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Novel milk-based oral formulations: Proof of concept

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ABSTRACT

The aim of this study is to develop milk-based formulations for ionized and unionized lipophilic drugs. Solubility studies of the following non-steroidal anti-inflammatory drugs (NSAIDs): mefenamic acid, tolfenamic acid, ketoprofen, meloxicam, tenoxicam and nimesulide in phosphate– and glycine–NaOH buffers at nominal pH 8–12, were performed. The solubilities of cyclosporine and danazol in water–ethanol solutions were studied. NSAIDs–, cyclosporine–, danazol–, aspirin–milk oral liquid formulations were prepared by adding the appropriate volume of (i) NSAIDs–alkaline buffer solutions, (ii) water–ethanol solutions of cyclosporine and danazol and (iii) aspirin aqueous solution to 150–200 ml of milk. All the non-steroidal anti-inflammatory drugs exhibited increased solubility in the alkaline buffers. The actual pH values (range 6.7–7.7) of the final NSAIDs–milk formulations were very close to milk pH. The higher ethanol content in ethanol–water mixtures increased the solubility of danazol and cyclosporine. A 15 mg meloxicam–, a 100 mg cyclosporine– and a 500 mg aspirin–milk formulation was administered orally to healthy volunteers. All these formulations showed a satisfactory *in vivo* performance. The strong buffering capacity of milk that was observed and the high solubility of unionized drugs in ethanol allow the preparation of drug–milk formulations with enhanced pharmacokinetic properties.

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1. Introduction

Lipophilic drugs are insoluble or practically insoluble in water. It is very well known that the aqueous solubility of a drug is a key parameter which governs its oral bioavailability. Therefore, the improvement of the rate and extent of absorption of lipophilic drugs is highly desirable. Various approaches have been utilized to increase the solubility of a drug by modifying its crystallinity and its non-polarity. The former approach is accomplished by micronization, spray drying or freeze drying (Rasenack and Müller, 2002; Rasenack et al., 2003a,b; Rogers et al., 2003a; Hu et al., 2003; Wu et al., 2009). The latter approach can be accomplished by modifying the media in which the drug is dissolved (Larrucea et al., 2002; Rogers et al., 2003b; Seedher and Bhatia, 2003; Mutalik et al., 2008; Larsen et al., 2008; Yeh et al., 2009). The modification of the media can be achieved by either adjusting the pH and/or the use of solubilizing agents, namely, cosolvents, surfactants, complexing agents and oil/lipids. However, a satisfactory effect with such methods cannot always be achieved. Besides, some of the solubilizing agents are used in quantities which can produce undesired effects. Overall, these approaches are limited in the range and quantity of drugs which they can accommodate and also in their ability to promote the access of drug throughout the gastrointestinal wall.

The present work focuses on the use of milk as the basic component for what we call milk-based formulations; milk is a natural, abundant and inexpensive carrier with the desired characteristics for oral drug delivery. In fact, synthetic emulsions are used for oral administration of sparingly soluble drugs, e.g. cyclosporine is formulated as a microemulsion (Neoral®). Milk, a daily ritual, is an oil-in-water natural emulsion since nearly all of the fat milk is in separate small globules (Walstra and Jenness, 1984). Several reports in literature suggest that the solubility of lipophilic drugs in milk is much higher than their aqueous solubility (Macheras et al., 1986, 1989, 1990, 1991). Also, the rate of dissolution of lipophilic drugs used in a powder form was found to be much higher in milk than in aqueous media (Macheras et al., 1986; Galia et al., 1998). A number of dissolution studies has shown that drug dissolution is higher from drug–milk dispersed systems than drug–milk physical mixtures (Topaloglou et al., 1998, 1999; Kumar and Mishra, 2006; Sahin and Arslan, 2007). A series of *in vitro* and *in vivo* studies with reconstituted freeze-dried drug–milk formulations have demonstrated their superiority in regard to solubility and dissolution (Macheras et al., 1986; Topaloglou et al., 1999) as well as the absorbability when compared to conventional capsule formulations of lipophilic drugs (Macheras and Reppas, 1986a,b; Macheras et al., 1991). Finally, a large number of drugs like non-steroidal anti-inflammatory drugs (NSAIDs) when taken orally in various dosage forms such as tablets, capsules, caplets, as well as chewable forms, create stomach irritation. A warning on the label of the commercial packages of NSAIDs refers “this product may cause stomach

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bleeding" while their official Summary of Product Characteristics (SPCs) recommend that they have to be taken with milk to prevent stomach irritation.

