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Research Paper

Monitoring of *In Vitro* Fat Digestion by Electron Paramagnetic Resonance Spectroscopy

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Purpose. The distribution of drugs between water, oil and mixed micelles after the oral application of lipid-based drug delivery systems affects their absorption rate. Since it has not been previously possible to monitor this process online during *in vitro* lipolysis, it was our aim to develop a suitable real-time method.

Materials and Methods. To follow the fate of a co-administered drug during fat digestion, the spin probe tempol benzoate was incorporated as a lipophilic model drug into a long-chain triglyceride (olive oil) and an *in vitro* digestion test was combined with electron paramagnetic resonance (EPR) spectroscopy (X-Band). Additionally the progression of digestion was determined by means of high performance thin layer chromatography (HPTLC).

Results. The spectral shape of the EPR spectrum changed significantly during the digestion process. EPR spectra at all times could be simulated with three species indicating a redistribution of the lipophilic model drug between olive oil, phosphate buffer and mixed micelles formed by bile salts and phospholipids.

Conclusion. This *in vitro* real-time analysis could be a very helpful tool to monitor the digestibility of novel lipid-based drug nanocarriers which is an important step to optimize and to predict drug delivery processes. In future the EPR monitoring of fat digestion will be transferred to *in vivo* experiments.

KEY WORDS: EPR; lipolysis; lipid-based drug delivery systems.

INTRODUCTION

Lipid based drug delivery systems play an important role for the improvement of the oral bioavailability of poorly water-soluble drugs. Formulations with natural or synthetic lipids favour the absorption by enhanced drug solubilisation and dissolution in the gastrointestinal tract (1,2). Lipid digestion plays a significant role in these processes. In the late 1990s the design of lipid-based formulations was empirical. Constantinides (1) and Khoo *et al.* (3) focused on the optimisation of the drug solubility in the lipid vehicle and the improvement of the *in vitro* dispersibility of the formulation. MacGregor *et al.* (4) worked on enhanced digestibility of the lipid vehicle. However, the fate of the co-administered drug remained unclear. In recent years several groups worked on a better understanding of the mechanisms by which lipids improve absorption. Simple *in vitro* tests were established that predict the complex series of events which occur on interaction of the lipid formulation and the gastrointestinal environment (4–7). Investigations based on titrimetric, high-performance thin-layer chromatography (HPTLC) and ultracentrifugation techniques showed that lipid digestion leads to the formation of new colloidal species, e.g., mixed micelles and vesicles formed by bile salts (BS) and phospholipids (8–12). The distribution of the drug between the aqueous, oil phase and mixed micelles will affect the absorption rate. Porter *et al.* (13) and Kaukonen *et al.* (5,6) assessed the distribution of co-administered drugs between the different phases after *in vitro* digestion of long and medium chain triglycerides and Porter *et al.* compared these data with *in vivo* results.

Until now it was not possible to perform an online study on the distribution of the drug between water, oil and colloidal species. The aim of the current study was to monitor the time-dependent fate of the co-administered spin probe tempol benzoate (TB) as a poorly water-soluble, lipophilic model drug in a long chain triglyceride (olive oil) during digestion. The *in vitro* lipid digestion test used here was recently established in our laboratory (14). Electron paramagnetic resonance (EPR) was applied as an online method to investigate the complex changes of the artificial gastrointestinal environment during digestion. EPR (or synonymously ESR, electron spin resonance) is a non-invasive method which requires the presence of paramagnetic molecules. Because most drug delivery systems are EPR silent, they require the addition of paramagnetic materials, e.g., nitroxides. Nitroxides can be used as model drugs and serve as

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ABBREVIATIONS: BS, bile salts; EPR, electron paramagnetic resonance; HPTLC, high performance thin layer chromatography; LCT, long chain triglycerides; MCT, middle chain triglycerides; TB, tempol benzoate.

reporter molecules to explore the molecular environment. A wide range of spin probes with different structures exist which permit the systematic variation of the physicochemical properties. EPR permits the quantitative measurement of micropolarity, microviscosity and (using special probes) microacidity and oxygen content. EPR spectroscopy and imaging experiments can be performed in nontransparent samples and recent developments permit the noninvasive monitoring of drug delivery processes in small animals. A recent review summarizes the EPR applications in the field of drug delivery (15). Lipolysis leads to the formation of new lipid structures with more polar characteristics (e.g., mixed micelles) and therefore, to a change of the of the spin probe environment. Because EPR spectra are sensitive to the micropolarity and microviscosity, the relocation of the spin probe can be monitored. Furthermore, the distribution of the model drug within the different lipid digestion products can be quantified by fitting the EPR spectra.

