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VALIDATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) METHOD FOR DELPHINIDIN-3-O-GLUCOSIDE CHLORIDE IN RAT PLASMA

(Pengesahan Kaedah Kromatografi Cecair Berprestasi Tinggi (KCPT) bagi Delfinidin-3-O-Glukosida Klorida dalam Plasma Tikus)

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Abstract

This study involved the development and validation of a high-performance liquid chromatography (HPLC) assay for the determination of delphinidin-3-O-glucoside chloride, specifically to be applied in future pharmacokinetic and bioavailability studies in rats supplemented with roselle extract. The HPLC method was validated following solid-phase extraction, with six validation parameters (linearity, recovery, precision, accuracy, limit of detection (LOD), and limit of quantification (LOQ) assessed according to Food and Drug Administration guidelines. Chromatographic separations were performed using Agilent Reverse Phase EC-C18 (4.6×150 mm; 2.7 µm particle size) and mobile phase acetonitrile with 0.1% trifluoracetic acid in an aqueous solution (81:19, v/v). The calibration curve of delphinidin-3-O-glucoside chloride had a coefficient determination (R^2) of ≥95.0% and recovery percentage of 80.37%. It also showed good precision and accuracy, and its method detection and quantification limits were 47 ng/mL and 213 ng/mL, respectively. In conclusion, the validated HPLC method in this study can be used to determine the pharmacokinetic of delphinidin-3-O-glucoside chloride or other type of anthocyanins in the future.

Keywords: delphinidin-3-o-glucoside chloride, rat plasma, high-performance liquid chromatography, method validation

Abstrak

Kajian ini melibatkan pembangunan dan pengesahan kaedah kromatografi cecair berprestasi tinggi (KCPT) bagi pengesanan sejenis antosianin, iaitu delfinidin-3-O-glukosida klorida, khususnya bertujuan untuk digunakan dalam kajian lanjutan untuk farmakokinetik dan bioavailibiliti antosianin di dalam tikus yang disuplementasi dengan ekstrak rosel. Kaedah KCPT ini disahkan menggunakan kaedah pengekstrakan fasa pepejal dan enam parameter pengesahan dinilai mengikut garis panduan Pentadbiran Makanan dan Dadah iaitu kelinearan, pemulihan, ketepatan, kejituan, had pengesanan, dan had kuantifikasi. Pengasingan kromatografik dilakukan menggunakan Agilent Fasa Berbalik EC-C18 (4.6×150 mm; 2.7µm saiz partikel) manakala fasa gerak adalah asetonitril bersama 0.1% trifluoroasetik asid di dalam air (81:19, v/v). Lengkung penentukuran delfinidin-3-0-glukosida klorida mempunyai penentuan pekali (R^2) ≥95.0% dan peratusan pemulihan delfinidin-3-0-glukosida klorida adalah 80.37%. Delfinidin-3-0-glukosida klorida juga menunjukkan ketepatan dan kejituan yang baik dan had pengesanan dan kuantifikasi kaedah untuk delfinidin-3-0-glukosida klorida masing-masing adalah 47 ng/mL dan 213 ng/mL. Kesimpulannya, kaedah HPLC yang disahkan dalam kajian ini boleh digunakan untuk menentukan tahap antosianin dalam kajian farmakokinetik pada masa depan.

Kata kunci: delfinidin-3-o-glukosida klorida, plasma tikus, kromatografi cecair berprestasi tinggi, pengesahan kaedah

Introduction

In recent years, anthocyanins have come into the interest of researchers from various fields due to their health-promoting properties. One major health benefit associated with anthocyanin intake is a reduced risk of coronary heart disease [1]. Anthocyanins are naturally occurring, water-soluble pigments found in the vacuoles of vascular plants that serve multiple functions, such as aiding in pollinating plants by luring insects and animals with their color and potent UV absorbance [2]. Anthocyanins give out red, purple, and blue pigments, resulting from the conjugated bonds in flowers, tubers, and fruits. They are classified under the flavonoid class and phenolic photochemical subclass [3]. While water-soluble, their stability varies according to pH, temperature, light, and structure [4]. As a result, anthocyanins appear red in acidic environments and blue in alkaline environments. Accordingly, they are commonly found in plants and the most prevalent anthocyanins in nature are cyanidin, delphinidin, and pelargonidin, which are present in 50% of flowers, 69% of fruits, and 80% of colored leaves [5]. However, in current study, we choose to focus on delphinidin since it is one of the most abundant types of anthocyanin found in roselle calyx extract, which is the interest of our research team.