In fact, the ability of dairy foods (whole milk 3.5% fat, skim milk nonfat, and cream 35% fat) to prevent bleeding and stress-induced gastric lesion has been shown in studies performed in rats by Dial et al. (1995). All three dairy products exhibited protection against stress-induced ulcer and bleeding in rats and there appeared to be a relationship between these side effects and the content of lipid in the products. Cream provided the greatest protection (35% fat), while the nonfat milk the lowest. However, as the nonfat milk exhibited a gastroprotective effect, indicated that other components of milk, besides lipids, contribute to that positive effect. The mechanism of protection by milk that was suggested in these studies, was related to the maintenance of a hydrophobic gastric surface that might enhance the barrier properties of the epithelium.

Several studies have shown that NSAIDs induce ulcer and bleeding due to topical mucosal injury, by dicreasing the surface hydrophobic barrier of the gastrointestinal (GI) mucosa, as a result of their ability to bind to zwitterionic phospholipids (Lichtenberger, 1995; Lichtenberger et al., 1995, 1996; Giraud et al., 1998). Moreover, multiple reports in the literature dissociate COX inhibition from mucosal injury (Ligumsky et al., 1982, 1983), although there are studies demonstrating that NSAID-induced ulceration is freequently associated with COX inhibition (Robert, 1981; Rainsford and Willis, 1982; Whittle et al., 1985; Lanza, 1989).

The principle behind the approaches described in this work is to prepare a pharmaceutical composition in order to present the drug in a dissolved form in the gastrointestinal tract, using milk as a dispersing medium and taking advantage of its gastroprotective characteristics. In the present work, the following three approaches are utilized: (I) preparation of a solution of an ionized acidic lipophilic drug in an alkaline buffer, (II) preparation of a water–ethanol solution of an unionized lipophilic drug and (III) use of a small water volume (≈20 ml) for the preparation of a drug solution of a moderately water soluble drug from an effervescent tablet. In all three cases (I, II and III) the prepared solution is dispersed in an appropriate volume of milk prior to oral administration.

A number of non-steroidal anti-inflammatory drugs were used as model ionized lipophilic drugs for the preparation of the drug solution in alkaline buffer following the approach designated as (I). In order to find the most appropriate composition of the drug–alkaline buffer in terms of the drug amount, pH value and ionic strength, solubility studies of NSAIDs in various buffer solutions were performed prior to the preparation of the drug–buffer solutions. Cyclosporine and danazol were used as model unionized lipophilic drugs for their solubility study in water–alcohol mixtures following the approach designated as (II). A commercially available effervescent aspirin tablet was used for the approach designated as (III). A meloxicam–milk formulation (prepared following approach I) and a cyclosporine–milk formulation (prepared following approach II) were administered to a volunteer. A comparative study of aspirin–milk formulation (following approach III) versus the aspirin–water solution was carried out in two volunteers based on a replicate design. The *in vivo* studies were performed to explore for the first time the *in vivo* performance of milk-based formulations.

2. Materials and methods

2.1. Materials

Ketoprofen, tolfenamic acid, meloxicam, tenoxicam and nimesulide were kindly provided by Kleva pharmaceutical industry (Athens, Greece). Mefenamic acid was provided by ELPEN pharmaceutical industry (Athens, Greece). Acetylsalicylic acid, salicylic acid and Salospir® effervescent tablets were obtained from Uni-Pharma Laboratories S.A. (Athens, Greece), cyclosporine A from RPG Life Sciences Limited, India, and danazol from Sanofi-Aventis, France. Ultra-high temperature processing (UHT) milk: full fat milk (3.5% fat, 3.3% proteins, 4.7% carbohydrates, Ennstal, Austria), semi skimmed milk (1.5% fat, 3.3% proteins, 4.8% carbohydrates, Ennstal, Austria) and skimmed milk (0% fat, 3.3% proteins, 4.9% carbohydrates, FAGE S.A., Greece). Methanol and acetonitrile HPLC grade were obtained from Merck (Darmstadt, Germany), ethanol (absolute), acetic acid (glacial) and hydrochloric acid (fuming 37%) from Panreac (Barcelona, Spain). Glycine, NaH_2PO_4 and NaOH were obtained from Merck (Darmstadt, Germany). Water was purified through a Labconco Water Pro PS water purification system. All other chemicals were of analytical grade.

