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Short communication

High-performance liquid chromatographic determination of the biologically active principle hypericin in phytotherapeutic vegetable extracts and alcoholic beverages

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Abstract

Hypericin was determined using an RP C_{18} (3 μm) column (8.3 × 0.4 cm I.D.), thermostated at 50°C. The separation was achieved with programmed elution using phosphate buffer (pH 7)–methanol (3:7) and water–methanol (3:7) as eluents. Fluorimetric detection was carried out with excitation at 470 nm and emission at 590 nm. The analytical sample was prepared by simple dilution in methanol of the phytotherapeutic extract or of the alcoholic beverage. Hypericin can be rapidly and accurately determined at concentrations down to 0.1 mg/kg without any interferences.

Keywords: Hypericum perforatum; Food analysis; Beverages; Pharmaceutical analysis; Hypericin

1. Introduction

Hypericum perforatum is a perennial herbaceous plant known to have medicinal properties since ancient times and it is widely used in phytotheraphy in several European countries. Many therapeutic properties are attributed to it: it acts on liver congestion and lung inflammation, it has cicatrizing and analgesic power and has antidepressant properties [1,2].

Interest in *Hypericum perforatum* has recently been revived because its vegetable extracts appear to have interesting antiviral properties [3]. This activity, which has been mainly attributed to hypericin, one of the components of the plant extract, has been shown in vitro against the human immunodeficiency virus (HIV) [4]. Studies on this are continuing in order to evaluate possible clinical applications.

As often happens with many vegetable species, *Hypericum perforatum* is also used both for therapeutic purposes and as a flavouring in the preparation of foods and alcoholic beverages. Both the drug and its derivatives (infusion, alcoholic tincture, fluid extract) are used in the flavouring industry to prepare liqueurs, especially digestive and tonic bitters [5].

In order to safeguard consumers, the EEC regulation for flavouring substances has established, with Directive 88/388 [6], that the hypericin content due to use of the extract of natural flavourings must not exceed limits of 0.1

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mg/kg in foods, 1 mg/kg in cakes and sweets and 10 mg/kg in alcoholic beverages.

No method for the determination of hypericin in food and beverages has been reported previously. As far as the phytotherapeutic extracts are concerned, the French Pharmacopoeia, for example, provides mainly for qualitative analyses and uses traditional techniques such as paper and thin-layer chromatography [7].

Two HPLC methods have recently been published for the determination of hypericin in plasma and biological fluids in a study of its antiviral properties [8] and in pharmaceutical capsules [9]. These methods were not suitable for our purpose because they took into account matrices very different from those which we intended to analyse and further they required the extraction and purification of the analytical sample.

For this reason, we though it would be useful to develop an HPLC method in order to determine hypericin both in aqueous–alcoholic extracts used in phytotherapy and in alcoholic beverages. The method proposed in this paper requires a simple dilution of the sample with methanol followed by reversed-phase liquid chromatography with fluorimetric detection.

2. Experimental

2.1. Equipment

A Perkin-Elmer (Norwalk, CT, USA) Series 4 liquid chromatograph equipped with a Rheodyne (Cotati, CA, USA) Model 8125 valve with a 20-μl loop and a Perkin-Elmer LS-4 spectrofluorimetric detector with a 3-μl microcell were used. The chromatographic data were recorded and processed by means of Chrom Card software (Fisons Instruments, Rodano, Milan, Italy).

2.2. Chemicals

Methanol was of HPLC grade (J.T. Baker, Deventer, Netherlands); water was obtained using a Milli-Q water purification system (Millipore, Bedford, MA, USA); 0.05 *M* phosphate buffer (pH 7) was prepared from disodium hydrogenphosphate dihydrate and potassium dihydrogenphosphate (RPE, Carlo Erba, Milan, Italy) and filtered through a HAWP filter (Millipore). Hypericin standard of purity >95% was purchased from Sigma (St. Louis, MO, USA).

2.3. Sample preparation

About 1 g of alcoholic beverage or phytotherapeutic aqueous–alcoholic extract was weighed exactly and then appropriately diluted with methanol, depending on the initial concentration. The dilutions generally used were 1:25 for aqueous–alcoholic extracts and 1:5 for alcoholic beverages.

The sample was ready for analysis after filtration through a GV_{13} Millex filter (Millipore). If kept at −18°C, it was stable for 2 weeks.

2.4. Chromatographic analysis

We used a Perkin-Elmer HS C_{18} (3 μm) (reversed-phase column (8.3 × 0.4 cm I.D.), connected with a Supelguard LC 18 (5 μm) guard column (2 × 0.46 cm I.D.) (Supelco, Bellefonte, PA, USA) thermostated at 50°C. For elution two mixtures of eluents were used: mobile phase A was phosphate buffer (pH 7)–methanol (3:7) and B was water–methanol (3:7). The elution programme was isocratic with 100% A for 3 min, followed by a linear gradient up to 100% B in 2 min, then isocratic again for 13 min. Re-equilibration of the column was achieved with a linear gradient from 100% B to 100% A (initial condition) in 2 min and followed by 10 min isocratic with 100% A before the next injection; the flow-rate was 1.5 ml/min. Fluorimetric detection was carried out with excitation at 470 nm and emission at 590 nm. Under these conditions, it took 30 min to complete a cycle of analysis. Hypericin was eluted at a retention time of 9 min.

