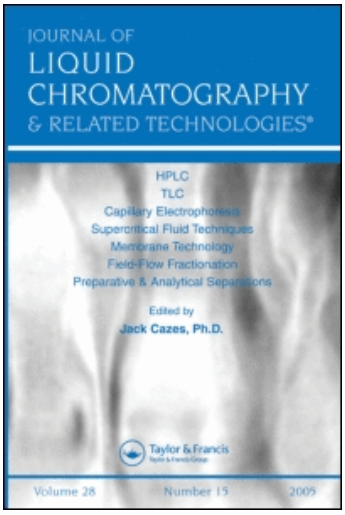

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Simultaneous Determination of Oleuropein and Tyrosol in Plasma Using High Performance Liquid Chromatography with UV Detection

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ABSTRACT

Oleuropein (O) and Tyrosol (T) are polyphenolic compounds with potent biological activities including, but not limited to, an antioxidant activity. An increased interest by many pharmaceutical companies has been shown to develop a formulation of O and/or T. In this research effort, a fast, simple, and reliable analytical method for the determination of O and T in plasma is required in order to carry out bioavailability studies. In this work, a reversed-phase isocratic high-performance liquid chromatographic (HPLC) method has been developed and validated for the simultaneous determination of O and T in plasma. The isolation of the polyphenolic analytes from plasma was carried out by liquid extraction

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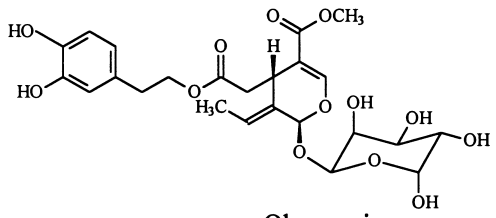
with ethyl acetate after the addition of Na_2SO_4 to the sample (salting out effect), followed by evaporation to dryness at 50°C, and reconstitution with mobile phase (4 × preconcentration). Chromatographic analysis was performed using a C8 column with MeOH/CH_3COOH 2% in water, 45 : 55 v/v as mobile phase, and UV detection at 280 nm. Vanillin (V) was used as internal standard. The recovery of the isolation procedure was 80% for O (CV = 6–18%) and 98% for T (CV = 3–8%). Retention times (min) were 4.6 for T, 6.2 for internal standard, and 8.9 for O, while endogenous plasma components were eluted before 4.3 min. Calibration curves of O and T in plasma were linear from 1–20 and 0.1–2.0 μg/mL at least, respectively ($r > 0.9996$ and 0.999, correspondingly). The detection limits of the method were 0.36 μg/mL for O and 0.09 μg/mL for T (4 × preconcentration). Precision and accuracy were determined from spiked plasma samples and were, for O CV = 5.7–8.3% and Er = 1.3–6.1% and for T CV = 6.1 − 0% and Er = 1.5–5%. The analytical methodology developed in this report is simple, rapid, accurate, and sensitive enough to be used in bioavailability studies.

Key Words: Oleuropein; Tyrosol; Plasma; HPLC; Bioavailability studies.

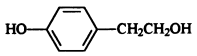
INTRODUCTION

The Mediterranean diet, rich in fresh fruits and vegetables, has been associated with a lower incidence of cardiovascular disease and cancer, partly because of its high proportion of bioactive compounds such as vitamins, flavonoids, and polyphenols. The major lipid component of such a diet is the drupe-derived olive oil in which several minor components including polyphenols,[1,2] such as oleuropein (O)[1,3] and tyrosol (T)[4] grant the oil its particular taste and aroma. Oleuropein, [2S–(2a,3E,4*β*)]-3-ethylidene-2-(*β*–D-glucopyranosyloxy)-3,4-dihydro-5-methoxycarbonyl)-2H-pyran-4-acetic acid 2-(3,4-dihydroxyphenyl) ethyl ester, (O), the most abundant among these polyphenols, is contained in the fruits, seeds, and leaves of the olive trees, as well as in the olive oil, and has been shown to be a potent antioxidant endowed with antiinflammatory and antimicrobial properties.[2,3,5–7] Tyrosol (*p*-hydroxy-phenyl-ethanol-2), (T), the antioxidant properties of which are weaker than those of O,[1,4–6] is also a polyphenol contained in the olive oil, the fruits, seeds, and leaves of the olive tree, as well as in wines and other alcoholic beverages. Olive leaves extract contains 6–25% w/v O and 7% w/v T and is used in the form of capsules as food supplement.

