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Short communication

Simultaneous determination of oleuropein and hydroxytyrosol in rat plasma using liquid chromatography with fluorescence detection

Hai-Wei Tan, Kellie L. Tuck, Ieva Stupans, Peter J. Hayball*

Centre for Pharmaceutical Research, School of Pharmaceutical, Molecular and Biomedical Sciences, University of South Australia, Adelaide, 5000, Australia

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Abstract

Oleuropein, the main glycoside present in olives, and hydroxytyrosol, the principal degradation product of oleuropein present in olive oil, have been linked to reduction of coronary heart disease and certain cancers. In the present study a direct and sensitive reversed-phase high-performance liquid chromatographic assay was developed for simultaneous quantification of both oleuropein and hydroxytyrosol. The plasma protein was precipitated with acetonitrile, samples were then centrifuged and supernatants were dried, and reconstituted with water prior to injection. The chromatographic analysis was carried out using a phenyl column and an isocratic elution of acidified water and acetonitrile with fluorescence detection at 281 and 316 nm for excitation and emission, respectively. The calibration curve was linear and limits of quantification were 30 ng/ml and 3 μg/ml for hydroxytyrosol and oleuropein, respectively. The method has been successfully applied to monitor oleuropein and hydroxytyrosol plasma levels in the rat.

Keywords: Oleuropein; Hydroxytyrosol

1. Introduction

Oleuropein and hydroxytyrosol are naturally occurring phenolic compounds found in olive (*Olea europea*) fruits. Whilst oleuropein is present in high amount in unprocessed olive fruit and leaves, hydroxytyrosol is more abundant in the processed olive fruit and olive oil [1,2]. Oleuropein is the main glycoside in olives and is responsible for the bitter taste of immature and unprocessed olives. Chemically, oleuropein is the ester of elenolic acid and 3,4-dihydroxyphenyl ethanol (Fig. 1).

3,4-Dihydroxyphenyl ethanol is more commonly known as hydroxytyrosol and it is the principal degradation product of oleuropein. During maturation of fruit or as a results of olive processing (such as oil production), chemical and enzyme reactions occur which reduce the concentration of oleuropein and raise the concentration of hydroxytyrosol.

Phenolic components of olive products, such as olive oil and nutriceuticals containing olive leaf extracts, have been reported to be beneficial to health. Numerous epidemiological studies have linked the consumption of olive oil, a fundamental constituent of the Mediterranean diet, to a reduction

*Corresponding author. Tel.: +61-8-8302-1646; fax: +61-8-8302-2389.

E-mail address: peter.hayball@unisa.edu.au (P.J. Hayball).

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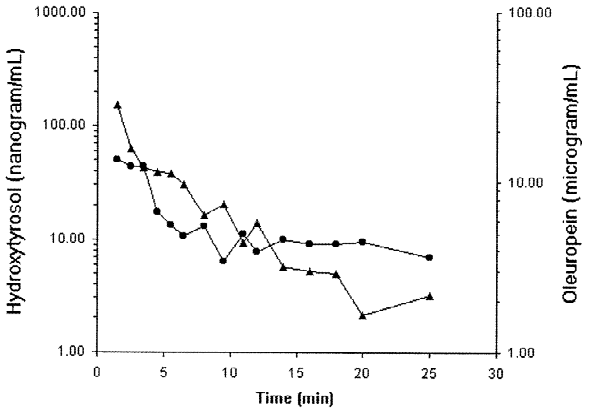

Fig. 1. Chemical structure of oleuropein.

in cardiovascular disease and various tumors [3,4]. Numerous studies have linked the antioxidant properties of olive-derived phenolic compounds to the health promoting properties of olive oil [5,6].

A number of studies of hydroxytyrosol quantification in biological fluids have been reported. The first reported assay was analysis of extracted rat plasma samples by GC–MS [7]. This method cannot be applied to oleuropein due to high molecular mass and low volatility. Recently, a method was reported for determination of hydroxytyrosol in rat plasma by HPLC [8]. This method incorporates a solid-phase extraction step; however, the quantification of oleuropein was not attempted. To our knowledge, there have been no reports of analytical methods describing the quantification of oleuropein in plasma. The aim of this study was to develop a direct and sensitive method for the simultaneous detection of oleuropein and hydroxytyrosol in plasma in order to define the plasma concentration–time profile of both compounds upon administration of oleuropein to rats.