To date, several analytical methods, especially liquid chromatography, are available for the detection of anthocyanins in biological fluid and tissue [6-10]. Liquid chromatography, including high-performance liquid chromatography (HPLC), allows the effective separation and identification of compounds in various biological samples, suitable for both qualitative and quantitative analysis. However, variability in experimental condition factors, such as extraction method, quantification, and parameter analysis, hinders the reproducibility of this method for obtaining the most efficient recovery of anthocyanins. Additionally, large sample volumes are required for the detection of anthocyanins, as shown in past studies [7, 11]. Although these protocols have been demonstrated to be efficient, none specifically focus on the anthocyanin delphinidin-3-O-glucoside chloride.

Despite these shortcomings, prior validation of HPLC provides documented evidence of a reliable analytical method for the sensitive quantification of compounds present in samples, thus providing valuable data. Therefore, the development and validation of a new HPLC protocol for the determination of anthocyanin, specifically delphinidin-3-O-glucoside chloride, is much needed as a potential reference protocol to be applied in future pharmacokinetic and bioavailability studies in rats supplemented with roselle extract. By employing six validation parameters according to FDA guidelines (linearity, recovery, precision, accuracy, limit of detection (LOD), and limit of quantification (LOQ), this study describes a rapid, sensitive, and specific HPLC protocol for the detection of delphinidin-3-O-glucoside chloride in small volumes of rat plasma.

Materials and Methods

Chemicals and reagent

Delphinidin-3-O-glucoside chloride (catalog no. CFN92038) and its internal standard, cyanidin-3-5-0-diglucoside chloride (catalog no. CFN92138) were purchased from ChemFaces (Wuhan China) (Figure 1). Acetonitrile (ACN), and methanol were of HPLC grade from Merck (Darmstadt, Germany) while sulfosalicyclic acid, trifluoroacetic acid (TFA) and formic acid (FA) were of analytical grade from Sigma-Aldrich (St Louis, USA). Water used was deionized and distilled using the Water Prodigy System (Labconco, MO, USA).

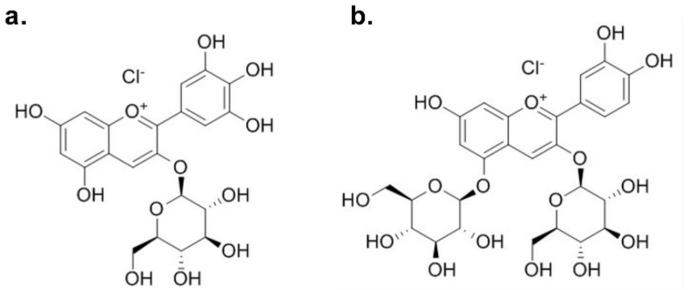

Figure 1 . Chemical structure for a. delphinidin-3-O-glucoside chloride with molecular formula of $C_{21}H_{21}CIO_{12}$, and b. cyanidin-3-5-O-diglucoside chloride with the molecular formula of $C_{27}H_{31}O_{16}C_{l}$

Chromatographic condition

The HPLC system consisted of a pump (Gilson 307), a column heater, an autosampler (Gilson GX-271) and detector (UV/VIS-155) from Gilson (Wisconsin, USA). Gilson Trilution® LC software was used for data acquisition. Chromatographic separations were performed on Agilent Reverse Phase EC-C18 (4.6 × 150 mm I.D, particle size 2.7 µm) (Agilent Technologies, California, USA) with flow rate of 0.5 mL/min, injection volume of 10 µL and detected at 525 nm. The mobile phase is composed of ACN with 0.1% of TFA in aqueous solution (81:19, v/v) and degassed in an ultrasonic bath (FB15063) from Fisher Scientific (USA). All solutions were filtered through a 0.45 µm membrane (Sartorious, Germany) prior to use.