Analytical methods, which have been described in the literature till now, have been referred to in the determination of O[1,8–13] and/or T[12–25] in olive seeds, olive leaf extracts, buds, olive oil, as well as in wines and other



Oleuropein



Tyrosol

alcoholic beverages, and are based on mass spectrometry,[8,10–13] fluorimetry,[14] capillary electrophoresis[15] or reversed phase high performance liquid chromatography (HPLC) with UV,[1,6–22] electrochemical,[23] or mass spectrometry detection.[24,25] Recently the determination of T in urine has been achieved by a GC-MS method, which includes a derivatization procedure.[26] Till now, there is no analytical method in the literature for the determination of O and/or T in plasma.

An increased interest by many pharmaceutical companies has been shown to develop a formulation of O and T. In this research effort, a fast, simple, and reliable analytical method for the determination of O and T in plasma is required, in order to carry out bioavailability studies of the development of O and T formulation. The aim of this study was to develop and validate an isocratic reversed phase HPLC method for the simultaneous quantitative determination of O and T in plasma.

EXPERIMENTAL

Apparatus

Chromatographic analysis was performed with a Waters Model 600E HPLC system consisting of a control system (Controller) Model 600E, a pump Model 600E fitted with a Ternary Gradient Unit, which was used under isocratic conditions (on manual mode), a Waters Model U6K manual injector, and a 20 μL sample loop. A Waters Model 486 variable wavelength UV/Vis detector was used set at 280 nm. Samples were chromatographed on a 5 μm Waters C8 Symmetry, 25 cm × 4.6 mm (I.D.) reversed-phase column connected with a 5 μm Waters C8 Symmetry guard column, 3.9 cm × 20 mm, which was used as a precolumn. The manipulation of chromatograms as well as the control of the chromatography system was achieved with a computer using the Waters Millenium 2010 program.

A Millipore filtration system (Millipore, Bedford, MA) with type HV Millipore filters (diameter 47 mm, pore size 0.45 μm) was used, for degassing

mobile phase under vacuum, while helium was purged through mobile phase during the analysis. Extraction organic phases were evaporated on a Bioblock Scientific isotherm dry bath under nitrogen.

A vortex (Genie, Model K-550 GE, Scientific Ind., Springfield, MA 01103) set at speed 4 was used for mixing of plasma samples and standards.

Reagents and Chemicals

Water used was HPLC grade and was prepared from distilled-deionized water using reverse osmosis Millipore Model Milli-Q RG apparatus. Methanol and ethyl acetate were HPLC grade and were obtained from Labscan. Analytical grade anhydrous sodium sulfate (99%), glacial acetic acid (99.8%), and hydrochloric acid were obtained from Merck. Pure oleuropein and tyrosol were obtained from Extrasynthese (Genay Cedex France), while vanillin (V) from Sigma. Pooled drug free plasma (blanc plasma) was used for the preparation of spiked plasma standards.

Chromatographic Conditions

The optimized mobile phase consisted of methanol/acetic acid 2% aqueous solution 45 : 55 v/v. A flow rate of 1.0 mL/min was used at ambient temperature, resulting in a pressure of about 158 kg/cm^2. Mobile phases were degassed by vacuum through filtration, after mixing.

