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Research Paper

Stability of oleuropein in the human proximal gut

Constantinos Markopoulos[a], Maria Vertzoni[a], Apostolos Agalias[b], Prokopios Magiatis[b] and Christos Reppas[a]

[a]Department of Pharmaceutical Technology, Faculty of Pharmacy and [b]Department of Pharmacognosy and Natural Products Chemistry, National & Kapodistrian University of Athens, Zografou, Greece

Abstract

Objectives We aimed to assess the intralumenal stability of oleuropein in human gastric and small intestinal contents. We additionally aimed to assess the stability characteristics of oleuropein in media simulating the intralumenal conditions.

Methods The intralumenal stability of oleuropein was assessed in aspirates from the stomach and the upper small intestine of healthy volunteers collected under both fasted and fed state conditions and in media simulating the intralumenal environment.

Key findings Oleuropein degraded in aspirates collected in the fasted state. When the initial concentration was about 50 μg/ml (close to expected intragastric concentration after single dose of commercially available products of oleuropein) the mean zero-order half-life of oleuropein in aspirates collected from the fasted small intestine was estimated to be 3.14 ± 0.08 h at 37°C (i.e. after oral administration in the fasted state, a substantial fraction of oleuropein degrades before reaching the intestinal mucosa). In contrast, oleuropein was stable in aspirates collected from the fed stomach; in small intestinal contents aspirated in the fed state the estimated zero-order degradation half-life was at least 12 h.

Conclusions These data suggest that oleuropein should not have substantial intralumenal stability problems when administered in the fed state. Data collected in media simulating the intragastric and intraintestinal environment suggest that pH affects the stability of oleuropein only at low pH values (of about 2). At higher pHs degradation characteristics are at least partly affected by the presence of other scavengers of reactive oxygen species in the medium.

Keywords antioxidants; dosing conditions; oleuropein; stability in gastric contents; stability in intestinal contents

Introduction

Animal studies suggest that phenols found primarily in the leaves of olive trees, such as oleuropein (Figure 1a), tyrosol (Figure 1b) and hydroxytyrosol (Figure 1c), lower susceptibility to oxidisation of ex-vivo low-density lipoprotein (LDH) particles or lower markers in urine of oxidative processes in the body[1] and also exhibit anti-ischaemic and hypolipidaemic effects.[2] However, results from various clinical studies in humans are equivocal in regard to these effects.[3] One reason might be that the phenol content of olive oil, the most frequently used vehicle for oral administration of these phenols, is low.[3] Another reason, however, might be the inconsistent and variable gastrointestinal absorption.

For tyrosol and hydroxytyrosol the absorption and disposition characteristics have been adequately studied.[4,5] For hydroxytyrosol, specifically, it is interesting to note the vast difference in disposition kinetics between animals and humans and the importance of the vehicle that is used for its oral administration (e.g. extra virgin olive oil vs yoghurt).[6] In contrast, the fate of oleuropein, the glucoside of elenolic acid with hydroxytyrosol (Figure 1a), after oral administration to humans, remains an ongoing objective.

Oleuropein is a hydrophilic compound (logP ~ 0.2),[7] and, at usually administered doses (with food or in specific dosage forms, e.g., http://www.burstingwithhealth.co.uk/pack-info/olive-leaf.html, http://store.agoodvitamin.com/amvi0ta.html or http://mindbodyhealth.com/oleuropein.htm (accessed 24 March 2008)), its absorption characteristics are not expected to be limited by the solubility in the gastrointestinal contents. Conversely, the

Correspondence: Dr Christos Reppas, Faculty of Pharmacy, Department of Pharmaceutical Technology, National and Kapodistrian University of Athens, Panepistimiopolis, 157 71 Zografou, Greece.
E-mail: reppas@pharm.uoa.gr

