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Simultaneous determination of diosmin and diosmetin in human plasma by ion trap liquid chromatography–atmospheric pressure chemical ionization tandem mass spectrometry: Application to a clinical pharmacokinetic study

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ABSTRACT

Diosmetin (3′,5,7-trihydroxy-4′-methoxyflavone) is the aglycone of the flavonoid glycoside diosmin (3′,5,7-trihydroxy-4′-methoxyflavone-7-ramnoglucoside). Diosmin is hydrolyzed by enzymes of intestinal micro flora before absorption of its aglycone diosmetin. A specific, sensitive, precise, accurate and robust HPLC assay for the simultaneous determination of diosmin and diosmetin in human plasma was developed and validated. Plasma samples were incubated with β-glucuronidase/sulphatase. The analytes were isolated by liquid–liquid extraction with *tert*-butyl methyl ether at pH 2, and separated on a C_{18} reversed-phase column using a mixture of methanol/1% formic acid (58:42, v/v) at a flow rate of 0.5 ml/min. APCI in the positive ion mode and multiple reaction monitoring (MRM) method was employed. The selected transitions for diosmin, diosmetin and the internal standard (7-ethoxycoumarin) at *m/z* were: 609.0 → 463.0, 301.2 → 286.1 and 191, respectively.

A good linearity was found in the range of 0.25–500 ng/ml ($R^2 > 0.992$) for both compounds. The intra-batch assay precision (CV) for diosmin and diosmetin ranged from 1.5% to 11.2% and from 2.8% to 12.5%, respectively, and the inter-batch precision were from 5.2% to 11.5% and 8.5% to 9.8%, respectively. The accuracy was well within the acceptable range the accuracies (from −2.7% to 4.2% and −1.6% to 3.5% for diosmin and diosmetin, respectively). The mean recoveries of diosmin, diosmetin and the internal standard were 87.5%, 89.2% and 67.2%. Stability studies showed that diosmin and diosmetin were stable in different conditions. Finally, the method was successfully applied to the pharmacokinetic study of diosmin in healthy volunteers following a single oral administration (Daflon®).

1. Introduction

Micronised purified flavonoid fraction (MPFF) [Daflon® 500 mg] is a well-established oral flavonoid pharmaceutical formulation with phlebotropic and venoprotective properties. It consists of 90% micronised of the pharmacologically active flavonoid diosmin and 10% flavonoids expressed as hesperidin. Diosmin (3′,5,7-trihydroxy-4′-methoxyflavone-7-ramnoglucoside, Fig. 1) is considered to be a vascular-protecting agent used to treat chronic venous insufficiency, hemorrhoids, lymphedema, and varicose veins. As a flavonoid, diosmin also exhibits anti-inflammatory, free-radical scavenging, and antimutagenic properties [1]. Diosmin was first isolated in 1925 from *Scrophularia nodosa*. Today can be manufactured by extracting hesperidin from citrus rinds, followed by conversion of hesperidin to diosmin.

Diosmin is clinically used as a micronised formulation. After oral administration, diosmin is rapidly hydrolysed by enzymes of the intestinal microflora into its flavone aglycone, diosmetin, which is subsequently absorbed into the systemic circulation. An in vitro study showed that ^{13}C- and ^{14}C-diosmin incubated with human gut flora were transformed to diosmetin, luteolin and phenolic acids [1]. Trace levels of diosmetin were detected in human plasma as its unconjugated form after oral administration of 10 mg/kg diosmetin to healthy volunteers [2].

Reversed-phase high-performance liquid chromatography combined with different detectors is the commonly used analytical method for separation of flavonoids [3–6]. However, only a few analytical methods, mainly HPLC-UV, have been reported for the quantitative determination of either diosmin and diosmetin in plant extracts, biological fluids, or pharmaceutical formulations. Some of these methods are not validated, are time-consuming, require laborious extraction techniques, using non-volatilie ion

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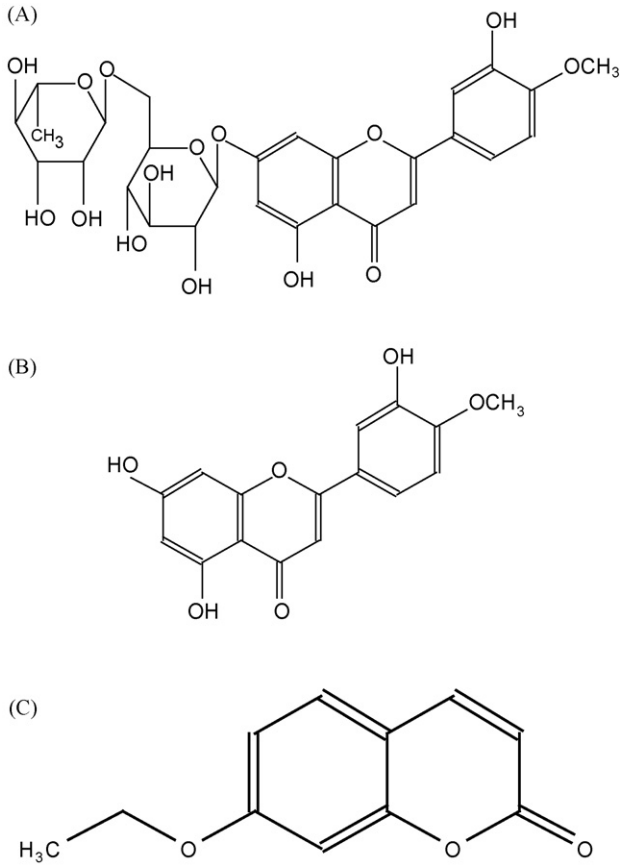