2.2. Solubility measurements

2.2.1. Alkaline buffer solutions

Mefenamic acid, tolfenamic acid, ketoprofen, meloxicam, tenoxicam and nimesulide are the non-steroidal anti-inflammatory drugs used for the solubility studies in various alkaline buffers. All substances were studied in phosphate buffer (NaH_2PO_4–NaOH) of various nominal pH (8, 9, 10, 11 and 12) and ionic strength (0.1 and 0.2 M). Meloxicam, tenoxicam and nimesulide were also studied in glycine buffer (glycine–NaOH) of various nominal pH (8, 9, 10, 11 and 12) and ionic strength (0.05 and 0.1 M).

The experimental procedure in all cases was as follows: excess amount of drug powder was added into a 25 ml conical flask containing 15 ml of the appropriate alkaline buffer. The flasks were placed in a thermostated water bath at 25 °C under constant shaking rate of 100 rpm for 48 h. The sample was filtrated through regenerated cellulose filter (17 mm, 0.45 μm Titan®, Wilmington, USA). The first 1 ml was discarded, in order to saturate the filter with the drug. All studies were performed in triplicate.

2.2.2. Solubility studies in water–ethanol solutions and in milk

The effect of temperature on the solubility of two lipophilic drugs, cyclosporine A and danazol was studied in water–ethanol solutions, using the shake-flask method described above. Danazol's solubility was also studied in milk in order to elucidate the effect of milk fat content and temperature on the solubility. Three types of ultra-high temperature processing (UHT) milk were used: full fat milk (3.5% fat), semi skimmed milk (1.5% fat) and skimmed milk (0% fat). The method was the same as the one described previously with the following modification: the samples were not filtrated, but they were placed in 10 ml plastic tubes and centrifuged at 10,000 rpm for 10 min in order to separate the excess solid from the milk. After the completion of the equilibrium phase, the supernatant was collected and analyzed as described in the drug analysis section. All studies were performed in triplicate.

2.3. Preparation of the drug–milk formulations

2.3.1. NSAIDs–milk formulations

The results of the solubility experiments were used to prepare a number of NSAIDs solutions in alkaline buffers for the therapeutic doses of NSAIDs used in practice. To this end, a weighted amount of drug, equal to the therapeutic dose, was dissolved in the appropriate volume of either NaH_2PO_4–NaOH 0.2 M pH 12 or glycine–NaOH 0.05 M pH 12 buffer, under magnetic stirring. The actual pH of the solutions prepared was measured. Table 1 shows the drug dose, the buffer used and its nominal pH, the drug concentration, the actual pH of the drug–buffer solutions, as well as the volume of these solutions containing the therapeutic dose. The NSAIDs–milk formulations were prepared by adding gradually at room tempera-

Table 1
Characteristics of the NSAIDs solutions in NaH_2PO_4–NaOH 0.2 M pH 12 or glycine–NaOH 0.05 M pH 12 buffer and pH of the final NSAIDs–milk formulations.

Dose (mg)	Concentration[a] (mg/ml)	Volume[b] (ml)	Actual pH[c]	pH of final drug/milk formulation[d]
Mefenamic acid (NaH_2PO_4–NaOH pH 12 0.2 M)				
500	40	12.5	10.15	7.35
500	25	20	11.01	7.73
50	10	5	11.62	6.81
100	10	10	11.62	7.01
Tolfenamic acid (NaH_2PO_4–NaOH pH 12 0.2 M)				
300	25	12	11.25	7.4
200	20	10	11.15	7.34
100	20	5	11.64	7.03
100	10	10	11.32	7.42
Ketoprofen (NaH_2PO_4–NaOH pH 12 0.2 M)				
200	50	4	7.11	6.71
100	50	2	8.16	6.72
Meloxicam (glycine–NaOH pH 12 0.05 M)				
15	6	2.5	10.39	6.73
15	3	5	11.38	6.79
7.5	1.5	5	11.73	6.74
Tenoxicam (glycine–NaOH pH 12 0.05 M)				
20	10	2	9.98	6.79
Nimesulide (NaH_2PO_4–NaOH pH 12 0.2 M)				
100	40	2.5	10.89	7.09

[a] The concentration of drug in the NSAIDs–buffer solutions.
[b] The volume of the NSAIDs–buffer solution containing the corresponding therapeutic dose.
[c] The actual pH of the NSAIDs–buffer solution.
[d] The pH of the final drug–milk formulation after the addition of the total volume of drug–buffer solution in 150 ml of 3.5% fat milk; the pH of the milk prior the addition of drug solution was 6.67 ± 0.076.

ture a measured volume of NSAIDs–buffer solutions, corresponding to the drug's therapeutic dose, in 150 ml of milk (3.5% fat) under continuous stirring and pH recording.

