International Journal of Pharmaceutics 444 (2013) 128–138

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Stability and physicochemical characterization of novel milk-based oral formulations

J. Kytariolos[a], G. Charkoftaki[a], J.R. Smith[b], G. Voyiatzis[c], A. Chrissanthopoulos[d], S.N. Yannopoulos[d], D.G. Fatouros[b,1], P. Macheras[a,*]

[a] Laboratory of Biopharmaceutics-Pharmacokinetics, Faculty of Pharmacy, University of Athens, Panepistimiopolis, GR-15771, Athens, Greece
[b] School of Pharmacy and Biomedical Sciences, University of Portsmouth, St Michael's Building, White Swan Road, Portsmouth PO1 2DT, UK
[c] FORTH ICEHT, Institute of Chemical Engineering & High Temperature Chemical Procedures, GR-26504 Patras, Greece
[d] University of Patras, Department of Materials Science, GR-26504 Patras, Greece

ARTICLE INFO

Article history:
Received 10 January 2013
Accepted 11 January 2013
Available online 20 January 2013

Keywords:
Milk-based formulations
Stability studies
NSAIDs
Cyclosporine
Danazol

ABSTRACT

Purpose: The purpose of this work was to assess the colloidal stability of novel milk-based formulations.
Methods: Milk-based formulations were prepared *in situ* by adding into milk alkaline- or ethanolic-drug solutions containing an array of drugs namely; ketoprofen, tolfenamic acid, meloxicam, tenoxicam and nimesulide, mefenamic acid, cyclosporine A, danazol and clopidogrel besylate. The produced formulations were characterized by means of dynamic lightscattering, ζ-potential studies, atomic force microscopy, fluorescence spectroscopy, Raman spectroscopy complemented with *ab initio* calculations and stability studies.
Results: The presence of the drugs did not induce significant changes in most cases to the particle size and ζ-potential values of the emulsions pointing to the colloidal stability of these formulations. Raman spectroscopy studies revealed interactions of the drugs and the milk at the intermolecular level. Complementary analysis with *ab initio* calculations confirmed the experimental observations obtained by Raman spectroscopy. Finally the produced drug containing alkaline/ethanolic solutions exhibited stability over a period of up to 12 months.
Conclusions: The current data demonstrate that milk is a promising drug carrier.

1. Introduction

Oral administration is the major route of drug delivery for the treatment of many diseases. However, poor solubility is one of the major problems encountered during formulation of orally administered drugs. There have been many approaches to improve solubility and dissolution rate, such as modification of drug's physicochemical properties (Hu et al., 2003; Rasenack and Müller, 2002; Rasenack et al., 2003a, 2003b; Rogers et al., 2003a, 2003b; Wu et al., 2009) or the media in which the drug is dissolved (Larrucea et al., 2002; Larsen et al., 2009; Mutalik et al., 2008; Rogers et al., 2003a, 2003b; Seedher and Bhatia, 2003; Yeh et al., 2009). However, all these methods have a variety of limitations.

In recent years, one of the most popular approaches for improving oral bioavailability of sparingly water-soluble drugs has been through the use of lipid-based drug delivery systems (Pouton, 2000). In earlier literature, there have been many studies that point to the beneficial effects of food or lipids on bioavailability of hydrophobic compounds (Humberstone and Charman, 1997; Pouton, 1997). When cyclosporine A was formulated as a microemulsion (Neoral®), it became clear that its formulation with lipids or surfactants had been crucial to the bioavailability improvement of the oral capsule product (Mueller et al., 1994a, 1994b, 1997; Pouton, 2000). Since then, there has been an increasing interest in the development of lipid-based formulations, such as oil solutions, self-emulsifying (SEDDS) and self-micro emulsifying drug delivery systems (SMEDDS). The primary mechanism of action, of the lipid-based formulations, is that the compound is presented in solubilized form *in vivo*, avoiding any dissolution process. This drug formulation leads to improved bioavailability of poorly water-soluble drugs (Humberstone and Charman, 1997; Mohsin et al., 2009; Porter et al., 2007; Pouton, 2000). However, the preparation of oral lipid-based synthetic emulsions is based on one or more of the following excipients: dietary oils composed of coconut oil, palm seed oil or long-chain triglycerides (*e.g.*, corn, olive, peanut, rapeseed, sesame, or soybean oils), lipid soluble solvents (*e.g.*, polyethylene glycol 400, ethanol, propylene glycol, glycerin), and various pharmaceutically acceptable surfactants (*e.g.*, Cremophor®

* Corresponding author. Tel.: +30 2107274026; fax: +30 2107274027.
E-mail address: macheras@pharm.uoa.gr (P. Macheras).
[1] Current address: Aristotle University of Thessaloniki, School of Pharmacy, Department of Pharmaceutical Technology, GR-54124 Thessaloniki, Greece.

0378-5173/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ijpharm.2013.01.022

EL, polysorbate, D-α-tocopherolpolyethyleneglycol 1000 succinate (TPGS®); Span 20; various Labrafils® and Gelucires®) (Hauss, 2007). As preparation of lipid-based delivery systems is relatively time consuming and a range from simple oil solutions to complex mixtures of oils, surfactants, co-surfactants and cosolvents are needed, recent work (Charkoftaki et al., 2012) focused on the use of milk as a drug delivery system, which is a ready, natural oil-in-water emulsion and could be applied for the development of paediatric formulations.

Since the adoption of Paediatric Regulations in the U.S. and E.U., there is a greater demand for age-appropriate medicines for children. However, despite this growing demand, paediatric drug formulation science is still at the beginning, as alternative drug delivery systems are needed since 'traditional' tablets and capsules cannot be swallowed by the very young. Liquid formulations are preferred for newborn infants and young children (below 6 years old) instead of solid oral dosage forms and particularly formulations who can deliver the drug whilst the baby drinks. The Dose Sipping Technology has been developed in order to deliver a single dose of small-sized pellets, overcoming swallowing issues (Breitkreutz and Boos, 2007). This technology incorporates small-sized pellets in a straw and when the child holds the straw in a beverage and sips, the drug is delivered in a 'user friendly' way. An alternative drug delivery vehicle, which is familiar to infants and older children, is milk, a natural oil-in-water emulsion. Dissolving ionized drugs (NSAIDs) in alkaline solutions or unionized drugs in ethanolic solutions and then dispersing them into milk is an interesting way to deliver drugs to children in a 'friendly' way (Charkoftaki et al., 2012); milk is a natural emulsion and a daily ritual for children, consisting of a variety of components, such as fat globules and caseins (Walstra and Jenness, 1984). Apart from the potential paediatric use, the milk-based formulations exhibited improved pharmacokinetic properties, content uniformity (Charkoftaki et al., 2012) and gastroprotective properties (unpublished data). The food–drug interactions may depend on several factors such as the physical and chemical characteristics of the drug, the size and the composition of the meal or the time of drug intake in relation with the meal (Singh, 1999). For instance certain lipid based formulations of poorly soluble drugs can diminish the food effect as has been demonstrated by Mueller et al. (1994a, 1994b).

The aim of the current work was to explore the physicochemical characteristics of the above mentioned milk-based formulations. We investigated any possible changes to the colloidal stability of the emulsion and to the particle size of milk's components, after the addition of drug-containing alkaline buffer or ethanolic solutions. The physicochemical characterization of the milk-based formulations was performed by measuring critical properties of the drug–milk system, such as ζ-potential and size distribution of emulsion components by means of dynamic light scattering (DLS) techniques. These studies were complemented with atomic force microscopy and fluorescence spectroscopy, Raman spectroscopy and computational investigations. Finally the stability of the drug-containing alkaline/ethanolic solutions was monitored over a period of up to 12 months and not the final milk-based formulation, as the aim was to develop a product that just prior use, would be mixed with milk.

2. Materials and methods

2.1. Materials

Long life milk (UHT) was commercially available, at three different fat concentrations: 3.6% (full fat), 1.8% (semi-skimmed) and 0.3% (skimmed). Ketoprofen (logP 3.2, MW 254.2), tolfenamic acid (logP 5.2, MW 261.7), meloxicam (logP 1.9, MW 351.4), tenoxicam (logP 1.9, MW 337.4) and nimesulide (logP 2.56, MW 308.3) were kindly provided by Kleva (Athens, Greece). Mefenamic acid (logP 4.2, MW 241.2) was provided by ELPEN (Athens, Greece). Cyclosporine A (logP, MW 1202.6) was provided from RPG Life Sciences Ltd, India, danazol (logP 3.46, MW 337.4) from Sanofi-Aventis, France and clopidogrel besylate (logP 2.5, MW 321.8) from Pharmathen S.A., Greece. Ethanol (absolute) was obtained from Thermo Fisher Scientific (Waltham, MA, USA), glycine, NaH_2PO_4 and NaOH were obtained from Merck (Darmstadt, Germany). Nile red (NR) was obtained from Sigma–Aldrich Inc. (St Louis, MO, USA). All other chemicals were of analytical grade.