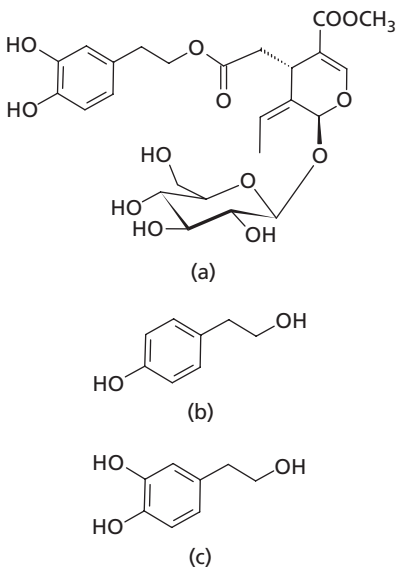

Figure 1 The structures of oleuropein (a), tyrosol (b) and hydroxytyrosol (c).

hydrophilic character of the compound combined with the relatively high molecular weight (540.5 Da) may create problems in the transport of oleuropein via the intestinal mucosa. In fact, in-situ rat perfusion data suggest that oleuropein is a low permeability compound.[7] Nevertheless, after administration of olive oil to humans, the total amount of hydroxytyrosol in urine is approximately twice the amount of administered hydroxytyrosol.[8] Therefore, after oral administration, oleuropein (or a derivative of it) reaches the general circulation and it is subsequently excreted in urine as hydroxytyrosol. However, to date, no plasma data of oleuropein or of its derivative(s) in plasma after oral administration of oleuropein to humans have been published.

With regard to intralumenal stability, specifically, available data are inconclusive. Vissers *et al.*[9] have found that at 37°C there is no degradation of oleuropein incubated for 2 h in the gastric juice of a fasted human subject that was diluted with water (1 : 11). Similarly there was no degradation of oleuropein incubated for 4 h in the duodenal juice of a fasted human subject that was diluted with water (1 : 4).[9] However, in another study where an olive oil polyphenolic fraction was incubated in HCl, pH 2 (37°C), a time-dependent increase of unconjugated phenols was observed.[10]

Based on the above data, after oral administration of oleuropein, degradation before reaching the general circulation could occur during residence in the gastrointestinal lumen, during transport via the gastrointestinal mucosa and during the first-pass from the liver. In this investigation we assessed the intralumenal stability of oleuropein in human gastric and small intestinal contents collected under fasting and fed state conditions. In an attempt to identify key factors that contribute to its instability, we additionally assessed the stability characteristics of oleuropein in media simulating the intralumenal conditions.

Materials and Methods

Materials

Oleuropein was extracted and isolated from *Olea europaea* leaves as follows: air-dried and pulverized leaves were extracted by mechanical stirring for 12 h with acetone. The extract was evaporated completely and washed with a mixture of CH_2Cl_2–methanol (98 : 2). The insoluble material was separated, dried and submitted to medium pressure LC with silicia gel 60 Merck (15–40 μm), using the CH_2Cl_2–methanol gradient as the eluent to isolate pure oleuropein. The purity of oleuropein was > 95%. The purity was determined by HPLC in comparison with a commercially available sample of oleuropein (Extrasynthese, Genay Cedex, France).

Pepsin from porcine stomach mucosa (0.064 mg pepsin/mg solid), and taurocholic acid (sodium salt, NaTc > 99% pure) were from Sigma Chemical (MO, US). Soya lecithin (Lipoid E PC, > 98% phosphatidylcholine) was from Lipoid GmbH (Ludwigshafen, Germany). Methanol of HPLC grade was from E. Merck (Darmstadt, Germany). Water purified with Labconco water pro ps system (Sigma Chemical, MO, US) was used in all procedures. All other chemicals were of analytical grade.

Human aspirates

The human aspirates, in which the stability of oleuropein was tested, were collected after administration of 250 ml water and after administration of 500 ml Ensure Plus to fasted healthy adults.[11] The composition of Ensure Plus is close to the composition of solid meals typically administered in bioavailability/bioequivalence studies.[11] All samples were kept at −70°C until used.

