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Determination of flavonoids from *Orthosiphon stamineus* in plasma using a simple HPLC method with ultraviolet detection

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Abstract

A simple liquid chromatographic method was developed for the simultaneous determination of flavonoids from *Orthosiphon stamineus* Benth, namely sinensitin, eupatorin and 3′-hydroxy-5,6,7,4′-tetramethoxyflavone, in plasma. Prior to analysis, the flavonoids and the internal standard (naproxen) were extracted from plasma samples using a 1:1 mixture of ethyl acetate and chloroform. The detection and quantification limits for the three flavonoids were similar being 3 and 5 ng/ml, respectively. The within-day and between-day accuracy values, expressed as percentage of true values, for the three flavonoids were between 95 and 107%, while the corresponding precision, expressed as coefficients of variation, for the three flavonoids were less than 14%. In addition, the mean recovery values of the extraction procedure for all the flavonoids were between 92 and 114%. The calibration curves were linear over a concentration range of 5–4000 ng/ml. The present method was applied to analyse plasma samples obtained from a pilot study using rats in which the mean absolute oral bioavailability values for sinensitin, eupatorin and 3′-hydroxy-5,6,7,4′-tetramethoxyflavone was 9.4, 1.0 and 1.5%, respectively.
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Keywords: Orthosiphon; Flavonoids; Sinensitin; Eupatorin; 3′-Hydroxy-5,6,7,4′-tetramethoxyflavone; HPLC

1. Introduction

Orthosiphon stamineus, Benth (Lamiaceae) is a popular medicinal plant in Southeast Asia and is widely used for the treatment of various diseases, especially those affecting the urinary tract [1]. Several classes of compounds have been identified in this plant and they included flavonoids, terpenoids, saponins, hexoses, organic acids, caffeic acids derivatives, chromene and myo-inositol [2–6]. Among these compounds, the flavonoids and caffeic acid derivatives were found to possess potential therapeutic properties, as they were shown to exert diuretic and uricosuric actions in rats [6]. The three main flavonoids found in *O. stamineus* were sinensitin, eupatorin and 3′-hydroxy-5,6,7,4′-tetramethoxyflavone [2,7,8] and they were also shown to possess cytotoxic [2], antifungal [9] and antioxidant activities [10,11]. Moreover, sinensitin has recently been reported to reverse the P-glycoprotein-mediated multidrug resistance in the absorption of drugs [12].

Despite the growing interest in these flavonoids, there is a paucity of information regarding to their absorption and disposition. Studies to yield such data would require sensitive and specific analytical methods for determination and quantification of these flavonoids in plasma. Several HPLC methods for the analysis of these flavonoids have been reported in the literature [3,8,10,13]. However, they were mainly developed for analysing the compounds in plant extracts and not in plasma. Moreover, the sensitivity of these methods, which was reported to be approximately 100 ng/ml [8,10,13], might not be sufficiently sensitive for their pharmacokinetic studies. Additionally, all these methods [3,8,10,13] have a long run time of 25–50 min. Therefore, the aim of this study was to develop a specific, sensitive and simple HPLC method using ultraviolet detection for simultaneous determination of sinensitin, eupatorin and 3′-hydroxy-5,6,7,4′-tetramethoxyflavone

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in plasma. We also demonstrated the applicability of this method in a bioavailability study of the compounds in rats.

2. Materials and methods

2.1. Materials

Standardized *O. stamineus* Benth (orthosiphon) extract (with 0.15, 0.21 and 0.05%; (w/w) of sinensitin, eupatorin and 3′-hydroxy-5,6,7,4′-tetramethoxyflavone, respectively) was a token from School of Pharmaceutical Sciences, Universiti Sains Malaysia. Sinensitin, eupatorin and 3′-hydroxy-5,6,7,4′-tetramethoxyflavone standards were purchased from Indofine (New Jersey, USA). Hydrochloric acid (HCl) was purchased from Mallinckrodt Bakers (Edo de Mex, Mexico). Sodium dihydrogen phosphate dihydrate ($NaH_2PO_4{\cdot}2H_2O$) was purchased from Merck (Darmstadt, Germany). All of the other solvents used were either of analytical reagent grade or of HPLC grade and were purchased from Merck (Auburn, Australia).