MATERIALS AND METHODS

Materials

Pancreatin (porcine) (P 7545, 8× USP specification activity), bile extract (porcine) (B 8631) and triolein, 1.2, 1.3-diolein, 2-monoolein and oleic acid were purchased from Sigma (St. Louis, USA). The bile extract was used as a heterogenic source of bile salts. The concentration of 3-hydroxy bile salts was 0.91 μmol/mg determined by means of the enzymatic test kit Ecoline S^+ (Diagnostic Systems, Germany) with Glycochenodeoxycholate as reference.

Olive oil was purchased from Caelo, Germany. Methanol, $CaCl_2$ and $Na_2HPO_4*H_2O$ of analytical grade, manufactured by Roth (Germany) were used. KH_2PO_4 was purchased from Merck, Germany. Chloroform of HPLC grade was purchased from Baker (Netherlands).

4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl, (tempol benzoate; TB; Fig. 1) was obtained from Aldrich Chem. Co, USA.

Methods

In Vitro Digestion

For the *in vitro digestion* experiments 1.5% (v/v) of olive oil, which is equivalent to 15.6 mM calculated as triolein, and 1 mM of TB were dispersed by Ultraturrax in 15 ml of digestion buffer (53.4% $Na_2HPO_4*2H_2O$ 1/15 M, 46.6% KH_2PO_4 1/15 M, 10 mM $CaCl_2*H_2O$, pH 6.8) containing 10 mM bile salts (14).

Experiments were initiated by the addition of 14.6 mg pancreatin extract containing 150 u/ml of pancreatic lipase activity. The *in vitro* digestion experiments were performed at 37°C in an end-over-end shaker. The pH was adjusted every 15 min. Digestion was monitored over 45 min using electron paramagnetic resonance (EPR). For EPR measurements a sample of the digest was filled into an EPR capillary which was measured with the X-Band spectrometer. The experiments were performed in quadruplicate.

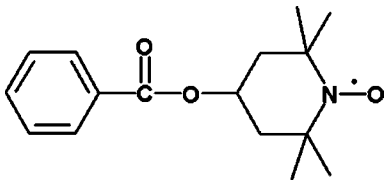

Fig. 1. Chemical structure of the EPR spin probe Tempol benzoate (TB, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl).

EPR Measurements

An EPR spectrometer of 9.5 GHz (X-Band; Miniscope MS 200) from Magnettech (Berlin; Germany) was used. The measurements were conducted with the following typical parameters: modulation frequency: 100 kHz; microwave power: 10 mW; scan range: 10 mT, scan time: 30 s, modulation amplitude: 0.1 mT.

Simulation of the EPR spectra was performed by means of Public EPR Software Tools (P.E.S.T.) from National Institutes of Health (National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709) (16). The optimization method used was LMB1. The recorded EPR spectra represent complex spectra. Spectral simulation was used to determine the distribution of the spin probe between the different species.

HPTLC Analysis

In order to estimate the extent of lipolysis *in vitro* digestion experiments were performed as stated above. Lipid concentrations were analyzed by HPTLC and spectrodensitometric analyses which were in general similar to the method described by Sek *et al*. (10). The experimental differences concern composition of the mobile phase, preconditioning of the HPTLC plates and detection wavelength for densitometric measurements. At 0, 5, 20 and 45 min, respectively, the samples of the digest were acidified and lipids extracted with chloroform. The extracted lipids were separated on a silica gel 60 HPTLC plate (Merck, Germany) by Automated Multiple Development (AMD) using an 11-step gradient based on hexane and ethylacetete (AMD 2, CAMAG, Switzerland). After post-chromatographic staining with an aqueous copper sulfate solution (10% $CuSO_4$, 8% H_3PO_4 and 5% CH_3OH) and heating to 150°C, the lipids were quantified by *in situ* spectrodensitometric measurements using a CAMAG TLC Scanner 3 at 675 nm as described previously (14). The optical densities were converted into masses using standard curves obtained from standard solutions containing triolein, diolein, monoolein and oleic acid which were separated simultaneously on each plate to avoid inter-assay variations. The masses of lipids obtained from scanning densitometric measurements were converted into concentrations using the molar masses of oleic acid and its derivates relative to the volume of the samples. The experiments were performed in triplicate.