The collection of aspirates was performed in the Red Cross Hospital of Athens after receiving approval by the Scientific and the Executive committee of the hospital. All subjects provided a written informed consent before their enrolment. Details of the administration and aspiration procedures have been published previously.[11]

Human gastric fluid (HGF) aspirated in the fasted state was divided into three categories, HGF^{20}_{fasted}, HGF^{40}_{fasted} and HGF^{60}_{fasted} (i.e. fluid aspirated 20 min, 40 min and 60 min after the administration of 250 ml of water, respectively). HGF^{20}_{fasted}, HGF^{40}_{fasted} and HGF^{60}_{fasted} were created by pooling aspirates (3 ml each) from 9, 6, and 5 individuals, respectively. Human intestinal fluids (HIF) in the fasted state were aspirated 30 min after the administration of 250 ml water. HIF_{fasted} was created by pooling aspirates (3 ml each) from 9 individuals.

In an attempt to take into account the varying conditions over the entire fed state duration, aspirates in the fed state were collected at various times and up to 3 h after meal administration. HGF aspirated in the fed state was divided into three categories, HGF^{30}_{fed}, HGF^{90}_{fed} and HGF^{180}_{fed} (i.e. fluid aspirated 30 min, 90 min and 180 min after the administration of 500 ml of Ensure Plus, respectively). HGF^{30}_{fed}, HGF^{90}_{fed} and HGF^{180}_{fed} were created by pooling aspirates (2 ml each) from 10, 13, and 14 individuals. Human intestinal fluids aspirated in the fed state were divided into three categories,

HIF^{60}_{fed}, HIF^{120}_{fed} and HIF^{180}_{fed} (i.e. fluid aspirated 30 min, 90 min and 180 min after the administration of 500 ml of Ensure Plus, respectively). HIF^{60}_{fed}, HIF^{120}_{fed} and HIF^{180}_{fed} were created by pooling aspirates (1 ml each) from 18, 18, and 15 individuals, respectively.

Media simulating the gastrointestinal fluids

Hydrochloric acid solution of pH 2.0 (10 mM), hydrochloric acid of pH 1.6 (25 mM) and fasting state simulating gastric fluid (FaSSGF) were used for simulating the conditions in the fasted stomach. FaSSGF consisted of 0.1 mg/ml pepsin, 80 μM NaTc, 20 μM soya lecithin, 34.2 mM NaCl and HCl 25 mM (pH 1.6).[12]

Fasted state simulating intestinal fluid (FaSSIF),[13] FaSSIF in which maleates were used as pH-buffering species ($FaSSIF_m$),[14] and FaSSIF of pH 7.1 ($FaSSIF_{7.1}$) were used for simulating the conditions in the fasted small intestine. FaSSIF consisted of (in mM) 3 NaTc, 0.75 lecithin, 28.66 NaH_2PO_4, 13.8 NaOH and 106 NaCl.

Cow's milk (0.75% fat, (Landesgenossenschaft Ennstal Molkereibetriebe, Steinach, Austria)), gradually digested with the addition of an acidic pepsin solution,[15] was used for simulating the conditions in the fed stomach.

Fed state simulating intestinal fluid (FeSSIF),[13] and FeSSIF in which citrates were used as pH buffering species ($FeSSIF_c$)[14] were used for simulating the conditions in the fed small intestine. FeSSIF consists of (in mM) 15 NaTc, 3.75 lecithin, 144 acetic acid, 101 NaOH and 173 NaCl.

Stability studies

The dose of oleuropein in products currently on the market is of the order of 25–50 mg (e.g., http://www.burstingwithhealth.co.uk/pack-info/olive-leaf.html, http://store.agoodvitamin.com/amvi0ta.html or http://mindbodyhealth.com/oleuropein.htm (accessed 24 March 2008)). Administration of the dose in the fasted state with a glass of water should result in initial intragastric volumes of about 300 ml[12] and, therefore, in intragastric oleuropein concentrations of 80–160 μg/ml. To take into account the greater degree of dilution in the fed state, a nominal initial oleuropein concentration of 50 μg/ml was chosen for all stability studies.

