1,2]. Combination therapy utilizing drugs with complementary mechanisms of action has become increasingly prevalent in simplifying dosing regimens, improving patient adherence, enhancing patient satisfaction, and reducing healthcare costs. In this context, novel glucose-lowering agents such as sodium-glucose cotransporter type-2 (SGLT-2) inhibitors have demonstrated significant improvements in β-cell function among patients with T2DM [3]. Dapagliflozin (DAP) is an effective medication that specifically targets hyperglycemia by selectively inhibiting SGLT-2. It works by reducing the reabsorption of glucose in the kidneys, independent of insulin secretion. When taken orally, DAP is quickly absorbed into the bloodstream, and food does not affect its potency. Therefore, it can be taken at any time, without regard to meals [4,5]. The compound DAP is known to undergo comprehensive metabolism via UDP glucuronosyltransferase-1A9 to form inactive DAP 3-O-glucuronide metabolites. It has also been observed to exhibit high plasma protein binding (91%) and has an oral bioavailability of approximately 78% [1,6]. DAP undergoes predominant elimination through urine with an elimination half-life of approximately 12-14 hours. Currently, there are scarce reported methods for the determination of DAP in biological fluids [7]. However, a recently developed method using liquid chromatography-tandem mass spectrometry (LC-MS/MS) can accurately determine DAP in normal Sprague-Dawley rats as well as in diabetic rats, in support of preclinical studies [8]. In the negative electrospray ionization mode, an LC-MS/MS assay was described for selected reaction monitoring (SRM) detection of DAP-acetate adduct ions in human plasma [9].

How to cite this article: Uner B. An LC-MS/MS method development for dapagliflozin-loaded nanostructured lipid carrier formulation in rabbit plasma. J Res Pharm. 2024; 28(2): 438-446.

ISSN: 2630-6344

http://dx.doi.org/10.29228/jrp.708

The assays conducted by He et al. And Ji et al. proved an effective tool for analyzing samples from critical clinical studies. It demonstrated remarkable accuracy, especially in studies involving pediatric patients. These studies were primarily aimed at assessing the pharmacokinetics and bioequivalence of immediate release (IR) and extended-release (XR) tablet formulations in healthy subjects, while also evaluating the impact of food on the pharmacokinetics of these drugs [8,10]. Overall, the assay's successful implementation underscores its importance as a valuable tool in clinical research. The methods presented here are lacking in detail regarding method development and validation. Furthermore, it should be noted that the lower limit of quantitation for DAP in these methods is only 0.2/1.0 ng/mL [10,11].

The aim of this study was to develop a novel LC-MS/MS method that is more sensitive and rapid in detecting DAP in plasma compared to existing methods. The newly developed method was subjected to rigorous validation in accordance with current regulatory guidelines. It was then utilized to investigate the pharmacokinetics of these drugs in healthy New Zealand rabbits using an NLC formulation under both fasted and fed states. The findings of this study could potentially contribute to advancements in the field of pharmacology.

