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Improved LC methods for the determination of diosmin and/or hesperidin in plant extracts and pharmaceutical formulations

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Abstract

Two improved, simple, selective and sensitive reversed-phase HPLC methods were developed for the determination of diosmin and hesperidin simultaneously or separately, using external or internal standard techniques, respectively. The methods were applied for determination of the diosmin and/or hesperidin in Buchu leaves and pharmaceutical dosage forms (three tablets). The methods involved very simple efficient extraction procedures, the use of a LiChrosorb RP-18 column, a mixture of methanol–water (60:40, v/v) as a mobile phase in an isocratic mode at a flow rate of 1.5 ml/min and UV detection at 345 nm. In all HPLC analysis the relative standard deviation did not exceed 1.9%. As low as 50 and 25 ng (per injection) could be detected in less than 6 min with a high degree of accuracy. This is the first report for the determination of the two flavonoids simultaneously in Buchu leaf and tablets containing them.

Keywords: High-performance liquid chromatography; Diosmin and hesperidin; Buchu leaves; Pharmaceutical formulations

1. Introduction

Diosmin is the major active constituent of Buchu leaf (*Barosma betulina* (Thunb.) Bartl. and Wendle, Rutaceae), which was introduced into Europe early in the 19th century as a diuretic and as a urinary antiseptic [1–3]. Diosmin **1** is chemically defined as the 7-rhamnoglucoside of 5,7,3′-trihydroxy-4′-methoxyflavone and its flavanone analog hesperidin **2**, are common flavonoids in Rutaceae plants. Both flavonoids are widely used for their phlebotonic and antioxidant properties and as vascular protectors [4–11]. Recent clinical studies demonstrated the use of diosmin and hesperidine for the treatment of venous leg ulcer and hemorrhoid [6], as chemopreventive agents in urinary-bladder [12] and colon [13] carcinogenesis. Both diosmin and hesperidin showed a very good tolerability and are considered quite safe, non-toxic drugs [6] (Scheme 1).

In view of the increasing interest in these bioflavonoids, especially for the treatment of chronic venous insufficiency, chronic hemorrhoids

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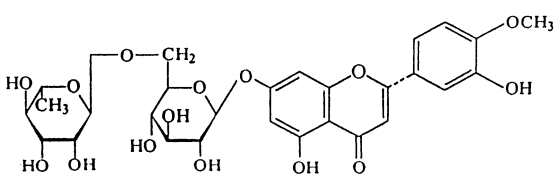
and as antioxidants, several methods have been reported for the determination of diosmin and/or hesperidine in plant extracts [14,15], biological fluids [15,16], and pharmaceutical formulations [14]. However, disadvantages of these methods as previously discussed in references [14] and [15] included lack of simplicity and accuracy as they involved extensive chromatographic procedures for purification followed by fluorimetric, colorimetric or GC assays, as previously discussed. Some HPLC methods have also been reported for the determination of diosmin or hesperidin as single components. Nevertheless, these methods suffer one or more of the following disadvantages: (a) the use of acids, other additives and/or tertiary or quaternary mixtures in the mobile phase [14,15], (b) they require inconvenient gradient elution and hence long elution time [15], (c) they do not involve internal standard [14,16], (d) they were applied only for biological fluids [16], (e) the use of strong alkali (e.g. NaOH) for extraction of the flavonoids.

Recently (in 1999), an HPLC method for the simultaneous determination of diosmin and hesperidin in flavonoid extracts and soft gelatin capsules was published [14]. This method can be criticized as it involves the use of sodium hydroxide solution for extraction, and sulphonic acid sodium salt in the mobile phase, which required pH adjustment with phosphoric acid, and also lacked the advantages of using internal standard [17].

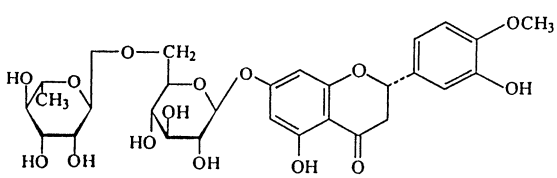
The use of strong alkalis (e.g. NaOH) for extraction may convert flavanones (e.g. hesperidin) to their corresponding chalcone derivatives [18,19] (this effect may be reversed by acids). This is also apparently applied for any flavanones that may exist in plant extracts (e.g. Buchu extract), causing misleading results. In addition, the use of acid in the mobile phase necessitated pH adjustment (the proposed methods do not involve alkalis or acids during the whole extraction and analysis process, using simple isocratic elution with no need for the inconvenient pH adjustment).