2.2. Instrumentation

The HPLC system comprised a Jasco PU-980 pump (Jasco, Hachioji City, Tokyo, Japan), a Gilson UV–vis 119 detector (Middleton, WI, USA) and a Hitachi D-2500 chromato-integrator (Hitachi, Tokyo, Japan). The detector was operated at an ultraviolet wavelength detection of 333 nm and the sensitivity of the detector was set at 0.005 AUFS. A Zorbax C18 (Agilent Technologies, USA) column (5 μm, 250 mm × 4.6 mm i.d.), fitted with a refillable guard column (Upchurch Scientific, Oak Harbour, WA, USA) and packed with Perisorb RP-18 (30–40 μm, pellicular, Upchurch Scientific, Oak Harbour, WA, USA), was used for the chromatographic separation. The temperature of the column was maintained at room temperature (25 °C). The mobile phase comprised isopropyl alcohol, acetonitrile and 0.02 M sodium dihydrogen phosphate (17:25:58, v/v) and was adjusted to pH 4 with 85% of phosphoric acid. Analysis was run at a flow rate of 0.8 ml/min and the samples were quantified using peak height.

2.3. Sample preparation

A 250 μl aliquot of plasma sample was measured into a microcentrifuge tube (Eppendorf, Hamburg, Germany), followed by the addition of 100 μl of 20 μg/ml of naproxen as the internal standard, 100 μl of 5 M HCl and 1.25 ml of a mixture of ethyl acetate and chloroform (1:1, v/v) extracting solvent. The mixture was vortex-mixed for 1 min using a vortex mixer (Thermolyne, Iowa, USA) and then centrifuged at 12,800 × *g* (Eppendorf, Hamburg, Germany) for 10 min. The organic layer was transferred into a new microcentrifuge tube and blow-dried under a gentle stream of nitrogen gas at ambient room temperature (approximately 25 °C). The residue was then reconstituted with 100 μl of mobile phase, vortex-mixed for 1 min and 50 μl was injected onto the column.

2.4. Assay validation

Samples were quantified using peak height ratio of each of the flavonoids over the internal standard (naproxen). Standard calibration curves were constructed by spiking flavonoid-free pooled human plasma with a known amount of sinensitin, eupatorin and 3′-hydroxy-5,6,7,4′-tetramethoxyflavone at a concentration range of 5–4000 ng/ml. These plasma standards were also used for the determination of the extraction recovery, within-day and between-day precision and accuracy ($n = 6$) of the method. The recovery of the extraction procedure for the flavonoids and internal standard was calculated by comparing the peak height of the flavonoids and internal standard obtained after extraction, with that of the flavonoids' standard solutions (in mobile phase) of the corresponding concentrations without extraction. The limit of detection was defined as the lowest concentration with detectable response while the limit of quantification was determined as the lowest quantifiable concentration with satisfactory between-day and within-day precision and accuracy of less than 20% for both coefficient of variation and percentage error [14]. The standards were stored at −20 °C in amber bottles and were found to be stable for at least 3 months without any appreciable decrease in the concentration of the flavonoids.

2.5. Evaluation of human plasma and rat plasma matrices

To determine the specificity of the method for analysing the flavonoids in rat as well as human plasma samples, blank plasma of both were analysed to demonstrate the absence of interfering endogenous peaks. In addition, standard calibration curves constructed from spiking both blank rat and human plasma with known concentrations of 10, 125, 500 and 2000 ng/ml of each of the flavonoids were compared. Both sets of calibration curves were prepared in triplicates. The purpose of such a comparison was to examine whether blank human plasma could be used to prepare the calibration curves in the analysis of plasma samples obtained from rats since human plasma is more available.