Standards for Calibration Graphs

Stock standard solutions of O, T, and V (internal standard) were prepared in methanol to give final concentrations of 1000, 500, and 500 μg/mL, respectively. A diluted aqueous standard stock solution of T of 100 μg/mL was prepared by diluting the stock standard 5-fold with distilled-deionized water. Six working solutions containing both O and T were prepared to give concentrations of O/T, covering the range from 10/1.0 to 200/20 μg/mL by appropriate dilutions of the stock standard solution of O (1000 μg/mL in methanol) and the diluted standard solution of T (100 μg/mL in water) with water. Plasma standards for calibration curves were prepared by spiking 1.0 mL aliquots of pooled drug free plasma with 100 μL of the above mentioned working solutions, to make plasma standards containing O to a concentration range of 1.0 to 20 μg/mL and T to a concentration range of 0.1 to 2.0 μg/mL. Namely, the plasma standards were, according to their O/T concentration, ranging from 1.0/0.1, to 20.0/2.0 μg/mL. A diluted methanolic

standard solution of internal standard (V) of 50 μg/mL was prepared by diluting the stock standard 10-fold with methanol. An aqueous working solution of internal standard (1.25 μg/mL) was prepared in a daily basis by dilution of 250 μL of the 50 μg/mL methanolic solution up to 10 mL with distilled water.

All stock, diluted stock and working solutions containing both O and T, when stored at 4°C were found stable for eight months. Only the aqueous working solution of the internal standard was unstable and was prepared daily.

Calibration graphs, based on the peak-area ratio of each compound to internal standard against analyte concentration, were prepared for each day of analysis to establish and check linearity and reproducibility of the method.

Extraction Procedure

In a 10-mL glass conical tube with a glass stopper, 1.0 mL of plasma samples, or the prepared plasma standard and 100 μL of internal standard aqueous solution 1.25 μg/mL (125 ng), were added and mixed for 15 s on the vortex. Consequently, 0.5 mL of hydrochloric acid 0.5 M was added in the tube and the whole was mixed for another 15 s on the vortex. Afterwards, 100 mg of anhydrous sodium sulfate was added in the tube and mixed on the vortex until dissolution. Each sample was extracted with 3.0 mL of ethyl acetate with vortexing for 2 min. The sample tube was centrifuged for 5 min at 2000 rpm. After separation and transfer of the upper organic layer into a 10-mL conical glass tube, the extraction procedure was repeated with 3.0 mL of ethyl acetate. The combined organic layers were evaporated to dryness at 50°C in the dry bath under a gentle stream of nitrogen. The residue was reconstituted in 250 μL of mobile phase and an aliquot of about 70 μL was injected onto the HPLC system.

RESULTS AND DISCUSSION

Oleuropein and T are substances with medium molecular mass and polarity, and reversed phase HPLC is the most suitable method for their analysis. Therefore, a C18 column is the first choice with a polar mobile phase. The addition of an acidic medium in the mobile phase is also necessary to keep the polyphenol analytes in the non-ionized form.

In a preliminary stage of the development of this method a C18 column was used (Nova-Pak 5 μm, 39 × 150 mm) in combination with methanol/acetic acid 2% v/v in water at different ratios in the range of 50 : 50–30 : 70 v/v, as mobile phase. The increase of the acidified water in the mobile phase resulted in

a dramatic increase of the retention time of O (2.4–17.5 min at the extreme ratios of the range studied) without a significant change in the retention time of T (1.4–2.1 min at the extreme ratios of the range studied). Varying concentrations of acetic acid in the aqueous constituent of the mobile phase were then tried (1–3%), as well as partial replacement of methanol with acetonitrile, but they had no influence on the chromatogram. Moreover, the constituents of a blank plasma extract were eluted at the same retention times with T.

In order to increase the retaining of the analytes in the stationary phase, a more polar than C18 column, such as C8, was chosen. For the optimization of the chromatographic conditions the so-called chromatographic response function (CRF) was used.[27] The best chromatographic response (combination of retention times and resolution) was achieved using methanol/acetic acid 2% in water, 45 : 55 v/v as mobile phase with which retention times (min) of 4.6 for T, 6.2 for internal standard, and 8.9 for O, were obtained. Endogenous plasma compounds were eluted before 4.3 min.