Moreover, no reports on HPLC methods for the analysis of diosmin and/or hesperidin in Buchu leaf (the original natural source of diosmin) or in the widely used tablet containing either one or both flavonoids are available.

This study is to establish an improved, simple, efficient and reliable HPLC method for the quantification of diosmin alone using an internal standard technique and another method for the simultaneous determination of diosmin and hesperidin in Buchu leaf and pharmaceutical dosage forms, using an external standard method. It should be stressed that this is the first report for the determination of diosmin and hespridin in Buchu leaf using HPLC technique. The official (Pharmacopial) methods [3,20] for evaluation of Buchu leaf are ambiguous (depending on the microscopical characters, extractive values or determination of the volatile oil percentage).



I, Diosmin



II, Hesperidin

Scheme 1.

2. Experimental

2.1. Materials

All solvents were of the HPLC grade and were purchased from Merck (Germany), and were degassed and filtered through Durapore filters 0.22 $\mu \times 47$ mm using a Waters solvent clarification kit. Diosmin and hesperidin were purchased from Aldrich Chemical Company and were checked for purity by determining their

physical constants and by spectral and HPLC analyses. All other reagents were commercial products of the analytical grade. Buchu leaf (*Barosma betulina*, Rutaceae) was kindly provided by Professor Dr. M. M. El-Sherei, Professor of Pharmacognosy, Faculty of Pharmacy, Cairo University.

2.2. Pharmaceutical dosage forms

2.2.1. Dioven® tablets

A product of Amriya Rhone-poulenc Pharmaceutical Industries Co. (Alexandria, Egypt). The stated composition is each tablet contains diosmin 150 mg.

2.2.2. Diosed® tablets

A product of SEDICO (South Egypt Drug Industries Co., 6 October City, Egypt). The stated composition is each tablet contains diosmin 150 mg.

2.2.3. Daflon® tablets

A product of Servier Egypt Industries Limited, 6th October City, Guiza, A.R.E.; under license of Les Laboratories Servier, France. The stated composition is each sugar-coated tablet contains 0.375 g flavonoid extract of Rutaceae (Aurantiae) equivalent to 150 mg diosmin excipient q.s.

2.3. Instrument and operating conditions

All chromatographic analyses were performed using a Waters HPLC system (Waters Associate Inc.), equipped with: Waters 515 HPLC pump attached to a Model 680 Automated Gradient Controller; a Waters 2487 Dual λ Absorbance detector; a Waters 746 data module and Rheodyne 7725*i* injector. The column was a reversed-phase LiChrosorb 10μ, RP 18 (Kontron Analytical) stainless steel column (25 cm × 4.6 mm i.d.). The mobile phase consisted of a mixture of methanol–water (60: 40, v/v), at a flow rate of 1.5 ml/min, and the column effluent was monitored at 345 nm. All chromatographic analyses were conducted isocratically and at ambient temperature.

2.4. Preparation of stock solutions

Stock solutions of diosmin and hesperidin were prepared separately at a concentration of 1 mg/ml, using 10 % dimethylsulfoxide in methanol.

2.4.1. Calibration graph for determination of diosmin alone using hesperidin as internal standard

Standard solutions containing diosmin at 5, 10, 25, 50 and 100 μg/ml and hesperidin as internal standard at 25 μg/ml were prepared using the previously prepared stock solutions. A 10 μl volume of each standard solution was injected in triplicates onto the HPLC column. Calibration for diosmin was constructed by plotting the ratio of peak area of diosmin to that of the internal standard versus the concentration of diosmin. This curve was used to determine the diosmin content in Diosed® and Dioven® tablets (both contain diosmin but no hesperidin).

2.4.2. Construction of calibration graph for simultaneous determination of diosmin and hesperidin using external standard technique

Standard solutions of diosmin (at concentrations of 5, 10, 25, 50, 100 μg/ml), and hesperidin (at concentrations of 5, 10, 25, 50, 75 μg/ml) were separately prepared using the previously prepared stock solutions. A 10 μl volume of each standard solution was injected in triplicates onto the HPLC column. Two calibration graphs, one for diosmin and the other for hesperidin, were constructed by plotting the peak area against concentration of diosmin and hesperidin, respectively.

