An in vitro approach to study the absorption of a new oral formulation of berberine

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Abstract

Background: Berberine (BBR) possesses several biological activities in humans, but the poor water solubility and low oral bioavailability preclude its pharmacological use. To overcome these limitations, several formulations have been prepared including encapsulation, micro- and nano-emulsion. The aim of this study was to develop a nanoemulsion delivery system of BBR and to evaluate its membrane permeability using Caco-2 cell model.

Methods: Nanoemulsions containing different ratios BBR:Compritol ATO 888 (a lipid excipient) were formulated at 25 and 80°C. The controls consisted of BBR and carboxymethylcellulose. Absorption of BBR nanoemulsions delivery systems was evaluated *in vitro* by using human colon adenocarcinoma cells (Caco-2) Transwell model. The amount of permeated BBR was determined by LC-HR-MS at time zero and every 30 min for 180 min.

Results: Nanoemulsions significantly improved apical-to-basal transport of BBR compared to the control formulation. Kinetics of BBR uptake showed that the maximum amount absorbed was reached after 90-120 min and the percentage of BBR absorbed by Caco-2 cells increased with increasing BBR-to-Compritol ratio (1:20 > 1:10 > 1:5 > 1:1). Moreover, the formulation prepared at 80°C showed a higher absorption rate (6-fold increment compared to control) than that developed at 25°C (4.5-fold increment compared to control). Furthermore, demethyl-BBR was detected after 120 min of incubation as partial metabolism of berberine in the intestine.

Conclusions: Overall, in our in vitro model, these new nanoemulsions seem to potentially improve the absorption of BBR. However, *in vivo* studies are necessary in order to demonstrate the bioavailability of BBR from this new formulation.

Keywords: berberine; nanoparticles; Caco-2 cells; *in vitro* absorption; mass spectrometry

1. Introduction

Berberine (BBR) is a natural alkaloid isolated from medicinal herbs, such as *Coptis chinensis* and *Hydrastis canadensis*, traditionally used for the treatment of diarrhea and gastroenteritis [1]. More recently, other biological functions have been attributed to berberine, including hypolipidemic, hypoglycemic, antiarrhythmic, anti-inflammatory, antimicrobial, and antineoplastic activities [2–5]. Regarding the hypolipidemic activity, it should be noted that the mechanism of action of BBR is different compared to statins. In fact, while statins act by inhibiting the synthesis of endogenous cholesterol and upregulating the low-density lipoprotein (LDL) receptor (LDL-R) in liver and peripheral tissues [6], BBR seems to reduce the amount of circulating cholesterol by increasing the expression and stabilization of LDL-R. Therefore, as a hypolipidemic molecule, BBR reduces cholesterol with a mechanism of action different from that of statins [7].

Despite the potential biological activities in humans and the low toxicity and cost, the therapeutic use of BBR has encountered several challenges. In particular, the main limitations are determined by its poor water solubility and bioavailability, which has been estimated to be less than 1% of the dose ingested [8]. In order to overcome this problem, a possible solution could be the use of a delivery system that can improve its bioavailability. The most common delivery systems consist of the use of polymeric nanoparticles, silica-based nanoparticles, micelles, liposomes, graphene, and lipid nanostructures [9]. Different lipid classes have been utilized as pharmaceutical excipients due to their negligible toxicity [9]. Among them, Compritol 888 ATO has been used in drug encapsulation, as a lubricating agent in the manufacture of oral preparations [10], and as a matrix-forming agent in the preparation of sustained-release tablets [11]. Compritol consists of a mixture of diacyl- (40–60%), monoacyl- (13–21%), and triacyl- (21–35%) glycerols [12], and this particular composition provides high drug entrapment efficiency [13].

Another strategy to enhance the efficiency of transport systems based on nanostructured lipid carriers is to use high process temperature [14]. In fact, it has been shown that temperature has an impact on the particle size and shape of nanosystems, features that can increase the solubility and dissolution rate of encapsulated drugs [15]. In particular, increased temperature leads to increased interfacial tension of the droplets, which may be responsible for the particle size and morphology [16]. In addition, low temperature induces consolidation of droplets in a non-spherical shape and a consequent low diffusion rate [16].