2.3.2. *Preparation and content uniformity of meloxicam–milk formulation*

15 mg of meloxicam were dissolved in 2.5 ml buffer glycine–NaOH 0.05 M pH 12. The meloxicam–milk formulation was prepared by adding the above solution into 147.5 ml 3.5% fat UHT milk followed by a gentle stirring with a spoon for 10 s. The pH of milk was measured before and after the addition of the meloxicam solution. Samples of milk were taken from surface, middle and bottom of the milk volume at 2 min, 1, 2 and 4 h. The experiment was run in triplicate at room temperature.

2.3.3. *Preparation and content uniformity of cyclosporine–milk formulation*

100 mg of cyclosporine were dissolved in 5 ml of a solution containing 60% ethanol and 40% water. The cyclosporine–milk formulation was prepared by adding this solution into 145 ml of 3.5% fat UHT milk followed by a gentle stirring with a spoon for 10 s. The pH of milk was measured before and after the addition of the cyclosporine solution. Samples of milk were taken from surface, middle and bottom of the milk volume at 2 min, 1, 2 and 4 h. The experiment was run in triplicate at room temperature.

2.3.4. *Preparation and content uniformity of aspirin–milk formulation*

Salospir®, a commercially available effervescent 500 mg aspirin tablet, was dissolved in 20 ml of water. After the effervescence was completed, the aspirin–milk formulation was prepared by adding 180 ml of 3.5% fat UHT milk into the aspirin solution, followed by a gentle stirring with a spoon for 10 s. The pH of milk was measured before and after the addition of the aspirin solution. Samples of milk were taken from surface, middle and bottom of the milk volume at 2 min, 1, 2 and 4 h. The experiment was run in triplicate at room temperature.

2.4. *In vivo studies*

All the procedures followed the tenets of the Declaration of Helsinki and were approved by the Committee of Medical Ethics of the University Hospitals Athens, Greece. All volunteers provided written informed consent to participate in this study.

2.4.1. *Meloxicam–milk formulation*

The meloxicam–milk formulation (2.5 ml glycine–NaOH buffer, containing 15 mg of meloxicam, added into 150 ml of milk) was orally administered to one healthy volunteer, after an overnight fast. The volunteer was maintained in the fasting state for 6 h after administering the drug. Venous blood samples (5 ml) were obtained before and at 15, 30, 45, 60, 90, 120, 150 min and at 3, 4, 6, 8, 10, 12, 24, 48 and 72 h after drug administration. The blood samples were immediately centrifuged for 10 min at 4000 rpm, the supernatant plasma was collected and transferred in glass vials of 5 ml. The plasma samples were stored at −20 °C until analysis.

2.4.2. *Cyclosporine–milk formulation*

The cyclosporine–milk formulation was orally administered to one healthy volunteer, after an overnight fast. The volunteer was maintained in the fasting state for 4 h after administering the drug. Venous blood samples (5 ml) were obtained before and at 15, 30, 45, 60, 90, 120, 150 min and at 3, 4, 6, 8, 10 and 12 h after drug administration. The blood samples were stored at −20 °C until analysis.

2.4.3. *Aspirin*

Two healthy male volunteers participated in a four-periods study. During the first and third period (R1 and R2) an effervescent tablet Salospir® of 500 mg acetylsalicylic acid dissolved in 200 ml of water was administered to the volunteers after an overnight fast. This was used as the reference (R) formulation. During the second and fourth period (T1 and T2) the aspirin–milk formulation (test,

T) was administered to the volunteers after an overnight fast. In all cases the volunteers were maintained in the fasting state for 4 h after administering the drug. Venous blood samples (8 ml) were obtained before and at 15, 30, 45, 60, 90, 120, 150 min and at 3, 4, 6, 8, 10 and 12 h after drug administration. The blood samples were immediately centrifuged for 10 min at 4000 rpm, the supernatant plasma was collected and transferred in glass vials of 5 ml. The plasma samples were stored at −20 °C until analysis.