2.5. Determination of diosmin and heperidin in Buchu leaves

One gram of powdered Buchu leaves was refluxed with 4 × 25 ml of methanol containing 10% dimethylsulfoxide, for 15 min each time. The combined extract was filtered and the volume was adjusted to 100 ml using methanol. Of this solution 1 ml was transferred to a 10 ml volumetric flask and the volume was adjusted to 10 ml with methanol. An appropriate volume (usually 10 μl) was injected onto the HPLC column. Diosmin

and hesperidin were calculated using the preconstructed calibration graphs (external standard method).

2.6. Determination of diosmin and hesperidin in Daflon® tablets

Ten Daflon® tablets were accurately weighed and finely powdered. A weight equivalent to one tablet was refluxed with 3 × 50 ml of 10% DMSO in methanol for 5 min each time. The combined extract was filtered and the volume was adjusted to 150 ml. Of the later solution 1 ml was transferred to a 10 ml volumetric flask and the volume was brought up to mark with methanol to yield a theoretical concentration of diosmin of 1 mg/ml. A suitable volume (usually 10 μl) of this solution was injected onto the HPLC column, concentrations of diosmin and hesperidin were obtained from the preconstructed calibration graphs.

2.7. Determination of diosmin in Diosed® and Dioven® tablets

The same procedure used for sample preparation of Daflon® tablet was used. However, hesperidin (as internal standard), was added to the final working solution at a concentration of 25 μg/ml. Diosmin content was calculated using the preconstructed calibration graph (internal standard technique).

The employed extraction procedures of diosmin and hesperidin from Buchu leaves or tablets were efficient, as no traces of either of the two flavonoids was detected in a further extraction (as tested by HPLC), a fact that confirmed complete extraction of diosmin and/or hesperidin.

3. Results and discussion

The initial purpose of this study was to develop an improved HPLC method for the determination of diosmin in its original natural source (Buchu leaf) and in pharmaceutical dosage forms containing diosmin as the only flavonoid (e.g. Diosed® and Dioven® tablets), using hesperidin as internal standard. This study, however, revealed the occurrence of hesperidin (in addition to diosmin) in Buchu leaf extract (it should be noted that there have been no previous reports indicating the presence of hesperidin in Buchu leaf). Therefore, we have also developed a second external standard HPLC method suitable for the simultaneous determination of both diosmin and hesperidin in Buchu leaf and in tablets containing them (e.g. Daflon®).

Diosmin **I** (a flavone glycoside) and hesperidin **II** (a flavanone glycoside) have similar structures and biological activities. Their solubility (especially diosmin) represented a problem for extraction and manipulation. Therefore, initial work was directed towards the selection of an efficient simple extraction procedure for diosmin and/or hesperidin from Buchu leaves and pharmaceutical tablets. It was evident that incorporation of 10% dimethylsulfoxide in methanol (instead of the methanolic NaOH used in a previous method [14] for determination of the two flavonoids in soft capsules) provided the most efficient solvent (among the several mixtures of solvents tried during this study) for complete extraction of the two flavonoids.

Regarding the selection of the mobile phase, a mixture of water–methanol (40:60, v/v) gave optimum chromatographic separation of diosmin and hesperidin (Fig. 1A). Previous HPLC methods involved the use of sodium hydroxide solution for extraction and acidic mobile phase requiring adjusted pH [14] and/or a gradient elution for determination of diosmin and/or hesperidin [15].

A typical chromatogram of a mixture of standard diosmin (t_R, 5.6 min) and hesperidin (t_R, 3.3 min), is shown in Fig. 1A. A mixture of water–methanol (40:60, v/v) as a mobile phase in a simple isocratic mode, provided symmetrical peaks for the two flavonoids with a good separation in less than 6 min (shorter time with respect to the previous methods [14,15]). The capacity factors (k') were 2.7 and 1.2 for diosmin and hesperidin, respectively and are optimum for both qualitative and quantitative analyses [17].