Fig. 1. Chemical structures of (A) diosmin, (B) diosmetin, and (C) 7-ethoxycoumarin.

pair reagents, or there were no internal standard [7–10]. Only two HPLC methods combined with ultraviolet [11], mass spectrometry detection [7] has been reported for the determination of diosmetin in biological fluids. Recently, Spanakis have reported a GC/MS method for the simultaneous determination of diosmetin and hesperetin in human plasma and urine [12] with a LOQ value of 2 ng/ml, too higher than evaluate the pharmacokinetic profile of diosmetin after oral administration of the micronised purified flavonoid fraction to human subjects.

To the best of our knowledge, no analytical method is available in the literature for the simultaneous determination of diosmin and its flavonoid aglycone. The objective of this paper was to develop and validate a novel HPLC method for the simultaneous determination of diosmin and diosmetin in human plasma, suitable for the development of pharmacokinetic studies after intake of foods or oral administration of pharmaceutical preparations that contain the micronised purified flavonoid fraction of diosmin in human subjects.

2. Experimental

2.1. Chemicals, reagents and solutions

Diosmin and diosmetin (purity >90%) were provided by Extrasynthese (Genay Cedex, France). 7-Ethoxycoumarin (purity > 99%) (molecule selected as Internal Standard) was purchased by Sigma–Aldrich (St. Louis, USA). Reagents and solvents such as acetic acid, methanol, *tert*-butyl methyl ether (HPLC grade), phosphoric acid (85%), sodium hydrogenphosphate monohydrate, sodium acetate, and dimetylsulphoxide were obtained from Merck (Darmstadt, Germany). Formic acid was purchased by Fluka (St. Louis, USA). Ultra-high quality water (obtained using a MilliQ apparatus, Millipore, Massachusetts, United States) was used for chromatography. Finally, β-glucoronidase HP-2S from *Helix pomatia*, 106,000 units/ml was provided by Sigma–Aldrich (St. Louis, USA).

2.2. Standard solutions and samples

Stocks solutions of diosmin, and diosmetin with a concentration of 1 mg/ml, were prepared separately by dissolving 10 mg of each analyte in a mixture of dimethylsulphoxide/methanol (50:50, v/v). Intermediate stock standards of 100, 10 and 1 μg/ml were prepared using methanol.

Nine standard solutions of each compound (5, 10, 20, 50, 100, 200, 1000, 2000, and 10,000 ng/ml) were made by further dilution of the intermediate stock solution with appropriate volumes of methanol. The standard solution of 7-ethoxycoumarin (0.5 μg/ml) was similarly prepared. Standard and stock solutions of diosmin, diosmetin and 7-ethoxycoumarin were stored at −80 °C.

Mixed calibration pools of diosmin and diosmetin were prepared by diluting appropriate volumes of each standard solution with plasma to achieve concentrations ranging from 0.25 to 500 ng/ml.

Pools of quality control samples were prepared by spiking drug-free human plasma with the different working standard solutions of diosmin and diosmetin. Finally, 0.25, 0.75, 3, 15 and 200 ng/ml were the plasma concentrations of diosmin and diosmetin in the quality control samples. Pools were stored into 1.2 aliquots in propylene tubes and frozen at −80 °C until use.

2.3. Sample preparation

Human plasma (0.5 ml) was thawed at room temperature and samples were processed after the addition of the internal standard (7-etoxycoumarin, Sigma, St. Louis, USA). Calibration standards, quality controls and collected samples were incubated under continuous shaking with 50 μl of 0.58 mol/l of acetic acid (pH 5) and 50 μl of β-glucuronidase for 2 h at 37 °C. The hydrolysed plasma samples acidified, and extracted with *tert*-butyl methyl ether (MTBE).