2.5. Drug analysis

2.5.1. NSAIDs

A Shimadzu UV-1700 spectrometer was used for spectrophotometric measurements of NSAIDs solubility studies. The filtrated sample of NSAIDs was transferred into a 1 cm path length quartz cuvette. Mefenamic acid was measured at 285 nm, tolfenamic acid at 289 nm, ketoprofen at 260 nm, meloxicam at 363 nm, tenoxicam at 370 nm and nimesulide at 392 nm. For all drugs, quantification of solubility data was made according to standard curves prepared in the corresponding solubility medium.

2.5.2. In vitro studies: analysis of danazol, cyclosporine, meloxicam and aspirin in multiple systems

All compounds were assayed with HPLC–UV methods. The chromatography system used was a GBC Scientific Equipment Pty Ltd., Melbourne, Australia HPLC consisted of a solvent delivery system LC 1120 HPLC pump, a UV/Vis detector LC 1210 UV/Vis Detector and Clarity® chromatographic software. Danazol and cyclosporine assays were based on previously published methods (USP 30-NF 25, 2007; Gad Kariem et al., 2000; Larsen et al., 2008). All the chromatographic conditions are presented in Table 2. For all drugs, quantification of solubility data was made according to standard curves prepared in the corresponding solubility medium. For all the compounds in the solubility studies in milk, a pretreatment step was necessary before the injection to the chromatograph. In certain volume of milk sample (usually 500 μl), 1000 μl of acetonitrile was added as a precipitation agent. The mixture was vortex-mixed for 1 min, placed into 1500 μl plastic centrifugation tubes and centrifuged at 15,000 rpm for 15 min. The supernatant was injected into the chromatograph. The method of danazol was linear from 0.2 to 30 μg/ml and the retention time was approximately 6 min. The method of cyclosporine was linear from 1 to 50 μg/ml and the retention time was approximately 11.5 min. The retention time for meloxicam was approximately 6.9 min and the method was linear from 15 to 3000 ng/ml. The analytical method for aspirin was similar to the one described above, except for the precipitation agent, as it was used HCl 2N instead of acetonitrile. For the quantification of aspirin, calibration curves were constructed for both acetysalysilic and salicylic acid and the retention time was approximately 4.2 and 6.5 min, respectively. The acetysalysilic acid method was linear from1 to 40 μg/ml while salicylic acid from 0.1 to 10 μg/ml.

2.5.3. In vivo studies: analysis of meloxicam, aspirin and cyclosporine

Meloxicam and aspirin. The above described HPLC–UV methods (Table 2) were applied to the *in vivo* study for both of the compounds and the preparation of the venous blood samples was the same as the milk samples.

Cyclosporine. The quantification of cyclosporine and the metabolites of the *in vivo* studies was performed by using a chemiluminescent automated assay (CMIA) with a manual specimen pretreatment step. The ARCHITECT Cyclosporine system was used for this purpose (ARCHITECT i2000 SR). The measurement range is 30 ng/ml (minimum reportable value based on functional sensitivity) to 1500 ng/ml. The limit of detection is less than 25 μg/ml and the recovery is 100 ± 10%.

2.6. Pharmacokinetics

The pharmacokinetic parameters of the *in vivo* studies were determined by standard noncompartmental methods. Maximum blood concentration (C_{max}) and time to peak concentrations (t_{max}) were noted directly from the individual blood concentration–time curve. The elimination constant k_{el} was calculated by log-linear regression in the terminal phase. The terminal half-life ($t_{1/2}$) was calculated according to $t_{1/2} = \ln 2/k_{el}$. The area under the curve to the last quantifiable concentration (AUC_{0-t}), was calculated by the linear trapezoidal rule. The area under the curve to infinity ($AUC_{0\to\infty}$) was determined using $AUC_{0\to\infty} = AUC_{0-t} + C_t/k_{el}$, where C_t is the last determined concentration.

