Journal of Pharmaceutical & Biomedical Analysis
Vol. 10, Nos 10–12, pp. 1089–1091, 1992
Printed in Great Britain

0731–7085/92 $5.00 + 0.00

Short Communication

Reversed-phase HPTLC densitometric evaluation of fraxin in *Fraxinus excelsior* leaves*

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Keywords: *Coumarins; fraxin; reversed-phase HPTLC densitometry;* Fraxinus excelsior *leaves; reversed-phase HPLC.*

Introduction

In France and Belgium, leaf extracts from *Fraxinus excelsior* are especially used for the treatment of rheumatic diseases and may owe their ethnopharmacological reputation to some diuretic and antiinflammatory properties [1–7]. Such properties may be at least partially explained by the ability of some coumarins to inhibit the formation of leukotrienes in polymorphonuclear leucocytes [8]. *Fraxinus excelsior* leaves contain some of these coumarins: fraxin, fraxetin, scopoletin and esculin. The present work describes a rapid and selective assay for fraxin based on its characteristic fluorescent properties by densitometry on reversed-phase HPTLC plates. Two chromatographic procedures (Silicagel and RP 18) have been assessed and the results compared with those obtained by reversed-phase HPLC on ODS-silica.

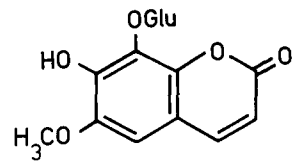

Figure 1
Structure of fraxin.

Experimental

Materials

Fraxin chemical reference substance (CRS) (cf. Fig. 1) was obtained from Roth GmbH (Karlsruhe, Germany). The ^1H and ^{13}C NMR signals of this CRS sample were identical to those reported for fraxin [9–10]. The NMR spectra were recorded on a Brucker spectrometer at 400 MHz for ^1H and at 100 MHz for ^{13}C NMR at CREMAN (Centre de Résonance Magnétique Nucléaire de l'Université de Liège).

The solvents were of analytical or HPLC grade and were used without further purification: acetonitrile, methanol, acetic acid and phosphoric acid were purchased from Merck (Darmstadt, Germany). Commercial batches of *Fraxinus* leaves were obtained from Denolin (Braine-l'Alleud, Belgium) and Pharmaflore (Lessines, Belgium). They were authenticated in the authors' Department according to the monograph of French Pharmacopoeia X [11].

Instrumentation

The densitometric system consisted of a CD 60 TLC-plate scanner (Desaga, Heidelberg, Germany). The solutions were spotted as bands by means of a TLC sampler AS 30.

The HPLC System consisted of a model 2249 liquid chromatograph (Pharmacia-LKB, Bromma, Sweden) equipped with a variable wavelength detector (Gilson, Villiers-le-Bel, France), set at 350 nm.

*Presented at the "Fourth International Symposium on Drug Analysis", May 1992, Liège, Belgium.
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Chromatographic conditions

The solutions used for the quantitative evaluation were the same for both the HPTLC and the HPLC methods: standard solution: 5 mg of fraxin CRS were dissolved in 100 ml methanol–water (1:1, v/v); sample solution: 0.250 g of *Fraxinus* leaves were extracted with 25 ml methanol at 40°C (90 min); the solution was filtered, evaporated and dissolved in 10 ml methanol.

HPLC separation. The separation was performed at room temperature on a Nucleosil-C_{18} prepacked cartridge (250 × 4 mm i.d.) from Macherey–Nagel (Düren, Germany), preceded by a pre-column (30 × 4 mm i.d.) packed with the same support material from Macherey–Nagel. Analytical samples were introduced into the column using a 20-μl loop valve. The mobile phase consisted of phosphoric acid (0.2% v/v)–acetonitrile (85:15, v/v). Absorbance of the effluent from the column was measured at 350 nm (cf. Fig. 2). The peak area method was used to calculate the concentration of fraxin.

HPTLC separation. Similar conditions were employed to those in HPLC. Migration was performed at room temperature on HPTLC plates (RP 18 WF 254s; 10 × 20 cm; Merck, Darmstadt, Germany, precoated with concentrating zone). Plates were developed to a distance of 7 cm (from the concentrating zone) with the following solvent system: phosphoric acid (0.4% v/v)–acetonitrile (60:40, v/v) in an unsaturated chamber. After development, the plate was dried for 60 min in a stream of cold air.

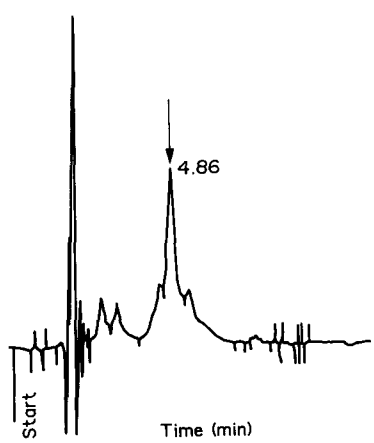

Figure 2
HPLC chromatogram of fraxin (t_R = 4.86 min) at 350 nm.

