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Determination of ipriflavone and its synthetic impurities by high-performance liquid chromatography using diode-array detection

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Abstract

A simple, accurate and rapid method for the determination of ipriflavone and its synthetic impurities has been developed. It consists in RP-HPLC separation and identification of the impurities from their UV spectra using photodiode-array detection. The method has been validated and shows good specificity, accuracy, precision and sensitivity.

1. Introduction

Ipriflavone (3-phenyl-7- isopropoxy-4*H*-1-benzopyran-4-one) is an isoflavone derivative used orally in the treatment of osteoporosis [1–4].

The accurate determination and control of the impurity profiles at the ppm level of orally administered drugs is of utmost importance [5–9], we have found high-performance liquid chromatography (HPLC) with photodiode-array (PDA) ultraviolet (UV) detection to be a very useful method for this task. The PDA detector has the capacity to acquire and store a great amount of spectral data from those UV-absorbing compounds in a chromatogram, thus making possible both spectral identification and individual analysis of the homogeneity/purity of each chromatographic peak.

Several methods for the determination of isoflavonoids have been described [10–13]. We describe in this paper a different and advantageous HPLC method for the determination of ipriflavone and its potential synthetic impurities.

2. Experimental

2.1. Chemicals

Synthetic-grade triethylamine (TEA), analytical-reagent grade 85% orthophosphoric acid and HPLC-grade acetonitrile were obtained from Merck (Darmstadt, Germany) and HPLC-grade methanol from Scharlau (Barcelona, Spain). Ultrapure water of Type I reactive degree was obtained using a Sation 9000 system (Sation, Barcelona, Spain).

Ipriflavone (**I**) and related impurities benzyl 2-hydroxy-4-isopropoxyphenyl ketone (**II**), 7-hydroxyisoflavone (**III**), benzyl 2,4-diisopropoxy-

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phenyl ketone (**IV**) and 7-ethoxyisoflavone (**V**) were obtained from the Research Department, FAES (Bilbao, Spain).

2.2. Apparatus

The assays were performed with a modular HPLC system consisting of two Model 510 solvent-delivery systems from Waters (Milford, MA, USA) and a Model 996 photodiode-array detector (Waters) with a range of 190–800 nm. Injections were accomplished with a Model 717 autosampler (Waters) equipped with a 250-μl syringe system and a 200-μl loop and an LC-101 column oven from Perkin-Elmer (Norwalk, CT, USA). The column used was ODS C_{18} (30 × 4.6 mm I.D.) of 3-μm particle size from Perkin-Elmer.

Data analysis was performed with a Millenium Software System (Waters) connected to a 486/66i computer (NEC, Boxborough, MA, USA). All the calculations concerning the quantitative analysis were performed with external standardization by measurement of peak areas.

2.3. Procedures

A 10-μl volume was injected on to the column in a mobile phase consisting of 0.01 *M* TEA (pH 2.5, adjusted with orthoposphoric acid)–acetonitrile (50:50) at a flow-rate of 1.2 ml/min. The chromatograms were recorded from 210 to 400 nm. Quantification was effected by measuring at the optimum wavelength of 225 nm as established from the three-dimensional chromatogram. The chromatographic run time was 12 min and the column void volume (the elution time of an unretained solute measured by injecting $NaNO_3$ solution) was 0.46 min.

To investigate the specificity of the assay, standard samples were prepared by spiking with standard methanolic solutions of the impurities (**II**, **III**, **IV** and **V**) to give final concentrations (each impurity) of 0.1, 0.2, 1 and 2% (w/w) in ipriflavone (**I**). These solutions were analysed and three-dimensional chromatograms (wavelength; time; absorbance) were obtained to select the optimum wavelength for detection of these potential impurities with maximum sensitivity.

To investigate linearity, duplicate methanolic solutions of five different concentrations (0.5, 1, 5, 10 and 25 μg/ml) were separately prepared in methanol for each impurity. Calibration graphs were constructed by plotting peak area against the concentration and the data were fitted to a straight line with zero intercept. Determination coefficients were calculated.

Limits of detection (LOD) were established at a signal-to-noise ration (S/N) of 3, measured at the 0.1% concentration level. Limits of quantifications (LOQ) were established at 3 × LOD or 9 × S/N. LOD and LOQ [14,15] were experimentally verified by ten injections of standard solutions of ipriflavone and the impurities at the LOD and LOQ concentrations. The instrumental precision was calculated at the LOQ level.

Throughout the study, the suitability of the chromatographic system was monitored by calculating the capacity factor (k'), the resolution (R_s) and the selectivity (α).