RESULTS

In order to understand the distribution process of the model drug TB (Fig. 1) between phosphate buffer, oil and mixed micelles (formed by bile salts and phospholipids), complex EPR spectra containing three different components had to be simulated. To reduce the amount of variable

simulation parameters during the simulation process, EPR spectra of TB in the separate environments were recorded and the belonging simulation parameters were determined.

The EPR spectrum of TB in phosphate buffer (Fig. 2a) shows a hyperfine coupling constant of 1.670 mT which is typical for polar environments. Due to the low viscosity the amplitude and the line width of the three EPR lines are almost equal. Under the more viscous conditions of TB in olive oil (Fig. 2c) the lines broaden, the hyperfine coupling constant is smaller (1.525 mT), indicating an apolar environment. The EPR spectrum shows three lines of decreasing height. This effect occurs when the spin probe has not a free mobility in all directions and can be attributed to the influence of the fatty acid chains of the olive oil. The hyperfine coupling constant of TB in mixed micelles (Fig. 2b) at 1.634 mT lies between the polar value of phosphate buffer and the apolar value of olive oil (Fig. 3). It indicates an intermediate polarity. Similar to the spectrum in olive oil, the EPR spectrum of TB in mixed micelles shows three lines of decreasing height. This effect can be attributed to TB molecules which experience a decreased mobility as they are incorporated into mixed micelles. The magnitude of this so-called "flue pipe" effect is depending on the bile salt concentration in the phosphate buffer. While TB in phosphate buffer containing 5 mM bile salts exhibits still a narrow third line (Fig. 4a), this line and also the other two lines broaden (Fig. 4c) with higher bile salt concentrations. The spectra can be simulated with two species. Species I with a coupling constant of 1.670 mT describes TB in phosphate buffer, species II (a_N = 1.634 mT) TB in mixed micelles. At a concentration of 5 mM bile salts 67% of the TB molecules are located in the mixed micelles, this fraction amounts 91% when increasing the bile salt concentration up to 40 mM. For the *in vitro* model a bile salt concentration of 10 mM (Fig. 4b) was used. Under these circumstances 22% of the spin probe are located in the phosphate buffer and 78% are incorporated into the mixed micelles.

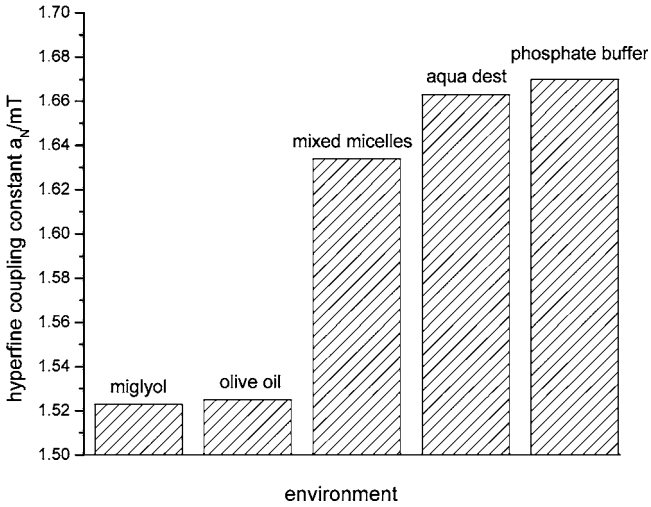

Fig. 3. Hyperfine coupling constant (a_N) values for TB depending on the polarity of the environment.

The EPR spectra of TB recorded at different times of the *in vitro* lipolysis (Fig. 5) were simulated with three species. The distribution of the model drug TB between phosphate buffer (a_N = 1.67 mT), olive oil (a_N = 1.525 mT) and mixed micelles (a_N = 1.634 mT) was determined.

Lipid digestion caused significant changes in the EPR spectra. In Fig. 5a the shape of the third peak is dominated by the TB species which is located in the olive oil (o). Before adding the digestion enzymes 82% of the TB molecules are located in olive oil, 12% in mixed micelles and 6% in phosphate buffer. After 5 min of digestion the spectral shape changes significantly. While the influence of the oily species on the third peak decreases, a more polar species (*) arises. At this state 67% of the TB molecules are still located in the oily phase, the amount of molecules in the mixed micelles increased to 26 and 7% are dissolved in phosphate buffer.