Oleuropein and T show λ_{max} at 279 and 274 nm, respectively, with molar absorptivities of 3727 and 2362 $M^{-1}\,cm^{-1}$, correspondingly, as calculated in the present study from the absorbance spectra, using standard solutions in the chosen mobile phase. The wavelength of 280 nm proved to be suitable for the identification and quantification of both compounds.

In order to choose a successful internal standard, the following compounds, which show similar structure with the substances under study were tried, resulting in the retention times shown in parenthesis: sulfosalicylic acid ($t_R = 1.8$ min), 4-hydroxy-3-methoxy-benzyl-alcohol ($t_R = 4.0$ min), 3-ethoxy-4-hydroxy-benzaldehyde ($t_R = 8.8$ min), amygdalic acid ($t_R = 6.0$ min), isoproterenol ($t_R = 2.0$ min), paracetamol ($t_R = 3.7$ min), and V ($t_R = 6.2$ min). Vanillin and amygdalic acid showed retention times at the desirable area, but the later showed very low molar absorptivity, while V at 0.5 μg/mL showed absorbance similar to that of 2 μg/mL T and 25 μg/mL O. Additionally, V was coextracted successfully with the extraction procedure developed for O and T from plasma. The only disadvantage observed with V is that aqueous dilute solutions of this compound are stable only for one day, however methanolic solutions are stable for at least one month. Consequently, the stock solution of the V internal standard was prepared in methanol, while the working aqueous solution of 1.25 μg/mL was prepared daily by dilution of the stock methanolic solution with water.

Figure 1 shows a typical chromatogram obtained from the injection of a test solution containing O/T/V at 25/2/0.5 μg/mL, respectively, (injected volume 20 μL, referring to 500, 40, 10 ng of O, T, V, correspondingly). Figure 2 shows a typical chromatogram obtained from extracted drug free plasma, spiked with O/T/V at 6.25/0.5/0.125 μg/mL, respectively. Resolu-

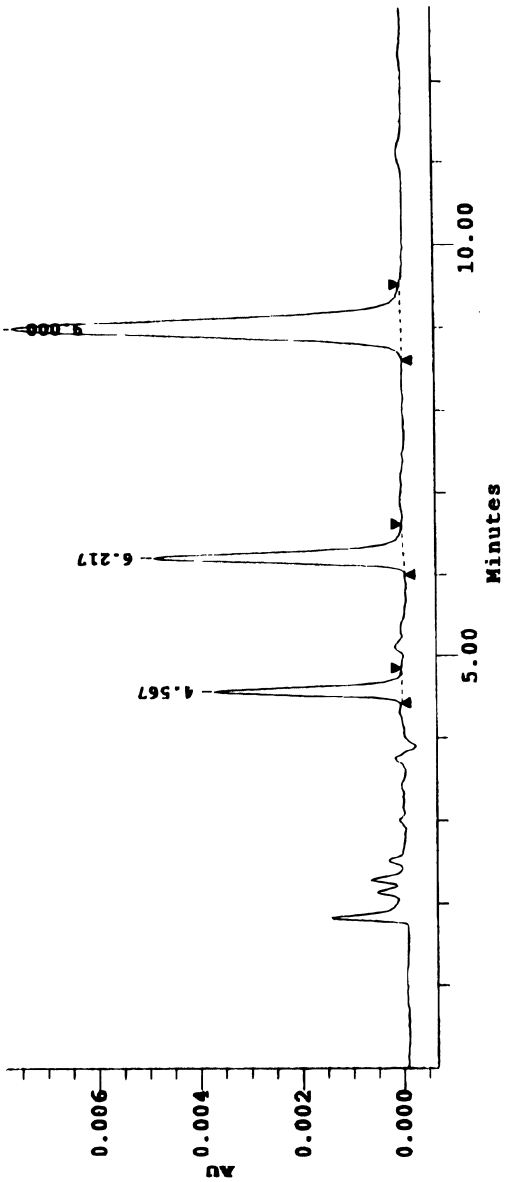


Figure 1. Typical chromatogram from a test solution containing oleuropein, tyrosol and vanillin in the mobile phase. Oleuropein 500 ng, tyrosol 40 ng, and vanillin 10 ng. Retention times: tyrosol 4.6 min, vanillin 6.2 min, oleuropein, 8.9 min. Resolution (Rs): Rs (tyrosol–vanillin) = 7.276, Rs (vanillin–oleuropein) = 8.652.