Quantitative analysis was effected by using linear regression lines, obtained by injecting solutions of known content of standard hypericin. The linear regression coefficient was 0.9997. The response of the detector was verified

to be linear in the concentration range 0.05–3.2 mg/kg.

3. Results

We used the proposed method to analyse aqueous–alcoholic extracts of *Hypericum perforatum* in phytotherapeutic preparations produced by European pharamaceutical companies. Random sampling was effected four times in 1 year so that we analysed 20 samples coming from different stocks. In the same way, commercial samples of alcoholic beverages were purchased at random in Italian grocery stores, amounting to 40 samples. Each sample was analysed three times.

Fig. 1 shows the chromatograms obtained for the hypericin standard, for a sample of a phytoterapeutic preparation and for an alcoholic beverage added with hypericin.

We identified hypericin by comparing the chromatographic behavior of the samples with that of the standard solution, and comparing the excitation and emission spectra of the standard which were registered directly by the "stopped-flow" technique with those obtained for the peak at the retention time of hypericin. In order to evaluate the accuracy of the proposed HPLC method, we added known amounts (from 1 to 12 mg/kg) of hypericin standard to ten commercial samples of alcoholic beverages and ten aqueous–alcoholic tinctures of *Hypericum perforatum* whose hypericin content had been determined. The recoveries for the alcoholic beverages ranged from 95% to 103.3%, with an average of 100.1%, and those for the aqueous–alcoholic tinctures ranged from 99.3% to 106%, with an average of 102.3%.

The precision of the method was determined by seven analyses of one sample of an aqueous–alcoholic extract of *Hypericum perforatum* and of one sample of alcoholic bitter without hypericin to which a known amount of hypericin standard was added. The R.S.D.s for hypericin were 0.5% and 0.4%, respectively.

Phytotherapeutic extracts produced by different pharmaceutical companies showed hypericin contents ranging from 9.1 to 12.5 mg/kg. This could be due to the neutral variability of the composition of the vegetable matter and not to unreliable methods of pharmaceutical preparation.

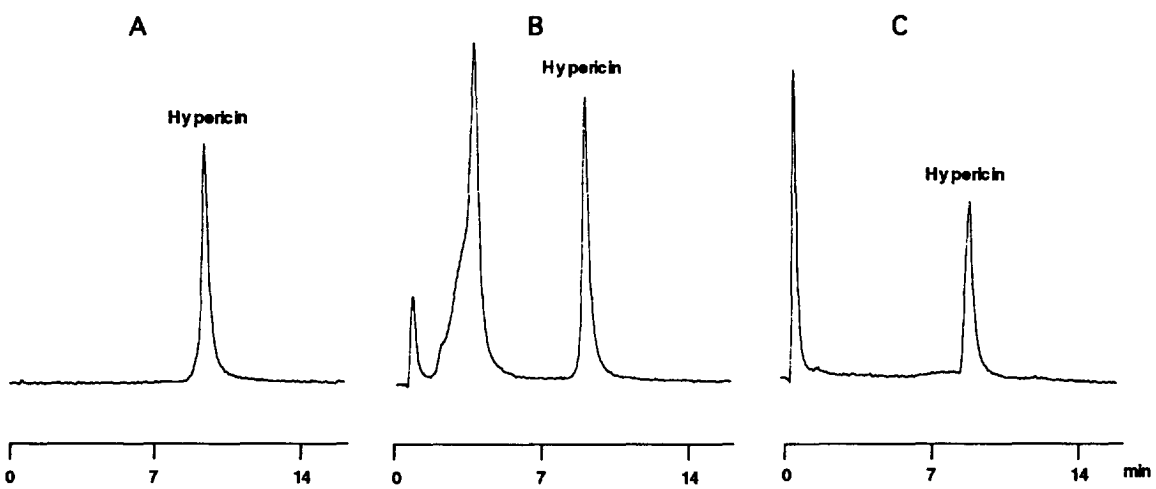

Fig. 1. Chromatograms obtained by injecting 20 μl of (A) 0.32 ppm hypericin standard, (B) phytotherapeutic aqueous–alcoholic extract of *Hypericum perforatum*, 0.4 ppm after dilution 1:25, and (C) bitter sample added with hypericin standard, 0.25 ppm after dilution 1:5. Column, RP 18 (3 μm) (8.3 × 0.4 cm I.D.) thermostated at 50°C; mobile phase, solution A = phosphate buffer (pH 7)–methanol (3:7), solution B = water–methanol (3:7), elution programme 100% A isocratic for 3 min, linear gradient up to 100% B in 2 min, then isocratic again for 13 min; flow-rate, 1.5 ml/min; fluorimetric detection at λ_{ex} 470 nm and λ_{em} 590 nm.

No hypericin was found in the samples of alcoholic bitters we analysed, which all came from the Italian market. This demonstrates that producers are conforming to European laws; in recent years, vegetable extracts used as flavourings in foods and beverages have been obtained by processes which eliminate or sharply reduce the amount of principle subject to limitation [10,11].

4. Conclusions

Owing to the high separative power of the chromatographic system adopted and to the selectivity of the detector, this method allowed us to obtain analytical samples by simple dilution with methanol. It also allowed us to determine hypericin rapidly and accurately, without any interferences, at concentrations down to 0.1 mg/kg both in phytotherapeutic extracts of *Hypericum perforatum* and in alcoholic beverages. It enabled us to control and standardize phytotherapeutic products and to verify whether the law had been applied correctly in the preparation of alcoholic beverages.

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