Preparation of stock, standard working solutions and plasma samples

Standard stock solutions of delphinidin-3-O-glucoside chloride (100 µg/mL) and its internal standard (100 µg/mL) were prepared by dissolving 1 mg of each in 10 mL methanol, respectively. A working solution of the internal standard was prepared by diluting the stock solution of cyanidin-3-5-0-diglucoside chloride with mobile phase to give a final concentration of 500 ng/mL. Five selected delphinidin-3-0-glucoside chloride concentrations of 250, 500, 1000, and 2000, 3000 ng/mL in buffer were prepared by further dilution of the stock solution with appropriate volumes of mobile phase accordingly.

Blank plasma was obtained from untreated rats and 500 µL of plasma spiked with delphinidin-3-O-glucoside chloride to achieve five concentrations of 250, 500, 1000, and 2000, 3000 ng/mL for calibration curve. Quality control (QC) samples of low, medium, and high concentration (350, 1500, 2500 ng/mL) were prepared similarly to the standard calibration. All stock solutions were stored at 4 °C and all working solutions were freshly prepared daily.

Solid phase extraction

The plasma samples spiked with delphinidin-3-O-glucoside chloride and its internal standard were subjected to solid phase extraction to remove unwanted analytes or impurities using Water Sep-Pak tC18 cartridge (Massachusetts, USA). Protein precipitation of plasma sample was done by adding 500 µL of 0.1 % TFA aqueous solution and 100 µL of 20 % sulfosalicylic acid aqueous solution in 500 µL of plasma, vortex mixed and centrifuged at 4000 × *g* for 20 mins at 4˚C and supernatant was kept for further separation. Following that, the cartridge was first pre-conditioned with 2 mL of methanol followed by 2 mL of 0.1% TFA in aqueous solution. The supernatant plasma sample was then loaded into the cartridge and then eluted with 1 mL of methanol. Eluted sample was then centrifuged at 5000 rpm for 10 minutes using an Ultrafree®-MC centrifugal filter (Merck, Germany) and the supernatant was evaporated to dryness using nitrogen gas. Dried residue then was reconstituted with 100 µL of mobile phase and then injected into HPLC for analysis.

Method validation

Plasma calibration curves were prepared and analyzed in triplicate for intra-day and inter-day to assess the linearity, precision, accuracy, recovery, LOD, LOQ, selectivity, sensitivity, and stability according to the

CDER [12].

Results and Discussion

Chromatographic condition

Based on our team's previous optimization study [13], for this study, we chose a composition of ACN with 0.1% TFA in an aqueous solution (81:19, v/v) as a mobile phase. Although in prior research rapid resolution liquid chromatography analysis was conducted for the identification of another type of anthocyanin (cyanidin-3-O-glucoside), this protocol still produced a good peak of delphinidin-3-0-glucoside chloride content and effectively separated it from its internal standard (cyanidin-3-5-0-glucoside chloride).

Solid-phase extraction

Several parameters, such as percentage of recoveries, chromatographic peak shape obtained, and peak tailing, were some of the factors that needed to be considered when choosing a suitable extraction method. In this experiment, we thus chose solid-phase extraction rather than liquid-liquid extraction based on our team's previous optimization study [13]. Furthermore, as Sep-Pak tC18 cartridges are widely employed to purify anthocyanin due to their simplicity and efficiency [14-17], we incorporated Sep-Pak tC18 cartridges into our SPE extraction method.

Recovery

In this study, we obtained delphinidin-3-O-glucoside chloride recoveries using two different methods: area ratio and slope ratio. We quantified recovery by finding the ratio of the slopes of the calibration curve for the extracted (in plasma) to non-extracted (in buffer) samples. The recovery of delphinidin-3-O-glucoside chloride revealed the mean recovery percentage to be 80.377%, while the slope ratio was 71.43%. Generally, recovery need not be 100% when 80–90% recovery is considered optimal. Tables 1 and 2 summarize the percentage recoveries and their standard deviations (SD) at different extract concentrations.