2. Experimental section

2.1. Materials

Hydroxytyrosol (3,4-dihydroxyphenyl ethanol) was synthesized by reduction of (3,4-dihydroxyphenyl) acetic acid with $LiAlH_4$ according to Baraldi et al. [9]. Oleuropein was purchased from Indofine Chemical (New Jersey, NJ, USA). Eserine (physostigmine), ascorbic acid and the internal standard (I.S.), 3,4-dimethoxyphenyl ethanol, were purchased from Aldrich (Milwaukee, WI, USA). Acetonitrile and acetic acid (HPLC graded) were obtained from BDH Laboratory Supplies (Poole, UK). Water used in all experiments was obtained from a milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Instrumentation

HPLC was performed on a Shimadzu HPLC system consisting of a pump (LC-10ADvp), auto sampler (SIL-10ADvp), degasser (DGU-12A), and a fluorescence detector (RF-551). Compounds were separated on a 250×4.6 mm (5 μm) Zorbax phenyl column at ambient temperature. The mobile phase consisted of acetonitrile–milli-Q water (consisting of 2% acetic acid) (15:85, v/v) and pH adjusted to 1.97 with 5.0 *M* hydrochloric acid. Optimum conditions were achieved by varying flow-rate. The initial flow-rate was 1.0 ml/min for 6.9 min, which then increased in a step-wise fashion to 1.5 ml/min from 7 to 10.4 min, and then to 2.0 ml/min from 10.5 to 26 min. Finally, the flow-rate was linearly reduced back to 1.0 ml/min for 2 min. The column eluate was monitored by fluorescence detection using wavelengths of at 281 and 316 nm for excitation and emission, respectively. Peak height integration was performed using a Merck–Hitachi D2500 Chromato-Integrator.

2.3. Sample preparation

To an aliquot (50 μl) of plasma sample, ascorbic acid (10 μl, 100 ng/ml) and eserine (10 μl, 200 ng/ml) were added for stabilization of both hydroxytyrosol and oleuropein. Acetonitrile (300 μl) was then added to precipitate proteinaceous material. After vortex mixing and centrifugation (1000 *g*) for 10 min, the supernatant was collected and dried under vacuum to dryness. The sample was then reconstituted with water (100 μl) prior to injection (50 μl) into HPLC for analysis.

2.4. Validation

Stock standard solutions of oleuropein and hydroxytyrosol were prepared by dissolving appropriate

amounts of the compounds in milli-Q water. These solutions were stored at 4 °C and used within 4 weeks because slight degradation of both compounds was observed in the subsequent week. Calibration curves of spiked plasma samples containing increasing concentrations of oleuropein (3–50 μg/ml), hydroxytyrosol (30–500 ng/ml) and a fixed concentration of 3,4-dimethoxyphenyl ethanol (10 μl, 100 ng/ml) were prepared daily and analysed according to the procedure described above.

2.5. Precision and accuracy

Quality control samples were prepared at 4, 20, 40 μg/ml and 40, 200, 400 ng/ml for oleuropein and hydroxytyrosol, respectively, by spiking pooled blank rat plasma. In order to reduce oxidation of hydroxytyrosol and hydrolysis of oleuropein, ascorbic acid (10 μl, 100 ng/ml) and eserine (10 μl, 200 ng/ml) was also added to the sample [8]. These samples were stored at −20 °C, and thawed prior to their use in an analytical run.

2.6. Sensitivity

The limit of quantification for each analyte was determined by replicate analyses and considered to be 10 times the standard deviation of baseline noise from six blank plasma samples.

2.7. In vivo study design

Written ethics approval was obtained from the local committee (IMVS, South Australia, Australia). A healthy male Sprague–Dawley rat (381 g) was dosed with 25 mg/kg (100 μl) of oleuropein dissolved in sodium chloride (0.9%, w/v) via a tail vein injection. Serial blood samples (300 μl) were withdrawn at appropriate time intervals via a catheter implanted in the right jugular vein and transferred to a heparinized tube. Plasma (150 μl) was immediately separated by centrifugation at 1000 *g* for 10 min. Plasma samples (50 μl) were immediately stabilized as indicated in samples preparation. Stabilized samples, if not possible to be analysed on the same day, were kept at −20 °C until the analyses were performed.