3. Results and discussion

3.1. Specificity

Three-dimensional chromatograms of the different mixtures of ipriflavone (**I**) and the impurities (Fig. 1) demonstrate a good separation of the ipriflavone (**I**) peak (t_R 3.4 min) from the impurities (t_R 0.8, 2.6, 5.6 and 9.1 min) and of the impurities from each other. Individual UV spectra of the impurities were measured (Fig. 2). A wavelength of 225 nm was found to be the most effective compromise to accomplish the detection and quantification of all four impurities in a single run.

3.2. Linearity

Table 1 presents the equation of the regression line, determination coefficient and standard error for each impurity. Using a zero intercept,

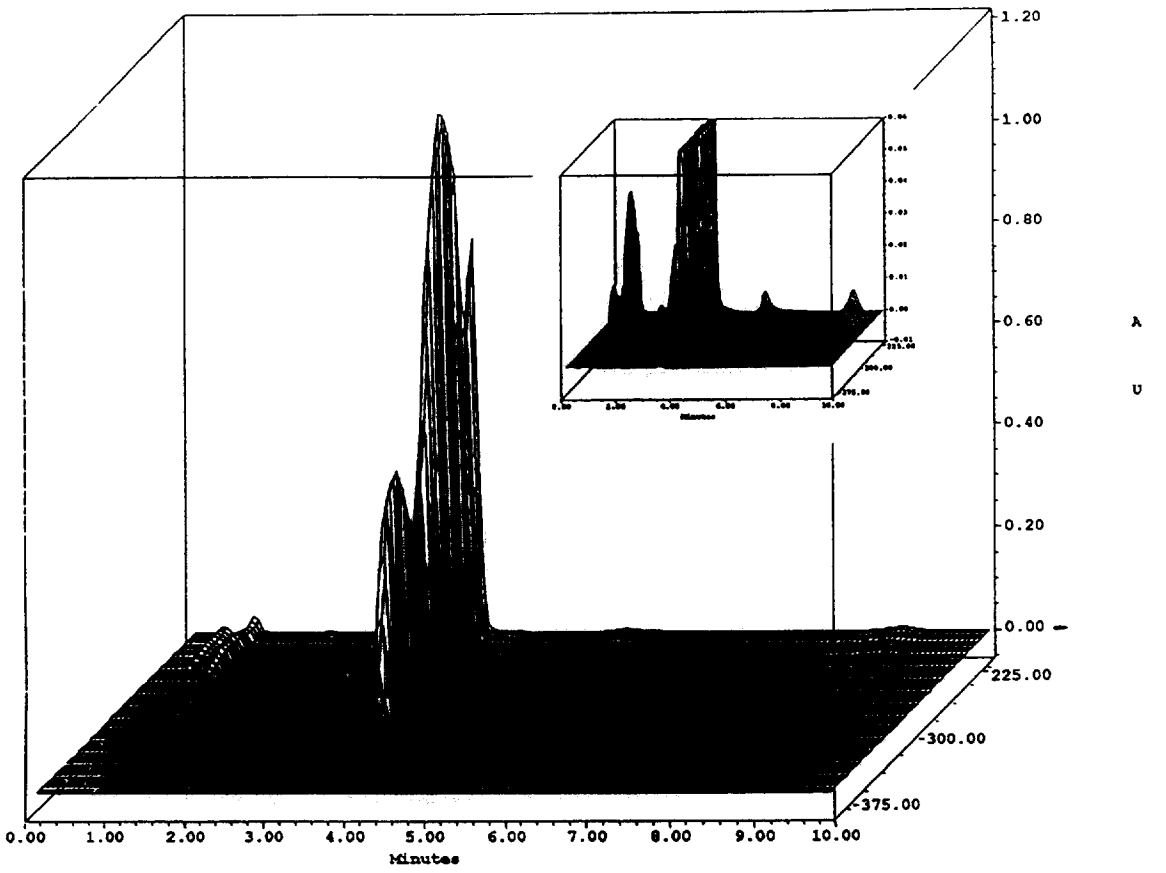

Fig. 1. Three-dimensional chromatogram of the mixture of ipriflavone and its four impurities at the 2% level, with an amplified detail in the inset. Chromatographic conditions as described under Experimental.

Table 1
Linearity results and limits of detection (LOD) and quantification (LOQ) calculated for each impurity

Compound	Equation[a]	R^2	Standard error of slope	No. of data points	LOD (ppm[b])	LOQ (ppm[b])
II	$y = 11551x$	0.9964	7711	8	446	1440
III	$y = 42900x$	0.9999	15418	10	20	80
IV	$y = 21763x$	0.9998	2836	9	300	1000
V	$y = 25997x$	0.9997	1465	10	74	240

Chromatographic conditions as described under Experimental.
[a] y = Concentration (μg/ml); x = peak area.
[b] With respect to ipriflavone (**I**) (w/w).

