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Assay of hydroxyfarrerol in biological fluids

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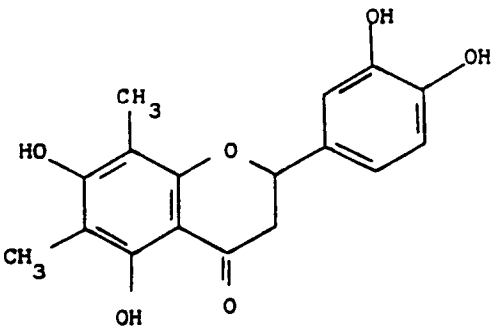
ABSTRACT

A high-performance liquid chromatographic method for the determination of hydroxyfarrerol (IdB 1031) in biological samples was developed. IdB 1031 was first extracted by liquid–solid partition and the extracts were evaporated and analysed on a reversed-phase column under isocratic conditions, using either an electrochemical or a UV detector. The detection limit was *ca.* 5 ng/ml. Preliminary pharmacokinetic data showed that rats treated orally with 500 mg/kg had an average peak plasma concentration (C_{max}) of 497 ng/ml after 2 h.

INTRODUCTION

In previous pharmacological investigations, (±)-3-hydroxyfarrerol (IdB 1031) was found to possess interesting mucokinetic properties [1]. For pharmacokinetic studies of IdB 1031, a high-performance liquid chromatographic (HPLC) method was developed and validated for quantitative assays in biological fluids.



IdB 1031

0021-9673/90/$03.50

Like other structurally related flavonoids, IdB 1031 can be easily extracted from plasma by liquid–solid partition, using methanol as extraction solvent, and determined by reversed-phase HPLC with eluents buffered to pH 2–3 with phosphoric acid [2–4]. The presence and the amounts of acetonitrile, methanol and 2-propanol in the eluent markedly influence the shape of the peak, the retention time and the selectivity for this kind of compound.

EXPERIMENTAL

Materials

IdB 1031 Lot 1/30 (Inverni della Beffa, Milan, Italy) was used as a reference product. Eriodictyol from Extrasynthèse (Genay, France) and naringenin from Aldrich (Steinheim, F.R.G.) respectively were used as internal standards, for electrochemical (ED) and UV detection. HPLC-grade solvents used for the preparation of samples and chromatographic eluents (methanol, acetonitrile, water, 2-propanol) were purchased from Merck (Bracco, Milan, Italy).

The liquid chromatograph consisted of a Millipore–Waters (Milford, MA, U.S.A.) Model 510 pump controlled by a Model 680 programmer equipped with an additional pulse damper, a Rheodyne (Calabasas, CA, U.S.A.) Model 7125 injector, an ESA Model 5100A two-cell electrochemical detector (Environmental Sciences, Bedford, MA, U.S.A.), a Varian (Sunnyvale, CA, U.S.A.) Model 2050 variable-wavelength UV detector and an HP 3390 integrator–recorder (Hewlett-Packard, Avondale, PA, U.S.A.). Data were analysed with an Apple II plus personal computer (Apple Computer, Cupertino, CA, U.S.A.).

The solid-phase extraction was carried out with Sep-Pak C_{18} (Waters Assoc., Milford, MA, U.S.A.).

Preparation of reference solutions

A stock solution (0.25 mg/ml) of internal standard was prepared by dissolving eriodictyol or naringenin in methanol containing phosphoric acid (0.5%, v/v). The solutions for the assay of IdB 1031 were prepared as follows: IdB 1031 (25 mg) was dissolved in methanol (100 ml) containing phosphoric acid (0.5%, v/v) (solution A). Solution A (1 ml) was diluted to 50 ml with the same solvent (solution B). Solution A (1 ml) and internal standard stock solution (1 ml) were diluted to 50 ml with the HPLC eluent. Thus, a solution containing 5 μg/ml of IdB 1031 and 5 μg/ml of internal standard was obtained. Solution B (5 ml) and internal standard stock solution (1 ml) were diluted to 50 ml with the HPLC eluent. Thus, a solution containing 0.5 μg/ml of IdB 1031 was obtained. The internal standard solution used for sample preparation was obtained by diluting the stock solution (1 ml) to 20 ml with water.