Table 1
Calibration data derived from linear least-squares regression analysis for hydroxytyrosol and oleuropein in rat plasma

Regression parameter	Mean (±SD) value (n=6)	
	Hydroxytyrosol	Oleuropein
Slope	0.011±0.006	0.065±0.033
y-Intercept	0.073±0.088	0.112±0.061
Correlation coefficient	0.9936±0.007	0.9667±0.0144
Concentration range	30–500 ng/ml	3–50 μg/ml

3. Results and discussion

3.1. Sample preparation and validation study

The analytical method described here incorporated variations to flow-rate within an analytical run. This allowed analysis of both hydroxytyrosol and oleuropein under isocratic elution conditions. The system pressure throughout the run was maintained below 3500 p.s.i. and eluate detection by native analyte fluorescence was not affected by the variation of pressure as excellent peak shapes and repeatability were achieved in each run. Fluorescence detection showed the better sensitivity and specificity over UV detection; the latter giving rise to overlapping peaks problem.

The assay was shown to be linear over a concentration range for both oleuropein and hydroxytyrosol. Calibration curves were generated by linear least-squares regression analysis (n=6) is given in Table 1. In addition, assessment of the assay precision and accuracy (Table 2) was made by calculating the coefficient of variation (C.V.), which ideally was

Table 2
Interday accuracy and precision of the analytical method for hydroxytyrosol and oleuropein in plasma (n=6 for each predicted concentration)

	Quality control	Mean (%C.V.) predicted concentration
Hydroxytyrosol (ng/ml)	40	38.71 (4.67)
	200	176.02 (4.13)
	400	363.25 (3.19)
Oleuropein (μg/ml)	4	4.65 (6.22)
	20	19.84 (4.82)
	40	39.86 (2.75)

to fall within 20% of stated concentrations for the lower concentration, and 10% of stated concentrations for the higher concentration. Accuracies at two different concentrations, for oleuropein and hydroxytyrosol were greater than 85%. High accuracy is achievable due to little sample handling needed for plasma processing.

3.2. In vivo study

Representative chromatograms of drug-free plasma, plasma spiked with oleuropein, hydroxytyrosol, internal standard, ascorbic acid and eserine, and plasma taken from a rat after a 25 mg/kg intravenous dose of oleuropein are shown in Fig. 2. No