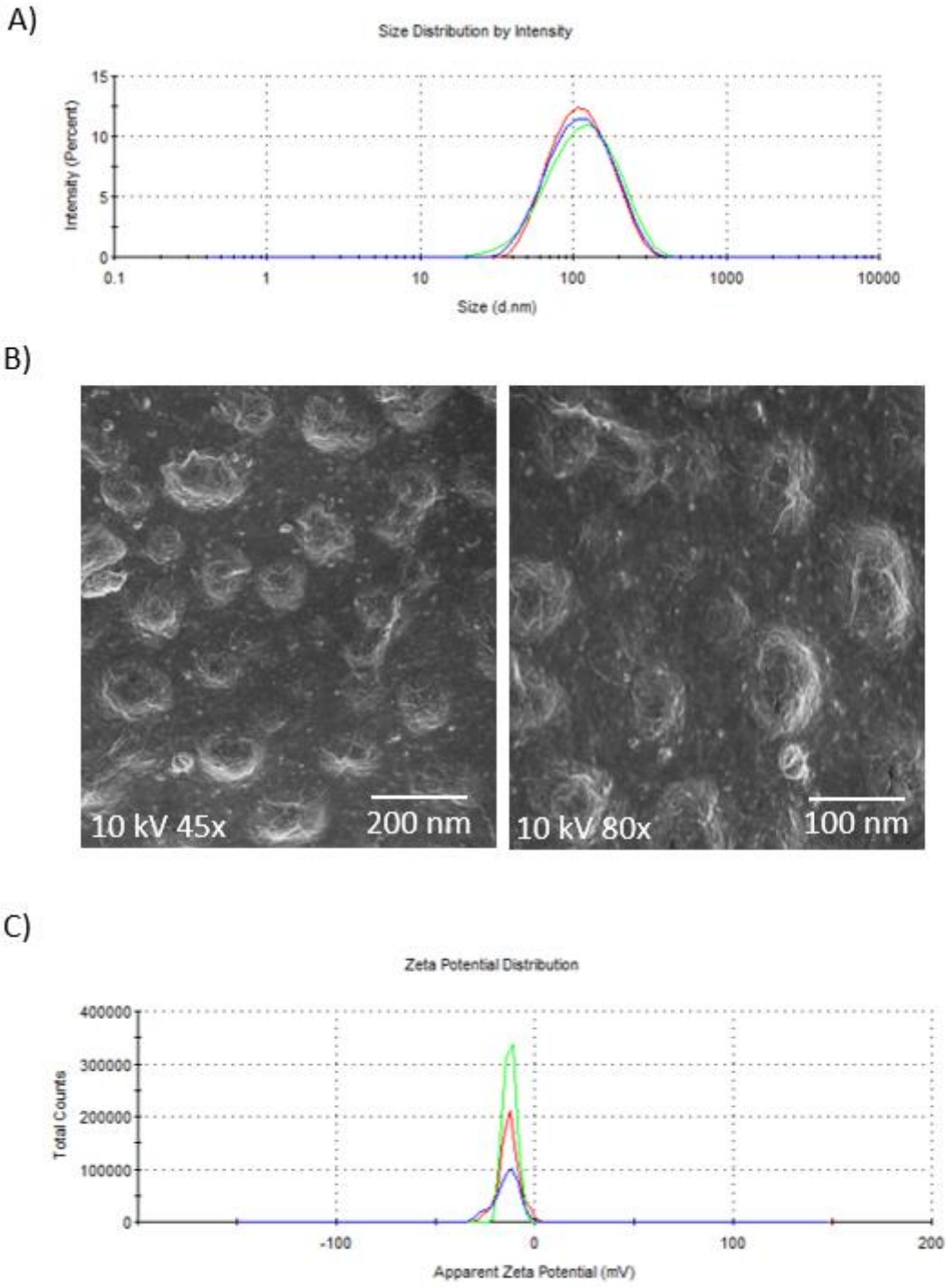
2. RESULTS AND DISCUSSION

2.1. Characterization of the nanoparticles

As per our findings, the size of particles in DAP-loaded NLCs was measured at 92.36 ± 1.28 nm (0.155, PDI) (Figure 1). This data is consistent and reliable, and we can confidently rely on it for further analysis. The lipid undeniably exerted an antagonistic effect on the NLCs' size. Hence, the presence of Poloxamer caused a more significant decrease in size than expected. The aggregation of particles unequivocally leads to an increase in their size. However, it is noteworthy that a decrease in the interfacial tension between two phases can effectively decelerate this process and reduce particle growth [12, 13]. A higher homogenization speed is essential for effective particle breakdown, resulting in a significant reduction in NLC size.

The form and surface morphology of optimized DAP-loaded NLCs was thoroughly examined using SEM. The analysis conclusively revealed that the particles had a perfectly spherical shape, with smooth surfaces and absolutely no aggregation. As illustrated in Figure 1-B, SEM is an effective tool for describing the surface morphology of both the medication and excipient [14]. The nanometric size of NLCs clearly indicates their potential for absorption by Peyer's patches and delivery to the intestinal lymphatic system, entirely bypassing the liver and significantly increasing the oral bioavailability of the drug [15]. Malvern's particle size measurement is significantly superior to SEM. The hydrodynamic size of a nanoparticle is accurately assessed through differential light scattering, which incorporates the nanoparticle size as well as the surrounding liquid layer. In contrast, SEM is used to determine the actual size of the nanoparticle without any ambiguity.