Initially, 5 mg of oleuropein were added to 2.5 ml of the medium to be studied. Due to the turbidity of most of the media used in this study and to be sure of the complete dissolution of oleuropein, all media were filtered at this stage. As aspirates collected from the small intestine and aspirates collected from the fed stomach could not be filtered through 0.45-μm filters, these media were filtered through 5.0-μm filters (Nylon filters, Titan; Scientific Resources Inc., NJ, US). All other media were filtered through 0.45-μm filters (regenerated cellulose filters Titan; Scientific Resources Inc., NJ, US). Fifty microlitres of the filtrate were diluted with 1950 μl of the medium and, after sealing with parafilm, the glass tube was transferred in a shaking water bath (37°C).

For all HGF_{fasted} media, samples were drawn at 0, 30, 60, 90, 120, 150, 180, 210 and 240 min. For all other aspirated fluids, samples were drawn at 0, 40, 80, 120, 160, 200 and 240 min. For all media simulating the gastrointestinal fluids, samples were drawn every 15 min for up to 4 h, except for $FaSSIF_m$ in which samples were drawn for up to 6 h.

In every medium, the stability of oleuropein was studied by performing three individual experiments.

Assay of oleuropein

Oleuropein was assayed with a HPLC-UV method that was based on a previously published method.[16] A Waters Symmetry C_8 (250 × 4.6 mm, 5 μm) column was used. For the analysis of HGF_{fasted} and HIF_{fasted} samples, the mobile phase was methanol–water–acetic acid (45 : 55 : 1.5 v/v/v). For HGF_{fed}, HIF_{fed}, and media simulating the conditions in the stomach and in the small intestine the mobile phase was methanol–water–acetic acid (40 : 60 : 1.5 v/v/v). In all cases flow rate of the mobile phase was 1 ml/min, the injection volume was 50 μl and the detection wavelength was 240 nm.

All HGF_{fasted} and HIF_{fasted} samples and all samples from media simulating the lumenal contents that were drawn from the incubator were analysed immediately with the HPLC-UV method. The HGF_{fed} and HIF_{fed} samples were treated with 10% $HClO_4$ before being injected into the HPLC, so that the proteins present in these fluids precipitated. In an Eppendorf tube, 250 μl of sample were mixed with 250 μl of 10% $HClO_4$. The mixture was stirred and subjected to centrifugation (11 000 rev/min, 10°C) for 10 min. Then, the supernatant was injected into the HPLC.

Oleuropein concentrations were quantified according to standard curves constructed in the corresponding stability medium at concentrations that ranged between 5 μg/ml and 50 μg/ml. It is important to note that standard curves were constructed by using exactly the same procedure after sampling from the incubator. Therefore, any degradation during the sample preparation for injection to the chromatographer was cancelled out by the standard curve characteristics. Determination coefficients (r^2) of the standard curves ranged between 0.98 and 0.999.

Data analysis

Although the nominal concentration of oleuropein at time zero of the stability studies was 50 μg/ml, the actual initial oleuropein concentration varied both between and within media. In experiments with media that, immediately after the addition of oleuropein, were filtered with 0.45-μm regenerated cellulose filters (n = 36), the measured initial oleuropein concentration in the filtrate showed minimal deviation from the nominal value and ranged from 45.5 to 55.4 μg/ml. In contrast, the measured initial oleuropein concentration after filtration through 5-μm nylon filters (n = 21) ranged from 20.1 μg/ml to 43.1 μg/ml. The manufacturer of the nylon filters (Titan; Scientific Resources Inc., NJ, US) indicates that proteins may be adsorbed onto these filters. This possibility was experimentally confirmed by assessing the binding of oleuropein on milk proteins by using low fat milk (0.75% fat). At a total concentration of 50 μg/ml, the percentage of oleuropein bound onto milk proteins was measured as 23.3 (2.6) (n = 3).