2.2. Preparation of the samples

2.2.1. Ionized drugs

Mefenamic acid, tolfenamic acid, ketoprofen, meloxicam, tenoxicam and nimesulide were used as model ionized NSAID compounds. The drugs were dissolved in either 0.2 M NaH_2PO_4–NaOH (pH 12) or 0.05 M glycine–NaOH (pH 12). The drug-containing buffer solutions were added into UHT milk (3.6%, 15 mL) and the size distribution of the fat globules and micelles were measured. Any alterations to the pH of the emulsions upon the addition of the drug containing solution were checked. To investigate the effect of different aqueous solutions on the physicochemical characteristics of milk, an array of drug-free solutions were added to UHT milk (3.6%, 15 mL), namely: (i) 0.1 M phosphate buffer (pH: 8, 10, 12), (ii) 0.2 M phosphate buffer (pH: 8, 10, 12), (iii) 0.05 M glycine–NaOH (pH: 8, 10, 12), and (iv) 0.1 M glycine–NaOH (pH: 8, 10, 12).

2.2.2. Lipophilic drugs

Cyclosporine A, danazol and clopidogrel besylate were used as model lipophilic compounds. They were initially dissolved in water–ethanol mixtures forming stock solutions and the appropriate amounts were added to milk (15 mL). The effect of the fat content was assessed by using full fat, semi skimmed and skimmed milk. Cyclosporine A and clopidogrel besylate were dissolved in aqueous–ethanolic mixtures containing 60% (v/v) of ethanol whilst danazol was dissolved in absolute ethanol. The concentration of cyclosporine A in the solution was adjusted to 20 mg/mL, the concentration of clopidogrel besylate to 112.1 mg/5 mL (equivalent to 75 mg clopidogrel base) and the concentration of danazol to 10 mg/mL. The dispersions were mixed for 15 s prior to analysis. In a similar manner, absolute ethanol was added to milk in various volumes to form milk–ethanol mixtures to study the effect of ethanol on the physicochemical characteristics of milk.

2.3. Dynamic light scattering (DLS) measurements

The particle size of undiluted milk formulations ($n = 3$) and the polydispersity index (PI) were measured by DLS (Zetasizer Nano ZS, Malvern Instruments, Malvern, UK) with fixed angle of 173°. The size distribution of the particles, expressed by volume distribution, was analysed by using the Dispersion Technology Software (DTS; v 5.10, Malvern Instruments, UK).

2.4. ζ-Potential measurements

The application of the M3-PALS technique (Smoluchowski approximation) allowed the determination of ζ-potential from measurements of electrophoresis (Malvern Nanosizer ZS, Malvern instruments, UK). The samples were diluted (1:100; $n = 3$) with distilled water, as described previously (Jack and Dahle, 1937). Data were analysed using the Dispersion Technology Software (DTS; v 5.10, Malvern Instruments, UK).

2.5. Atomic force microscopy studies (AFM)

AFM studies (MultiMode/NanoScope IV Scanning Probe Microscope, Digital Instruments, Santa Barbara, CA, USA) were performed in air under ambient conditions (T = 23 °C, RH = 21%) using the J-scanner (max. xy = 200 μm). Scanning was performed in tapping mode using Si cantilevers with integrated tips (t = 3.5–5.6 μm, l = 140–180 μm, w = 48–52 μm, ν_0 = 315–327 kHz, k = 12–103 N m^{-1}, R < 10 nm; model: OTESPA, Bruker, France), and an rms amplitude of 0.8 V was used. Images were processed using NanoScope software (v7.10, Digital Instruments, Santa Barbara, CA, USA). Specimens for imaging were prepared by placing diluted dispersions (1:100 in distilled water; 10 μL) of each sample onto a freshly cleaved surface of muscovite mica (Agar Scientific, Stansted, Essex, UK; mounted on a nickel disc), leaving for 2 min and then drying the surface in a stream of N_2. Dimensions (heights and widths) of features were obtained from line-profiles using NanoScope software from three independent sample preparations.

2.6. Fluorescence spectroscopy

The hydrophobic fluorescent probe Nile red (NR) was used as a model drug. A final concentration of 3 μM NR in ethanol was added to the milk formulations and emission fluorescence spectra were obtained with a Cary Eclipse Fluorescence Spectrophotometer (Varian Inc., USA). The spectra were recorded at room temperature with both slit widths set at 5 nm. The excitation wavelength was fixed at 546 nm, and the emission spectra were recorded from 550 to 700 nm with a scanning speed of 120 nm/min. The spectra were analysed using the Cary Eclipse Scan Application (v1.1) and saved as ASCII files.

2.7. Raman spectroscopy studies

Stock solutions of meloxicam (15 and 30 mg/mL) were prepared in glycine–NaOH buffer (pH 12, 0.05 M). An aliquot (1 mL) from each solution was transferred into a volumetric flask (10 mL) and diluted with full fat milk. The meloxicam/milk samples with volumes of 1.5 and 3.0 mg/mL, respectively, were frozen for 24 h at −80 °C and then were freeze dried for 48 h (Christ Alpha I-5, Germany).

Raman spectra of the freeze dried meloxicam/milk samples were recorded with a Fourier Transform (FT) Raman spectrometer (Bruker, model FRA 106/S) with a resolution of 2 cm^{-1}, and over the broad spectral range 100–4500 cm^{-1}. The 1064 nm laser line was used as the excitation source. The power level was set to 250 mW and the microscope was loosely focused (~150 μm) on the sample to avoid heating. The signal was detected by a liquid-nitrogen-cooled CCD (Ge-detector). Each spectrum was accumulated over 300 scans for a good signal-to-noise ratio. A few milligrams of each sample was put in an aluminium can and pressed gently to form a pellet. At least three pellets were measured for each sample. The Raman spectra of the meloxicam diluted in a NaOH solution (pH 12) were obtained using an excited Ar^+ ion laser operating at 514.5 nm and measured using a JobinYvon (T64000) spectrometer (right-angle scattering).

2.8. Computational studies

Hartree–Fock (HF) molecular orbital calculations were carried out within the GAUSSIAN 03 program package (Frisch et al., 2004). The fully optimized configurations of meloxicam (neutral and anionic forms) and vibrational spectra were calculated. The ability of meloxicam to bind Ca^{2+} and its structural rearrangement were also studied. The electronic structure of the atoms participating in the investigated structures are described by the TZVp basis set, which are full-electron contracted Gaussian basis sets of triple-ζ quality group included polarization functions (Schaefer et al., 1994).

2.9. Stability studies

Long term and accelerated stability studies were conducted for the alkaline solutions of meloxicam, ketoprofen and nimesulide. Accelerated stability studies were conducted for the ethanolic solutions of danazol, cyclosporine and clopidogrel. Cyclosporine was also tested under intermediate and long-term conditions.

Aqueous alkaline NSAID solutions were prepared as follows: (i) therapeutic doses of ketoprofen (200 mg) and nimesulide (100 mg) were dissolved in phosphate buffer (0.2 M, pH 12, 4 and 2.5 mL, respectively) and (ii) meloxicam (15 mg) dissolved in glycine–NaOH (0.05 M, pH 12, 2.5 mL) buffer.

Ethanolic solutions containing the appropriate amount of the active compounds corresponding to their therapeutic doses were prepared for solubility studies.

Stock solutions were prepared corresponding to the following final concentrations: (i) 100 mg of danazol in 10 mL of absolute ethanol, (ii) 100 mg of cyclosporine in 5 mL of 60% ethanol, and (iii) 112.1 mg of clopidogrel besylate, corresponding to 75 mg of base, in 5 mL of 60% ethanol.

Appropriate volume of the stock solutions was filled into glass vials, sealed, weighted and stability studies were conducted, according to the conditions specified within the ICH Q1A (R2) guideline (EMA, 2003). Samples were prepared in triplicate for each time point. Three of the samples for all of the final formulations were quantified on the day of manufacturing, to serve as reference.

For long-term stability studies, glass vials were stored at 25 ± 2 °C, 60± 5% RH and samples were withdrawn at 3, 6, 9 and 12 months. For intermediate stability studies, storage conditions and withdrawal times were 30 ± 2 °C, 65 ± 5% RH and 1, 3 and 6 months. For accelerated stability studies, 40 ± 2 °C, 75 ± 5% RH storage conditions were used and samples were withdrawn after 1, 3 and 6 months.

2.10. Statistical analysis

The effect of the drug-free and the drug-containing alkaline/ethanolic solutions on the particle size of milk was examined statistically by paired t-test. A significance level of p < 0.01 denoted significance in most cases. All DLS studies and ζ-potential measurements were performed in triplicate. Statistical analysis was performed using Sigma plot 10 statistical software.

3. Results and discussion

3.1. DLS studies

The DLS footprints from samples from long life milk (UHT) of full fat, semi-skimmed and skimmed are shown in Fig. 1. Fat globules and free casein micelles were apparent in each sample. For full fat and semi skimmed milk, a third feature in the DLS footprint, attributed to the presence of a fraction of large particles, most probably aggregates, could be seen; this may have arisen due to the milk processing procedure.