The zeta potential unequivocally represents the electric potential differential present throughout the ionic layer encircling a positive ion in colloids. A lower zeta potential value unambiguously indicates a higher degree of aggregation.The zeta potential assessment is a highly effective and efficient method for reducing the need for stability investigations and significantly improving the shelf-life of DAP-NLCs [16]. It is crucial to maintain a zeta potential range of -12.2 to -18.6 mV in DAP-loaded NLCs to guarantee their stability (Figure 1-C).



	Size (nm)	PDI	Zeta Potential	Encapsulation (%)
DAP-NLC	92.36 ± 1.28	0.155 ± 0.068	-15.4 ± 3.2	96.75 ± 0.66

Figure 1. Characterization studies of DAP-loaded NLC formulations A) particle size graphic B) morphological examination C) electricity charge of the particle

2.2. Mass spectroscopy

Considering the fundamental nature of DAP, it was imperative to investigate both positive and negative ionization modes as they are extensively studied in reported methods [9,17]. It is a well-established fact that

the nature of the mobile phase has a significant impact on the type and intensity of ion formation. When ammonium acetate or ammonium formate is used in the positive ionization mode, it leads to the formation of a major precursor ion corresponding to DAP-ammonium ion adduct [M+NH4]+. While it was challenging to achieve a consistent MS product ion with an intensity in the range of 1.2-1.4 cps, there may be potential strategies or techniques that could be explored to improve the outcome. The deprotonated molecular ion [M-H]- (m/z 411) has been confirmed as the primary ion in negative mode for DAP in water/acetonitrile, according to El-Zaher et al [18]. It has been firmly established that the adduct experiences a significant increase in abundance upon the introduction of formate or acetate ions, leading to a considerable reduction in the sensitivity of detection. The results of Surendran et al. demonstrate with utmost clarity that the intensity of acetate ion adducts of DAP (m/z 465) in negative mode is significantly higher than that of [M-H]- ions [19]. The present study confidently employed the polarity switching approach to accurately determine DAP simultaneously.

The mass parameters for DAP were precisely fine-tuned in negative ionization mode using ammonium acetate/formate as a mobile phase additive. After careful consideration, it was determined that acetate adduct ion [M+CH3COO]- (m/z 467) is the superior choice over formate adduct ion [M+HCOO]- (m/z 453) due to its significantly stronger signal intensity and minimal background response. It is important to note that the most stable and intense product ions were unequivocally observed at m/z 329.1 and 335.1 for DAP and DAP-loaded NLC, respectively (as illustrated in Figure 2). For unambiguous identification of the analytes, it was imperative to study qualifying peaks at m/z 409.0 and 407.2 for DAP-loaded NLC and DAPA, respectively in Figure 2.

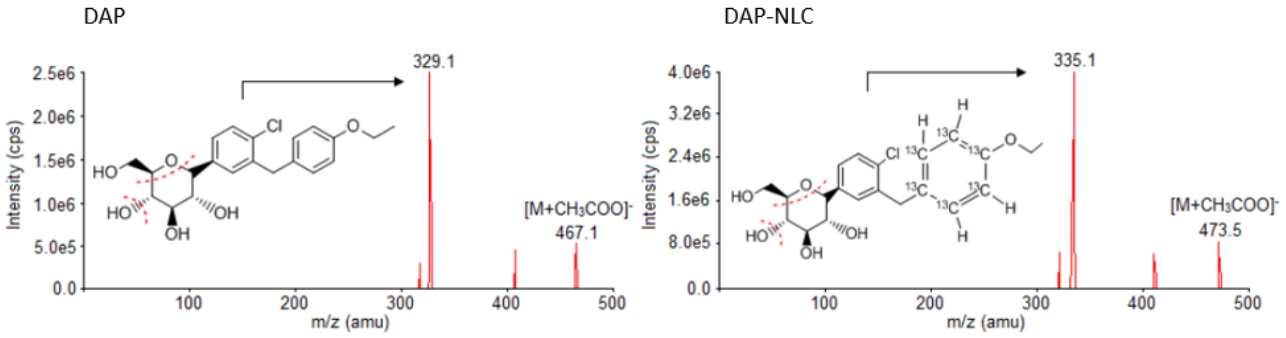


Figure 2. Product ion mass spectra of DAP & DAP-NLC (m/z 473.5 → 335.1) in the negative ionization mode