Sample preparation

The extraction of plasma was carried out as follows. Internal standard (40 μl, corresponding to 0.5 μg of product) and water (1 ml), containing phosphoric acid (0.5%, v/v), were added to 0.5 ml of plasma. After mixing on a vortex mixer, this suspension was loaded onto a Sep-Pak C_{18} cartridge, previously equilibrated with methanol (2 ml), water (5 ml) and water containing 0.5% (v/v) phosphoric acid (0.5 ml). After elution of the plasma suspension, the cartridge was washed with 1 ml of

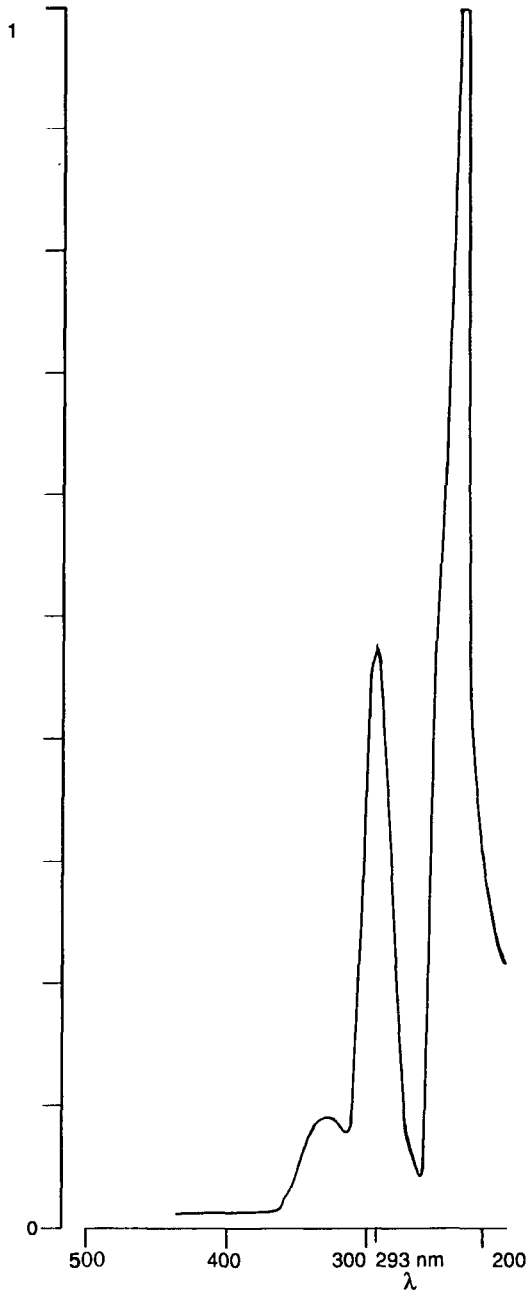


Fig. 1. UV spectrum of IdB 1031. Scan, 500–190 nm; scan rate, 100 nm/min; chart speed, 2 cm/min; λ_{max}, 293 nm; absorbance, 0.494; $E^{1\%}_{1\,cm}$, 617; ε, 19 500 l/mol · cm.

water, containing 0.5% (v/v) phosphoric acid, and then with 0.5 ml of methanol in order to remove the water absorbed by the stationary phase. The eluates were discarded and the cartridge was washed with 0.5-ml portions of methanol twice. The eluates were collected and concentrated to a small volume at 40°C under a gentle stream of nitrogen. The residue was resuspended in 100 μl of the HPLC eluent and filtered through filters of 0.45-μm porosity.

Chromatographic conditions

The UV spectrum of IdB 1031 in methanol showed λ_{max} = 293 nm with ε = 19 500 l/mol · cm (Fig. 1). The presence of a catechol moiety makes the molecule electrochemically active and oxidizable at a low potential (0.3 V *vs.* H_2/H^+), so both ED and UV detection can be performed on this molecule.

The eluent was prepared by mixing 30 volumes of acetonitrile, 8 volumes of 2-propanol and 62 volumes of water. Phosphoric acid (2 ml/l) was added and the pH was adjusted to 2.5 with a few drops of 1 *M* sodium hydroxide solution. The column (250 mm × 4.6 mm I.D.) was packed with Nucleosil C_8 (5 μm) (Macherey, Nagel & Co., Düren, F.R.G.). The flow-rate was 0.6 ml/min. The electrochemical detector was set to a potential of +0.01 V in the first cell (used as background noise filter) and to 0.30 V in the second cell. The UV detector was set at 293 nm. The retention times of eriodictyol, naringerin and IdB 1031 were 7, 8.5 and 10 min, respectively.