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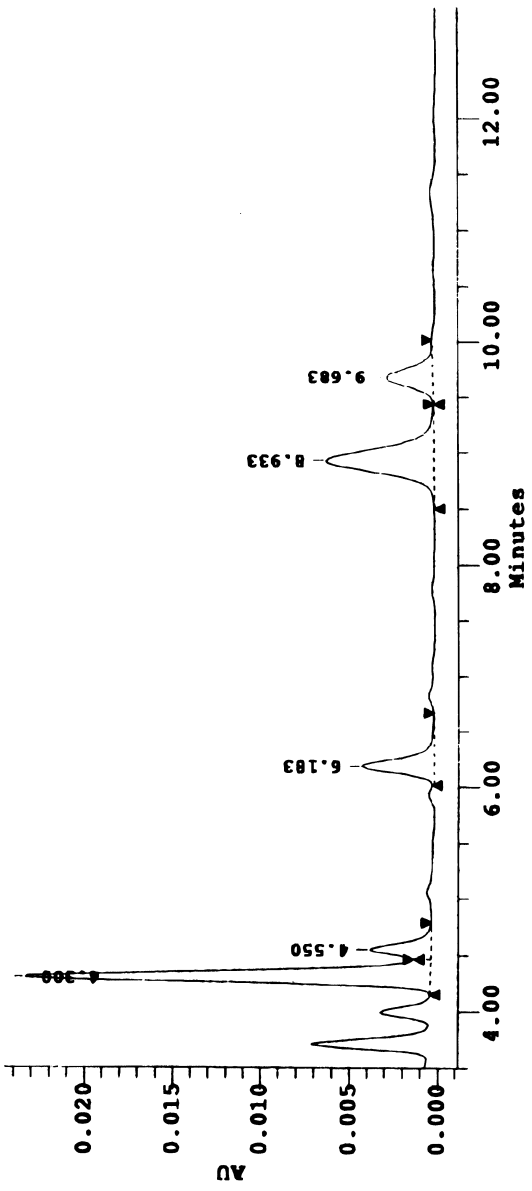

Figure 2. Typical chromatogram from an extracted plasma sample spiked with oleuropein, tyrosol, and vanillin. Theoretically: oleuropein 500 ng, tyrosol 40 ng, and vanillin 10 ng. Retention times: tyrosol 4.55 min, vanillin 6.18 min, oleuropein 8.93 min. Resolution (Rs): Rs (plasma-tyrosol) = 1.265, Rs (tyrosol–vanillin) = 7.276, Rs (vanillin–oleuropein) = 8.652, Rs (oleuropein–plasma) = 2.098.

tion of peaks in all cases was excellent and no interfering peaks of sample matrix were observed by analyzing a great number of plasma samples.

Extraction Procedure

In order to develop a successful extraction procedure, several solvent systems were tested for the extraction of O and T from aqueous solutions, after pH adjustment with HCl 0.5 M. Most of the solvent systems tested gave extremely low extraction recoveries (Table 1). The solvent systems with the highest recoveries were then tested for the extraction from spiked plasma samples (Table 2). Chloroform–isopropanol 70 : 30 v/v, which gave the best results for the extraction of O and T from aqueous solutions, proved to be unsuitable for the extraction of the compounds from spiked plasma samples, since a solid cake was created in the upper aqueous phase, due to the precipitation (denaturation) of proteins, which made the separation of the organic layer difficult. The salting out effect was then used by adding inorganic salt in plasma prior to the extraction. High extraction recoveries were achieved from spiked plasma samples when anhydrous sodium sulfate (100 mg) was added to the sample prior to the extraction with ethyl acetate. Therefore, the salting out effect caused by sodium sulfate proved to be the only way to succeed in extraction of the analytes and the internal standard from plasma samples. It was surprising that, with the extraction procedure finally chosen, higher extraction recoveries were achieved from plasma samples than for aqueous solutions. A possible explanation could be that soluble ionic ingredients of plasma contribute to the transport (extraction) of the analytes to the organic layer.