For each replication, both the zero-order and the first-order model were fitted to the data using Sigmaplot 2000 for Windows version 6.00 (SPSS Inc.). Based on the values of coefficients of determination, the predicted residual error

sum of squares (a gauge of how well a regression model predicts new data), and the values of Durbin-Watson statistic (a measure of correlation between the residuals), a conclusion on which of the two models was the most appropriate for describing all data sets could not be drawn. However, since, for every replication, the degradation half-life estimated with the zero-order model was similar to that estimated with the first-order model, it was decided to report the half-lives estimated by using the simplest model (i.e. the zero-order model). When the slope of the regression line was significant, the zero-order degradation half-life of oleuropein in the incubation medium, t½, was estimated using the following equation:[17]

$$t_{\frac{1}{2}} = C_0/2k \quad (1)$$

where C_0 is the measured concentration at time zero, and k is the slope of the regression line that was fitted to the concentration-vs-time data set.

Comparisons of half-lives estimated in HGF_{fasted}, in HGF_{fed}, and in various media simulating the gastric conditions in the fasted state were performed, after passing the equal variance and normality tests, with one-way analysis of variance (Sigmastat for Windows 2.03, 1992-1997 SPSS Inc.). $P < 0.05$ was considered significant.

Results

Oleuropein degraded in aspirates collected from the fasted stomach and from the fasted small intestine with apparent zero-order kinetics. Two example sets of stability profiles are presented in Figure 2. Although this study was not specifically designed to quantify the degradation products, one of the degradation products in HGF was hydroxytyrosol; after 4 h incubation in HGF^{60}_{fasted}, 8.6% of the oleuropein content had been transformed to hydroxytyrosol (data not shown). These data are not in agreement with the earlier data of Vissers *et al.*,[9] which indicated that oleuropein is stable in the fasted upper gastrointestinal lumen. In that previous study, however, the aspirates were diluted with water prior to stability testing and had been collected from a single individual. In the fed state, oleuropein was found to be stable in gastric aspirates but degraded in small intestinal aspirates, again with apparent zero-order kinetics. Figure 3 shows two example sets of stability profiles in aspirates collected in the fed state.

Table 1 shows the pH of the aspirates used in the stability studies and the estimated half-lives of oleuropein in the various aspirates. Although data from HGF_{fasted} samples suggest that degradation characteristics in the fasted stomach are sensitive to gastric composition and perhaps to the intragastric pH (differences between HGF^{20}_{fasted} and HGF^{40}_{fasted} and between HGF^{20}_{fasted} and HGF^{60}_{fasted} are both significant ($P < 0.001$ in both cases)), data in HIF_{fed} suggest the opposite; degradation characteristics are not sensitive to nutrient content or the pH of the small intestine, because differences among the degradation half-lives in the various HIF_{fed} samples were not significant ($P = 0.829$).

In media simulating the conditions in the human upper gastrointestinal lumen, stability profiles of oleuropein varied dramatically with medium composition and, whenever degradation was observed, it occurred according to apparent zero-order kinetics. Two example sets of stability profiles are