The effect of different amounts of ethanol on the particle size distributions of the milk emulsions was further evaluated (SC Fig. 1S). The experimental data yielded bimodal distributions of particle sizes where the hydrodynamic diameter of the majority of casein micelles (1st peak) lies in the region of 60–100 nm and the fat globules (2nd peak), in the region 300–800 nm. A decrease to the intensity of the 1st peak accompanied with a gradual increase to that of the 2nd peak was observed with an increasing proportion of ethanol present in the emulsions. The latter might be attributed

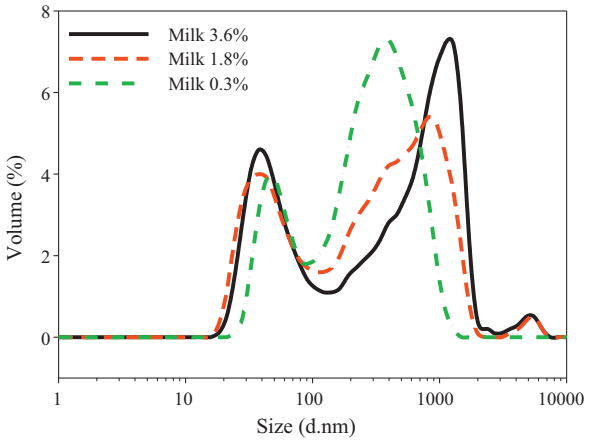

Fig. 1. The DLS footprint of samples from long life milk (UHT) of full fat (3.6%, solid line), semi-skimmed (1.8%, dashed line) and skimmed (0.3%, dashed-dotted line).

to possible disruption of casein micelles due to the presence of the solvent. The DLS footprints for formulations containing 5%, 20% and 30% of ethanol gave evidence of a 3rd peak indicating the formation of micron-sized entities. Moreover, a shift towards higher particle size diameters for both casein and fat globules was noticed. The same trend was also observed for full fat and semi-skimmed milk.

Table 1 summarizes the corresponding particle sizes for dispersions of compositions with different fat contents and different drugs. As mentioned previously, a bimodal distribution was obtained for the milk-based formulations. The DLS evaluation of the plain emulsions revealed that in moving from full fat to skimmed milk, the relative proportions of each feature changes significantly (t-test, $p < 0.05$), with a decrease to the size of fat globules as the fat content is reduced. This effect can be directly correlated to the composition of the vehicle formulation: a decreased fat content of the vehicle results in a decrease in the droplet size. The particle sizes of the droplets arising from the milk based formulations were virtually unaffected by the addition of the drugs (Table 1). This was with the exception of danazol when added to semi-skimmed milk, where significantly higher particle sizes diameters (t-test, $p < 0.01$) were obtained for the fat globules compared with the other two drug-containing emulsions (Table 1). With skimmed milk samples, significantly higher particle size diameters (t-test, $p < 0.01$) were found compared to the control obtained for the fat globules with the drug clopidogrel.

The reported polydispersity index (PI) values, ranging from 0 (ideally monodispersed) to 1 (system with very broad size distribution), were *ca.* 0.44 and *ca.* 0.23 for full fat and skimmed milk, respectively. The systematic PI improvement was probably the result of the decrease of fat content.

For emulsions loaded with NSAID drugs, similarly, particle size measurements showed two peaks corresponding to casein micelles and fat globules (Table 2). The addition of mefenamic and tolfenamic acid increased significantly the mean diameters of fat globules of the produced emulsions compared with the control (t-test, $p < 0.01$) with the exception of one concentration of tolfenamic acid (100 mg). No obvious relationship between particle size diameter and the amount of drug loaded to the emulsions could be established.

3.2. ζ-Potential measurements

Consistent with previous studies (Michalski et al., 2001), all milk formulations were found to carry a negative net charge (Table 3). The ζ-potential of skimmed milk (-29.6 ± 5.2 mV), however, was lower in absolute values than that of samples of full fat milk (-34.2 ± 4.8 mV) and semi-skimmed milk (-35.0 ± 5.7 mV). This might be attributed to less lipid being present. The presence of ethanol exerted little influence on ζ-potential. Although the ζ-potential measurements were not modified significantly upon addition of the lipophilic drugs in full fat nor semi-skimmed milk, the surface charge was affected with the ζ-potential moving towards lower negative values in skimmed milk.

In an attempt to further investigate how the solvent (ethanol) affects the electrokinetic properties of the emulsions, different amounts of ethanol were added (0–50%, w/w) and the produced dispersions were further assessed. The presence of ethanol did not induce any significant changes to the ζ-potential of the produced emulsions (data not shown).

The effect of NSAID drugs to the electrical properties of formulations containing full fat milk is shown in Table 4. The choice of the buffer solutions, varying in their ionic strength (NaH_2PO_4–NaOH 0.2 M pH 12 and glycine–NaOH 0.05 M pH 12), had little effect on the surface charge of the produced emulsions of full fat milk (data not shown). The ζ-potential of the emulsions was significantly increased (t-test $p < 0.05$) in the presence of mefenamic acid (40 mg/mL, 500 mg; -38.0 ± 1.3 mV) and mefenamic acid (10 mg/mL, 100 mg) compared with the control (-34.2 ± 0.4 mV), implying an association of the drugs with the casein or the fat globules. The results showed that the addition of the drugs had little

Table 1
The effect of lipophilic drugs on the particle size of full fat, semi-skimmed and skimmed milk formulations, respectively, containing 3% of ethanol.

Drug dissolved in ethanolic solution added into milk	Peak 1 (nm)[a]	Peak 2 (nm)[b]	PI
Medium: full fat milk (3.6%)			
Full fat milk (3.6%)	52.7 ± 2.0	837.0 ± 63.7	0.468
Cyclosporine	52.1 ± 16.5	844.2 ± 21.2	0.448
Danazol	44.4 ± 11.6	860.5 ± 98.7	0.453
Clopidogrel	64.0 ± 12.2	842.6 ± 70.5	0.470
Medium: semi-skimmed milk (1.8%)			
Semi-skimmed milk (1.8%)	45.2 ± 12.9	630.6 ± 126.0	0.374
Cyclosporine	53.70 ± 12.8	597.6 ± 90.5	0.338
Danazol	77.50 ± 6.0	758.0 ± 34.6	0.368
Clopidogrel	68.60 ± 13.8	648.0 ± 43.5	0.364
Medium: skimmed milk (0.3%)			
Skimmed milk (0.3%)	53.91 ± 4.30	392.8 ± 11.7	0.250
Cyclosporine	54.27 ± 7.5	404.0 ± 22.0	0.236
Danazol	56.59 ± 1.5	419.0 ± 25.0	0.239
Clopidogrel	50.8 ± 4.1	358.3 ± 16.2	0.234

[a] Attributed to free casein micelles.
[b] Attributed to fat globules.

Table 2
The effect of antiinflammatory non-steroidal (NSAID) drugs on the particle size of full fat milk formulations.

Amount of drug dissolved in alkaline buffer added into full fat milk		Peak 1 (nm)[a]	Peak 2 (nm)[b]
Full fat milk (3.6%, control)		52.7 ± 2.0	837.0 ± 63.7
Medium: phosphate buffer 0.2 M pH 12			
Mefenamic acid	500 mg	63.7 ± 9.3	974.3 ± 8.9
	50 mg	43.9 ± 16.6	940.3 ± 45.8
	100 mg	48.9 ± 18.3	927.6 ± 27.5
Tolfenamic acid	100 mg	51.67 ± 18.6	861.6 ± 22.8
	200 mg	48.9 ± 7.7	973.1 ± 53.6
	300 mg	70.5 ± 17.6	947.6 ± 21.5
Ketoprofen	200 mg	51.3 ± 17.3	868.0 ± 70.5
	100 mg	47.7 ± 7.2	800.2 ± 59.5
Nimesulide	100 mg	39.5 ± 8.0	821.1 ± 75.6
Medium: glycine buffer 0.05 M pH 12			
Meloxicam	15 mg	61.0 ± 5.7	736.4 ± 61.0
	7.5 mg	52.9 ± 6.2	828.8 ± 45.7
Tenoxicam	100 mg	67.6 ± 19.0	871.7 ± 98.1

[a] Attributed to free casein micelles.
[b] Attributed to fat globules.

Table 3
The effect of lipophilic drugs on the ζ-potential (mV) of full fat, semi-skimmed and skimmed milk formulations, respectively, containing 3% of ethanol.

Formulation	Full fat milk (3.6%)	Semi-skimmed milk (1.8%)	Skimmed milk (0.3%)
Untreated	−34.2 ± 4.8	−35.0 ± 5.7	−29.6 ± 5.2
Ethanol 3%	−32.2 ± 4.8	−34.9 ± 5.0	−29.9 ± 4.7
Cyclosporine	−29.9 ± 4.3	−34.8 ± 5.0	−27.3 ± 5.2
Danazol	−31.8 ± 4.0	−35.6 ± 4.5	−24.8 ± 6.9
Clopidogrel	−34.4 ± 5.1	−37.1 ± 4.3	−24.6 ± 7.0

or no effect to the overall charge of the produced milk emulsions which in turn is not altering the colloidal stability of the formulations.