Recovery

Absolute extraction recovery data, reproducibility of recovery (CV%), from plasma samples spiked with O/T/V at 1/0.1/0.125 μg/mL, 6.25/0.5/0.125 μg/mL, and 20/2/0.125 μg/mL, as well as relative extraction recovery data, are presented in Tables 3, 4, (means of five experiments) and 5, respectively. For the calculation of the absolute extraction recovery, the peak areas from extracted samples were compared to those obtained from the direct injection of the corresponding working standard in mobile phase, taking into account the 4× preconcentration (Table 3), or the ratios of the slopes of the corresponding calibration curves in the absence of internal standard, which were estimated (Table 5). For the calculation of the relative recovery of the extraction procedure, the ratio of the slopes of calibration curves constructed

Table 1. Absolute extraction recoveries of O, T, and V from aqueous solutions containing O/T 6.25/0.5 μg/mL or O/T/V 6.25/0.5/0.125 μg/mL with various extraction solvent systems.

	Solvent system	Solvent volume (mL)	HCl 0.5 M (mL)	Evaporation (°C)	Recovery (%)		
					O	T	V
1	CH_2Cl_2	2 mL × 2	0.5 mL	Room temp.	8.3	21	—
2	CH_2Cl_2	2 mL × 2	0.5 mL	50°C	—	8	—
3	$CHCl_3$–isopropanol 90 : 10 v/v	3 mL × 2	0.5 mL	Room temp.	19	48	—
4	$CHCl_3$–isopropanol 90 : 10 v/v	3 mL × 2	1.0 mL	Room temp.	6	50	—
5	CH_2Cl_2–isopropanol 90 : 10 v/v	3 mL × 2	1.0 mL	Room temp.	8	53	—
6	CH_2Cl_2–isopropanol 90 : 10 v/v	3 mL × 2	1.0 mL	50°C	4	47	—
7	Ethyl acetate–acetone 2 : 1 v/v	3 mL × 2	0.5 mL	Room temp.	2	73	—
8	Ethyl acetate–acetone 90 : 10 v/v	3 mL × 2	0.5 mL	Room temp.	7.5	64	—
9	Ethyl acetate–acetone–isopropanol 80 : 10 : 10 v/v	3 mL × 2	0.5 mL	Room temp.	35	60.4	—
10	Ethyl acetate–acetone–isopropanol 70 : 10 : 20 v/v	3 mL × 2	0.5 mL	Room temp.	—	45.3	—
11	Ethyl acetate–acetone–isopropanol 60 : 30 : 10 v/v	3 mL × 2	0.5 mL	Room temp.	14.8	55	—
12	Ethyl acetate–isopropanol 80 : 20 v/v	3 mL × 2	0.5 mL	Room temp.	16.8	67.2	—
13	Ethyl acetate–isopropanol 70 : 30 v/v	3 mL × 1	0.5 mL	Room temp.	—	62.0	—
14	Ethyl acetate–isopropanol 60 : 40 v/v	3 mL × 1	0.5 mL	Room temp.	—	73.9	—
15	$CHCl_3$–isopropanol 80 : 20 v/v	3 mL × 2	0.5 mL	Room temp	45.4	63.3	—
16	$CHCl_3$–isopropanol 70 : 30 v/v	3 mL × 2	0.5 mL	Room temp.	70.9	92.6	—
17	$CHCl_3$–isopropanol 60 : 40 v/v	3 mL × 2	0.5 mL	Room temp.	61.9	92.0	—
18	Ethyl acetate	3 mL × 2	0.5 mL	Room temp.	9.5	44.7	—
19	Ethyl acetate + 100 mg Na_2SO_4	3 mL × 2	0.5 mL	Room temp.	11.1	83.7	42.9
20	Ethyl acetate + 100 mg Na_2SO_4	3 mL × 2	0.5 mL	50°C	7.9	71.6	25.3

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Table 2. Absolute extraction recoveries of O, T, and V from spiked plasma samples containing O/T/V 6.25/0.5/0.125 μg/mL with various extraction solvent systems.