2.6. Pilot bioavailability study

This study was approved by the Ethics Committee on Animal Studies, Universiti of Sains, Malaysia. The experiment was conducted using six adult male Sprague-Dawley rats (mean = 249 g, S.D. = 18) according to a two-way crossover study design with a wash out period of 1 week. On the first occasion, one group of the rats was administered orally with the standardized orthosiphon extract, while the other group received intravenous administration of a mixture containing the three flavonoids. After a washout period of 1 week, the

two groups received the alternative preparations with the respective routes of administration. The oral dose was approximately 15, 21 and 5.0 mg/kg of sinensitin, eupatorin and 3′-hydroxy-5,6,7,4′-tetramethoxyflavone, respectively, while the intravenous dose was approximately 2.5 mg/kg for each of the flavonoids.

3. Results

Fig. 1A–C shows the chromatograms of the blank human plasma, blank rat plasma and human plasma spiked with 250 ng/ml of each of the sinensitin, eupatorin and 3′-hydroxy-5,6,7,4′-tetramethoxyflavone, while Fig. 1D shows the chromatogram of the rat plasma after intravenous administration of 2.5 mg/kg of each of the pure sinensitin, eupatorin and 3′-hydroxy-5,6,7,4′-tetramethoxyflavone. It can be seen that the peaks of all the flavonoids were well separated and free of interference from endogenous compounds in both the human and rat plasma. Moreover, Fig. 1C shows that no major peak was detected after the naproxen peak, which eluted at approximately 17.7 min, when the chromatogram was run until 35 min. Thus, there would be no carryover peaks from one sample to the next. Mean retention times for 3′-hydroxy-5,6,7,4′-tetramethoxyflavone, sinensitin, eupatorin and naproxen were 7.39 ± 0.03, 9.62 ± 0.06, 11.15 ± 0.02 and 17.72 ± 0.03 min, respectively. The total run time for each injection was approximately 19 min, being shorter than those of the other reported methods [3,8,10,13].

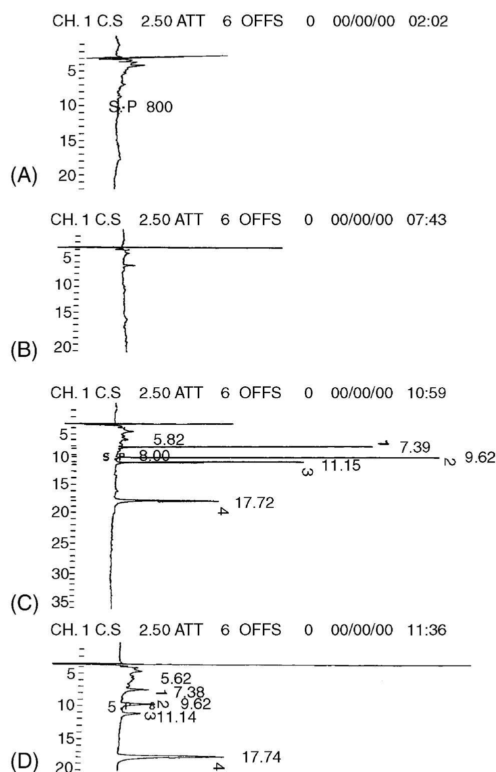

Fig. 1. Chromatograms for the analysis of sinensitin, eupatorin and 3′-hydroxy-5,6,7,4′-tetramethoxyflavone in plasma. (A) Blank human plasma; (B) blank rat plasma; (C) human plasma spiked with 250 ng/ml of sinensitin, eupatorin and 3′-hydroxy-5,6,7,4′-tetramethoxyflavone with the run time extended to 35 min; (D) a rat plasma containing 26.7, 19.5 and 20.4 ng/ml of sinensitin, eupatorin and 3′-hydroxy-5,6,7,4′-tetramethoxyflavone, respectively 1.5 h after intravenous administration of 2.5 mg/kg of mixed sinensitin, eupatorin and 3′-hydroxy-5,6,7,4′-tetramethoxyflavone (y-axis, attenuation = 6; x-axis, chart speed = 2.5 mm/min; peaks: 1, 3′-hydroxy-5,6,7,4′-tetramethoxyflavone; 2, sinensitin; 3, eupatorin; 4, naproxen).