3.3. AFM studies

Representative AFM images of full fat milk in the absence and presence of 5% ethanol are shown in Fig. 2a and b, respectively.

Table 4
The effect of ionized drugs (NSAIDs) on the ζ-potential (mV) of full fat milk formulations containing buffers of different ionic strengths.

Formulation		ζ-Potential (mV ± sd)
Full fat milk (control)		−34.2 ± 0.4
Medium: phosphate buffer 0.2 M pH 12		
Mefenamic acid	500 mg	−38.0 ± 1.3*
	50 mg	−31.1 ± 4.7
	100 mg	−30.07 ± 2.2*
Tolfenamic acid	100 mg	−34.2 ± 1.1
	200 mg	−35.3 ± 1.6
	300 mg	−35.4 ± 1.3
Ketoprofen	200 mg	−35.0 ± 1.2
	100 mg	−34.43 ± 2.4
Nimesulide	100 mg	−39.5 ± 8.1
Medium: glycine buffer 0.05 M pH 12		
Meloxicam	15 mg	−34.2 ± 1.2
	7.5 mg	−33.9 ± 0.9
Tenoxicam	100 mg	−33.2 ± 1.2

*The asterisk indicates statistically different values compared to full fat milk formulation (control).

Spherical droplets of fat (fat globules) and casein micelles are the main structural features of milk. The presence of alkaline buffers or ethanol did not induce any changes to the morphology of the colloids (data not shown).

Small globular structures (mean diameter *ca.* 120 nm, indicated by white arrow; Fig. 2a) were assumed to be casein micelles, whilst the larger, spherical structures (mean diameter <800 nm; indicated by black arrow) are consistent with lyotropic order (fat globules). These micelles are located close to the surface of the fat globules forming a corona around these spherical droplets. As can be seen in particle size distribution histogram in Fig. 2S (SC), globular structures were obtained for different conditions tested. Vesicles ranged from 120 nm up to 1500 nm suggesting that structural features correspond to casein micelles and fat globules (SC Fig. 2S). The results obtained from AFM analysis are in broad agreement with the data obtained from DLS measurements.

3.4. Fluorescence spectroscopy studies

In an attempt to gain insights for the localization of the drugs in the emulsions droplets a model compound (Nile red [NR]) with very low aqueous solubility and high lipophilicity was added to the milk emulsions and the fluorescence spectra of NR were recorded. Owing to its hydrophobic nature, NR has been used as a lipid probe (Greenspan and Fowler, 1985). The emission spectra of NR added to milk samples of various fat contents are shown in Fig. 3a. NR fluorescence in water is very weak and red shifted ($\lambda_{max} \approx 660$ nm; Fig. 3a) and therefore full fat and semi-skimmed milk samples showed similar spectral positions and shapes; however, the relative fluorescence yield in semi-skimmed milk was higher than that of full-fat milk. This suggests that NR can penetrate deeper into either the casein micelles or the fat globules when present in

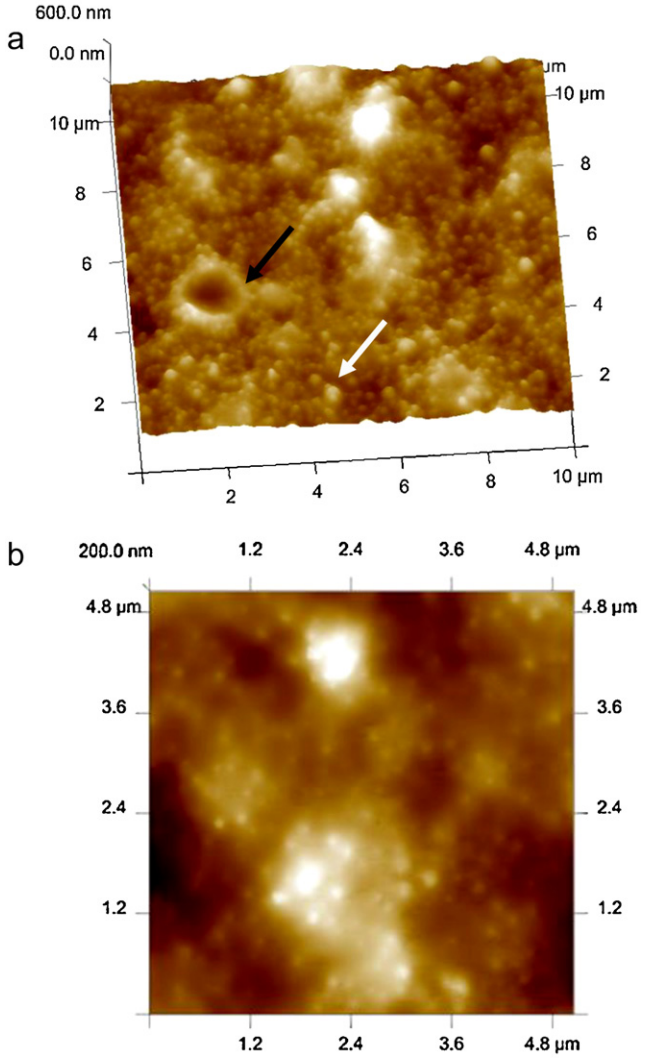

Fig. 2. AFM images of full fat milk (a) and full fat milk containing 5% EtOH (b). Fig. 2a shows small globular structures with average diameter of *ca.* 120 nm (indicated by white arrow), which are assumed to be casein micelles, and other spherical structures (indicated by black arrow) with diameter of *ca.* 800 nm, which are consistent with lyotropic order (fat globules).

semi-skimmed milk than in whole milk, thus being less exposed to water.

When the NR was added to skimmed milk samples, the emission maximum shifted ($\lambda_{max} \approx 620$ nm) closer to the value in water, indicating that the probe was located in a water-rich environment (Fig. 3a). This might be due to skimmed milk containing less lipid material and therefore accommodating lower numbers of NR molecules in the emulsion.

Full fat samples in up to 30% ethanol showed the mixture had little effect on the fluorescence intensity (Fig. 3b). Between 30 and 50% ethanol, a steep decrease in the intensity was observed. Semi-skimmed and skimmed milk samples followed the same pattern, with small changes in the intensity values when ethanol was present up to 20%. A decrease was observed, however, when the organic solvent increased up to 50%, emphasizing its important role in the polarity of the system.

A significant red shift in the emission spectrum was seen when the amount of ethanol in all formulations was increased (Fig. 3c), suggesting NR to be located in the more hydrophilic environment. Notably, the NR emission maximum for skimmed milk containing 50% of ethanol was $\lambda_{max} \approx 645$ nm which is close to the value in water, indicating that the probe was located in a water-rich environment. The presence of ethanol provides a lipid environment with lower incorporation therefore the model compound is exposed to water. The latter might be attributed to the ability of ethanol to dissolve the lipid content in the emulsion.

The red shift to the spectrum when the amount of ethanol increases corroborates with the fluorescence intensity changes previously observed (Fig. 3b).

3.5. Raman studies

A Raman spectrum of meloxicam (pH 12) and freeze dried milk mixed with meloxicam (1.5 and 3 mg/mL) over a limited wave number range is shown in Fig. 4a. The spectrum of milk contains several bands assigned to milk proteins and fat globules (Udenfriend, 1962). A weak sharp band at 1002 cm^{-1} originates from the breathing mode of phenylalanine indicative of the milk protein content. The modes at 1301 cm^{-1} (CH_2 twisting), 1452 cm^{-1} (CH_2 scissoring) and 1745 cm^{-1} (C=O) represent vibrations of the milk fat globules.

Due to intermolecular interactions between meloxicam and milk constituents, the vibrational modes of neat meloxicam were expected to appear appreciably modified in the Raman spectra of the mixtures. In practice, only the 1595 cm^{-1} peak remained unchanged in the spectra of meloxicam/milk powders (denoted by asterisks in Fig. 4a). On the other hand, the addition of meloxicam in milk caused mild spectral changes at various wave number ranges, which are briefly discussed below.

In the C–C bond stretching region, the relative intensity of the 1065, 1082, and 1121 cm^{-1} peaks, is an indicator of the *gauche* fraction in the acyl chains. The prevalence of the 1082 cm^{-1} band in milk powder indicates dominance of the *gauche* conformation. The relative intensity of these three Raman bands changed gradually with meloxicam addition and, as the spectra reveal, the 1121 cm^{-1} band became dominant in the sample containing meloxicam (3 mg/mL). The bands at 1655 and 1670 cm^{-1} represent vibrational modes of C=C double bonds in *cis* and *trans* conformations, respectively. The intensity ratio of these two bands I^{1655}/I^{1670} was *ca.* 2.0–2.5 for fat globules (Forrest, 1978), which indicates ~40% unsaturation (*cis*) conformation. In the present case, this ranged from 1.6 to 1.8 for the milk powder and milk/meloxicam mixtures, indicating rather low unsaturation (*cis* conformation) of ~30%. In addition, the intensity ratio I^{2931}/I^{2894} has been demonstrated to be a sensitive measure of both inter-chain and intra-chain order-disorder processes in the bilayer acyl chains (O'Leary et al., 1984). Details of the C–H stretching modes are shown in Fig. 4b. The spectra were normalized to coincide with the 2895 cm^{-1} band assigned to anti-symmetric methyl stretching vibrational motion. The 2931 cm^{-1} mode was assigned to Fermi resonance interaction with binary combinations of C–H bending (MacPhail et al., 1984). The 2931 cm^{-1} band exhibited a systematic increase in relation to the 2895 cm^{-1} band with the meloxicam content. Fig. 4b shows that the addition of meloxicam caused a small but directional change to the bilayer acyl group.