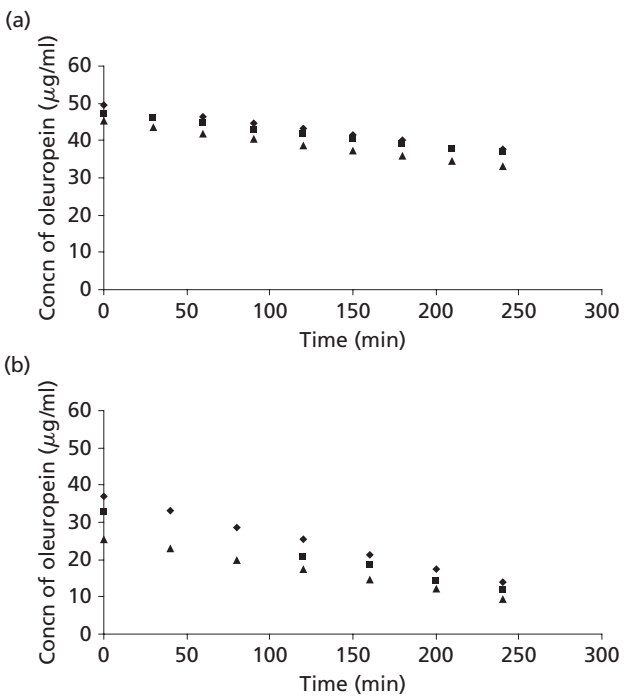

Figure 2 Concentration vs time data of oleuropein incubated at 37°C in HGF^{40}_{fasted} (a) and in HIF_{fasted} (b) media. Data are means ± SD of 3 independent experiments.

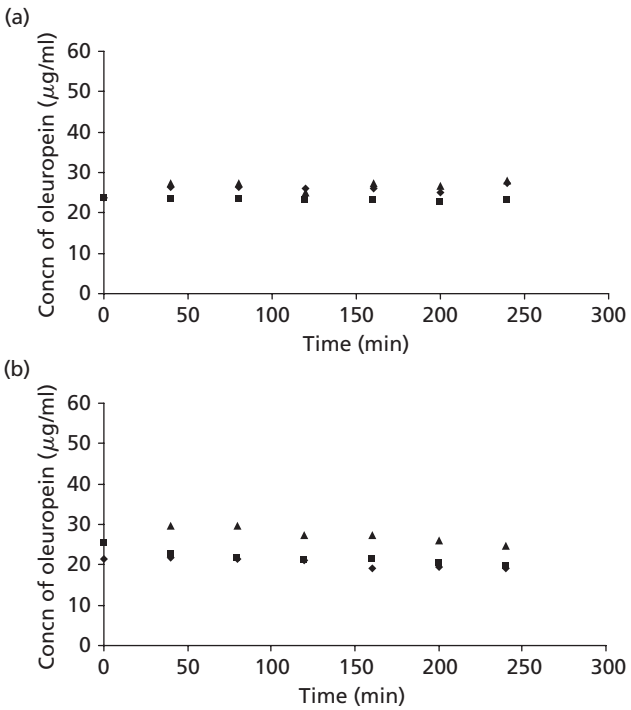

Figure 3 Concentration vs time data of oleuropein incubated at 37°C in HGF^{90}_{fed} (a) and in HIF^{60}_{fed} (b) media. Data are means ± SD of 3 independent experiments.

Table 1 The human aspirates used in this study, their pH values, and the respective degradation half-lives (t½) of oleuropein at 37°C[a]

Human aspirates[b]	pH	t½ (h)
HGF^{20}_{fasted}	2.3	29.3 ± 3.76
HGF^{40}_{fasted}	2.1	8.18 ± 0.73
HGF^{60}_{fasted}	2.0	7.04 ± 1.14
HIF_{fasted}	7.1	3.14 ± 0.08
HGF^{30}_{fed}	6.1	NS[c]
HGF^{90}_{fed}	5.4	NS[c]
HGF^{180}_{fed}	4.2	NS[c]
HIF^{60}_{fed}	6.3	12.3 ± 2.06
HIF^{120}_{fed}	5.8	13.9 ± 7.16
HIF^{180}_{fed}	5.1	15.5 ± 5.54

[a]Data are presented as mean ± SD. Since the stability experiments in this study lasted for up to 4 h, the exact values of half-lives that are estimated to be much longer than 4 h could deviate from the values presented in this table.
[b]Numbers indicate the number of minutes after administration of water (fasted) or Ensure plus (fed) that a sample was aspirated. HGF, human gastric fluid; HIF, human intestinal fluid.
[c]Oleuropein was stable for at least 4 h in this medium (i.e. the slope of the regression line was statistically not significant (NS)).