The following procedure for application of bands was programmed on the TLC sampler: width of band, 3 mm; distance between bands, 10 mm; step volume, 0.5 μl; rate of application, 40 s μl^{-1}; break between applications, 15 s. Aliquots of 1 and 2 μl were applied for the standard, and 2 μl for the samples.

Quantification. The chromatogram was scanned by means of a CD 60 scanner supplied with user-friendly software operating via a personal computer under the following conditions: scanning mode, remission, fluorescence (Mercury lamp); excitation wavelength, 360 nm; emission wavelength cut-off filter, 450 nm; width of slit, 0.2 mm; height of slit, 3 mm; spot optimization mode utilized; resolution, 0.100 mm; number of measurements per position, 32; signal factor, 5. Peak area measurement was employed (cf. Fig. 3).

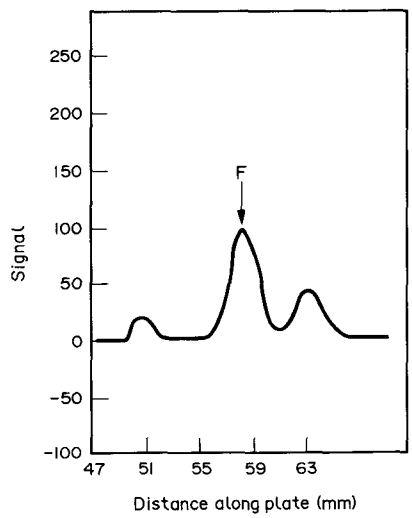

Figure 3
Densitometric scan of fraxin (fluorescence excitation wavelength, 360 nm; emission monitored above 450 nm). F: fraxin.

Results and Discussion

Choice of the HPTLC system

A chromatographic system previously described for the quantitative evaluation of fraxin was tested using HPLTC silicagel, and as mobile phase: ethylacetate–2-butanone–water–formic acid (5:3:2:1, v/v/v/v) [12]. The 'Partridge phase' was also tested on these plates: butanol–water–acetic acid: (40:50:10, v/v/v). Unfortunately, the separation was incomplete and the quantitative results were higher than those obtained by HPLC. For this reason, it was decided to use eluent conditions

similar to those for HPLC and this gave results comparable to those for HPLC. Acetic acid (10% v/v) was also tested as a mobile phase on HPTLC RP 18 plates. The results were similar to those obtained for both the present HPTLC and the HPLC methods.

Validation data

Good linearity was obtained for the calibration curve of fraxin by HPTLC with the phosphoric acid–acetonitrile mobile phase. By plotting the peak area vs the fraxin concentration, the following regression equation was found: $y = 18.487 + 5759.6\,x$ (λ_{ex} = 360 nm; $\lambda_{em} > 450$ nm; concentration range, 25–125 μg ml^{-1}; $r^2 = 0.9953$; $n = 26$).

The precision of the method was assessed by calculating the RSD values for the results obtained at a fraxin concentration of 50 μg ml^{-1}. The within-day reproducibility was 1.07% ($n = 6$) and the between-day reproducibility was 1.59% ($n = 8$).

The HPLC method was also characterized by good linearity, the following regression equation being found: $y = 79.026 + 2007.0\,x$ (λ_{ex}, 350 nm; concentration range, 25–100 μg ml^{-1}; $r^2 = 0.9869$; $n = 13$). The HPTLC and HPLC results for one of the batches compare well, confirming the accuracy of the method. The amount of fraxin found by the proposed HPTLC method was 0.142% (w/w) (RSD = 0.85%; $n = 4$) while that by the HPLC was 0.140% (w/w) (RSD = 0.98%; $n = 4$). By HPTLC using 10% (v/v) acetic acid as mobile phase the amount found was 0.141% (w/w) (RSD = 0.79%; $n = 4$). This shows that the results obtained with different chromatographic procedures are similar and gives some evidence for the purity of the fraxin peak in HPTLC.

Conclusions

The amount of fraxin found in six commercial batches of *Fraxinus excelsior* leaves (based on dry weight) ranged from 0.1 to 0.2%. The HPTLC method is a rapid method which could be successfully used in future for the quantification of fraxin in *Fraxinus excelsior* leaves and extracts.

Acknowledgements — This work was supported by the FNRS (National Fund for Scientific Research of Belgium). The authors are indebted to Dr R. Warin for his skilful assistance in spectrometric measurements.

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[Received for review 5 May 1992; revised manuscript received 24 June 1992]