Plasma samples were transferred to 13 mm × 100 mm conic tubes and spiked with the internal standard (20 μl of 0.5 μg/ml of 7-ethoxycoumarin). Then, 1 ml phosphate buffer (pH 2, 0.01 M) was added to the tubes. After vortex-mixed well for 10 s, 6 ml of *tert*-butyl methyl ether was added to the sample tubes. The tubes were capped, vortex-mixed for 1 min, and centrifuged at 2000 × *g* and 1 °C for 10 min. Finally, the obtained supernatant was transferred to a clean tube and dried under vacuum pressure (Vortex evaporator, Labconco) at 40 °C for 15 min. The dried extracts were reconstituted in 200 μl of mobile phase, transferred to limited volume autosampler vials, capped and placed on the HPLC autosampler. A 25-μl aliquot of the supernatant was injected onto HPLC column.

2.4. Apparatus and chromatographic conditions

The apparatus used for the HPLC analysis was a Model 1100 series LC coupled with a MSD Ion Trap XCT Plus detector (Agilent, Waldbronn, Germany). Data acquisition and analysis were performed with a Hewlett–Packard computer using the ChemStation G2171 AA program (LC) and LC/MSD Trap Software 5.2 (MS). Separation was carried out at 40 ± 0.1 °C on a reversed-phase, 150 mm × 3 mm column packed with C_{18}, 5 μm silica reversed-phase particles (Gemini®) obtained from Phenomenex (Torrance, USA). The mobile phase was a mixture of methanol and 1% formic acid (58:42, v/v). Detection of diosmin, diosmetin and the inter-

nal standard was achieved by multiple reaction monitoring at m/z transitions of 609.0 → 463.0, 301.2 → 286.1 and 191, respectively. Data were acquired using the following settings: APCI capillary voltage was set at 4500 V (+) ion mode. The liquid nebulizer was set to 60 psig and the nitrogen drying gas was set to a flow rate of 4 l/min. Drying gas temperature was maintained at 350 °C. Data was acquired at a rate of 20,000 Da/s with a stored mass range of m/z 50–1000.

Separation was achieved by isocratic solvent elution at a flow rate of 0.5 ml/min.

2.5. Quantitation

Each calibration curve of diosmin or diosmetin consisted of nine calibration points (0.25, 0.5, 1, 2.5, 5, 10, 50, 100 and 500 ng/ml). Calibration curves were determined by least square linear regression analysis (weighting $1/X^2$). Peak area ratios of diosmin or diosmetin and 7-ethoxycoumarin versus the corresponding calibrator concentration were plotted.

2.6. Validation

A thorough and complete method validation of diosmin and diosmetin in human plasma was done following the US FDA guideline [13]. The method was validated for selectivity, sensitivity, interference check, linearity, precision and accuracy, recovery, matrix effect, cross-specificity, stability and dilution integrity.

The method was validated by analysis of human plasma quality control samples prepared at five concentrations spanning the calibration range (0.25, 0.75, 3, 15 and 200 ng/ml) and calibrator samples prepared as it is above indicated. Three samples of each quality control pool, and nine calibration samples were analysed on six different analytical batches. On batch 1 the number of samples for each quality control pool was 5. The linearity of the method was assessed by along six different analytical batches. To be acceptable the calibration curve had to have a correlation coefficient (r) of 0.995 (determination coefficient of 0.990) or better and the back-calculated concentration of the calibrator samples were ±15% deviation from the nominal value except at the lower limit of quantitation, which was set at ±20%. Precision of a method was expressed as the percentage of the coefficient of variation of replicate measurements. Intra-batch precision was determined by analysing three sets of spiked plasma samples at each QC level in a batch. Inter-batch precision was determined by analysing three sets of spiked plasma samples at each QC level in six consecutive batches. Accuracy was measured according to the following equation:

$$\text{Percentage difference from theoretical value} = \left[\frac{X - C_T}{C_T}\right] \times 100$$

where X is the determined concentration of a quality control and C_T is the theoretical concentration. To be acceptable, the measures should be lower than 15% at all concentrations.

The overall recovery for diosmin, diosmetin and 7-ethoxycoumarin was calculated by comparing the peak area ratios of spiked samples before and after the extraction in different lots of plasma at four concentration levels.

The matrix effect over diosmin, diosmetin and 7-ethoxycoumarin was evaluated by comparing the peak area of the analyte dissolved in the reconstituted residues of processed blank plasma with that standard solutions at the same concentration dissolved in mobile phase. Matrix effect was evaluated at four different concentration levels of diosmin and diosmetin, with three samples analysed at each set. The matrix effect of the internal standard was evaluated at the concentration in plasma samples using the same method.

The selectivity of the assay was determined by the individual analysis of blank samples. The retention times of endogenous compounds in the matrix were compared with those of diosmin, diosmetin and 7-ethoxycoumarin.

LOD was defined as the sample concentration resulting in a peak area of three times the noise level. LOQ was defined as the lowest drug concentration, which can be determined with an accuracy and precision <20%. In this work LOD of the assay method was determined by analysis of the peak baseline noise in 10 blank samples.