The most important feature that emerged in the meloxicam/milk Raman spectra was the appearance of a new band at 1396 cm^{-1} that grew proportionally with the meloxicam content. The presence of the band was the result of intermolecular interactions between meloxicam and milk constituents. Resolving the origin of this new Raman band is, however, not straightforward. A possible source could be the interaction between the C=O and the OH groups with the Ca^{2+} ions of milk proteins. Such an interaction would cause appreciable changes in the heteroatom ring where the OH group is attached. It is therefore plausible to consider that the

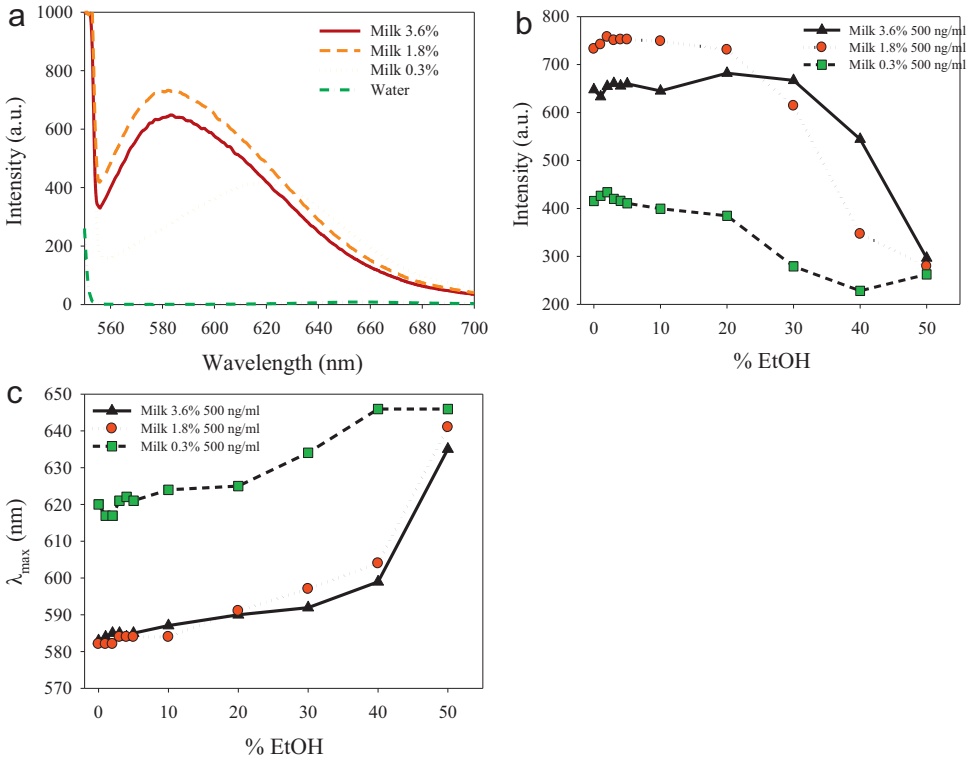

Fig. 3. (a) Nile red emission spectra full fat milk (solid line), semi-skimmed milk (dashed line), skimmed milk (dotted line) and water (dashed-dotted line), (b) relation between the fluorescence intensity and the amount of EtOH present in these formulations and (**c**) relation between the peak maximum and the amount of EtOH present in these formulations.

new Raman band at 1396 cm^{-1} arises from the shift of a band in the meloxicam Raman spectrum due to changes in the electronic distribution in this heteroatom ring. This band may have originated from a blue-shift of the 1302 cm^{-1} band of the neutral meloxicam molecule. To induce perturbation to the six-membered heteroatom ring, the Raman spectrum of a dilute aqueous basic (pH 12) solution of the anionic form of meloxicam was studied; the spectrum exhibited a large shift of the 1302 cm^{-1} band to 1418 cm^{-1}, implying a de-protonation of the OH group taking place in the basic environment.

3.6. Computational studies

To understand the origin of the 1302 cm^{-1} Raman band in neutral meloxicam and the shift of this band in various environments, *ab initio* calculations were carried out at the Hartree–Fock level. This enabled the determination of the equilibrium structure and the harmonic vibrational frequencies of the neutral meloxicam molecule (Fig. 5a), and three variants of this molecule, *i.e.*, two anionic species (charge: −1) where two different H atoms were removed from the molecule (Fig. 5b and c), and the anionic species interacting with a Ca^{2+} cation (net charge +1) (Fig. 5d). The neutral meloxicam molecule adopts various conformations and it is well known to exist in the anionic form in aqueous solutions at neutral pH. The optimized molecular structure of the neutral form Fig. 5a is the energetically most stable neutral form as reported elsewhere (Snor et al., 2009).

Fig. 6 shows the calculated Raman spectra of the various meloxicam structures. The spectra were obtained using the harmonic frequencies with a full-width broadening of 10 cm^{-1} and the Raman activities of each frequency. The calculated spectra were scaled on the wave number axis by a factor of 0.88 to coincide with the experimental data. The value emerged here is within the typical range (Check et al., 2001) for *ab initio* simulations performed with the HF method. Comparison of the calculated and experimental Raman spectra revealed the following striking similarities:

(i) The intense band at 1595 cm^{-1} remained at fixed energy irrespective of the meloxicam molecule form, *i.e.*, neutral or charged. This was true for both the experimental spectra of neat meloxicam and meloxicam/milk mixture and the four calculated molecular structures of meloxicam. Analysing the normal modes of the meloxicam molecule, as given by the *ab initio* calculations, showed that this band could be assigned to the —C═O vibrational motion.

(ii) The experimental spectra showed that the band located at ∼1302 cm^{-1} for neutral meloxicam shifted to higher energy for the meloxicam/milk mixtures as a result of the de-protonation of the molecule. In a similar way, the calculated harmonic frequency of the corresponding band in neutral meloxicam shifted from ∼1300 to ∼1343 for the anionic form in Fig. 5b and to ∼1312 cm^{-1} for the anionic form in Fig. 5c. A moderate shift to ∼1317 cm^{-1} was observed for the meloxicam/Ca^{2+} species. The magnitude of the shift in the experimental spectra was much higher (∼90 cm^{-1}) than the calculated one; this reflects the limitations of the calculation procedure that considers only one meloxicam molecule and ignores also other intermolecular interactions. Normal mode analysis showed that the 1302 cm^{-1} Raman band of the meloxicam molecule, which suffers the strongest changes when changing environment, is a combined vibration motion of the six-membered heteroatom ring where the OH group is attached. Specifically, this mode includes vibrational motion of the C═C and the C—N bonds. This finding complies with the removal of the H atom affecting the vibrational properties of the adjacent atoms.

(iii) In both the experimental and calculated Raman spectra of the neutral meloxicam molecule, the intensity of the C═O band at 1595 cm^{-1} was stronger than that of the heteroatom ring band

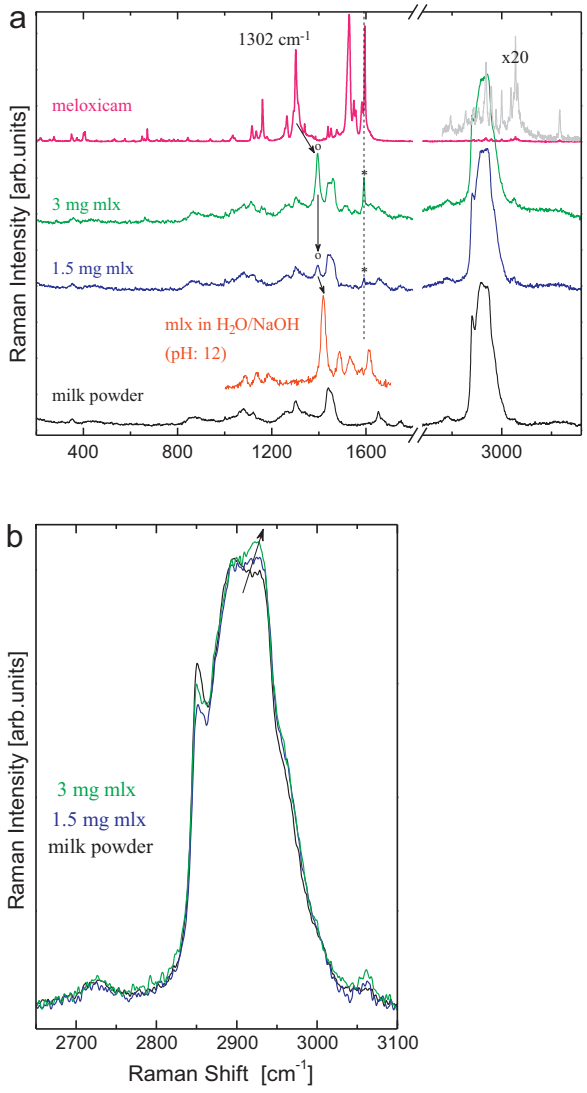

Fig. 4. (a) Stokes-side Raman spectra of milk powder, crystalline meloxicam (mlx) and meloxicam/milk dried mixtures. The spectrum of meloxicam diluted into an aqueous solution of NaOH is also shown. The wave number axis is broken to hide a range free of vibrational lines; (b) Raman spectra of the high frequency bands (C—H stretching modes). The spectra have been normalized to coincide at the 2900 cm^{-1} band.

at 1302 cm^{-1}. Interestingly, the intensity ratio of these two bands changed in the same direction for the charged meloxicam forms for both experimental and theoretical spectra. Thus, the *ab initio* calculations seem to also capture changes in the Raman activity of the various harmonic frequencies of meloxicam at various conformations of the molecule.