2.3. Chromatographic evaluation

To achieve optimal separation of DAP from the NLC components, the selectivity of the Poroshell 120 column was finely tuned by adjusting the proportion of organic modifier, pH and ionic strength of the eluent. Our findings demonstrate that the composition (aqueous and organic part) and pH of the mobile phase play a crucial role in determining the efficiency of separation and sensitivity of the method [20]. The sensitivity was considerably enhanced in the presence of ammonium acetate at pH 4.5, utilizing acetonitrile as the organic modifier. The analyte was effectively resolved (resolution factor 2.11) within 2.5 minutes, with sharp peak shapes and an adequate response. The method's selectivity is clearly demonstrated by the ion chromatograms of DAP in blank plasma, blank plasma with internal standard working solution, and plasma with DAP-NLC (Figure 3).

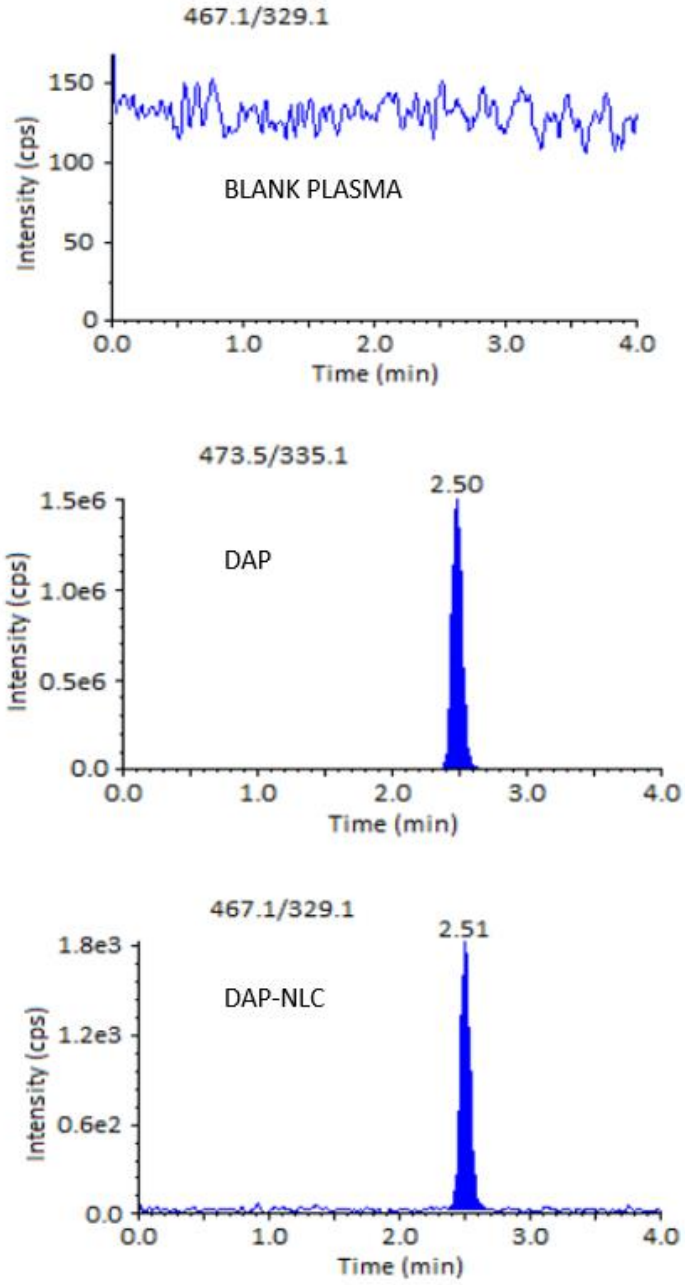


Figure 3. Chromatograms of DAP in blank plasma (drug free), blank plasma with DAP, and DAP-NLC