The stability of diosmin and diosmetin in both frozen plasma samples (−80 °C) over 3 months, and in processed samples left at room temperature (20 ± 3 °C) over 24 h, was also studied.

2.7. Application of the method

The developed method has been applied to a pharmacokinetic study in which the concentrations of diosmetin were measured in more than 1216 plasma samples. The study was performed according to the revised Declaration of Helsinki. Ethics Committee and Spanish Medicine Agency approved the study protocol. In this study 32 healthy volunteers received a single oral dose of Micronised purified flavonoid fraction (MPFF) [Daflon® 500 mg]. Blood samples were collected in 10-ml sterile citrate tubes predose and at the following times after dosing: 0.33, 0.67, 1.0, 1.33, 1.67, 2.0, 2.33, 2.67, 3.0, 3.5, 4.0, 6.0, 9.0, 12.0, 72.0, 120.0 and 144.0 h post-dose, after each administration. Samples were immediately centrifuged for 5 min at 2000 rpm. Following centrifugation, plasma samples were transferred to polyethylene tubes and stored at −20 °C until analysis. Pharmacokinetic parameters were calculated by noncompartmental methods. All calculations were carried out using WinNonlin Professional Version 5.1 (Scientific Consulting, Inc., Mountain View, USA). The area under the curve for the time of administration to the last measured concentration (AUC_{0-t}) was calculated by trapezoidal integration. The total area under the curve from administration to infinity ($AUC_{0-\infty}$) was calculated as the sum of AUC_{0-t} and residual area (C_t divided by k_e, with C_t as the last measured concentration and k_e as the apparent terminal elimination rate, estimated by linear regression of the terminal portion of the log-transformed concentration-time profiles). Half-life ($t_{1/2}$) was calculated by dividing 0.693 by the k_e. Maximal plasma concentration (C_{max}) and the time to attain peak (t_{max}) were obtained directly from the raw data.

3. Results and discussion

3.1. Liquid/MS chromatographic conditions

The objective of this study was to develop an HPLC method for the determination of diosmin and its bioactive derivative compound diosmetin in human plasma. Liquid chromatography–mass spectrometry is recognized as a powerful tool for the quantitative determination of an active compound in biological samples due to the selectivity, sensitivity, robustness and sample throughput. Especially, LC–MS^n allows the sequential fragmentation of a given molecular ion, provide substantial information for the identification of selected molecule based on their fragmentation patterns. This information is very useful to differentiate compounds with similar chromatographic and ultraviolet–visible spectral features.

Diosmin and diosmetin are very weak basic compounds with three pK_a values for the aglycone (corresponding to the three phenolic OHs) and eight for the glycoside (two phenolic and six

alcoholic OHs). Moreover, the aglycone possessed lower apparent ionisation constants than the corresponding glycosilated flavonoid [14]. Therefore, the APCI source produced the highest S/N ratios for the two analysing compounds. According to the full-scan APCI (+) mass spectra, the protonated molecule ion $[M+H]^+$ m/z 609.0 for diosmin, $[M+H]^+$ m/z 301.1 for diosmetin and $[M+H]^+$ m/z 191 for 7-etoxycoumarin (IS) were selected as the precursor ion to obtain the product ion. The most sensitive mass transition from the precursor ion to the product ion was m/z 609.0 → 463.0 for diosmin, 301.2 → 286.2 for diosmetin and m/z 191.0 → 162.9 for the internal standard.

The product ion spectra of diosmin and diosmetin are very similar. Although the positive ion APCI-MS spectrum of diosmin and diosmetin, shows peaks at 609.0 and 301.1 for the molecular mass [M+H], both show the loss of methyl as most prominent fragmentation, producing m/z 286.2 after MS^2 (diosmetin) and MS^3 (diosmin) fragmentations (Figs. 2(B) and 3). The MS–MS spectrum of the diosmin parent ion (Fig. 2(A)) presents some interesting evidence regarding the chemical structure. The observed loss of [M+H−148] fragment, a rhamnose ring, appear characteristic of a rutinoside presence. Therefore, fragments m/z 301.2 are related to the loss of the rutinosyl unit (glucose + rhamnose).

The chromatographic conditions described in this analytical procedure were achieved after investigating different C_{18} or C_8 reversed-phase columns and several mobile phases. Symmetrical, sharp and well-resolved peaks were observed for diosmin, diosmetin, and internal standard using a Gemini C_{18} column and a mixture of methanol–water as mobile phase. The chromatographic behaviour showed a significant improvement when formic acid was added to methanol–water mixture. The addition of formic acid may suppress the ionization of phenol groups lead to improve the peak shape and the separation of the analytes. A 1% formic acid–methanol (58:42, v/v) mobile phase composition was fixed since adequately balances the chromatographic separation efficiency and MS sensitivity. Moreover, the selected package is organo-silica grafting process which incorporates highly stabilizing ethane cross-linking, into the grafted layers on the silica surface. This not only provides resistance to high pH attack, but also maintains the high efficiency and mechanical strength of a silica particle.