3.7. Stability studies

3.7.1. Appearance/visual inspection

3.7.1.1. NSAIDs in alkaline solutions. No discoloration or precipitation was observed in any of the samples during the long term, intermediate or accelerated stability studies.

3.7.1.2. Danazol, cyclosporine and clopidogrel in ethanolic solutions. No discoloration or precipitation was observed for the danazol and the cyclosporine samples during the accelerated stability studies. In contrast, the colour of the clopidogrel samples changed during the stability testing, from colourless to pale yellow. These results

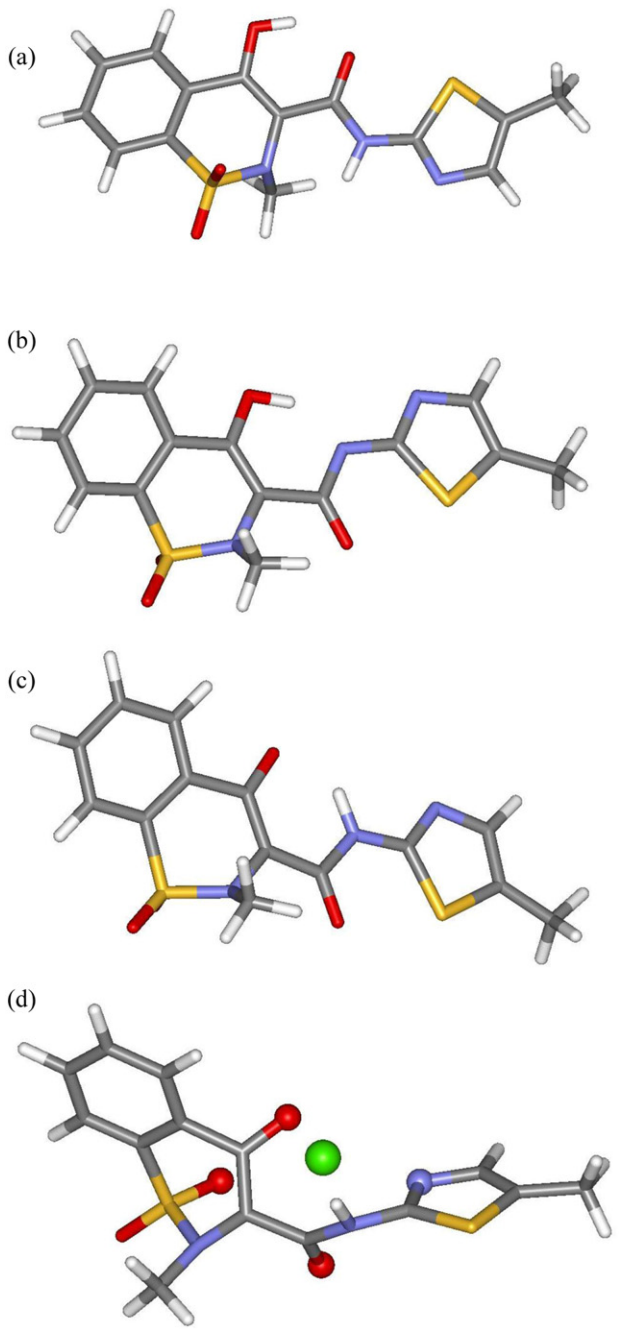

Fig. 5. Optimized geometries of the meloxicam molecule at various conformations: (a) neutral; (b) anionic, de-protonated with net charge −1, a H atom has been removed from the N atom adjacent to the 5-membered ring; (c) anionic, de-protonated with net charge −1, a H atom has been removed from the OH group; (d) anionic (de-protonated as in case (c)) in the presence of a Ca^{2+} cation (net charge +1). The de-protonated forms of meloxicam show significant orientational changes of the five-membered heteroatom ring in comparison with the neutral form. The four atoms marked by spheres, exhibit strong interactions with Ca^{2+} and are positioned at distances in the range 2.5–2.7 Å. Colour code: light grey: H, grey: C; yellow: S, purple: N, red: O, green: Ca^{2+}. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

are in accordance with the quantification results and the impurity profiles as presented below.

3.7.2. Quantification studies

3.7.2.1. NSAIDs in alkaline solutions. For ketoprofen, the total amount of drug for the long-term studies at 25 °C (3, 6 and 9

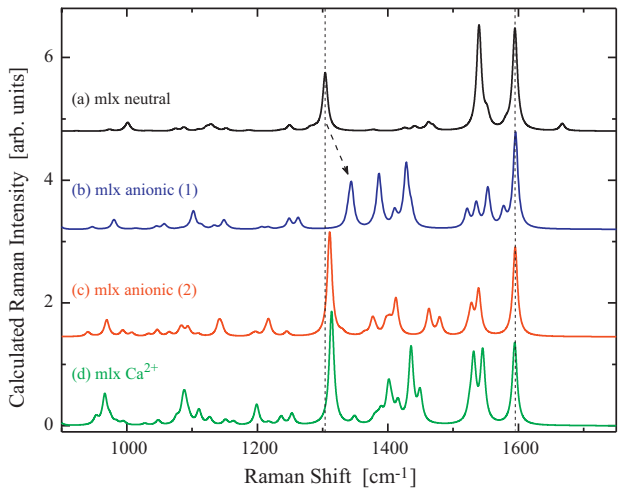

Fig. 6. Calculated Raman spectra of the four meloxicam structures shown in Fig. 5, as denoted in the legends.

months) ranged from 101 to 105% compared with the initial value. For the accelerated stability studies, the total amount ranged from 99.9 to 106% and 99.7 to 105% for 30 °C and 40 °C compared with the initial value, respectively (Fig. 7a). For nimesulide, the total amount of drug for the long-term studies ranged from 96.5 to 107%, whilst at the accelerated stability studies ranged from 99.7 to 100.2% and 101 to 118% for 30 °C and 40 °C, respectively, compared with the initial value (Fig. 7b). For meloxicam, for the long-term studies, the concentration ranged from 99 to 103%, whilst that for the accelerated stability studies ranged from 96 to 103% for both 30 °C and 40 °C, compared with the initial value (Fig. 7c). In all the stability studies, the initial and the final pH values of the drug-containing alkaline solutions were measured. The pH changes, for all three solutions, were minor and ranged from 98.8 to 102% (SC Fig. 3S).

For all three solutions, the results of the preliminary stability studies were very promising. Although a trend was observed for ketoprofen and nimesulide (Fig. 7a and b), this could be explained by the evaporation of the solution and the variability of the analytical technique.

3.7.2.2. Danazol, cyclosporine and clopidogrel in ethanolic solutions. Surprisingly, the most stable compound, based on the quantification approach, was cyclosporine A. At 6 months at the accelerated conditions, the mean amount of cyclosporine was 101.5% (range 96.9–104.9%) (Fig. 8a). In contrast, the assays for danazol and clopidogrel were significantly lower than the initial concentration, *i.e.*, 90.5% (range 88.9–93.5%) (Fig. 8b) and 81.6% (range 80.4–84.4%) (Fig. 8c), respectively.