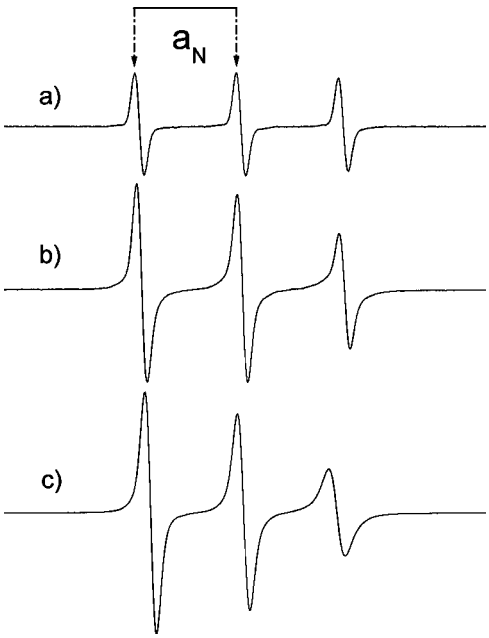

Fig. 2. EPR spectra of TB loaded. (a) Phosphate buffer, (b) mixed micelles, (c) olive oil. Note the change of the a_N value (hyperfine coupling constant) depending on the environment.

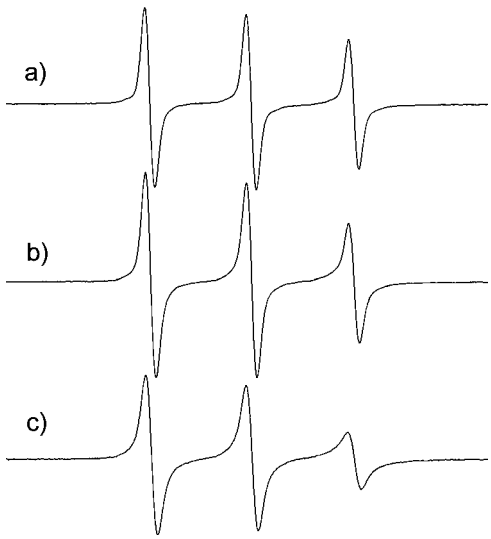

Fig. 4. EPR spectra of TB loaded. (a) Mixed micelles (5 mM) in phosphate buffer, (b) mixed micelles (10 mM) in phosphate buffer, (c) mixed micelles (40 mM) in phosphate buffer.

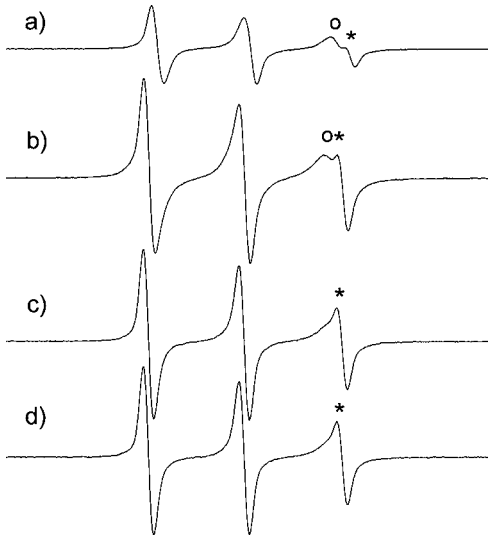

Fig. 5. EPR spectra of TB loaded olive oil. (a) After 0 min of digestion, (b) after 5 min of digestion, (c) after 20 min of digestion, (d) after 45 min of digestion. (o marks the lipophilic and * the hydrophilic species).

After 20 min of digestion (Fig. 5c) the outer line of the third peak becomes dominant indicating that an increasing number (50%) of TB molecules is now integrated into the more polar species of mixed micelles and only 33% are still located in olive oil. At the end of the experiment, after 45 min, the majority of the TB molecules are located in the mixed micelles (60%), 26% are dissolved in olive oil and 14% in phosphate buffer.

In summary the redistribution of TB between oil and mixed micelles was fastest during the first 5 min and proceeded with high speed up to 20 min (Fig. 6). In the following 25 min the rate of redistribution decreased considerably. The amount of spin probe in the phosphate buffer was always below 16% (Table I).

Table I. Distribution of TB into an Oil Phase, Mixed Micelles and Phosphate Buffer

Time [min]	Percent of TB in oil phase	Percent of TB mixed micelles	Percent of TB in phosphate buffer
0	81.85 (±2.90)	11.74 (±1.73)	6.41 (±1.38)
5	67.47 (±5.08)	26.01 (±3.38)	6.78 (±1.51)
20	33.19 (±1.04)	50.26 (±1.19)	15.06 (±2.65)
45	25.54 (±2.72)	60.34 (±2.39)	14.12 (±2.69)

Data are present as mean ± SD (n = 4).