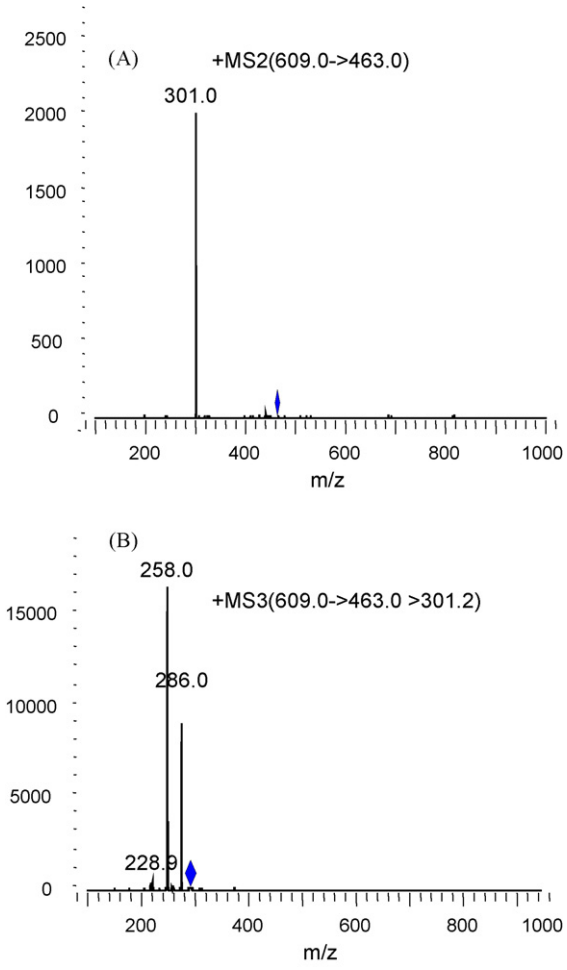

Fig. 2. APCI (A) MS–MS and (B) MS–MS–MS spectrum of diosmin.

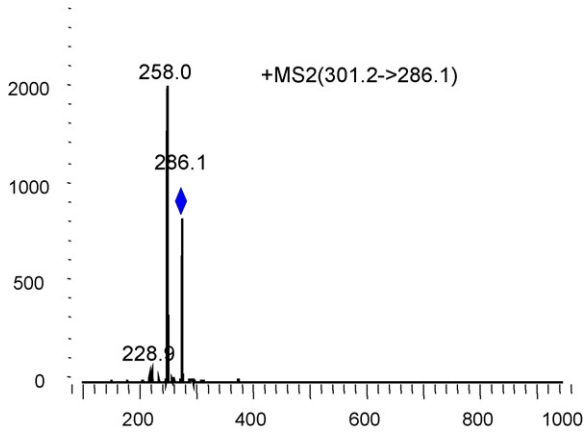

Fig. 3. APCI-MS–MS spectrum of diosmetin.

Although diosmin and diosmetin have similar structures their physicochemical properties represented a problem for the extraction from biological samples. Liquid–liquid extraction has become one of the most widely used biological sample pretreatment methods for flavonoids. However, previous HPLC methods involved the use of sodium hydroxide solution for diosmin extraction. The use of strong alkalis for extraction may convert flavanones to the corresponding chalcone derivatives, causing misleading result [9]. The solvent solubility also represents a problem for diosmin extraction. To obtain a clean chromatogram and achieve a sufficient extraction recovery, three types of solvents (diethyl ether, *tert*-butyl methyl ether and cyclohexane) were evaluated. *tert*-Butyl methyl ether in acidic media was proved to be the best in terms of higher extraction recovery, negligible matrix effects, and absence of endogenous interference at the retention times in the chromatogram. Moreover, the shaking time employed for the diosmin and diosmetin liquid–liquid extraction was significantly diminished about the used in the previously published methods.

In vivo and in vitro studies carried along with diosmetin in rats demonstrated that diosmetin undergoes a rapid glucuronidation to different glucuronides at the level of the intestinal mucosa. Four glucuronides were identified in rat blood after 100 mg/kg p.o. administration of a diosmetin suspension [15]. Only diosmetin trace levels (lower than 5 ng/ml) were detected in human plasma without treatment of β-glucuronidase/sulphatase. Therefore, it is essential to release diosmetin from its glucuronic acid conjugate via enzymatic hydrolysis to carry out accurate pharmacokinetic studies. The amount of enzyme (β-glucuronidase H2S) required and the length of incubation for the complete hydrolysis were optimized using various amounts of the enzyme (500, 1000, 2000, 3000 and 5000 units) and different lengths of time (15, 30, 60, 90 and 120 min at 37 °C in a water bath). A pooled sample with an unknown amount of diosmetin glucoronides obtained from a human pharmacokinetic study was used for the optimization