The assay sample was calculated from the following equation 1:

$$\text{Recovery (\%)} = (\text{mean area ratio extracted} / \text{mean area ratio non-extracted}) \times 100 \quad (1)$$

Table 1. Recovery of delphinidin-3-O-glucoside chloride by area ratio

Concentration (ng/mL)	**Mean Area Ratio (Non-Extracted)**	**SD**	**Mean Area Ratio (Extracted)**	**SD**	**Percentage Recovery (%)**
3000	2.068	0.246	1.514	0.161	73.199
2000	1.387	0.275	1.035	0.258	74.662
1000	0.776	0.050	0.507	0.259	65.365
500	0.294	0.065	0.277	0.175	94.350
250	0.203	0.052	0.191	0.046	94.311
Mean					80.377 ±11.823

Table 2. Recovery of delphinidin-3-O-glucoside chloride by ratio of slopes

	Combined Standard Curve (Non-Extracted)	**Combined Standard Curve (Extracted)**
Intercept, a	-0.0097	0.0437
Slope, b	0.0007	0.0005
R^2	0.9648	0.9653
Recovery by the ratio of slope	0.0005/0.0007 = 71.43 %	

Calibration curve, precision, accuracy, and linearity
The concentration of a drug in an unknown sample can be determined using calibration curves to compare the unknown concentration to a set of standard samples with known concentrations [18]. The calibration curve of delphinidin-3-O-glucoside chloride was linear in the concentration range (250 ng/mL, 500 ng/mL, 1000 ng/mL, 2000 ng/mL, and 3000 ng/mL) in rat plasma. Five concentrations were selected to produce a calibration curve for delphinidin-3-O-glucoside on intraday (Figure 2) and inter-days (Figure 3). Five to eight points should make up the calibration curve to cover the expected analyte concentration range in the test samples [19-20].