	Solvent systems	Solvent volume (mL)	HCl 0.5 M (mL)	Evaporation (°C)	Recovery (%)		
					O	T	V
1	$CHCl_3$–isopropanol 70 : 30 v/v	3 mL × 2	0.5 mL	Room temp.	—	—	—
2	Ethyl acetate–isopropanol 80 : 20 v/v + 100 mg Na_2SO_4	3 mL × 2	0.5 mL	50°C	62.5	108	—
3	Ethyl acetate + 100 mg Na_2SO_4	3 mL × 2	0.5 mL	50°C	83.8	97	83.8

Table 3. Absolute extraction recoveries and CV% of O, T, and V from spiked plasma samples ($n = 5$).

Spiked plasma standards O/T (μg/mL)	Oleuropein (O)		Tyrosol (T)		Vanillin (V)	
	Rec.% ± SD	CV%	Rec.% ± SD	CV%	Rec.% ± SD	CV%
1.0/0.1	73.54 ± 13.0	18	146.0 ± 11.27	7.7	73.34 ± 11.22	15
6.25/0.5	77.32 ± 6.70	8.7	98.8 ± 3.15	3.2	86.29 ± 9.64	11
20/2.0	79.52 ± 4.81	6.0	101.42 ± 5.42	5.4	73.50 ± 7.48	10

Note: All spiked plasma standards contained vanillin (internal standard) at 0.125 μg/mL.

Table 4. Reproducibility of extraction procedure and CV% of O and T from spiked plasma samples ($n = 5$).

Spiked plasma standards O/T (μg/mL)	Oleuropein		Tyrosol	
	PAR ± SD	CV%	PAR ± SD	CV%
1.0/0.1	0.402 ± 0.04	10	0.24 ± 0.03	12
6.25/0.5	2.338 ± 0.20	8.6	0.68 ± 0.06	8.8
20/2.0	8.098 ± 0.47	5.8	2.50 ± 0.15	5.9

Note: PAR, Peak area of extracted analyte to peak area of extracted internal standard. All spiked plasma standards contained vanillin (internal standard) to 0.125 μg/mL.

in the presence of internal standard was used, taking into account the 4× preconcentration (Table 5). For the calculation of the reproducibility of extraction procedure, the ratio of peak areas of analytes to that of the internal standard was used (Table 4).

Linearity

The peak area ratios for O/internal standard and T/internal standard were linearly related to plasma concentrations of oleuropein and tyrosol, respectively, from 1 to 20 and from 0.1 to 2 μg/mL at least, respectively.

The slopes of eight calibration curves of oleuropein in plasma, prepared over a period of two months, had a CV of 5.6%. The average regression equation is shown in Table 5. The correlation coefficients for each individual standard curve was greater than 0.9996, and all intercepts were not statistically different from zero (t experimental $= 0.48 < t$ theoretical $= 2.776$).

The slopes of eight calibration curves of tyrosol in plasma, prepared over a period of two months, had a CV of 6.5%. The average regression equation is shown in Table 5. The correlation coefficients for each individual standard curve was greater than 0.9989 but the intercepts were statistically different from zero (t experimental $= 7.33 > t$ theoretical $= 2.776$), due to an insufficient resolution of T from the last plasma peak.

The detection and the quantification limits for the determination of O in plasma were estimated from the average regression equations of the corresponding calibration curves, based on the standard deviation of intercepts (3.3 and 10 times the SD), respectively. The detection and the quantification limits for the determination of O in plasma, according to the described method, were 0.36 and 1.08 μg/mL, respectively, while for the

Table 5. Calibration curves of O and T in plasma and in standard solutions prepared in mobile phase with and without the presence of internal standard and estimation of the absolute and relative recovery % through the values of the corresponding slopes (4× preconcentration).