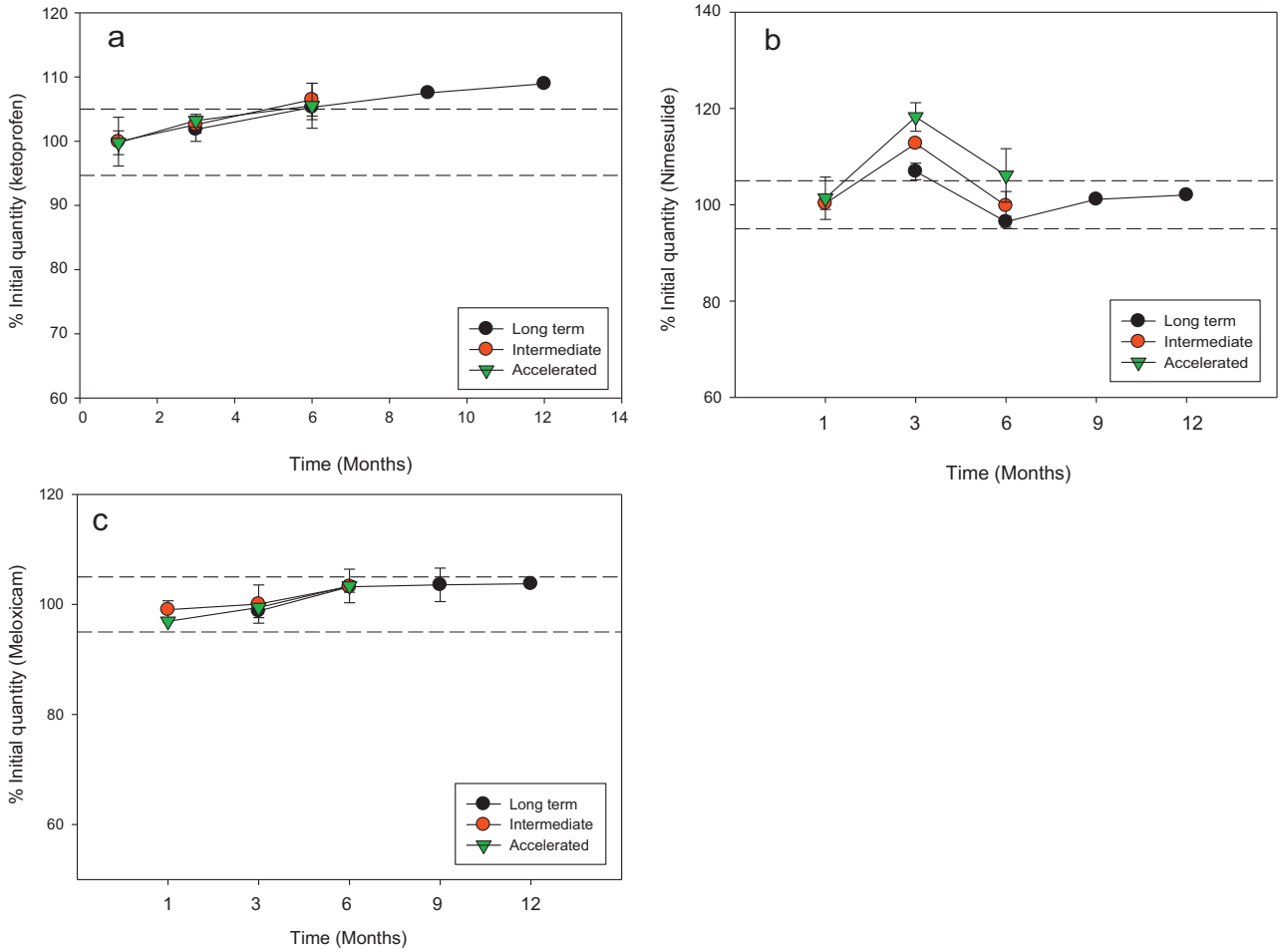

Fig. 7. (a) Long-term (25 °C), intermediate (30 °C), and accelerated (40 °C) stability studies of ketoprofen (200 mg) in phosphate buffer (4 mL, 0.2 M, pH 12); (b) long-term (25 °C), intermediate (30 °C), and accelerated (40 °C) stability studies of nimesulide (100 mg) in phosphate buffer (2.5 mL, 0.2 M, pH 12); (c) long-term (25 °C), intermediate (30 °C), and accelerated (40 °C) stability studies for meloxicam (15 mg) in glycine–NaOH buffer (2.5 mL, 0.05 M, pH 12). Horizontal dashed lines indicate values ± 5% of the initial concentration.

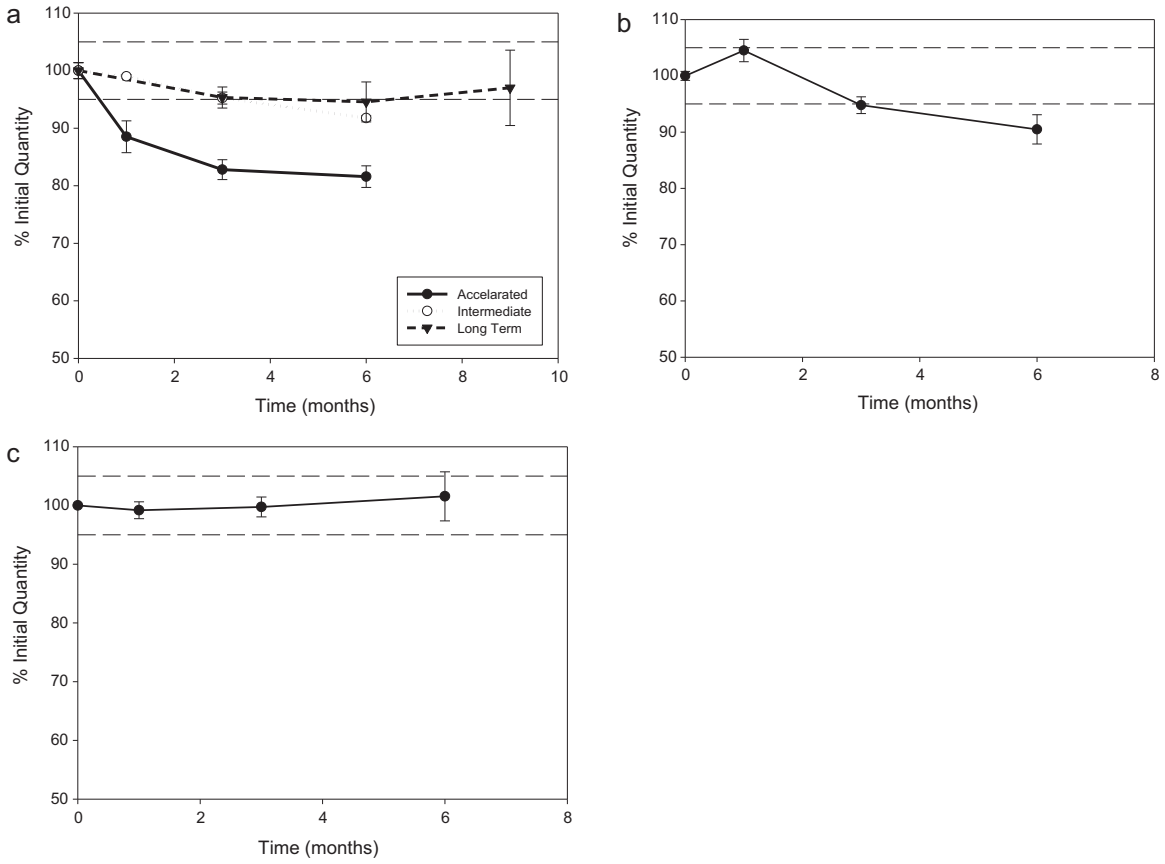

Fig. 8. (a) Long-term (25 °C), intermediate (30 °C), and accelerated (40 °C) stability studies of cyclosporine (100 mg) in 5 mL of 60% ethanol; (b) accelerated (40 °C) stability studies of danazol (100 mg) in 10 mL of absolute ethanol; (c) accelerated (40 °C) stability studies for clopidogrel besylate (112.1 mg) in 5 mL of 60% ethanol. Horizontal dashed lines indicate values ±5% of the initial concentration.

4. Conclusions

Towards the development of milk-based formulations for oral delivery to special populations such as children, a systematic series of formulations have been formulated and investigated. Alterations to the particle size of the milk droplets in the presence of the different drugs are rather case specific and no trend could be established between the physicochemical properties of the drugs and their mean diameter.

AFM studies revealed the presence of small globular structures which are assumed to be casein micelles, and larger, spherical structures assigned as fat globules. The AFM studies were in a broad agreement with the DLS measurements. It is known that emulsions with low ζ-potential values are prone to aggregation resulting to poor performance. The ζ-potential data pointed to the colloidal stability of these emulsions which is prerequisite for a stable formulation.

The changes to the fluorescence intensity values in formulations containing ethanol indicated that the solvent can induce structural changes to the system affecting the polar environment of the emulsions and eventually the loading capacity of the formulation. Raman spectroscopy studies revealed that the addition of meloxicam in milk caused spectral changes at various wave number ranges giving evidence of the interactions between the milk components and the drug. All formulations, with the exceptions of danazol and clopidrogel, were stable. It should be emphasized, however, that these formulations are intended to be used within 2 h after their preparation. These initial studies demonstrate that milk possesses the stability required to be used as an alternative drug delivery system for the development of formulations to be administered to paediatric populations.

Acknowledgements

Part of this work was funded by COST Action: B25/Short-Term Scientific Mission (STSM), (COST-STSM-B25-04376).

The authors would like to thank professor Alexios-Leandros Skaltsounis and his group for kindly providing the freeze dryer equipment (Department of Pharmacognosy and Natural Product Chemistry, Faculty of Pharmacy, Athens, Greece).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpharm.2013.01.022.