71 Therefore, the aim of the present study was to design and optimize BBR transport systems
72 based on nanostructured lipid carriers (NLCs) consisting mainly of Compritol and, in
73 lesser amounts, lecithin, developed at different temperatures (25 vs. 80 °C). Then, test the
74 different formulations *in vitro* to evaluate the kinetics of BBR uptake using Caco-2 cells
75 grown on the Transwell diffusion system as a model of intestinal absorption [17].
76

77 **2. Materials and Methods**

78 *2.1. Materials*

79 Standards of berberine (BBR), methanol, acetonitrile, and formic acid were provided by
80 Sigma-Aldrich (St. Louis, MO, USA). Berberis extract at 90% BBR was from Vivatis
81 Pharma (Gallarate, VA, I). Compritol 888 ATO, or glyceryl dibehenate according to the
82 European Pharmacopeia, was supplied by Pharmalabor (Canosa di Puglia, BT, I). Water
83 was obtained from an Arium pro apparatus (Sartorius, Milan, I). Human Caucasian colon
84 adenocarcinoma (Caco-2) cells (Cat. No 09042001-1VL), tested for intestinal permeability
85 characteristics, were from the European Collection of Authenticated Cell Cultures
86 (ECACC) and purchased by Sigma-Aldrich (St. Louis, MO, USA). Minimum Essential
87 Medium Eagle (MEM; Cat. No. M5650-500mL), penicillin–streptomycin solution (Cat.
88 No. P4333-100mL), MEM Non-Essential Amino Acid Solution (100×) (Cat. No. M7145-
89 100mML), and sodium pyruvate (100 mM) (Cat. No. 11360070) were provided by Sigma-
90 Aldrich (St. Louis, MO, USA). Millicell® tissue culture plate well inserts (Cat. No.
91 PIHP01250) were from Merck (Darmstadt, D).
92

93 *2.2. Preparation of lipid nanostructures containing berberine*

94 Approximately 50, 100, 200, and 500 mg of berberis extract was mixed with 850, 800,
95 700, and 400 mg of Compritol, respectively, and then 100 mg of lecithin was added to
96 each mixture at room temperature (RT; 25 °C). In this way, the ratio of BBR to emulsifiers
97 was in the range of 1:20–1:1 (w/w). A further hot preparation (80 °C) was made by
98 heating Compritol 5 °C above its melting point, and then adding 100 mg of previously
99 heated lecithin at the same temperature. The mixture was kept in mild agitation and the
100 temperature was fixed to ensure that the lipid material did not solidify. After 10 min,
101 berberis extract was added and the emulsion was obtained by stirring the mixture at 16,000
102 rpm for 20 min. The emulsion was quickly cooled and reduced to a fine powder. All
103 samples were stored in a refrigerator at 4 °C. The amount of BBR in the formulations was
104 determined as described by Wang [5]. The controls consisted of the same amounts of BBR