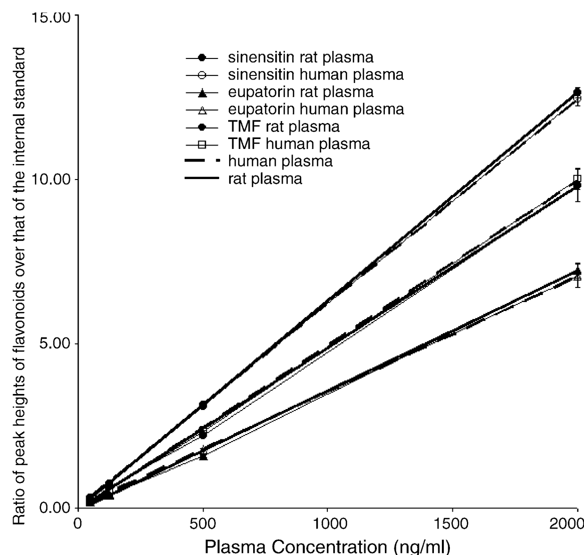

Fig. 2. Mean standard calibration curves of sinensitin, eupatorin and 3′-hydroxy-5,6,7,4′-tetramethoxyflavone (TMF) (mean ± S.D., $n = 3$) prepared using blank rat plasma and blank human plasma.

Fig. 2 shows the mean standard calibration curves of the flavonoids prepared using blank rat and human plasma, respectively. It can be seen that for all three flavonoids, the plots obtained with both matrices were essentially superimposable. Hence, blank human plasma could be used to prepare the calibration curves for the analysis of rat plasma samples and vice versa.

Tables 1–3 show the extraction recovery, within-day and between-day accuracy and precision values for sinensitin, eupatorin and 3′-hydroxy-5,6,7,4′-tetramethoxyflavone, respectively. It can be seen that the accuracy of the assay method for the three flavonoids at all the concentrations evaluated were between 95 and 107%, while the corresponding precision, expressed as the coefficient of variation (C.V.), were all less than 14%. Moreover, the mean extraction recovery values for the flavonoids at the concentrations studied were between 92 and 114%, while that of the internal standard (naproxen) was 88.4 ± 5.6%. The lowest concentration detectable for the three flavonoids was similar, being approximately 3 ng/ml and the limit of quantification for the three flavonoids was also similar being 5 ng/ml. The standard calibration curves ($n = 6$) were found to be linear over the concentration range of 5–4000 ng/ml for all the flavonoids with mean slopes of 311.3 ± 18.8, 522.1 ± 7.7 and 380.5 ± 18.0, respectively for sinensitin, eupatorin and 3′-hydroxy-5,6,7,4′-

Table 1
Extraction recovery, within-day and between-day precision and accuracy values ($n = 6$) for sinensitin

Concentration (ng/ml)	Recovery		Within-day		Between-day	
	Mean (%)	C.V. (%)	Accuracy (% of true value)	Precision (C.V.) (%)	Accuracy (% of true value)	Precision (C.V.) (%)
5	103.4	3.9	105.5	7.3	99.7	5.7
10	95.4	8.8	103.7	8.1	106.1	6.9
50	95.1	2.8	98.5	1.0	96.5	3.8
250	102.7	4.1	96.0	2.0	99.6	2.9
500	104.4	3.5	96.4	1.8	98.0	2.9
1000	94.3	3.3	95.6	1.6	101.6	1.6
2000	103.8	3.0	100.1	2.4	99.4	1.4
4000	102.0	4.8	95.6	2.7	99.4	2.7

Table 2
Recovery, within-day and between-day precision and accuracy values ($n = 6$) for eupatorin