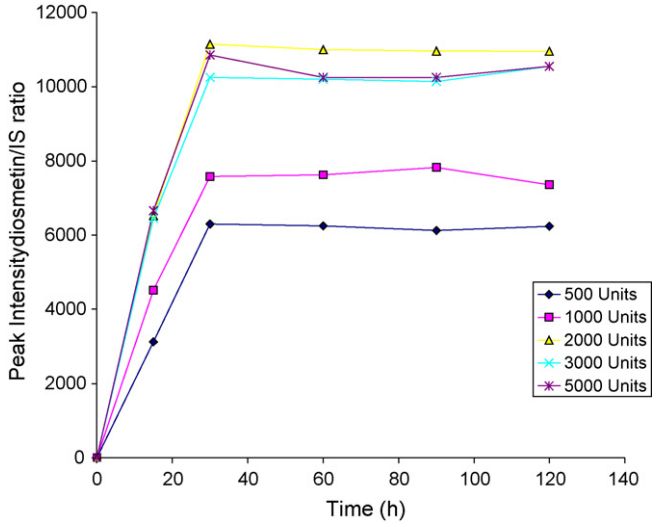

Fig. 4. Effect of varying incubation times (15–120 min) at 37 °C and enzyme concentration (500–5000 units per ml of plasma) on peak height ratio of diosmetin to internal standard (PE/IS).

procedure. After incubation for an appropriate time with an appropriate concentration of enzyme, the samples were extracted as described in the sample preparation procedure and analysed by HPLC/MS/MS. The results of the optimization procedure are shown in Fig. 4. It was concluded that those 2000 units of enzyme incubated with 1 ml of plasma for 30 min was sufficient for complete hydrolysis of diosmetin glucoronides. Reproducibility of the hydrolysis was estimated as the R.S.D. (precision) obtained during the optimization procedure. An R.S.D. of 9.8% indicated that the hydrolysis of diosmetin glucoronides was being carried out reproducible.

Under these chromatographic conditions the retention time of diosmin, diosmetin and the internal standard were 2.1 ± 0.2, 5.5 ± 0.3 and 4.4 ± 0.3 min, respectively. The R_s values between diosmin and internal standard, and between the internal standard and diosmetin were 2.3 and 2.1, values that reflected the high degree of selectivity that show the chromatographic developed method. The symmetry factor of all peaks ranged from 0.8 to 0.97, values near to 1. Fig. 5 displays the chromatograms of plasma extracts of a healthy volunteer given orally Daflon®. The absence of interfering matrix peaks at the retention times of diosmin, diosmetin and the internal standard in blank plasma extracts allows to show the higher selectivity degree of the developed chromatographic method. Moreover, the total run time was only 7 min, much lower than the previously described values in the works of Kanaze and Spanakis [11,12].

3.2. Validation

Assay performance of the present method was assessed by all the following criteria: linearity, accuracy, precision, LOD, LOQ, recovery, matrix effect evaluation, stability, and applicability to pharmacokinetic studies. The assays exhibited linearity between the response (y) and the corresponding concentration of diosmin and diosmetin (x), over the 0.25–500 ng/ml range in plasma samples. The mean standard curve was typically described by the equation $y = 0.021(\pm 0.004)x - 0.00056(\pm 0.00009)$, $r = 0.9971$ for diosmin and $y = 0.013(\pm 0.003)x - 0.00023(\pm 0.00005)$, $r = 0.9959$ for diosmetin (mean values of slope and intercept, ±S.D.). For each point of calibration standards, the concentrations were back-calculated from the equation of the regression curves, and R.S.D. were computed. The calibration standard curves had a reliable reproducibility over the standard concentrations across the calibration range over six different batch analysis. The average regression ($n = 6$) was found to be ≥0.995. The % accuracy observed for the mean of back-calculated concentrations for six calibration curves was within −4.77 to 6.05% for diosmin, and −1.51 to 8.02% for diosmetin. The % precision values ranged from 5.2 to 7.9 for diosmin and from 6.1 to 9.9 for diosmetin. The LOD of diosmin and diosmetin in plasma were 0.13 and 0.16 ng/ml (S/N = 3) and the estimated LOQs were calculated as low as 0.24 and 0.25 ng/ml