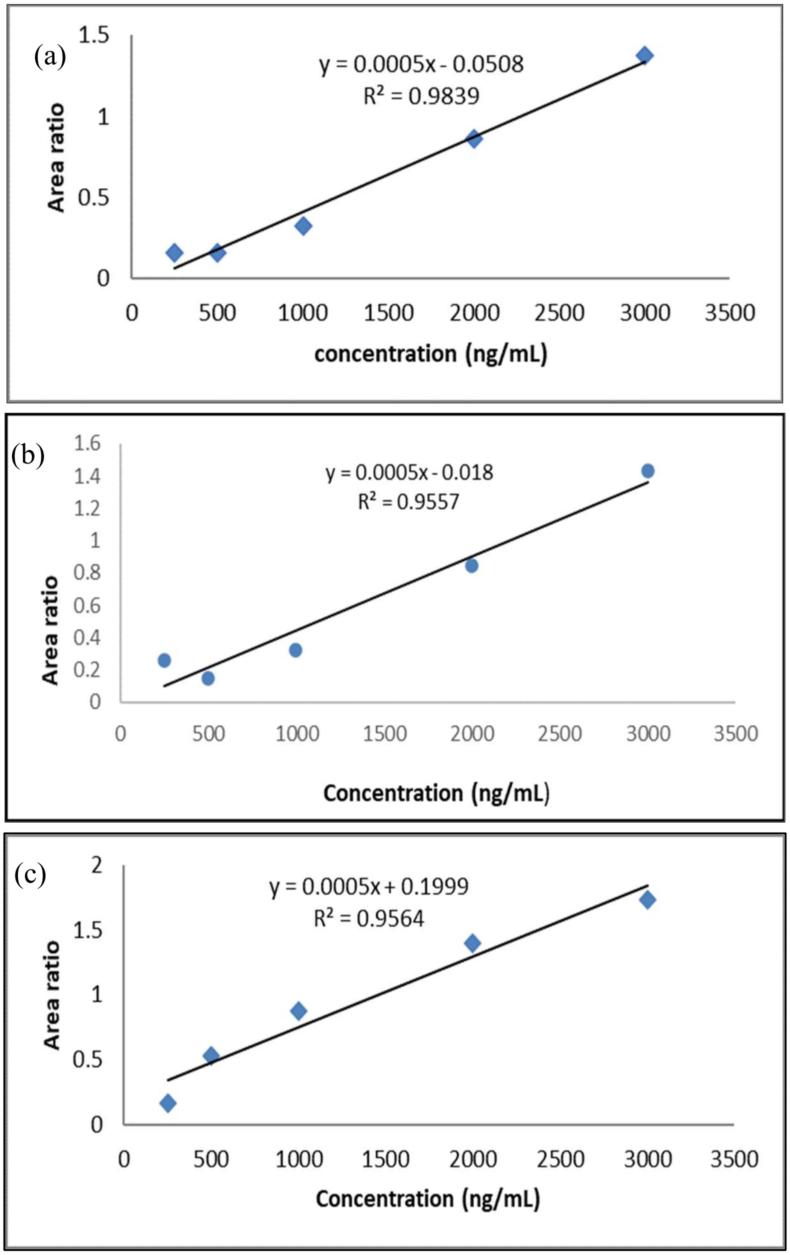

Figure 2. Intra-day validation: calibration curves of the area ratio of delphinidin-3-O-glucoside chloride to cyanidin-3-5-0-glucoside chloride versus delphinidin-3-O-glucoside chloride's concentrations a) validation 1, b) validation 2, and c) validation 3.