2.4. Plasma extraction

To extract DAP from plasma, solid phase extraction or a combination of SPE with protein precipitation have been the only reported methods thus far [18,21]. Despite the existence of literature on DAP extraction from plasma, it is important to note that there has not been any method reported yet for simultaneous extraction. In order to streamline and accelerate the extraction process, we conducted initial trials using PP with acetonitrile and methanol. However, we found that the recovery was consistently inadequate and not quantitative, particularly at LOD (0.07 ng/mL) and LOQ (0.35 ng/mL) levels for both analytes. Solid phase extraction of plasma samples was effectively carried out using Oasis HLB cartridges, with and without the use of ion-pair reagent (SDS), for optimal sample cleanup. Table 1 summarizes the results of the trials conducted on both cartridges. The cartridge consistently recovered the analytes in the presence of an ion-pairing agent. This clearly demonstrates the efficacy and reliability of the cartridge.Different concentrations of SDS (5, 7, and 10 mM) were tested to conclusively determine the optimal SDS concentration for maximum DAP extraction

recovery. Through rigorous experimentation, it has been unequivocally established that 5 mM SDS is the most effective concentration.

The recovery was significantly enhanced through optimization of the washing solution and eluent volume. With the use of 7% methanol, we were able to achieve consistent results that effectively eliminated any endogenous substances. Trust the accuracy of our findings - choose the reliability of 7% methanol.Methanol and acetonitrile were thoroughly investigated as elution solvents to ensure the complete recovery of analytes in the presence of 7% acetic acid. Upon thorough analysis, it was conclusively determined that the optimal solvent system for elution is 750 µL of 7% (v/v) acetic acid in acetonitrile. The extraction recovery of DAPA remained highly consistent and reproducible across all QC levels, affirming the accuracy and reliability of the results obtained.

Table 1. Average of extraction recovery of DAP (n = 6)

Cartridge	Analyte	Average recovery (%)			
		non ion-pair reagent		ion-pair reagent	
		LQC	MQC	LQC	MQC
Oasis	DAP	42.76 ± 2.42	49.13±1.92	87.77±1.56**	89.1±1.4**

DAP: Dapagliflozin; LQC: Low quality control; MQC: Medium quality control (**represents p<0.01)

2.5. Validation

The linear regression equation for peak area ratios of DAP and its corresponding concentrations is y = (0.9209 ± 0.0023)x + (0.1129 ± 0.0105), which was obtained with high confidence and can be relied upon for accurate predictions. Based on the statistical analysis, the correlation coefficient (r^2) value is 0.9997, indicating a strong correlation between the variables. Moreover, the precision (%CV) and accuracy for the calibration curve standards demonstrate exceptional results, ranging from 0.24-3.69 and 98.9-101.2%, respectively (Table 2). These results confidently demonstrate the accuracy and reliability of the calibration methodology. On the top of that, the results indicate an impressive intra-batch and interbatch precision ranging from 1.1 to 4.3%, and an accuracy within 99.3 to 103.1%. The data clearly demonstrates that the optimized extraction procedure has resulted in a mean extraction recovery rate of 86.82% and 84.95% for DAP and DAP-NLC, respectively. Furthermore, results provide indisputable evidence of the stability of both DAP and DAP-NLC at two QC levels.

Table 2. Batch pericision and accuracy for DAP (n = 6)

Quality control (ng/mL)	Intra-batch			Inter-batch		
	Average concentration (ng/mL)	% CV	%Accuracy	Average concentration (ng/mL)	% CV	%Accuracy
LQC (2)	2.035	0.24	100.7	1.977	3.69	98.9
MQC (100)	100.49	2.29	99.5	100.79	2.15	101.1
HQC(2000)	1977	0.31	98.9	2019	1.15	101.2

DAP: Dapagliflozin; LQC: Low quality control; MQC: Medium quality control; HQC: High quality control

3. CONCLUSION

The development of DAP-NLC involved the use of hot homogenization, followed by an ultrasonication method that utilized CSA as the lipid and Poloxamer 188 as the surfactant. The DAP-NLC exhibits a mean particle size of 92.36 nm, an EE of 96.75%, a PDI value of 0.155, and a zeta potential of -15.4 mV. These values unequivocally demonstrate the superior performance of DAP-NLC. Furthermore, fully validated a novel LC-MS/MS method has been successfully developed using polarity switching approach for simultaneous quantification of DAP and DAP-NLC in rabbit plasma. The optimized ion-pair solid phase extraction protocol, utilizing 5 mM SDS, ensures accurate and precise recoveries of both drugs from 75 µL of plasma samples.The method demonstrates a high degree of selectivity, as confirmed by the matrix effects assessment conducted in plasma. By utilizing acetonitrile: 25 mM ammonium formate buffer, pH 4.1 (65:35, v/v) as the mobile phase, it is possible to perform chromatographic analysis for the simultaneous separation of analytes within a mere 4.0 minutes.