Table 2 Media simulating the environment in the stomach and the small intestine, their pH values and the respective degradation half-lives (t½) of oleuropein at 37°C[a]

Medium[b]	pH	t½ (h)
HCl 10 mM	2.0	14.7 ± 0.18
HCl 25 mM	1.6	6.16 ± 0.26
FaSSGF	1.6	6.00 ± 0.18
FaSSIF	6.5	NS[c]
$FaSSIF_m$	6.5	NS[c]
$FaSSIF_{7.1}$	7.1	23.7 ± 1.14
Digested cow's milk (0.75% fat)	6.5 → 3.0	NS[c]
FeSSIF	5.0	1.96 ± 0.10
$FeSSIF_c$	5.0	NS[c]

[a]Data are presented as mean ± SD. Since the stability experiments in this study lasted for up to 4 h, the exact values of half-lives that are estimated to be much longer than 4 h could deviate from the values presented in this table.
[b]FaSSGF, fasted state simulating gastric fluid; FaSSIF, fasted state simulating intestinal fluid; FeSSIF, fed state simulating intestinal fluid; composition of FaSSGF, FaSSIF and FeSSIF as well as meanings of the rest of symbols are provided in the Materials and Methods section.
[c]Oleuropein was stable for at least 4 h in this medium (i.e. the slope of the regression line was statistically not significant (NS)).

presented in Figure 4. Estimated degradation half-lives are presented together with the pH of the media in Table 2. The degradation of oleuropein in HCl solution of pH 2.0 is in agreement with earlier data.[10] Analysis of variance of degradation half-lives in the media simulating the gastric composition in the fasted stomach indicated that pH is indeed a key factor; the difference between half-lives in HCl 10 mM (pH 2.0) and HCl 25 mM (pH 1.6) or FaSSGF (pH 1.6) was significant ($P < 0.001$ for both comparisons).

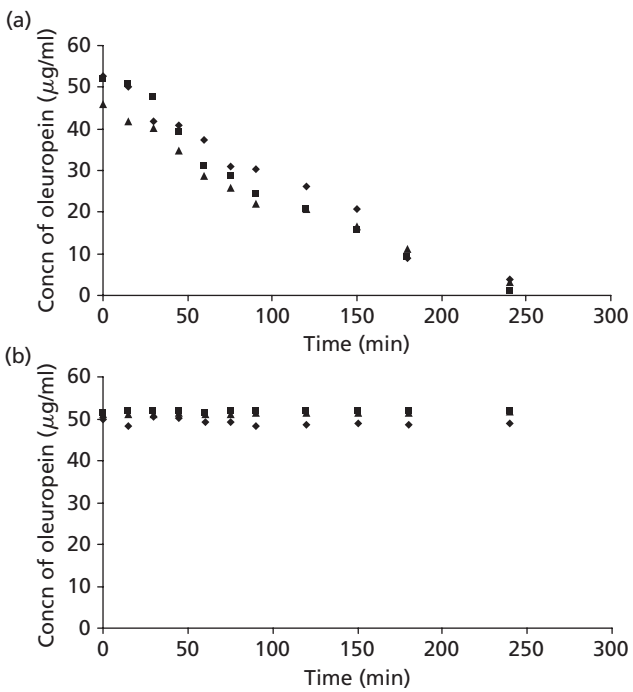

Figure 4 Concentration vs time data of oleuropein incubated at 37°C in FeSSIF (a) and in $FeSSIF_c$ (b) media. Data are means ± SD of 3 independent experiment.