105 used in the test formulations, while Compritol and lecithin were replaced by
106 carboxymethylcellulose.
107
108 *2.3. Cell culture*
109 Caco-2 cells were cultured in minimum essential medium (MEM) supplemented with 10%
110 (v/v) fetal bovine serum, antibiotics (50 U per mL penicillin, 50 μg per mL streptomycin),
111 1% (v/v) of 100× non-essential amino acids, and 1 mM sodium pyruvate. Cell cultures
112 were maintained at 37 °C and 5% (v/v) CO_2 atmosphere. The medium was replaced every
113 3 days during cell growth and differentiation. For the experiments, cells were used 21 days
114 after reaching confluence to allow for differentiation into intestinal epithelial cells. Cells
115 were used between passages 4 and 10.
116
117 *2.4. Viability assay*
118 The toxicity of the compounds was tested on Caco-2 cells by Trypan blue exclusion assay
119 using a TC20TM automated cell counter and dual-chamber cell counting slides (BIORAD,
120 Segrate, Milan, I). Caco-2 cells with 80% confluence grade were treated with BBR at
121 different concentrations for 24 h and in the presence of Compritol, lecithin, and cellulose.
122 Subsequently, cells were trypsinized, resuspended, and used for Trypan blue exclusion
123 assay. Three independent experiments were performed in which each condition was tested
124 in triplicate.
125
126 *2.5. Transepithelial electrical resistance measurement*
127 In order to measure transepithelial electrical resistance (TEER), Caco-2 cells with 80%
128 confluence grade were seeded on Transwell® 24-well permeable media (12 mm, 0.4 μm
129 pore polyester membranes) at a density of approximately 2×10^5 cells per well. Cells were
130 differentiated into polarized monolayers by growing on Transwell inserts for 18–21 d. The
131 volume of medium added to the upper and lower compartments was 0.4 and 0.6 mL,
132 respectively. TEER was measured using a Millicell-ERS Resistance System (Millipore,
133 Bedford, MA, USA) that includes a dual-electrode volt-ohm-meter. TEER was calculated
134 as follows: TEER = (Rm − Ri) × A, where Rm is transmembrane resistance, Ri is intrinsic
135 resistance of cell-free media, and A is the surface area of the membrane in cm^2.
136 Monolayers were used when TEER values were between 350 and 450 $\Omega\ cm^2$.
137
138

139 *2.6. Berberine permeability across Caco-2 cell monolayer*

140 In the apical (AP) to basolateral (BP) experiments, 0.5 mL of test solution (25 µg BBR)
141 was added to the AP side of the monolayer at the beginning of the test, and after 30, 60,
142 90, 120, and 180 min, 25 µL was taken from the basal side. To the different aliquots, 25
143 µL of methanol was added, the mixture was centrifuged at 6000 *g* for 2 min, and the
144 supernatants were stored at –20 °C before LC-HR-MS analysis.

145

146 *2.7. Berberine determination by UHPLC-Orbitrap MS*

147 The analysis was performed on an Acquity UPLC system (Waters, Milford, MA, USA)
148 coupled with an Orbitrap high-resolution Fourier transform mass spectrometer, Exactive
149 model (Thermo Scientific, Rodano, I), equipped with an HESI-II probe for ESI and a
150 collision cell (HCD). The operative conditions were as follows: spray voltage +4.0 kV,
151 sheath gas flow rate 55 (arbitrary units), auxiliary gas flow rate 20 (arbitrary units),
152 capillary temperature 350 °C, capillary voltage +60 V, tube lens +100 V, skimmer +26 V,
153 and heater temperature 130 °C. A 1.7 µm Kinetex XB C18 column (150 × 2.1 mm,
154 Phenomenex, Torrence, CA, USA) maintained at 45 °C was used for separation. The flow
155 rate was 0.7 mL/min, and the eluents were 0.05% formic acid in water (A) and acetonitrile
156 (B). UPLC separation was achieved by the following linear elution gradient: 5–50% of B
157 for 5 min, then increased to 90% B for 5 min. The acquisition was made in full-scan mode
158 in the range (m/z)+ 100–1000 u, using an isolation window of ± 2 ppm. The AGC target,
159 injection time, mass resolution, energy, and gas in the collision cell were 1×10^5, 50 ms,
160 50 K, 20 V, and N2, respectively. The MS data were processed using Xcalibur software
161 (Thermo Scientific). The peak identity was ascertained by evaluating the accurate mass
162 and the fragments obtained in the collision cell. Berberine stock solutions (0.1 mg/mL)
163 were prepared in methanol and stored at –20 °C. Working solutions (n = 5) were prepared
164 in the range of 2–200 ng/mL and stored at 4 °C. Analysis was carried out in duplicate.