Additionally performed HPTLC analyses of the digestion samples showed a fast initial decrease of triglycerides. Within 5 min there were only 66% of the triglyceride left. The initial phase was followed by a more slow degradation. At the end of the experiment there was 33% of the triglyceride present in the digestion mixture. Experimental data also showed a transient accumulation of diglyceride and a successive increase in monoglycerides and fatty acids (Fig. 7).

The triglyceride concentration in samples without addition of pancreatin was 8.8 mM. Since olive oil is a heterogeneous mixture of triglycerides with differing fatty acid composition and derivatisation by charring and *in situ* quantification is strongly dependent from the nature of the substrate (e.g. saturation and chain length) this value was set 100%. Total lipid mass calculations were performed after the following equation (10):

$$3 * TG_{remaining} + 2 * DG_{formed} + MG_{formed} + FA_{formed} = FA_{blank} + 3 * TG_{initial}.$$

Lipid recovery was between 104 and 119% (data not shown) which can be attributed to a slightly increased densitometric response of the lipolytic products.

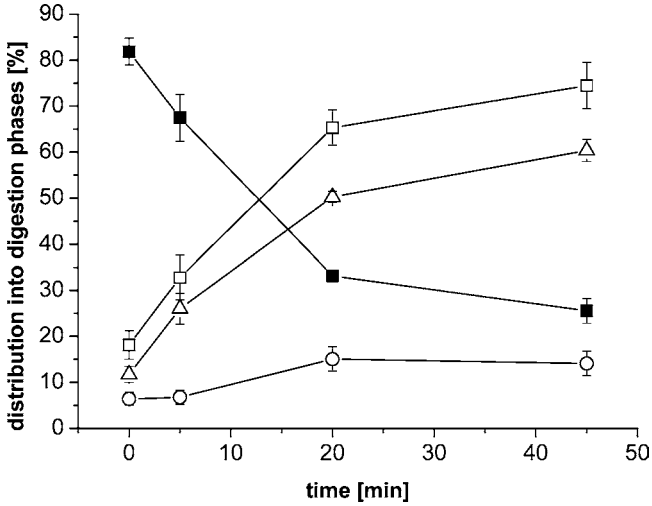

Fig. 6. Distribution of TB into oil phase (■), mixed micelles (△), phosphate buffer (○) and mixed micelles + phosphate buffer = dispersed aqueous phase (□)after 0, 5, 20 and 45 min digestion of 1.5% (v/v) of a long-chain triglyceride under mediate (10 mM) bile salt conditions. Data are present as mean ± SD (n = 4).

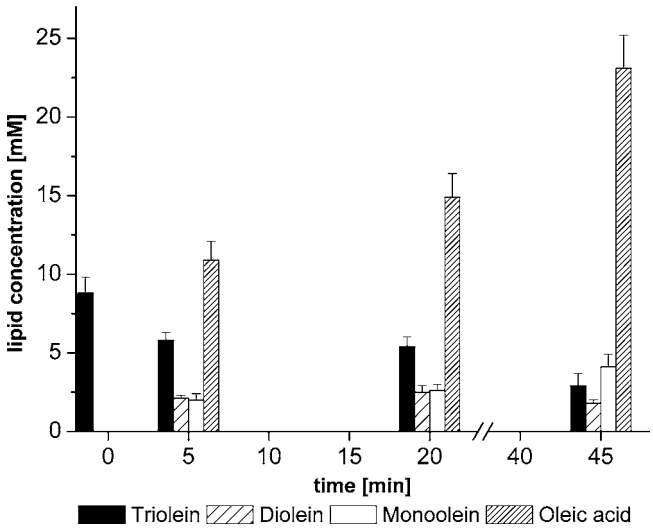

Fig. 7. Changes in lipid concentrations calculated as Oleic acid derivates during a 45 min *in vitro* digestion period determined by means of HPTLC and *in situ* spectrodensitometric analyses. Data are present as mean ± SD (n = 3).

DISCUSSION

The major goal of our studies was to develop a methodology to be able to non-invasively monitor *in vitro* fat digestion by EPR. In detail we wanted to study the distribution of a lipophilic paramagnetic model drug between the aqueous, oil phase and mixed micelles after the oral application of TB in a long chain triglyceride (olive oil). While there have been extensive prior studies investigating the formation of different phases and colloidal species during *in vitro* digestion, no prior group has reported the online monitoring of fat digestion. EPR spectroscopy offers the unique possibility of performing non-invasive measurements even in non-transparent samples (15,17,18). We performed spectral quantification of the EPR absorption function by fitting the individual spectral components of phosphate buffer, olive oil and mixed micelles consisting of bile salts and phospholipids.