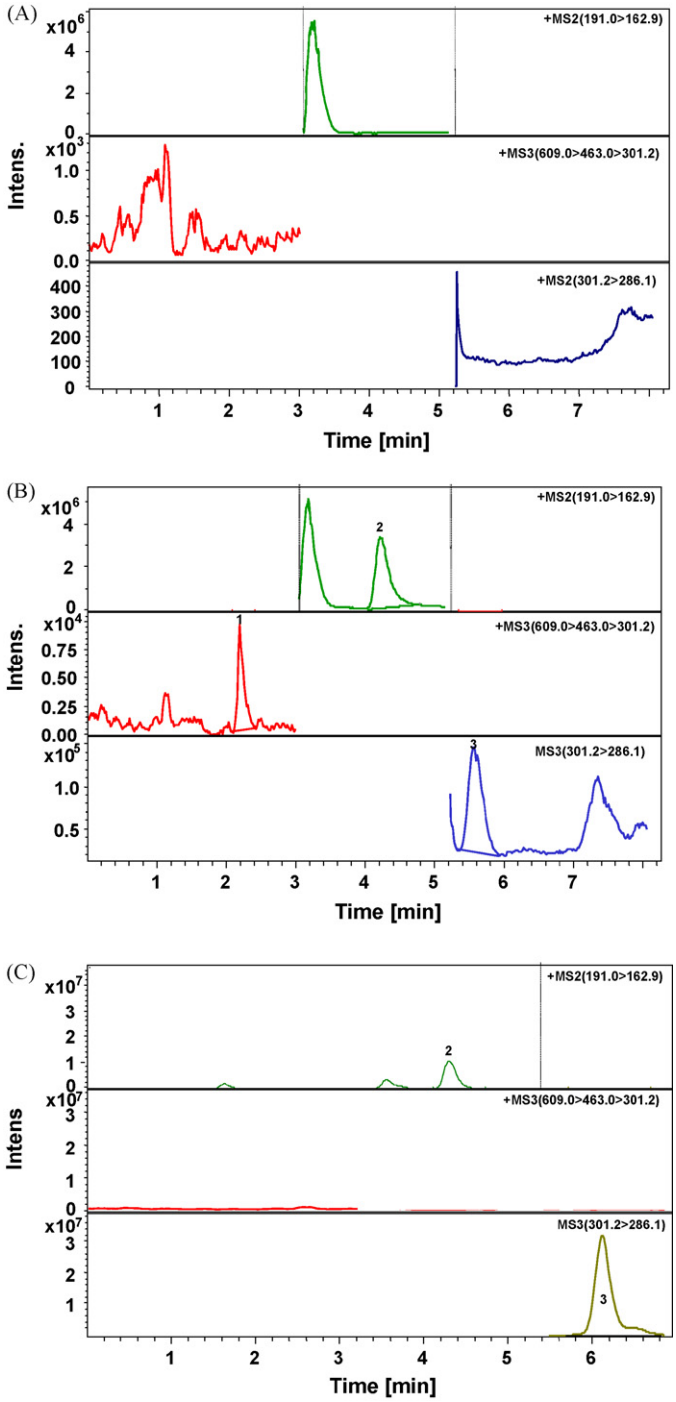

Fig. 5. Chromatograms of (A) blank human plasma, (B) blank human plasma spoked with diosmin (0.25 ng/ml, 1), diosmetin (0.5 ng/ml, 3), and 7-ethoxycoumarin (internal standard, 2), and (C) resulting from the analysis human plasma samples obtained at 24 h (0 ng/ml diosmin, 2; 347.28 ng/ml diosmetin, 3) from a subject who received a single oral dose of Daflon® (450/50 mg).

Table 1
Accuracy ($n = 6$) of the method, expressed as relative error in %, for the determination of diosmin and diosmetin in plasma.

Theoretical concentration (ng/ml)	Measured concentration, mean ± S.D. (ng/ml)	Accuracy (%)
Diosmin		
0.25	0.25 ± 0.02	1.2
0.75	0.76 ± 0.02	1.33
3	3.02 ± 0.25	0.67
15	14.25 ± 1.25	−5.00
200	198.25 ± 3.55	−0.88
Diosmetin		
0.25	0.25 ± 0.02	1.6
0.75	0.74 ± 0.03	−1.33
3	2.90 ± 0.36	−3.25
15	15.53 ± 1.41	3.52
200	196.71 ± 12.40	−1.65

(S/N = 10), which are better than the previously obtained values from plasma samples [11,12]. The obtained values were confirmed with a result of 0.24 ± 0.02 and 0.25 ± 0.02 ng/ml for plasma samples ($n = 6$).

The pretreatment recoveries of diosmin and diosmetin in plasma samples were 85.2 ± 8.35% and 87.75 ± 9.17%, respectively. Similar values were obtained for the internal standard (67.18 ± 10.51%).

The evaluation of matrix effect from the influence of coeluting components on analyte ionization is needed from all LC–MS/MS method. The effect of the matrix on ionization efficiency, expressed as the ratio of the peak area of analytes spiked after pretreatment to that of the neat standard solutions in the mobile phase, were negligible with less than 2.65, 3.22 and 2.87% of loss in the recovery values of diosmin, diosmetin and the internal standard, respectively.