165

166 *2.8. Statistical analysis*

167 Statistical analysis was performed by means of Statistica software (Statsoft Inc., Tulsa,
168 OK, USA). Analysis of variance (ANOVA) was used to assess the effects of the different
169 formulations on BBR uptake in the Caco-2 cell culture model. Post hoc analysis of
170 differences between treatments was assessed by the least significant difference (LSD) test
171 with $p \leq 0.05$ as the level of statistical significance. Data were derived from three

172 independent experiments in which each condition was tested in triplicate. Results were
173 statistically expressed as mean ± standard error of mean.
174

175 **3. Results**

176 *3.1. Effect of BBR on Cell Viability*

177 Table 1 presents the effects of BBR on cellular viability assessed by Trypan blue
178 exclusion assay at all concentrations tested for 24 h and in the presence of Compritol,
179 lecithin, and cellulose solution. The control condition is represented by cells in their
180 normal growth medium without BBR and nanoemulsion formulation. BBR nanoemulsion
181 and unencapsulated BBR did not affect cell viability, which remained higher than 90%.
182

183 *3.2. Effect of nanostructured lipid carrier (NLC) transport system on BBR intestinal*
184 *absorption*

185 To assess whether the presence of emulsifiers in formulations containing BBR could
186 improve its absorption, a delivery system consisting of nanostructured lipid carriers was
187 prepared. The effect of different formulations on the absorption of BBR was evaluated by
188 a Caco-2 cell transport model, which comprises a monolayer of cells expressing analogue
189 morphological and functional features of intestinal epithelium such as microvilli and tight
190 junctions [18]. The results reported in Table 2 suggest that fatty acid esters contained in
191 Compritol could significantly improve the transport of BBR. Indeed, formulations
192 containing the tested emulsifiers showed increased BBR uptake compared to controls
193 without lipid carriers. Moreover, the increased uptake determined by emulsifiers was
194 confirmed at all-time points analyzed (30 to 180 min).
195 In addition, we observed that the absorption of BBR increased in a time-dependent manner
196 for both nanoemulsions and control formulation without emulsifiers. However, the control
197 formulation reached a plateau after approximately 120 min, while the new nanoemulsions
198 containing emulsifiers showed continuous uptake without reaching a plateau until 180 min
199 (Table 2). In addition, the ratio of BBR to emulsifiers had a relevant influence: 50 mg of
200 BBR in the formulation with a ratio of 1:20 led to a higher permeate amount than the
201 control containing 500 mg of BBR (ratio 1:1) without emulsifiers.
202 Relative to the rate of BBR permeating through Caco-2 cells in the Transwell diffusion
203 system, we found a positive correlation with the BBR to emulsifier ratio. In particular, the
204 highest proportion of BBR uptake was obtained with a ratio of 1:20 (1:20 > 1:10 > 1:5 >
205 1:1) (Figure 1). In particular, the rate raised from 0.74% (ratio 1:1) to 2.3% (ratio 1:20) for

206 the RT-prepared formulation and from 0.9% (ratio 1:1) to 3.1% (ratio 1:20) for the hot-
207 prepared formulation. The same results cannot be translated to the control preparations,
208 since the rate of BBR permeating through Caco-2 cells remained stable between the
209 different ratios of BBR and cellulose tested, particularly around 0.5%.
210 Furthermore, we also observed that the formulation prepared at high temperature (80 °C)
211 led to better absorption than the formulation developed at 25 °C for all BBR-to-emulsifier
212 ratios tested (Figure 1). Specifically, the maximum difference in the rate of absorption was
213 0.8% (hot- vs. RT-prepared solution, 3.1% vs. 2.3%) and it was observed at the highest
214 ratio (1:20).
215
216 *3.3. Berberine and its metabolites identification by High-Resolution Mass Spectrometry*
217 Figure 2A shows the ions extracted at $(m/z)^+$ 336.1230 u, corresponding to BBR, in a sample
218 obtained after 180 min of incubation with a solution containing BBR:Compritol in a 1:5 ratio.
219 In some basal solutions, obtained after 120 min of incubation with Berberine, the untargeted
220 analysis revealed the presence of a compound with Rt 2.35 min and $(m/z)^+$ 322.1070 u
221 ($C_{19}H_{16}NO_4$), which was 14 kDa less than BBR (Figure 2B). The unknown compound (M1)
222 was then fragmented at different collision energies (30-60 V) and it produced ions with
223 *m/z* 307.0845 u by losing a methyl moiety and the *m/z* 292.0970 by loss of a further hydroxyl
224 group and 278.0015 u by further loss of methyl moiety. Figure 3 reports the fragmentation
225 pattern of BBR and M1. Of note that the fragments with *m/z* 292.0970 and 278.0015 were in
226 common with BBR. Thus, M1 was tentatively identified to be 9-demethyl-BBR
227 (berberrubine) or 10-demethyl-BBR (thalifendine).
228