References

Breitkreutz, J., Boos, J., 2007. Paediatric and geriatric drug delivery. Expert Opin. Drug Deliv. 4, 37–45.
Charkoftaki, G., Kytariolos, J., Macheras, P., 2012. Novel milk based oral formulations: proof of concept. Int. J. Pharm. 390, 150–159.
Check, C.E., Faust, O.T., Bailey, M.J., Wright, J.B., Gilbert, M.T., Sunderlin, S.L., 2001. Addition of polarization and diffuse functions to the LANL2DZ basis set for p-block elements. J. Phys. Chem. A 105, 8111–8116.
EMA (European Medicines Agency) London, 2003. Stability Testing of New Drug Substances.
Forrest, G., 1978. Raman spectroscopy of the milk globule membrane and triglycerides. Chem. Phys. Lipids 21, 237–252.

Frisch, M.J., Trucks, G.W., Schlegel, H.B., Scuseria, G.E., Robb, M.A., Cheeseman, J.R., Montgomery Jr., J.A., Vreven, T., Kudin, K.N., Burant, J.C., Millam, J.M., Iyengar, S.S., Tomasi, J., Barone, V., Mennucci, B., Cossi, M., Scalmani, G., Rega, N., Petersson, G.A., Nakatsuji, H., Hada, M., Ehara, M., Toyota, K., Fukuda, R., Hasegawa, J., Ishida, M., Nakajima, T., Honda, Y., Kitao, O., Nakai, H., Klene, M., Li, X., Knox, J.E., Hratchian, H.P., Cross, J.B., Bakken, V., Adamo, C., Jaramillo, J., Gomperts, R., Stratmann, R.E., Yazyev, O., Austin, A.J., Cammi, Pomelli, C., Ochterski, J.W., Ayala, P.Y., Morokuma, K., Voth, G.A., Salvador, P., Dannenberg, J.J., Zakrzewski, V.G., Dapprich, S., Daniels, A.D., Strain, M.C., Farkas, O., Malick, D.K., Rabuck, A.D., Raghavachari, K., Foresman, J.B., Ortiz, V., Cui, Q., Baboul, A.G., Clifford, S., Cioslowski, J., Stefanov, B.B., Liu, G., Liashenko, A., Piskorz, P., Komaromi, I., Martin, R.L., Fox, D.J., Keith, T., Al-Laham, M.A., Peng, C.Y., Nanayakkara, A., Challacombe, M., Gill, P.M.W., Johnson, B., Chen, W., Wong, M.W., Gonzalez, C., Pople, J.A., 2004. Gaussian 03, Revision D.01. Gaussian, Inc., Wallingford, CT.

Greenspan, P., Fowler, S.D., 1985. Spectrofluorometric studies of the lipid probe, Nile red. J. Lipid Res. 26, 781–789.

Hauss, J.D., 2007. Oral lipid-based formulations. Adv. Drug Deliv. Rev. 59, 667–676.

Hu, J., Johnston, K.P., Williams 3rd, R.O., 2003. Spray freezing into liquid (SFL) particle engineering technology to enhance dissolution of poorly water soluble drugs: organic solvent versus organic/aqueous co-solvent systems. Eur. J. Pharm. Sci. 20, 295–303.

Humberstone, A.J., Charman, W.N., 1997. Lipid based vehicles for the oral delivery of poorly water soluble drugs. Adv. Drug Deliv. Rev. 25, 103–128.

Jack, E.L., Dahle, C.D., 1937. The electrokinetic potential of milk fat. I. General electrophoretic studies. J. Dairy Sci. 20, 551–556.

Larrucea, E., Arellano, A., Santoyo, S., Ygartua, P., 2002. Study of the complexation behavior of tenoxicam with cyclodextrins in solution: improved solubility and percutaneous permeability. Drug Dev. Ind. Pharm. 28, 245–252.

Larsen, A., Holm, R., Pedersen, M.L., Müllertz, A., 2009. Lipid-based formulations for danazol containing a digestible surfactant, Labrafil M2125CS: in vivo bioavailability and dynamic in vitro lipolysis. Pharm. Res. 25, 2769–2777.

MacPhail, R.A., Strauss, H.L., Snyder, R.G., Elliger, C.A., 1984. Carbon—hydrogen stretching modes and the structure of *n*-alkyl chains. 2. Long, all-trans chains. J. Phys. Chem. 88, 334–341.

Michalski, M.C., Michel, F., Sainmont, D., Briard, V., 2001. Apparent-potential as a tool to assess mechanical damages to the milk fat globule membrane. Colloids Surf. B: Biointerfaces 23, 23–30.

Mohsin, K., Long, M.A., Pouton, C.W., 2009. Design of lipid-based formulations for oral administration of poorly water-soluble drugs: precipitation of drug after dispersion of formulations in aqueous solution. J. Pharm. Sci. 98, 3582–3595.

Mueller, E.A., Kovarik, J.M., van Bree, J.B., Tetzloff, W., Grevel, J., Kutz, K., 1994a. Improved dose linearity of cyclosporine pharmacokinetics from a microemulsion formulation. Pharm. Res. 11, 301–304.

Mueller, E.A., Kovarik, J.M., van Bree, J.B., Grevel, J., Lücker, P.W., Kutz, K., 1997. Influence of a fat-rich meal on the pharmacokinetics of a new oral formulation of cyclosporine in a crossover comparison with the market formulation. Pharm. Res. 11, 151–155.

Mueller, E.A., Kovarik, J.M., van Bree, J.B., Grevel, J., Lucker, P.W., Kutz, K., 1994b. Influence of a fat-rich meal on the pharmacokinetics of a new oral formulation of cyclosporine in a crossover comparison with the market formulation. Pharm. Res. 11, 151–155.

Mutalik, S., Anju, P., Manoj, K., Usha, A.N., 2008. Enhancement of dissolution rate and bioavailability of aceclofenac: a chitosan-based solvent change approach. Int. J. Pharm. 28, 279–290.

O'Leary, T.J., Ross, P.D., Levin, I.W., 1984. Effects of anesthetic and nonanesthetic steroids on dipalmitoylphosphatidylcholine liposome: a calorimetric and Raman spectroscopic investigation. Biochemistry 23, 4636–4641.

Porter, C.J., Trevaskis, N.L., Charman, W.N., 2007. Lipids and lipid-based formulations: optimizing the oral delivery of lipophilic drugs. Nat. Rev. Drug Discov. 6, 231–248.

Pouton, C.W., 1997. Formulation of self-emulsifying drug delivery systems. Adv. Drug Deliv. Rev. 25, 47–58.

Pouton, C.W., 2000. Lipid formulations for oral administration of drugs: non-emulsifying, self-emulsifying and 'self-microemulsifying' drug delivery systems. Eur. J. Pharm. Sci. 11, S93–S98.

Rasenack, N., Hartenhauer, H., Müller, B.W., 2003a. Microcrystals for dissolution rate enhancement of poorly water-soluble drugs. Int. J. Pharm. 26, 137–145.

Rasenack, N., Müller, B., 2002. Dissolution rate enhancement by in situ micronization of poorly water-soluble drugs. Pharm. Res. 19, 1894–1900.

Rasenack, N., Steckel, H., Müller, B.W., 2003b. Micronization of anti-inflammatory drugs for pulmonary delivery by a controlled crystallization process. J. Pharm. Sci. 92, 35–44.

Rogers, T.L., Johnston, K.P., Williams 3rd, R.O., 2003a. Physical stability of micronized powders produced by spray-freezing into liquid (SFL) to enhance the dissolution of an insoluble drug. Pharm. Dev. Technol. 8, 187–197.

Rogers, T.L., Overhoff, K.A., Shah, P., Santiago, P., Yacaman, M.J., Johnston, K.P., Williams 3rd, R.O., 2003b. Micronized powders of a poorly water soluble drug produced by a spray-freezing into liquid-emulsion process. Eur. J. Pharm. Biopharm. 55, 161–712.

Schaefer, A., Horn, H., Ahlrichs, R., 1994. Fully optimized contracted Gaussian basis sets for atoms Li to Kr. J. Chem. Phys. 97, 2571–2578.

Seedher, N., Bhatia, S., 2003. Solubility enhancement of Cox-2 inhibitors using various solvent systems. AAPS PharmSciTech 4, E33.

Singh, B.N., 1999. Effects of food on clinical pharmacokinetics. Clin. Pharmacokinet. 37, 213–255.

Snor, W., Liedl, E., Weiss-Greiler, P., Viernstein, H., Wolschann, P., 2009. Density functional calculations on meloxicam–cyclodextrin inclusion complexes. Int. J. Pharm. 381, 146–152.

Udenfriend, S., 1962. Fluorescence Assay in Biology and Medicine. Academic Press, New York/San Francisco/London.

Walstra, P., Jenness, R., 1984. Dairy Chemistry and Physics. John Wiley & Sons, New York.

Wu, K., Li, J., Wang, W., Winstead, D.A., 2009. Formation and characterization of solid dispersions of piroxicam and polyvinylpyrrolidone using spray drying and precipitation with compressed antisolvent. J. Pharm. Sci. 98, 2231–2422.

Yeh, M.K., Chang, L.C., Chiou, A.H., 2009. Improving tenoxicam solubility and bioavailability by cosolvent system. AAPS PharmSciTech 10, 166–171.