For the determination of diosmin in pharmaceutical tablets free of hesperidin (e.g. Diosed® and Dioven®), hesperidin was used as internal standard as it fulfilled the requirement of an ideal

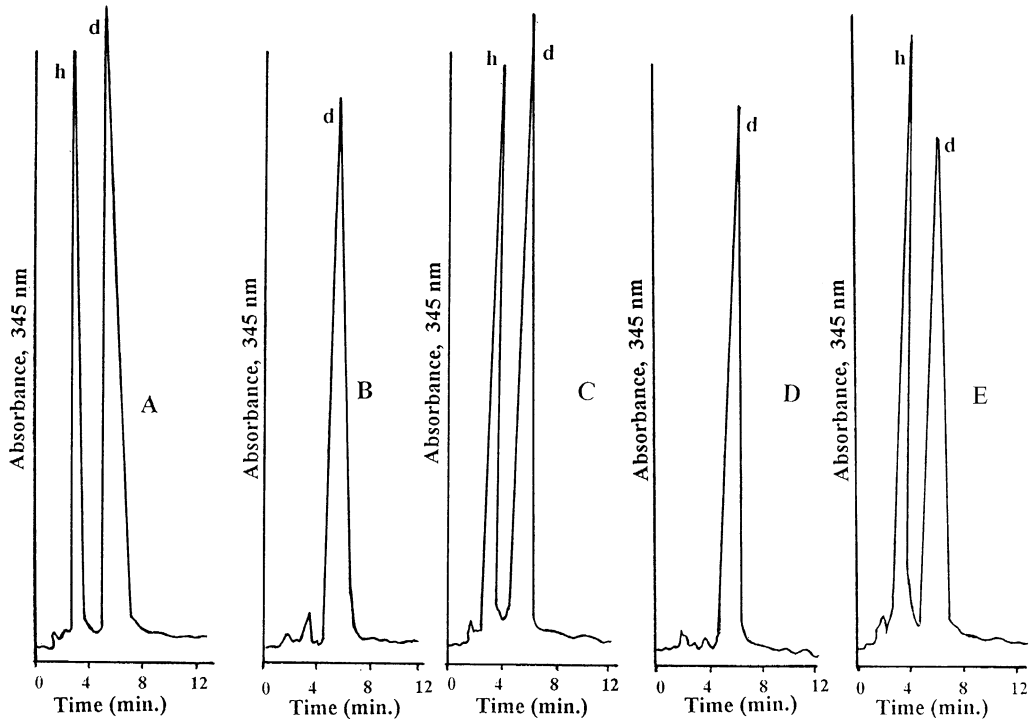


Fig. 1. HPLC chromatograms of: (A) a mixture of pure hesperidin and diosmin; (B) Diosed® tablet; (C) Diosed® tablet containing the internal standard (hesperidin); (D) Dioven® tablets; (E) Dioven® tablets containing the internal standard. The ordinate is the absorbance at 345 nm, and the abscissa is t_R (min). (h) Internal standard (hesperidin); (d) diosmin, operating conditions were described in the text.

internal standard [17]. A calibration graph for diosmin was constructed in the range 5–100 μg/ml, by plotting the concentration of diosmin versus the peak area ratio of diosmin to hesperidin. The regression analysis of experimental data points showed a linear relationship with excellent correlation coeffecient ($r = 0.99$) and the linear regression equation for diosmin was:

$$Y = 2.525 \times 10^{-2} X - 3.458 \times 10^{-3}$$

Where Y is the peak-area ratio of diosmin to that of the internal standard and X is the concentration of diosmin. This system allowed accurate detection of as low as 50 ng for an injection volume of 10 μl. Subsequently, this method was applied to analyse diosmin in two pharmaceutical products, Diosmin® and Dioven® tablets with a very simple extraction process. The HPLC chromatograms (Fig. 1B, C, D and E) indicated that, peaks due to diosmin and the internal standard were free from interference from formulation excipients. The assay results of six separate determinations of each tablet are summarized in Table 1. The results provided a mean potency ($\pm$S.D.) of 98.8% ($\pm$1.7) and 99.3% ($\pm$1.2) for Dioven® and Diosed® tablets, respectively.

For the simultaneous determination of diosmin and hesperidin, two calibration graphs were obtained over the ranges of 5–100 μg/ml (for diosmin) and 2.5–75 μg/ml (for hesperidin); by plotting the concentrations of each flavonoid against the resultant peak areas. The regression equations were:

$$Y = 1.781 \times 10^{-1} X - 1.921 \times 10^{-1}$$

(for diosmin)

$$Y = 6.603 \times 10^{-1} X - 4.282 \times 10^{-1}$$

(for hesperidin)

Table 1
Determination of diosmin in Dioven® and Diosed® tablets by HPLC using the internal standard method

Tablet	Diosmin (mg/tablet)[a]		Percentage of label claim ± S.D.%
	Label claim	Found (± S.D.%)	
Dioven®	150	148.2 (± 1.4%)	98.8 ± 0.9
Diosed®	150	149.0 (± 1.2%)	99.3 ± 0.8

[a] Results are the average of six separate determinations.