3. Results

3.1. Solubility studies

3.1.1. NSAIDs in alkaline buffer solutions

As expected, the solubility of all non-steroidal anti-inflammatory drugs studied, was found to increase as function of pH, Fig. 1. The highest solubility values were observed at nominal pH 11 and 12. Mefenamic acid's solubility was 46.91 ± 2.3 mg/ml in phosphate buffer 0.2 M pH 12 (Fig. 1a), while tolfenamic acid's and ketoprofen's was 35.15 ± 0.36 mg/ml and 73 ± 0.78 mg/ml, respectively, in the same buffer solution (Fig. 1b and c, respectively). Mefenamic and tolfenamic acid exhibited a 375- and 130-fold solubility increase, respectively, compared to the solubility values obtained from experiments performed in phosphate buffer 0.2 M pH 8. The actual pH values (pH of the filtrate) for mefenamic and tolfenamic acid were lower than the nominal pH of the buffer solutions, except for the pH 8 which remained constant. The two compounds exhibited increased solubility in phosphate buffer 0.1 M too; the highest values were observed in pH 12. However, the solubility increase in phosphate buffer 0.1 M was not as high as in 0.2 M; the final pH of the system at equilibrium had similar values to the 0.2 M phosphate buffer. Ketoprofen did not exhibit a great solubility increase in phosphate buffer 0.1 and 0.2 M pH 12.

Table 2
The chromatographic conditions used for the analysis of drugs in multiple systems.

Chromatographic conditions	Cyclosporine A	Danazol	Meloxicam	Aspirin
Column	ODS2 RP-C18 (250 mm × 4.6 mm, 5 μm)	ODS RP-C18 (150 mm × 4.6 mm, 5 μm)	ODS RP-C18 (150 mm × 4.6 mm, 5 μm)	ODS RP-C18 (150 mm × 4.6 mm, 5 μm)
Mobile phase (v/v/v)	acetonitrile:water 70:30	acetonitrile:water 55:45	acetonitrile:sodium acetate buffer (pH 3.3, 170 mM) 38:62	methanol:water:acetic acid 450:550:16
Flow rate (ml/min)	2	1.3	1	1
Temperature (°C)	50	Ambient	Ambient	Ambient
Detection wavelength (nm)	210	287	355	237
Injection volume (μl)	50	20	50	20

Fig. 1. Solubility values of an array of non-steroidal anti-inflammatory drugs in phosphate buffer 0.1 M (●), 0.2 M (○) and glycine buffer 0.05 M (▼), 0.1 M (▽) at various nominal pH values (8, 9, 10, 11 and 12). On the plots are the actual (final) pH values of the system at equilibrium. *Key*: (a) mefenamic acid, (b) tolfenamic acid, (c) ketoprofen, (d and e) meloxicam, (f and g) tenoxicam, (h and i) nimesulide. Data shown as mean ± SD in all graphs; SD is very small and it is not visible in most of the graphs.

The increase is about 2.5-fold compared to pH 8 for both buffer solutions and the final pH remained unaltered within the range 6.2–6.6 (Fig. 1c). For the two oxicams (meloxicam and tenoxicam) the solubility increase was moderate in the experiments performed with phosphate buffer (Fig. 1d and f) and the actual pH remained lower than the nominal buffer pH. In the experiments performed in glycine–NaOH buffer the solubility values were increased in both ionic strengths, 0.05 and 0.1 M. More specifically, meloxicam reached 15.4 ± 0.17 mg/ml and 21.2 ± 0.3 mg/ml in glycine buffer 0.05 and 0.1 M pH 12, respectively; the final pH ranged from 7.7–9.1 and 7.3–10.1, respectively (Fig. 1e and g). Tenoxicam exhibited even higher than meloxicam's solubility in glycine buffers and the final pH range was lower than the nominal. Nimesulide was more soluble in phosphate than in glycine buffer (Fig. 1h and i). In fact, it exhibited a 348- and a 520-fold solubility increase in phosphate buffer 0.1 and 0.2 M pH 12, respectively, in comparison to the same buffer solutions pH 8.

3.1.2. Danazol and cyclosporine in water–ethanol solutions

The solubility of danazol increased by increasing the amount of ethanol in water–ethanol solutions (Fig. 2). Similarly, danazol solubility was found to increase as a function of temperature. The highest solubility observed was 33.91 ± 0.54 mg/ml in absolute ethanol at 37 °C.