Accuracy values were within acceptable limits (Table 1). The results for within-day and between-day precision for our sample are presented in Table 2 and the values ranged between 1.79% and 8.77%, and 3.75 and 12.01% for diosmin; and between 4.05% and 12.54%, and 3.75–12.01% for diosmetin, respectively.

Diosmin and diosmetin were stable in plasma samples stored at −20 °C for at least 3 months. The stability of diosmin and diosmetin in processed samples left at 4 ± 0.3 °C over 48 h was also studied from our laboratory quality control samples. Diosmin and diosmetin and the internal standard were also stable in these conditions. No significant degradation of diosmin and diosmetin were observed after three freeze/thaw cycles (−80 °C), and in plasma samples and stock solutions over 24 h at 18 °C.

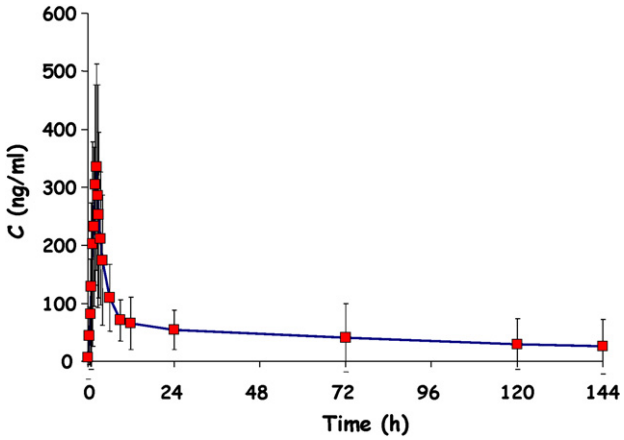

Fig. 6. Mean concentration–time profile of diosmetin after an oral dose of Daflon® in healthy volunteers.

Table 3
Summary of diosmetin pharmacokinetic parameters for volunteers receiving 450 mg of diosmin by oral route.

	Test, mean ± S.D.
Diosmetin	
$AUC_{0-t\,h}$ (mg h/l)	7163.54 ± 6973.9
C_{max} (mg/l)	397.17 ± 202.48
t_{max} (h)[a]	2.33 (1.33–3)
$t_{1/2}$ (h)	70.34 ± 54.16

[a] Median (range).

3.3. Application of the method

The applicability of this method has been demonstrated in vivo by the determination of diosmin and diosmetin in plasma samples from healthy subjects receiving a single oral dose of micronised purified flavonoid fraction (MPFF) [Daflon® 500 mg] (Fig. 6). Diosmin was not detected in human plasma with and without both β-glucuronidase/sulphatase treatment of plasma samples whereas diosmetin was measurable at the first sample time (0.33 h) in the majority of volunteers after β-glucuronidase treatment. The sensitivity of the analytical method was adequate for the determination of diosmetin along 4 times of their respective mean half-life, and the short retention time made possible the diosmin and diosmetin quantitation in a lot of samples in a single analytical batch. The pharmacokinetic parameters are summarised in Table 3.

Table 2
Between- and within-day batch variability of the HPLC method for the determination of diosmin and diosmetin in plasma.

Theoretical concentration (ng/ml)	Intra-batch variability ($n = 5$)		Inter-batch variability ($n = 18$)	
	Measured concentration, mean ± S.D. (ng/ml)	CV (%)	Concentration found, mean ± S.D. (ng/ml)	CV (%)
Diosmin				
0.25	0.25 ± 0.02	8.00	0.24 ± 0.02	8.33
0.75	0.76 ± 0.02	2.63	0.72 ± 0.03	4.17
3	3.02 ± 0.25	8.28	2.95 ± 0.35	11.86
15	14.25 ± 1.25	8.77	15.22 ± 1.36	8.94
200	198.25 ± 3.55	1.79	202.12 ± 17.85	8.83
Diosmetin				
0.25	0.25 ± 0.02	8.01	0.25 ± 0.03	12.01
0.75	0.74 ± 0.03	4.05	0.75 ± 0.05	3.75
3	2.90 ± 0.36	12.54	3.05 ± 0.30	9.77
15	15.53 ± 1.41	9.10	14.76 ± 1.33	8.98
200	196.71 ± 12.40	6.30	198.11 ± 16.82	8.49

4. Conclusions

A sensitive, accurate and precise bioanalytical method involving a simple liquid–liquid extraction of plasma samples and LC–MS/MS determination of diosmin and diosmetin was developed and validated to meet the requirements of the pharmacokinetic investigations. The liquid–liquid extraction procedure is much simpler than the time-consuming liquid–liquid extraction procedure needed by other LC-UV or GC–MS methods. These advantages would make it efficient for the analysis of large numbers of plasma samples obtained from pharmacokinetic studies.

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