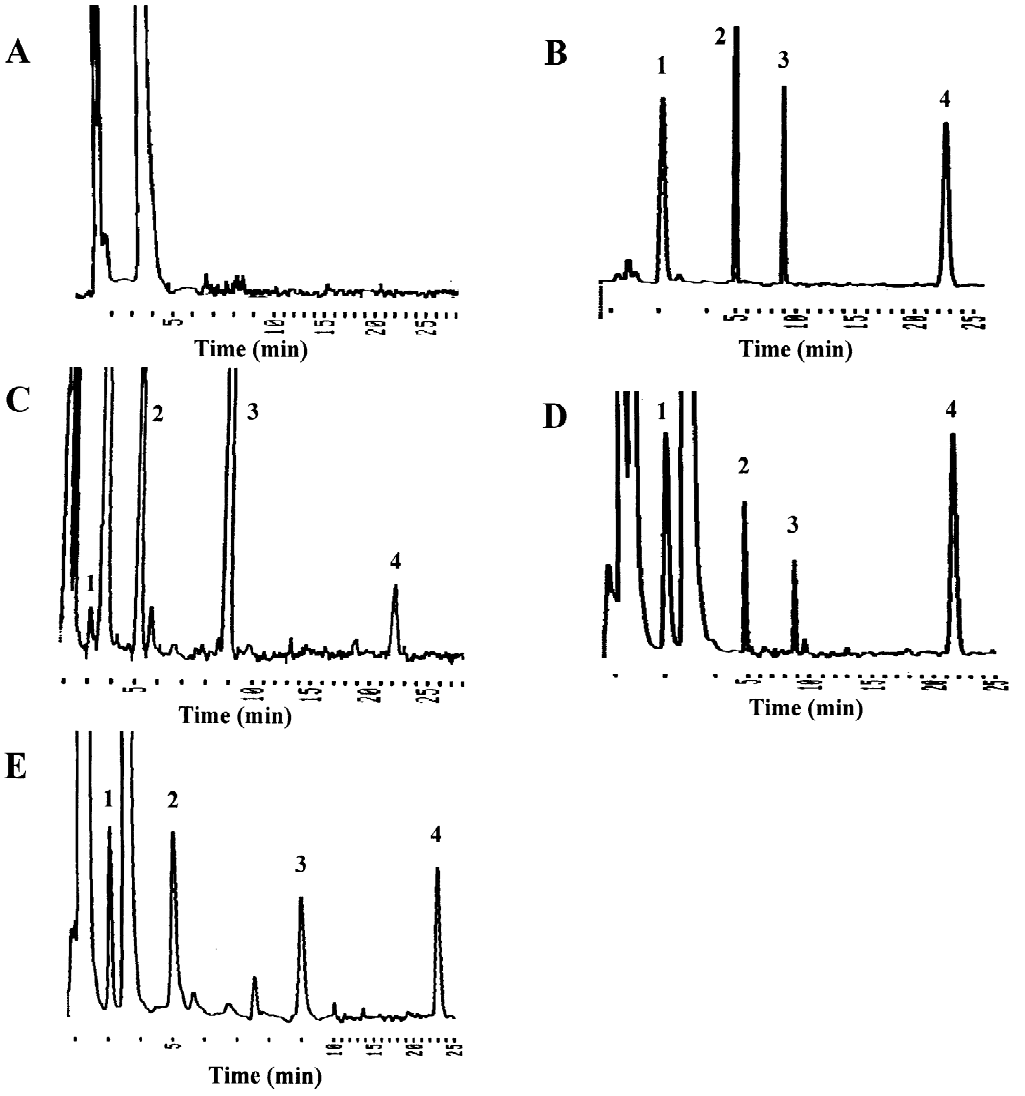

Fig. 2. Representative chromatogram under different conditions. (A) Drug-free plasma. (B) Standards: hydroxytyrosol (100 ng/ml); oleuropein (10 μg/ml) and preservatives[#] in water. (C) Plasma spiked at the LOD levels of hydroxytyrosol (15 ng/ml); oleuropein (1.5 μg/ml) and preservatives. (D) Plasma spiked with hydroxytyrosol (200 ng/ml); oleuropein (20 μg/ml) and preservatives. (E) Plasma sample taken at 1.5 min after a single intravenous 25 mg/kg dose of oleuropein. Peaks: (1) hydroxytyrosol; (2) eserine (200 ng/ml); (3) 3,4-dimethoxyphenyl ethanol (I.S., 100 ng/ml); (4) oleuropein. [#]Ascorbic acid (100 ng/ml) was not detected using these wavelengths.

overlapping peaks were detected for hydroxytyrosol and oleuropein with endogenous peaks. In addition, the plasma volume required for analysis was only 50 μl. This is very much smaller than the previously reported method, where the plasma volume used was 1000 μl [7,8].

The dosing profile shows that at 10 min, both oleuropein and hydroxytyrosol were rapidly distributed with less than 25 and 10% remaining in the systemic compartment (Fig. 3). On the basis of our previous reported results, hydroxytyrosol is likely to be metabolized, distributed and eliminated as unchanged compound and also as monosulfate and 3-*O*-glucuronide conjugated forms [10].

Herein is described a simple procedure which is direct, sensitive and reproducible. This procedure utilises a simple workup procedure and it does not require the use of solid-phase extraction. Furthermore, this analytical procedure describes simultaneous quantification of both oleuropein and hydroxytyrosol. An analytical procedure that accomplishes this has not been previously described in the literature. This method has been successfully applied to monitor oleuropein and hydroxytyrosol plasma levels in the rat after intravenous dosing of oleuropein.

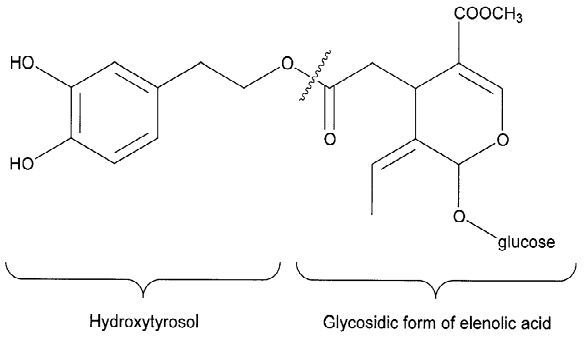

Fig. 3. Semilogarithmic plasma concentration–time profile for oleuropein (●) and hydroxytyrosol (▲) following a single intravenous 25 mg/kg dose of oleuropein.

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