Treatment schedule

IdB 1031 was administered by gastric gavage to rats fasted for 18 h at 500 mg/kg.

TABLE I

LIQUID–SOLID EXTRACTION RECOVERY OF IdB 1031 FROM RAT PLASMA

UV detection was used, although ED detection gave identical results.

IdB 1031 added ($=x$)[a] (ng/ml)	IdB 1031 recovered ($=y$)[a] (ng/ml)	Recovery (%)
10	10.343	103.4
	9.328	93.3
25	17.781	71.3
	18.317	73.3
50	47.222	94.4
	47.175	94.3
100	80.682	80.7
	87.944	94.4
300	243.815	81.3
	259.872	86.6
500	441.269	88.2
	430.118	86.0
Mean:		87.3
S.D.:		9.44
R.S.D. (%):		10.81

[a] Linear correlation gave the relationship $y = -0.880 + 0.865x$ ($r^2 = 0.9986$).

Animals, divided into eight per group, were killed before and 0.25, 0.5, 1, 2, 4, 6, 8, 24 and 48 h after treatment; heparinized blood was collected and the resulting plasma was immediately separated and processed as described above.

RESULTS AND DISCUSSION

The results obtained led to the conclusion that IdB 1031 can be evaluated by HPLC by using both ED and UV detection with standards enabling the assay to be performed in biological samples.

Owing to its structure (it is a weakly acidic compound), IdB 1031 can be extracted from previously acidified plasma by liquid–solid partition, with a mean recovery of about 87% (Table I). HPLC can be carried out on reversed-phase columns with a mobile phase buffered to pH 2.5 with phosphoric acid (Fig. 2). Good results are obtained if IdB 1031 is dissolved in aqueous–alcoholic solutions, acidified to pH 2–3 in which it is fairly stable. The extraction recovery was linear in the range 10–500 ng/ml in plasma with both ED and UV detection. The reproducibility, expressed as relative standard deviation (R.S.D.), was 6.05% with ED and 10.81% with UV detection. The lowest detectable concentration was 5 ng/ml with both detection methods. Both methods seem to be equivalent for pharmacokinetic purposes, even though ED allows a better signal-to-noise ratio to be achieved.

Data for IdB 1031 plasma levels in rats after a single oral dose (500 mg/kg) indicate rapid absorption (t_{max} = 2 h), followed first by a fast and then by a slower elimination phase. The peak concentration (C_{max}) was, on average, 497 ng/ml (Fig. 3). HPLC recordings also revealed the presence of two unknown peaks, possibly corresponding to two metabolites (M_1, M_2) (Fig. 4).

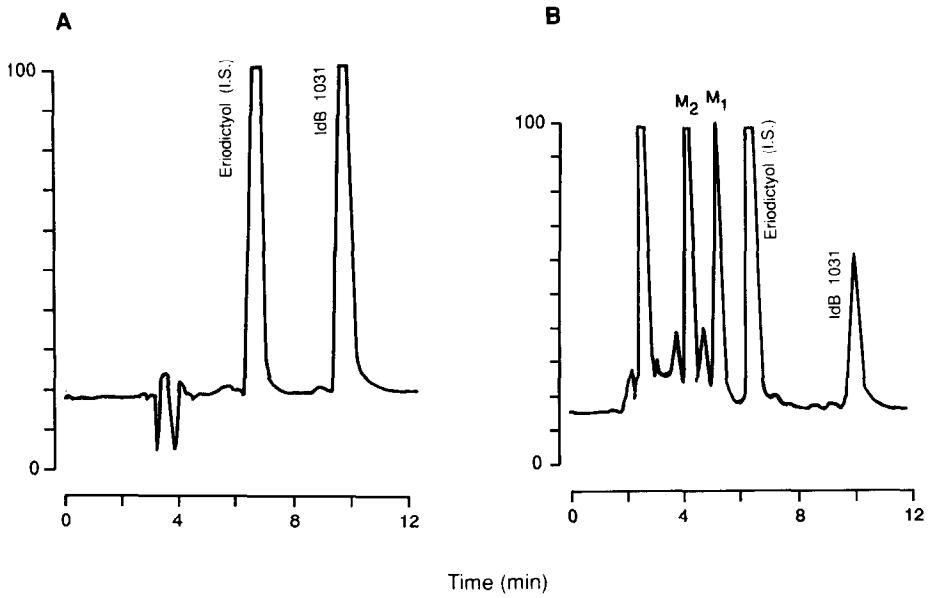


Fig. 2. Typical HPLC recordings obtained with ED. (A) IdB 1031 and eriodictyol as authentic standards. (B) Rat plasma 2 h after oral administration of IdB 1031 (500 mg/kg).