229 **4. Discussion**

230 The aim of the present study was as follows: First, to compare the absorption of BBR from
231 two different formulations (nanostructured lipid carrier vs. carboxymethylcellulose
232 excipient), and second, to evaluate the kinetics of uptake by modifying the temperature for
233 preparing the emulsifier (25 vs. 80 °C) and the ratio of BBR to emulsifier (from 1:1 to
234 1:20). Absorption was tested by using a Caco-2 cell Transwell model. The results obtained
235 document that the formulations containing emulsifiers increased the rate of BBR
236 absorption compared to the control supplement. The rate of absorption increased with an
237 increased BBR-to-emulsifier ratio.
238 The absorption of BBR from different formulations has been evaluated in several studies
239 in order to identify new transport systems (such as nano-based carriers) able to enhance its

240 bioavailability [19]. This interest has been mainly attributed to the biological properties of
241 BBR and its potential application in different therapeutic areas, including the treatment of
242 dyslipidemia [20–22]. In fact, it has been recognized that the low lipophilic property of
243 BBR determines its scarce uptake and consequently the biological effect. Thus, the use of
244 a lipid-based complex could represent a valid solution to improve BBR absorption.
245 However, phospholipids could lead to the formation of phytosome, leading to a reduction
246 in the dissolution rate and consequent absorption [23].
247 Our findings show that an increased BBR-to-Compritol ratio induced an improvement of
248 BBR absorption. These results are probably attributable to the capacity of the surfactant
249 Compritol to act as solid dispersion by enhancing the stability and dissolution rate of BBR,
250 as also documented by Zhang [23] in a study in which they processed complex BBR-
251 phospholipids with the carrier TPGS100 and SiO_2. This solid dispersion led to a higher
252 rate of dissolution and stability of the BBR complex. Our results seem to be in accordance
253 with other in vitro studies testing the role of different nanoemulsions in the enhancement
254 of BBR intestinal absorption. For example, Deng [24] used in their experiments a transport
255 system consisting of Compritol and other excipients such as olive oil, cremophor EL, and
256 d-α-tocopheryl polyethylene glycol 1000 succinate, documenting improved BBR uptake.
257 This nanoemulsion was tested in both Caco-2 cells and RAW 264.7 macrophages,
258 showing improved BBR absorption compared to free BBR. In another study, Kwon [25]
259 developed a berberine-loaded mixed micelle formulation by using two surfactants,
260 Pluronic 85 and Tween 80. The authors reported that administration of the berberine-
261 loaded mixed micelle formulation increased BBR solubility and absorption in Caco-2
262 cells.
263 Absorption of BBR-loaded nanoemulsions was also assessed in in vivo studies. Elsheikh
264 [26] found that their formulation, BBR-loaded cremochylomicrons, enhanced the rate and
265 extent of BBR absorption compared to free BBR in Sprague-Dawley rats. Further, Sun
266 [27] assessed the effect of Gelucire 44/14 (composed of polyethylene glycol monoesters
267 and diesters, monoglycerides, diglycerides, and triglycerides) on the transport of BBR
268 using an in situ closed-loop method in rats. The authors found that Gelucire 44/14 was
269 able to significantly increase plasma concentration of BBR compared to the control group,
270 and in particular enhanced absorption in the ileum.
271 Overall, from these studies the important role of the type of excipient in BBR absorption
272 became clear. This effect can be attributed to the capacity of surfactants, used as excipient,
273 to inhibit the P-glycoprotein involved in the efflux of BBR and the CYP2D6 and CYP3A4