4. MATERIALS AND METHODS

4.1. Materials

The experiment utilized the following chemicals, all of which were acquired from Sigma Aldrich (St. Louis, MO, US): Dapagliflozin (DAP) (≥98%, SML2804), Poloxamer-188, cetostearyl alcohol (CSA), oleic acid (OE), and sodium dodecyl sulphate (SDS). Additionally, HPLC grade methanol, acetonitrile, ammonium formate, ammonium acetate, and analytical reagent grade formic acid and acetic acid were obtained from Merck (Milwaukee, WI, US), while the water used in the entire analysis was prepared from the highly reliable Milli-Q water purification system purchased from Millipore (Tempe, AR, US). Blank New Zealand rabbit plasma was also acquired after taking the ethical approval from Washington University Animal Care and Used Committee in St. Louis, MO US (Protocol number: 10-9218) and kept at a temperature of −70 °C until use.

4.2. Preparation of NLC

The process to create DAP-loaded NLCs involved a hot homogenization technique with the incorporation of an ultrasonic phase.DAP (100 mg) was promptly dissolved in bulk-lipid and then quickly dissolved in 15 mL of chloroform and methanol (1:2) at a high temperature of 80°C. The aqueous process utilized the surfactant Poloxamer-188, which was dissolved in 10 mL of distilled water to make a 2% solution and heated to the same temperature. After collecting the clear and homogeneous lipid phase in a beaker, heated aqueous phase solution was added to it. The mixture was then subjected to homogenization using Ultra Turrax T40 from IKA, Königswinter, Germany, for 10 minutes at 15,000 rpm in a high-speed homogenizer. Later, the pre-emulsion was ultra-sonicated at 500W for 7.5 minutes with a probe sonicator (Ultrasonic processors, Z511471, Denver, CO, US). The size and encapsulated NCL were precisely measured to ensure optimal results. The formulation was then rapidly lyophilized using MSE PRO Lab (MSE Supplies, Tucson, AR, US) and stored in a refrigerator at 4°C to preserve the highest quality.

4.3. Particle size, morphology, zeta potential, and encapsulation

The Particle size and distribution (PDI) of the NLC formulations were precisely measured using the Malvern Zeta Sizer Nano ZS instrument (Malvern, Freehold, NJ, US). The measurements were taken at a temperature of 25 ± 2 °C and an angle of 173°, which is known to effectively detect the maximum number of particles. It is imperative that formulations be diluted with distilled water at a ratio of 1:10 (v/v) and then completed to a final volume of 1 mL prior to conducting any measurements. For highly accurate zeta potential readings, it was imperative to conduct the measurement in electrode cuvettes with a dielectric constant of 78.5, which was determined to be the only optimal option for the diluted sample.

The size and morphology of the produced particles were analyzed using a Zeiss EVO 25 (Oberkochen, Germany) scanning electron microscope (SEM). Microscopy was utilized to thoroughly analyze the dispersion patterns of nanoparticles subsequent to their deposition on a thin carbon sheet and prompt removal from the chamber. Prepare the lyophilized DAP-NLCs by spreading a minute quantity onto carbon-coated tape and drying it at room temperature. Then, sputter the sample with a thin layer of gold under vacuum to ensure accurate results.

Centrifugation was the method of choice to determine the drug load of DAP-NLCs, as the formulation used was in a dried form. For this purpose, the formulation was dissolved in purified water. The sample was rigorously centrifuged for a duration of 20 minutes at a speed of 15,000 rpm using the ELMI CM-7S Plus Benchtop Centrifuge from MSE Supplies based in Tucson, AR, US. Once the supernatant was retrieved, the NLC particles were thoroughly rinsed with water and assertively dispersed in methanol (1:1, v/v). The material was sonicated for 10 minutes using a probe sonicator and then centrifuged again. After that, the supernatant was collected and the content of DAP was measured three times using a UV spectrophotometer set to 237 nm [14]. To determine the encapsulated drug, the following formula was used:

$$\text{EE. (\%)} = \frac{\text{Experimental drug content in NLC}}{\text{Theoretical drug content of NLC}} \times 100 \qquad (1)$$