In the literature the separation of the long chain triglyceride (LCT) digests into a floating oil phase, a dispersed aqueous phase and a pellet phase after ultracentrifugation has been described (11). Since in our experiments simulation of the EPR spectra was at all times successful with the simulation parameters of oil, buffer and mixed micelles, the presence of TB molecules in the pellet phase can be excluded. Kaukonen *et al*. (5) also reported a very low drug content in the pellet phase when using LCT and drug molecules as diazepam with a log D in the range of TB.

The distribution of TB in phosphate buffer containing bile salts and phospholipids shows that with increasing bile salt concentrations the amount of TB solubilized in mixed micelles increases. This finding is in accordance with results from HPLC analysis on drug solubilisation during *in vitro* digestion. Zangenberg *et al*. (7) reported an increase of Class II drug solubility in the aqueous phase in the presence of bile salts. In studies performed by Kaukonen *et al*. (5) an increase of drug solubility corresponded with an increase in BS/PL concentrations. It has also been previously reported, that the initial lipolysis rate of LCT is BS dependent (12) and increases with higher concentrations. These findings have to be taken into account for the setup of *in vitro* digestion tests. Since several groups have reported the concentration of BS in the duodenum in the postprandial state with mean values between 5 and 15 mM with peaks up to 40 mM depending on the time after ingestion of the meal (9,19–22) and 3–7 mM during fasted state (9,19,23), varying concentrations are used for *in vitro* tests (4,10,13,24) leading to different distribution profiles of co-administered drugs during *in vitro* digestion. The drug distribution will also play a role in food-dependency of drug absorption. Depending on the different bile salt concentrations for fasted and fed state, the distribution of a drug (with a special log *P*) between the oil, water and micellar phases might vary leading to different rates of absorption. In our case when looking at TB with a log *P* of 2.5 the *in vitro* variations of the solubilisation capacity of mixed micelles in phosphate buffer were 67% for fasted state (5 mM), 87% for fed state (20 mM) and up to 91% for BS peaks in fed state (40 mM).

Upon addition of olive oil to the phosphate buffer/mixed micelles system 82% of the spin probe were located in olive oil due to the moderate lipophilicity of TB. After addition of digestion enzymes a rapid redistribution of the spin probe within the first 5 min of digestion occurred indicating a fast degradation of olive oil. These fast changes in lipid concentrations during the first 5 min of *in vitro* lipolysis could be confirmed by HPTLC analyses. The results showed that about one third of triglycerides were degraded initially. After 20 min up to 45 min of digestion only small quantitative changes in the distribution of TB were visible. In accordance with that, the quantification of LCT and lipolytic products reflected a slower digestion during this period. These findings are confirmed by Sek *et al*. (10,11) who reported a decrease of the rate of diglyceride and triglyceride hydrolysis after the first 10 min. At the end of the digestion experiment after 45 min still 26% of the spin probe molecules were present in the oil phase indicating an incomplete digestion of the long chain triglyceride. The undigested triglyceride was quantified to be 33% by means of HPTLC analyses.

The EPR results are also confirmed by findings in the literature (5,10), where it is reported, that the digestion of LCT compared with MCT progresses more slowly and after 30 min undigested lipid is present.

The distribution of TB after the digestion experiment was quantified with 74% of the spin probe in the dispersed aqueous phase and 26% in olive oil. These data are close to HPLC results from Kaukonen *et al*. (5) who measured a distribution of diazepam (log $P = 3$) with 80% in the dispersed aqueous phase and 20% in the LCT phase.

CONCLUSION

An EPR method was developed that enabled the first online monitoring of the distribution of a paramagnetic lipophilic model drug between buffer, oil and mixed micelles after *in vitro* digestion. We were able to follow the redistribution of the model drug between the different phases by clear changes in the spectra which could be quantified by spectral simulation. In future the *in vitro* results will be supplemented by corresponding *in vivo* digestion studies. The here presented online method could be a helpful tool to study both the uptake of lipophilic model drugs from a lipid formulation after oral administration and to monitor the interaction of model drugs with lipid-rich meals. Comparison of *in vitro* and *in vivo* data will lead to a better understanding of the *in vitro-in vivo* correlation of lipid-based drug nanocarriers. This will be an important step to optimize and to predict drug delivery processes.

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