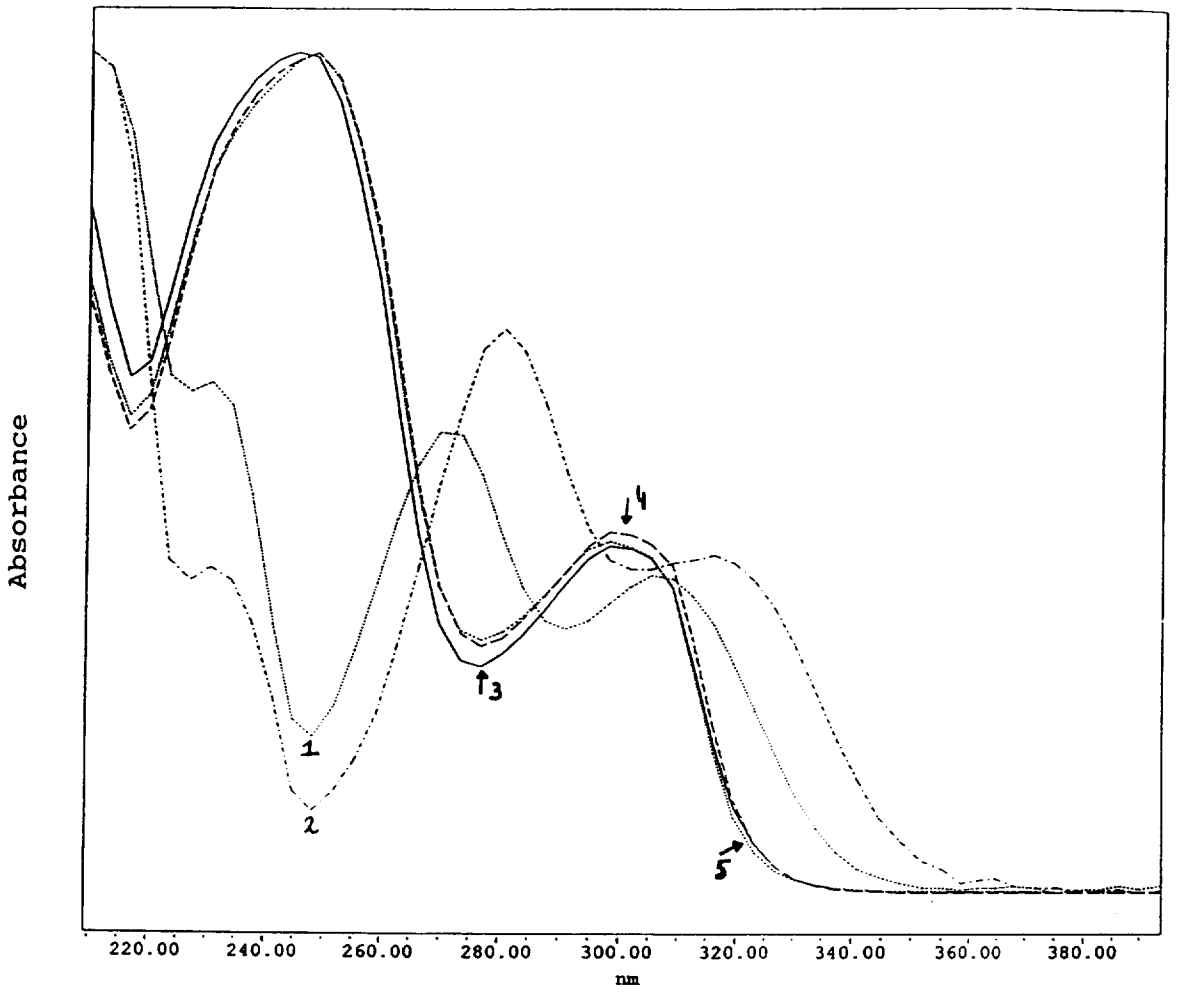

Fig. 2. Photodiode-array spectra of the middle of the peak corresponding to the retention times of compounds **I** (4), **II** (2), **III** (3), **IV** (1) and **V** (5). Chromatographic conditions as described under Experimental.

excellent linearity was obtained for compounds **III**, **IV** and **V** ($r^2 < 0.999$ in all cases). Compound **II** also provided good linearity although slightly lower than that obtained for the other impurities ($r^2 > 0.996$).

3.3. Limits of detection and quantification

The LOD and LOQ obtained for each impurity are given in Table 1. Table 2 gives the precision of the method, expressed as the rela-

Table 2
Precision of the assay of impurities at the LOQ level ($n = 10$)

Compound	R.S.D. (%)[a]
II	4.08
III	10.63
IV	4.85
V	2.25

Chromatographic conditions as described under Experimental.

[a] R.S.D. (%) = (standard deviation/mean) · 100.