4.4. Analytical conditions

For the chromatographic analysis of DAP, we utilized the LC-VP HPLC system (Shimadzu, Kyoto, Japan) in conjunction with the InfinityLab Poroshell 120 column (50 × 3.5 mm, 5 µm). This method was chosen for its high precision and accuracy in producing reliable results. The mobile phase was delivered at a

consistent flow rate of 1.2 mL/min and composed of acetonitrile-25 mM ammonium acetate in pH 4.1 water at a ratio of 65:35 [17]. The temperature of the column oven was set to 40 °C while the autosampler temperature was set to 5 °C. The system pressure was maintained at 1900 psi, and the injection volume was kept at 5 µL.

The LC system was expertly connected to the EVOC LC-TQ triple quadrupole mass spectrometer (Bruker, Billerica, MA, USA), and skillfully operated using a turbo ion spray ionization source in both positive and negative ion mode for DAPA.Quantitation was executed with utmost precision using multiple reaction monitoring (MRM) to closely monitor the ion transitions. DAPA and DAPA-13C6 were quantified by monitoring the ion transitions m/z 467.1/329.1 and m/z 473.5/335.1, respectively, with a non-negotiable and highly accurate dwell time (210 ms).

4.5. Quality control and calibration

In order to ensure accuracy, separate stock solutions of DAP were carefully prepared by dissolving accurately weighed amounts in methanol. To create calibration standards and quality control (QC) samples, appropriate volumes of working solutions, which were prepared from intermediate solutions for both the analytes, were added to blank plasma. This method guarantees that the samples are of the highest quality. Prepare internal standard stock solutions (50.0 µg/mL) by dissolving 1.0 mg of reference standards in 20.0 mL methanol. Prepare the DAP (DAP-13C6) working solution by combining it in methanol:water (60:40, v/v). Make calibration standards ranging from 0.05 to 200 ng/mL. It is essential to note that DAP QC samples were prepared at three concentration levels, which include the lower limit of quantification quality control at 2.00/0.20 ng/mL, medium limit quality control at 100.0/100.00 ng/mL, and high limit quality control at 2000/200.0 ng/mL. These concentration levels have been carefully chosen to ensure the accuracy and reliability of the results. It is imperative that standard stock and working solutions for spiking be stored at 4°C, while calibration standards and QC samples in plasma must be kept at -70°C until use.

4.6. Extraction

To prepare the sample, a precise amount of 75 µL of spiked plasma was added to a vial. Then, 50 µL of a carefully mixed solution containing the internal standard was added to the sample and vortexed vigorously for 45 seconds. The sample was then centrifuged at 2500 g for exactly 10 minutes. After that, the resulting solution was loaded onto Oasis HLB extraction cartridges, manufactured by Water, Milford, MA, US. The cartridges had been treated beforehand with 0.5 mL of methanol, followed by 1.5 mL of 2.5 mM SDS in water. After washing the cartridge with 5.0 mL of water, it was further washed with 2.5 mL of 7% methanol in water [22]. In order to ensure proper collection, the analyte and its corresponding internal standard were eluted using a solution of 7% (v/v) acetic acid in acetonitrile, with a volume of 750 µL, and subsequently placed into vials for further processing. The samples were dried using nitrogen at 40°C and reconstituted with 250 µL of mobile phase. 10 µL was injected into the chromatographic system.

4.7. Statistics

All data collected in this study were analyzed using GraphPad Prism version 11.0.3 (Boston, MA, US). To conduct parametric analysis, the plasma DAP data were converted to logarithms. The t-test was used to investigate two-group normally distributed data, while the Mann-Whitney U test was employed to analyze non-normally distributed data. When comparing more than two groups, the one-way ANOVA were utilized.

Acknowledge: The author would like to express special thanks to the Institutional Animal Care and Use Center in St. Louis, MO, USA.

Author contributions: Concept – B.U. Supervision – B.U.; Resources – B.U.; Materials – B.U.; Data Collection and/or Processing – B.U.; Analysis and/or Interpretation – B.U.; Literature Search – B.U.; Writing – B.U.; Critical Reviews – B.U.

Conflict of interest statement: The authors declared no conflict of interest.

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