Discussion

In the fasted stomach, pH seems to be one of the factors contributing to the degradation of oleuropein (Tables 1 and 2). In contrast, pH as well as bile salts and phospholipids are not important for the observed degradation of oleuropein in aspirates collected from the fasted small intestine (Tables 1 and 2). The latter observation contradicts previous data showing that oleuropein degrades at pH 7.[7] However, no additional information is given in that relevant article on the exact composition of the solution. Taken together, these observations suggest that an important factor for the stability of compounds that are scavengers of reactive oxygen species (e.g. oleuropein) may be, apart from the acidity of the medium, the presence of other components in the medium. Evidence for the correctness of such a hypothesis is provided by the stability data in HIF_{fasted} and in $FaSSIF_{7.1}$ (Tables 1 and 2). The two media have similar pH values but differ in composition (unlike HIF_{fasted}, $FaSSIF_{7.1}$ contains high amounts of phosphates). The estimated degradation half-life of oleuropein in HIF_{fasted} and in $FaSSIF_{7.1}$ is 3.14 h and 23.7 h, respectively (Tables 1 and 2). It would be interesting to assess the stability of oleuropein in a biorelavant medium that also contains the physiological buffer system. However, the in-vitro use of bicarbonates, the buffer system that mostly operates in the fasted small intestine, is associated with various practical issues and often leads to irreproducible results.[18]

Data collected in fed aspirates and in media simulating the lumenal contents in the fed state suggest that oleuropein is stable in stomach, regardless of the pH ($3.0 < pH < 6.5$; Tables 1 and 2). In contrast, oleuropein degraded in aspirates

collected from the fed small intestine but, in this case, degradation seems to be independent of pH (Table 1), and, since nutrient composition in fed intestinal aspirates varies among the tested samples,[11] it is also independent from nutrient composition. Data in media simulating the composition in the fed small intestinal contents show that in FeSSIFc (pH 5.0) oleuropein was stable but in FeSSIF (pH 5.0) oleuropein degraded (Figure 4, Table 2). Presumably, citrates (that are reactive oxygen species scavengers)[14] compete with oleuropein and, therefore, they improve the stability characteristics of oleuropein. A similar argument has been introduced in the past for explaining the different dissolution profiles of troglitazone (another reactive oxygen species scavenger) in $FeSSIF_c$ and in FeSSIF).[14] Again, it would be desirable to perform relevant experiments by using the physiological buffer system, but in the fed small intestine the dominating buffering species varies with the type and the amount of ingested food (i.e. it is not possible to define).

Degradation of oleuropein during gastric residence should be minimal, because in the fasted state gastric emptying is rapid (e.g. Macheras *et al*.)[17] whereas in the fed stomach oleuropein is stable (Table 1). However, since, small intestinal transit time is (regardless of dosing conditions) approximately 3 h[19] and oleuropein is a low permeability compound,[7] about half of an orally administered amount should be degraded during residence in the fasted small intestine (Table 1). In contrast, estimated degradation half-lives in aspirates collected from the fed small intestine indicate no major intralumenal stability issues. Consequently, the contradictory data to date on the ability of oleuropein and other phenols to produce a measurable effect on LDL oxidisability or other oxidation markers in humans after oral administration in the fed state (i.e. after administration of olive oil)[3] should be primarily attributed to the low phenol content of olive oil and much less to a problematic arrival of oleuropein at the intestinal mucosa. Literature data suggest that, after oral administration of oleuropein in the fed state, hydroxytyrosol (a derivative of oleuropein, Figure 1) was measured in urine.[5] Whether derivatisation of oleuropein begins at the intestinal mucosa (e.g. with deglycosylation) as is the case with some other glycosides[20] or occurs during first-pass from the liver is difficult to investigate for various reasons (e.g. oleuropein is not stable in plasma (Markopoulos *et al.* unpublished data)) and a single form of its aglycone cannot be isolated or quantified, due to the complex pathways of oleuropein hydrolysis.[21]

Conclusions

This study shows that oleuropein (a scavenger of reactive oxygen species) has substantial lumenal stability problems after oral administration in the fasted state. In conjunction with data collected in media simulating the conditions in the upper gastrointestinal lumen it seems that degradation characteristics in the fasted stomach are partially related to the acidity of the contents (pH values of about 2). At higher pH, however, degradation characteristics are affected by the presence of scavengers of reactive oxygen species in the medium but not by the pH.

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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