Concentration (ng/ml)	Recovery		Within-day		Between-day	
	Mean (%)	C.V. (%)	Accuracy (% of true value)	Precision (C.V.) (%)	Accuracy (% of true value)	Precision (C.V.) (%)
5	104.1	5.3	106.6	6.9	106.7	5.3
10	99.4	12.6	104.4	13.3	103.3	10.2
50	95.9	3.9	100.0	9.5	94.7	2.0
250	101.7	4.8	98.3	3.5	97.9	3.4
500	102.2	3.7	100.1	1.6	97.6	5.2
1000	92.8	5.0	95.2	2.1	100.0	3.3
2000	102.2	3.3	99.3	2.5	100.0	1.5
4000	99.2	5.1	96.6	2.9	99.5	3.5

Table 3
Recovery, within-day and between-day precision and accuracy values ($n = 6$) for 3′-hydroxy-5,6,7,4′-tetramethoxyflavone

Concentration (ng/ml)	Recovery		Within-day		Between-day	
	Mean (%)	C.V. (%)	Accuracy (% of true value)	Precision (C.V.) (%)	Accuracy (% of true value)	Precision (C.V.) (%)
5	101.9	3.6	105.4	4.7	108.4	5.4
10	113.3	6.5	100.4	12.8	104.1	6.7
50	98.1	3.2	97.4	6.1	96.9	2.6
250	104.0	3.0	97.4	2.6	99.4	2.1
500	106.1	3.1	97.7	0.7	98.0	2.9
1000	93.6	3.3	93.8	1.6	100.7	1.3
2000	103.6	2.9	100.1	3.1	99.9	1.6
4000	102.2	4.6	96.7	2.6	100.0	3.0

tetramethoxyflavone, while their respective mean intercept values were -0.59 ± 1.28, -0.51 ± 0.99 and 0.01 ± 0.57. In addition, the mean correlation coefficient values of the three flavonoids were always greater than 0.9999.

Figs. 3 and 4 show the mean plasma profiles of the sinensitin, eupatorin and 3′-hydroxy-5,6,7,4′-tetramethoxyflavone after intravenous (i.v.) and peroral administration of 2.5 mg/kg and 10.0 g/kg, respectively of each of the flavonoids. It can be seen that the plasma levels of the flavonoids given intravenously were much higher than those after oral administration, even though the i.v. doses were much lower, indicating that the oral bioavailability of the three flavonoids was low and incomplete. The calculated mean absolute oral bioavailability value for sinensitin was $9.4 \pm 2.6\%$, while that for eupatorin and 3′-hydroxy-5,6,74′-tetramethoxyflavone were very low being $1.0 \pm 0.9\%$ and $1.5 \pm 2.5\%$, respectively.

4. Discussion

From the results, it is clearly shown that the method is specific with no interference from endogenous peaks or carryover peaks for both rat and human plasma in the analysis of the three flavonoids. Moreover, the standard calibration curves prepared in both rat and human plasma were almost superimposable and hence could be substituted with one another. Since human plasma is more readily available, it was

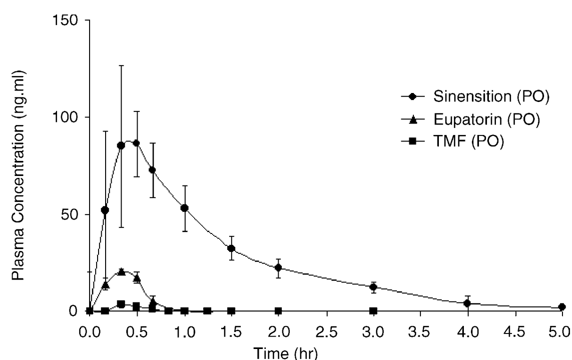

Fig. 3. Mean plasma concentration–time profiles (mean ± S.E.M., $n = 6$) of sinensitin, eupatorin and 3′-hydroxy-5,6,7,4′-tetramethoxyflavone (TMF) after oral administration of 10.0 g/kg standardized *O. stamineus* extract (equivalent to 15.0, 21.0 and 5.0 mg/kg of sinensitin, eupatorin and TMF, respectively) (PO: peroral administration).

used to prepare standard calibration curves and samples for validation of this method.