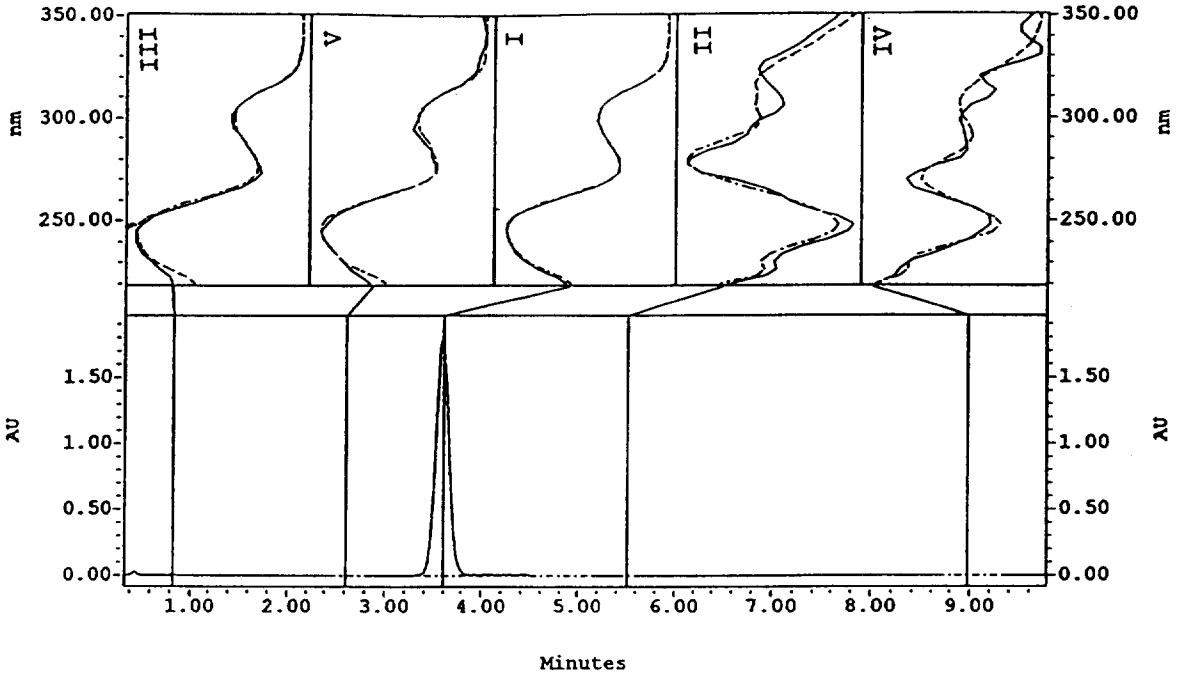

Fig. 3. Chromatogram of the mixture of ipriflavone (**I**) standard and its impurities (**II**, **III**, **IV** and **V**) at LOD concentration levels. Spectral identification of the peaks (solid lines) was made by comparison with a data base (dashed lines). Chromatographic conditions as described under Experimental.

tive standard deviation (R.S.D.) at the LOQ; it is acceptable for all the impurities. An excellent match of the UV spectra of the impurities obtained at the limits of detection with those obtained at other concentrations (Fig. 3, Table 3) was observed.

3.4. Suitability of the method

The chromatographic parameters such as capacity factor, resolution and relative retention were satisfactory for these compounds (Table 3). The retention was low for compound **III** ($k' < 1$),

Table 3
Chromatographic parameters of ipriflavone and its impurities and matching spectral results at LOD concentration levels

Compound	Retention time (min)	Area (μV s)	Resolution (R_s)	k'	Selectivity	Match angle[a] (°)	Threshold angle[b] (°)
III	0.843	3802	–	0.83	–	2.727	4.176
V	2.610	1651	11.27	4.67	5.61	2.026	6.966
I	3.610	17 241 875	4.50	6.85	1.47	1.743	3.001
II	5.510	858	6.17	10.98	1.60	6.743	13.916
IV	8.977	2118	5.53	18.51	1.69	8.118	14.873

Chromatographic conditions as described under Experimental.
[a] Match angle = the spectral contrast between the two peaks.
[b] Threshold angle = the spectral difference attributed to noise and solvent effects.

as would be expected from its structure and physico-chemical characteristics, but we believe these results to be satisfactory. The selectivity and specificity of the system have been demonstrated.

4. Conclusions

The method described is suitable for the identification and quantification of the four potential impurities in the synthetic procedure for ipriflavone [16]. The method exhibits good sensitivity, with detection limits of the order of <100 ppm for compounds **III** and **V** and <500 ppm for **II** and **IV**. The spectral study allows the identification of the peaks at the levels of the detection limits and the purity of the peaks at the quantification limits. All four impurities exhibit good linearity in the range 0–5% (w/w) with respect to ipriflavone, which demonstrates the suitability of the method for the determination of these compounds in this range.

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