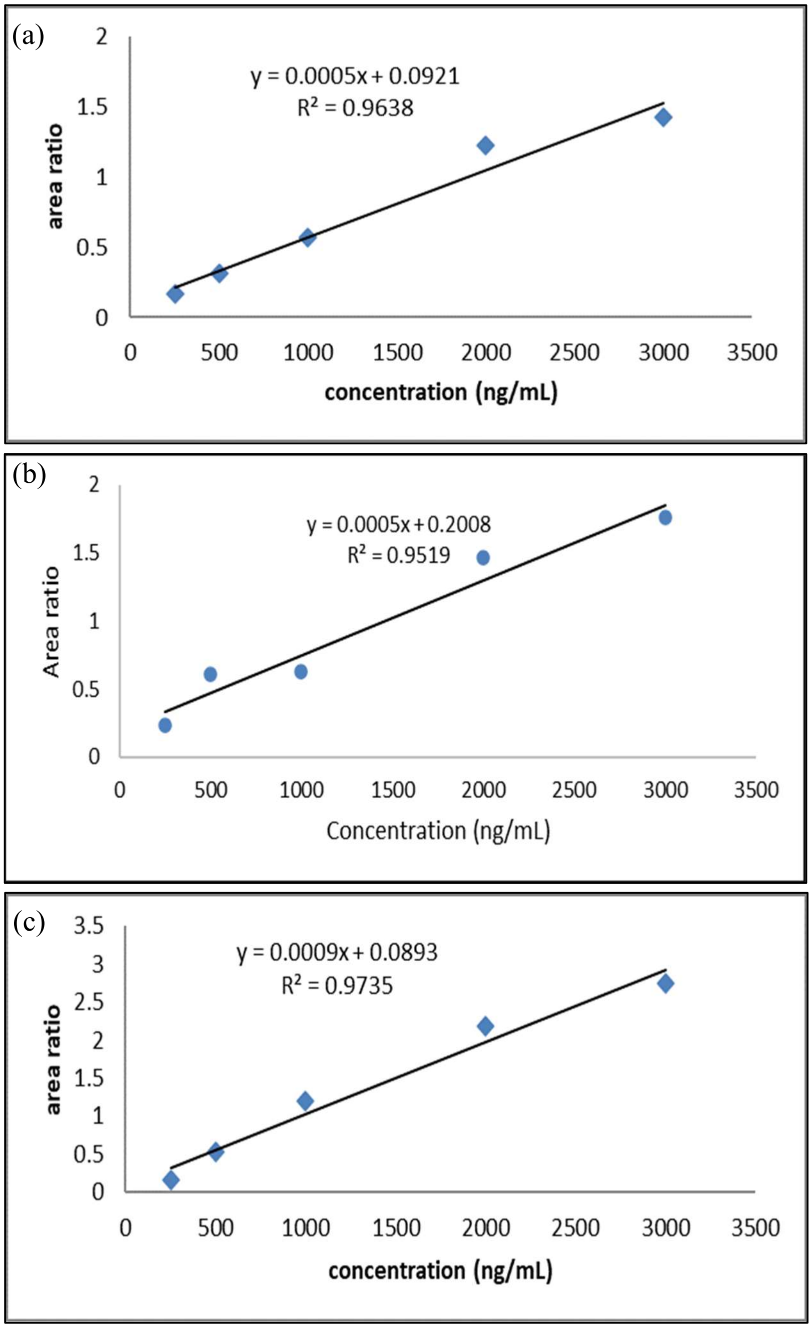

Figure 3. Inter-day validation: calibration curves of the area ratio of delphinidin-3-O-glucoside chloride to cyanidin-3-5-0-glucoside chloride versus delphinidin-3-O-glucoside chloride's concentrations a) validation day, 1 b) validation day 2, and c) validation day 3

The precision and accuracy of the assay were determined from the low (350 ng/mL), medium (1500 ng/mL), and high (2500 ng/mL) QC plasma samples. Intra- and inter-day were determined for each QC rat plasma sample, with each sample in triplicates for each day, and expressed as a percentage of the coefficient of variation (CV). The assay's mean analyte recovery rate served as the accuracy definition. The precision determined at each concentration should not exceed 15% CV, except for LOQ, which should not exceed 20%

CV, according to the CDER (2018). It also strongly advises against using accuracy and precision data independently, especially when it comes to drugs. The precision and accuracy results determined for the HPLC method's intra- and inter-day values are shown in Table 3.

Table 3. Precision and accuracy of delphinidin-3-O-glucoside chloride in intraday

Concentration (ng/mL)	**Mean (ng/mL)**	**SD**	**Precision (CV)**	**Accuracy (%)**	**Percentage Difference**
Delphinidin-3-O-glucoside chloride intraday (within batch) (*n*=3)					
2500	2495	0.03	2.72	99.8	- 0.2
1500	1305	0.05	8.12	87.0	- 13.0
350	389	0.005	3.36	87.1	12.86
Delphinidin-3-O-glucoside chloride intraday (between batch) (*n*=9)					
2500	2315	0.12	9.84	92.6	- 7.4
1500	1616	0.07	7.63	92.3	7.73
350	341	0.01	4.89	97.4	- 2.57

The lowest analyte concentration in a sample that may be quantitated is known as LOD, although it is not always possible to determine it under certain experimental conditions. In contrast, the LOQ in the calibration curve can be consistently measured with relative standard deviation (RSD; 20%) and precision (80-120%). In this study, LOD and LOQ were 47 ng/mL and 213 ng/mL, respectively. However, some research findings on delphinidin's LOD and LOQ are far different from these values. For instance, a recent study on the determination of delphinidin-3-O-glucoside in plasma using ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC-MS) found LOD to be 2.3 ng/mL and LOQ to be 8.1 ng/mL [21]. Another study proposing the determination of anthocyanin and non-anthocyanin in Jussara extracts via UPLC-MS showed that the LOD and LOQ of delphinidin-3-O-glucoside were 5.60 µg/L and 18.50 µg/L, respectively [22]. A third study found the LOD to be 0.14 mg/kg and LOQ to be 0.42 mg/kg for delphinidin-3-O-glucoside in grape extract [23].

Conclusion

In conclusion, this developed and validated HPLC method coupled with SPE for sample preparation is rapid and convenient for the quantification of delphinidin-3-O-glucoside chloride in rat plasma samples. The calibration curve of delphinidin-3-O-glucoside chloride was linear in the concentration range. Intra- and inter-day validation showed a good coefficient of determination above 0.95. The mean recovery was 80.377%, while the slope ratio was 71.43%. This study therefore showed good precision and accuracy data for delphinidin-3-O-glucoside chloride in rat plasma. Except for LOQ, which should not exceed 20% of the CV, CDER [15] states that the precision determined at each concentration should not be greater than 15% of the CV. The LOD and LOQ were 47 ng/mL and 213 ng/mL, respectively. To conclude, the validations in this study indicate that these findings, as determined via HPLC, are achievable, reproducible, and reliable for further studies of anthocyanin pharmacokinetics and bioavailability in rat plasma.

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