Naproxen was chosen as the internal standard as it had an optimal absorbance at the wavelength used and a suitable elution time. To date, no author has reported a suitable internal standard for their extraction method. The extraction procedure involved liquid–liquid extraction using ethyl acetate and chloroform (1:1, v/v) as the extraction solvent. Ethyl acetate alone was sufficient for extracting the three flavonoids from the plasma but gave a poor recovery of about 20% for naproxen (internal standard). On the other hand, chloroform with 5 M hydrochloric acid yielded a recovery of more than 90% for naproxen but resulted in poor recoveries of approximately 20–40% for the three flavonoids. A mixture of ethyl acetate and chloroform at a ratio of 1:1 (v/v) with 5 M hydrochloric acid was found to be most suitable as it was able to achieve optimal recovery of more than 90% for all the flavonoids and the internal standard.

Initially, when a mobile phase comprising 30% of acetonitrile and 70% of sodium dihydrogen phosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$) buffer at pH 4 was used, a long run time of more than 25 min was required for satisfactory separation of the flavonoids. Also, the peaks of the three flavonoids were found to be broad. Varying pH of the mobile phase from 3.5 to 5 has no effect on the retention times as well as the peak shape of the flavonoids. Increasing the acetonitrile content in the mobile phase to 50% was able to reduce the retention times of the flavonoids but the peak shapes of the three flavonoids remained broad with severe tailing observed for the eupatorin peak. A further increase in the acetonitrile content to more than 50% in the mobile phase resulted in poor resolution of the three flavonoid peaks. The addition of 20% of isopropyl alcohol to the initial composition of the mobile phase of 30% of acetonitrile and 70% of buffer, was able to shorten the elution time of the three flavonoids without affecting the resolution of the three flavonoid peaks. Moreover, the peak shape of all three flavonoids especially that of eupatorin was greatly improved. However, further increase in the isopropyl alcohol content to 25%, resulted in poor resolution of the three flavonoid peaks, as well as a sharp increase in the backpressure of the column. Therefore, a composition of isopropyl alcohol:acetonitrile:buffer at a proportion of 17:25:58 (v/v) was deemed optimal and chosen in the present study.

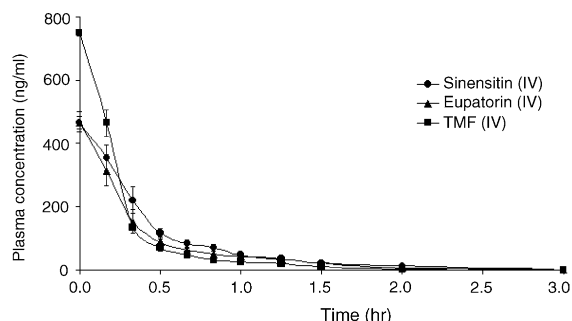

Fig. 4. Mean plasma concentration–time profiles (mean ± S.E.M., $n = 6$) of sinensitin, eupatorin and 3′-hydroxy-5,6,7,4′-tetramethoxyflavone (TMF) after intravenous administration of 2.5 mg/kg of each pure flavonoids, respectively. (IV: intravenous administration).

From the pilot bioavailability study in rats, it was demonstrated that the present method is simple and suitable for the analysis of sinensitin, eupatorin and 3′-hydroxy-5,6,7,4′-tetramethoxyflavone in plasma. It was also observed that the oral bioavailability of the three flavonoids was very poor and erratic being approximately 10% for sinensitin and less than 2% for both eupatorin and 3′-hydroxy-5,6,7,4′-tetramethoxyflavone.

In conclusion, the present HPLC method is simple, sensitive and specific for simultaneous determination of sinensitin, eupatorin and 3′-hydroxy-5,6,7,4′-tetramethoxyflavone in plasma obtained from bioavailability/pharmacokinetic studies.

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