274 in enterocytes, responsible for BBR metabolism [25–27]. Moreover, a mixed micelle
275 formulation consisting of surfactants could increase intestinal absorption by affecting cell
276 integrity, paracellular transport, and macropinocytosis transmembrane mechanisms [28].
277 Another aim of the study was to evaluate the effect of two temperatures (25 vs. 80 °C),
278 used for the preparation of nanoemulsions, on BBR bioavailability. In our experimental
279 conditions, we found that heating the Compritol, as excipient, at about 80 °C resulted in a
280 further improvement of BBR uptake compared to the same formulation developed at room
281 temperature (25 °C). The contribution of temperature to drug delivery and bioavailability
282 has been evaluated in different studies [14, 29, 30]. For example, Barthelemy [29]
283 demonstrated that the dissolution rate of coated drug-loaded beads using Compritol
284 increased with the use of high temperature (54 °C). More recently, a review showed that a
285 nanoemulsion based on Compritol had better drug release when prepared under hot
286 conditions compared to the cold analogue formulation. The authors attributed their
287 findings to the positive impact of high temperature on the reduction of vesicular diameter,
288 the entrapment efficiency, and the zeta potential of the nanoemulsions [14]. Furthermore,
289 He [30] evaluated the absorption of silymarin-loaded solid lipid nanoparticles prepared by
290 using cold and hot (85 °C) Compritol. The results showed an increased rate of silymarin
291 release with the hot-prepared formulation, in line with our findings on BBR.
292 Regarding BBR metabolism, it should be noted that we detected the presence, in small
293 amounts, of demethyl-BBR in some samples after 120 min of incubation of Caco-2 cells
294 with berberine. Xu [31] also detected this metabolite, in addition to other derivatives from
295 phase 2 transformations, in rat plasma after oral ingestion of berberine. Thus, our data
296 appear to support the hypothesis that already at the level of enterocytes there is a partial
297 metabolization of berberine to give demethyl-BBR, a more polar compound than the
298 starting product.
299

300 **5. Conclusions**

301 In our experiments, two new formulations prepared at 25 and 80 °C induced a significant
302 increase in BBR absorption compared to the traditional formulation (4.5-fold and 6-fold,
303 respectively). Since we excluded a possible cytotoxicity effect of the emulsifiers, we may
304 assume that the use of Compritol 888 ATO as excipient may represent a useful alternative
305 for the development of transport systems able to deliver and absorb BBR in a more
306 efficient way. However, since the data were obtained in vitro, it is highly recommended to
307 substantiate these findings with human studies aimed at evaluating BBR bioavailability.

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309
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438 **Figure 1**. Effect of berberine-to-emulsifier ratio and method of preparing emulsifying
439 solution on rate of BBR uptake at 180 min. BBR, berberine; RT, room temperature.
440 Results derived from three independent experiments in which each condition was tested in
441 triplicate. Values with different letters are significantly different ($P < 0.05$). Data are reported
442 as mean ± SEM.
443
444 **Figure 2**. The extracted ion chromatogram of (A) BBR and (B) its metabolite M1 in a basal
445 solution after 180 min incubation with a solution containing BBR:Compritol in a ratio 1:5.
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447 **Figure 3**. The fragmentation pattern of (A) BBR and (B) its metabolite M1.
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Table 1. Percentage of cell viability following supplementation with berberine evaluated by Trypan blue exclusion assay.

	MEM	C1	C2	C3	C4	Test 1	Test 2	Test 3	Test 4
Viability %	91.9 ± 1.1	92.9 ± 0.9	94.8 ± 0.5	92.3 ± 0.7	92.3 ± 0.7	90.4 ± 0.8	91.7 ± 1.3	93.1 ± 0.7	91.3 ± 0.9

MEM, negative control; C1, BBR 50 mg + cellulose 950 mg; C2, BBR 100 mg + cellulose 800 mg; C3, BBR 200 mg + cellulose 800 mg; C4, BBR 500 mg + cellulose 500 mg; test 1, BBR 50 mg + Compritol 850 mg + lecithin 100 mg; test 2, BBR 100 mg + Compritol 800 mg+ lecithin 100 mg; test 3, BBR 200 mg + Compritol 700 mg + lecithin 100 mg; test 4, BBR 500 mg + Compritol 400 mg + lecithin 100 mg. Results derived from three independent experiments in which each condition was tested in triplicate. Data are reported as mean ± SEM. MEM, minimum essential medium; BBR, berberine.