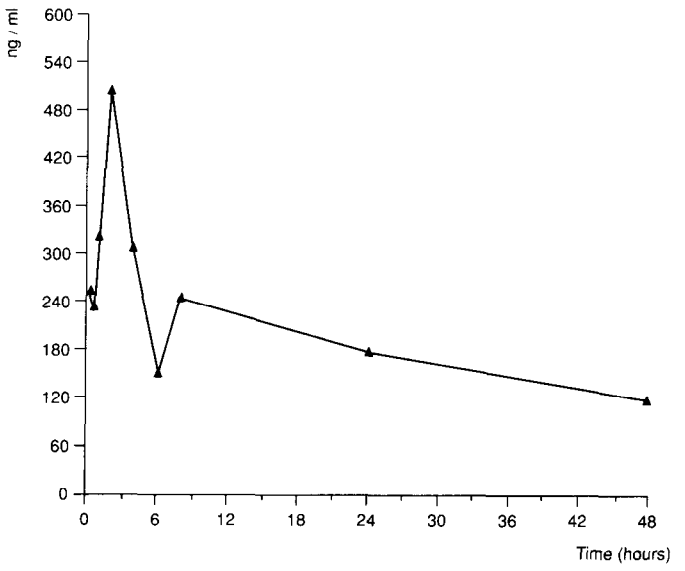


Fig. 3. Mean plasma levels (n = 8) of IdB 1031 in rats after oral administration (500 mg/kg) of IdB 1031. The standard deviations were on average 66% of the mean value, ranging from 50 to 80%.

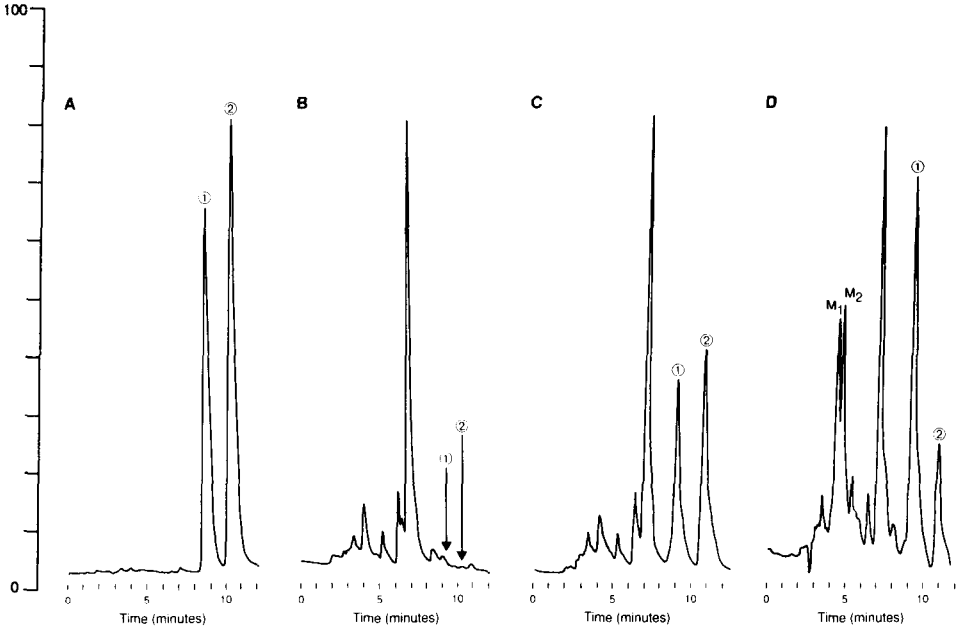


Fig. 4. Chromatograms showing the separation between IdB 1031 and naringenin. (A) Standard solution; (B) blank plasma; (C) plasma spiked with IdB 1031 and naringenin; (D) plasma sample after oral administration of IdB 1031 (500 mg/kg) to rats. UV detection, 293 nm. Peaks: 1 = naringenin; 2 = IdB 1031; M_1, M_2 = unknown metabolites.

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