Calibration curve	Average regression equations
Oleuropein	
Standard solutions in mobile phase (without internal standard)	$y = (4576.6 \pm 59.0)C_O + (1534.6 \pm 1630)$
Spiked plasma standards (without internal standard)	$y = (14597.7 \pm 478.5)C_O - (2373.8 \pm 4926.7)$
Standard solutions in mobile phase (with internal standard)	$y = (0.1075 \pm 0.0014)C_O + (0.0324 \pm 0.0405)$
Spiked plasma standards (with internal standard)	$y = (0.4155 \pm 0.0044)C_O - (0.1034 \pm 0.0449)$
Absolute recovery = 79.7%	
Relative recovery = 96.7%	
Tyrosol	
Standard solutions in mobile phase (without internal standard)	$y = (12106.9 \pm 97.4)C_T + (434.5 \pm 262.5)$
Spiked plasma standards (without internal standard)	$y = (43058.6 \pm 498.7)C_T + (3715.7 \pm 506.6)$
Standard solutions in mobile phase (with internal standard)	$y = (0.2845 \pm 0.0021)C_T + (0.0091 \pm 0.0058)$
Spiked plasma standards (with internal standard)	$y = (1.2201 \pm 0.0338)C_T + (0.1039 \pm 0.0343)$
Absolute recovery = 88.9%	
Relative recovery = 107.3%	

Note: Absolute recovery% = (slope of plasma calibration curve without internal standard:4)/slope of calibration curve of standard solutions not including internal standard prepared in mobile phase.
Relative recovery% = (slope of plasma calibration curve with internal standard:4)/slope of calibration curve of standard solutions including internal standard prepared in mobile phase.
y = peak area of analyte (calibration curves without internal standard).
y = peak area ratio of analyte/internal standard (calibration curves with internal standard).

Table 6. Within run precision and accuracy data of O and T from spiked plasma samples ($n = 5$).

	Concentration added (μg/mL)	Concentration found, mean ± SD (μg/mL)	CV%	Relative error % (Er %)
Oleuropein	1.00	1.21 ± 0.10	8.3	21
	6.25	5.87 ± 0.49	8.3	−6.1
	20.00	19.74 ± 1.12	5.7	−1.3
Tyrosol	0.100	0.116 ± 0.024	20	16
	0.500	0.477 ± 0.050	10	−4.6
	2.00	1.97 ± 0.12	6.1	−1.5

determination of T in plasma they were 0.09 and 0.28 μg/mL, correspondingly (4 × preconcentration).

Precision and Accuracy

Precision and accuracy were determined by analyzing plasma samples spiked with O and T at 1.0/0.1, 6.25/0.5, and 20.0/2.0 μg/mL, and the results are presented in Table 6. Within-run CV% for O was ranging from 5.7 to 8.3% all over calibration curve, while for T from 6.1% to 20%. Between-run CV% was 9.5% and 10.2% for O and T, respectively, at O/T 6.25/0.5 μg/mL spiked plasma samples ($n = 10$) over a period of two months. At the low concentrations tested (O/T 1.0/0.1 μg/mL), the within run precision and accuracy of the method developed, validated from the CV% and the relative error %, respectively, was not as high as for the median and high concentrations, but the low concentrations tested were very close to the quantification limits of the method.

CONCLUSIONS

The analytical method developed in this report is simple, rapid, accurate, sensitive, and moreover, is the first method described for the determination of O and T in plasma. It can be used for monitoring O and T plasma levels in bioavailability studies. Since there are not any reports in the literature concerning the plasma concentrations of O and T in humans, the method developed can be further modified to correspond to the desirable concentration range.

ABBREVIATIONS

O	Oleuropein
T	Tyrosol
V	Vanillin
HPLC	High performance liquid chromatography
CV%	Coefficient of variation %

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