Table 2. Absorption kinetics of berberine nanoemulsions (T1, T2, T3, and T4) and their controls (C1, C2, C3, and C4) from apical to basal in human Caucasian colon adenocarcinoma cell (Caco-2) model (n = 3). Results derived from three independent experiments in which each condition was tested in triplicate. Data are reported as mean ± SEM.

min	30	60	90	120	150	180
formulation	μM BBR					
C1	0.00 ± 0.00[a]	0.07 ± 0.00[a]	0.13 ± 0.0[a]	0.20 ± 0.0[a]	0.21 ± 0.0[a]	0.24 ± 0.01[a]
T1 RT	0.04 ± 0.00[b]	0.14 ± 0.0[b]	0.28 ± 0.0[b]	0.47 ± 0.02[b]	0.46 ± 0.02[b]	0.51 ± 0.02[b]
T1 HOT	0.03 ± 0.00[b]	0.20 ± 0.0[b]	0.30 ± 0.0[b]	0.52 ± 0.02[b]	0.58 ± 0.02[b]	0.61 ± 0.02[b]
C2	0.03 ± 0.00[a]	0.09 ± 0.00[a]	0.22 ± 0.0[a]	0.33 ± 0.02[a]	0.38 ± 0.02[a]	0.39 ± 0.01[a]
T2 RT	0.06 ± 0.00[b]	0.33 ± 0.01[b]	0.60 ± 0.03[b]	0.82 ± 0.04[b]	0.90 ± 0.04[b]	1.02 ± 0.04[b]
T2 HOT	0.22 ± 0.0[c]	0.73 ± 0.03[c]	0.95 ± 0.04[c]	1.07 ± 0.04[b]	1.19 ± 0.05[b]	1.23 ± 0.05[b]
C3	0.06 ± 0.00[a]	0.20 ± 0.01[a]	0.27 ± 0.0[a]	0.34 ± 0.01[a]	0.34 ± 0.0[a]	0.35 ± 0.02[a]
T3 RT	0.30 ± 0.0[b]	0.79 ± 0.03[b]	0.93 ± 0.04[b]	1.12 ± 0.04[b]	1.18 ± 0.04[b]	1.37 ± 0.06[b]
T3 HOT	0.30 ± 0.0[b]	1.13 ± 0.05[c]	1.26 ± 0.05[b]	1.42 ± 0.05[c]	1.67 ± 0.06[c]	1.78 ± 0.08[c]
C4	0.05 ± 0.00[a]	0.22 ± 0.01[a]	0.30 ± 0.0[a]	0.34 ± 0.02[a]	0.34 ± 0.0[a]	0.36 ± 0.01[a]
T4 RT	0.58 ± 0.02[b]	1.01 ± 0.03[b]	1.12 ± 0.04[b]	1.31 ± 0.05[b]	1.42 ± 0.05[b]	1.57 ± 0.05[b]
T4 HOT	0.51 ± 0.0[b]	1.48 ± 0.05[c]	1.64 ± 0.06[c]	1.82 ± 0.06[c]	1.98 ± 0.07[c]	2.12 ± 0.06[c]

C, control; T, test; RT, nanoemulsion prepared at room temperature; HOT, nanoemulsion prepared by heating emulsifiers to ~80 °C. C1, BBR 50 mg + cellulose 950 mg; C2, BBR 100 mg + cellulose 800 mg; C3, BBR 200 mg + cellulose 800 mg; C4, BBR 500 mg + cellulose 500 mg; test 1, BBR 50 mg + Compritol 850 mg + lecithin 100 mg; test 2, BBR 100 mg + Compritol 800 mg + lecithin 100 mg; test 3, BBR 200 mg + Compritol 700 mg + lecithin 100 mg; test 4, BBR 500 mg + Compritol 400 mg + lecithin 100 mg. Results derived from three independent experiments in which each condition was tested in triplicate. Data are reported as mean ± SEM. Values with different letters within the same column are significantly different ($P < 0.05$).