Where Y is the peak area and X is the concentration. These results showed good linear relationships between peak area and concentration within the above ranges, for each of diosmin and hesperidin. This method was used to analyze diosmin and hesperidin simultaneously in Buchu leaf and in Daflon® tablets, using quite simple extraction procedures. Typical chromatograms of Daflon® tablets and Buchu leaf extracts are shown in Fig. 2A and 2B, respectively. Peaks due to the two flavonoids were well resolved from each other and from other minor coextracted materials. Six determinations were made for each sample, and the standard deviation was always below ± 1.9%, in all cases, and the results are summarized in Table 2.

The selectivity of the proposed methods was evaluated by the analysis of Daflon® tablets (which contains flavonoids extract) and Buchu leaf extract. Their chromatograms (Fig. 2) show well developed and separated peaks for diosmin and hesperidin, while the other minor constituents did not interfere. This indicated that the proposed methods can be applied for the selective determination of both flavonoids in the tablets and in Buchu extract.

To determine the recovery and to ensure the validity and reproducibility of the proposed methods, repeated injections (six injections, each of 10 μl) of the same sample of each of the studied tablets and Buchu extracts were used. These samples were prepared by addition of known amounts of standard diosmin and/or hesperidin to exact weights of previously assayed tablets and Buchu leaf. The obtained results indicated that both diosmin and/or hesperidin were almost quantitatively recovered from the three studied tablets (the mean recoveries were 99.3 ± 0.4% and 99.6 ± 0.3%, for diosmin and hesperidin, respectively). The mean recoveries (± S.D.%) of diosmin and hesperidin from Buchu leaf were 96.9(± 1.2) and 98.1(± 0.7), respectively. The results revealed the presence of 0.8% diosmin; hesperidin is also reported for the first time in Buchu leaf, although in a small percentage (0.12%).

In summary the HPLC methods established in this study comprise the first report for the simultaneous determination of diosmin and hesperidin in Buchu leaves and the widely used tablets containing them. Compared with other reported

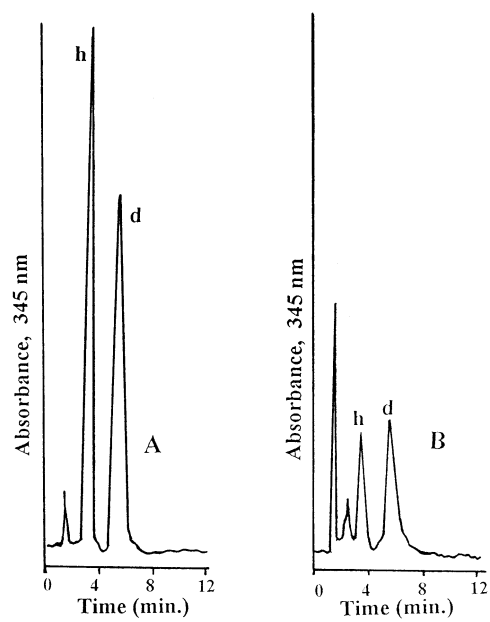


Fig. 2. HPLC chromatograms of: (A) Daflon® tablet; (B) Buchu leaf extract. The ordinate is the absorbance at 345 nm, and the abscissa is t_R (min). (h) Hesperidin, (d) diosmin, operating conditions were described in the text.

Table 2
Diosmin and hesperidin in Buchu leaf (g%) and Daflon® tablets (mg/tablet), as determined simultaneously by the external standard HPLC method

Drug	Diosmin[a]	Hesperidin[a]
Buchu leaf	0.82 (± 0.06%)	0.12 (± 0.07%)
Daflon® tablets	138.9 (± 1.9%)	14.8 (1.6%)

[a] Results are the average of six different determinations, and S.D.% values are given in parenthesis.

HPLC methods, these methods provide significant advantages in terms of simplicity, convenience, time of analysis and sensitivity. A straight forward simple extraction using inert solvents, in which both diosmin and hesperidin are quite stable, and the use of alkalis or acids was completely avoided. Buchu leaf, which is a natural drug used mainly for its diosmin content, has never before been analyzed by HPLC for its flavonoid contents.

These reversed-phase HPLC methods should also find application in the quality control of diosmin and heperidin, as well as in the pharmacokinetic studies of the two drugs.

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