The solubility of cyclosporine in water–ethanol solutions has been previously studied for concentrations of ethanol up to 40% (Ran et al., 2001). These results showed that by increasing the percentage of ethanol in water–ethanol mixtures the solubility of cyclosporine increased. In the present study the concentration of ethanol was deliberately increased to 60% to induce a further increase in the solubility of cyclosporine. However, complete dissolution of solid cyclosporine was observed, at all temperatures

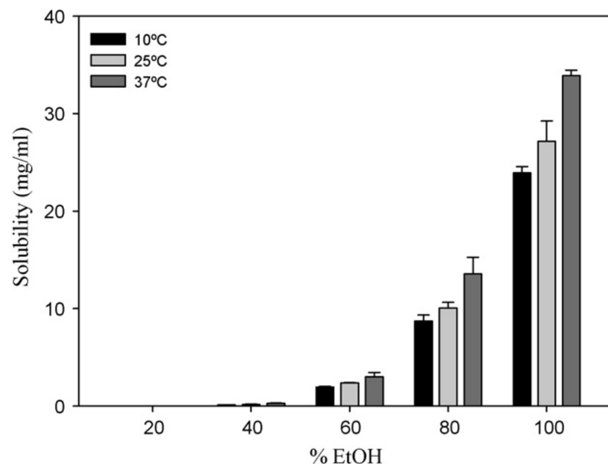

Fig. 2. Saturation solubilities of danazol in water–ethanol solutions at 3 different temperatures (10, 25 and 37 °C).

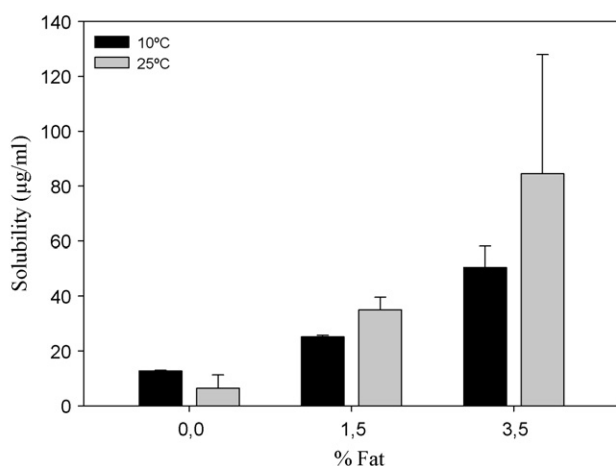

Fig. 3. Danazol solubilities in milk at 10 and 25 °C.

studied. Based on the amount of solid cyclosporine and the volume of solvent used the solubility of cyclosporine in 60% ethanolic solutions at 5, 25 and 37 °C was found to be higher than 60 mg/ml.

3.1.3. Danazol in milk

The saturation solubility of danazol in milk was significantly higher than water, especially in full fat milk (Fig. 3). Furthermore, the solubility (y) of danazol in milk correlates nicely with the percent of fat in milk (x), proving that the fat globules of milk act as solubilizing agents for danazol (Fig. 3). Linear regression analysis to the solubility data produced the following results: $y = 4.57 + 22.44x$, $R^2 = 0.995$ at 25 °C and $y = 11.35 + 10.86x$, $R^2 = 0.987$ at 10 °C.

3.2. Drug–milk formulations

3.2.1. NSAIDs–milk formulations

The 3.5% milk that was used for the experiments had a range of pH from 6.6 to 6.8. The addition of all alkaline buffer NSAIDs solutions caused a slight or moderate pH increase of milk. The pH changes vary according to the amount of drug used, the volume and the ionic strength of the buffer, Table 1 and Fig. 4. The pH value of mefenamic/milk formulation after the addition of 12.5 ml phosphate buffer solution (NaH_2PO_4–NaOH 0.2 M pH 12) containing 500 mg mefenamic acid was 7.35 and compared to the initial milk pH 6.66 there is a 9.4% increase of the pH value, Fig. 4a. The solution containing tolfenamic acid (dose 100 mg, buffer volume 10 ml) increased the milk pH up to 7.42 (Fig. 4b), while ketoprofen buffer solutions (dose 200 and 100 mg, respectively) did not cause any significant increase (final milk pH 6.71 and 6.72, Fig. 4c and d, respectively). The increase of milk pH after the addition of the glycine buffer solutions containing meloxicam (Fig. 4e and f, dose 7.5 and 15 mg, respectively) or tenoxicam (Fig. 4g) is so small, which is negligible. The milk pH, after the nimesulide buffer solution addition, increased only 3.5% (Fig. 4h). All data clearly demonstrate that milk strongly resists to pH changes, even after the addition of so strong alkaline buffer solutions.

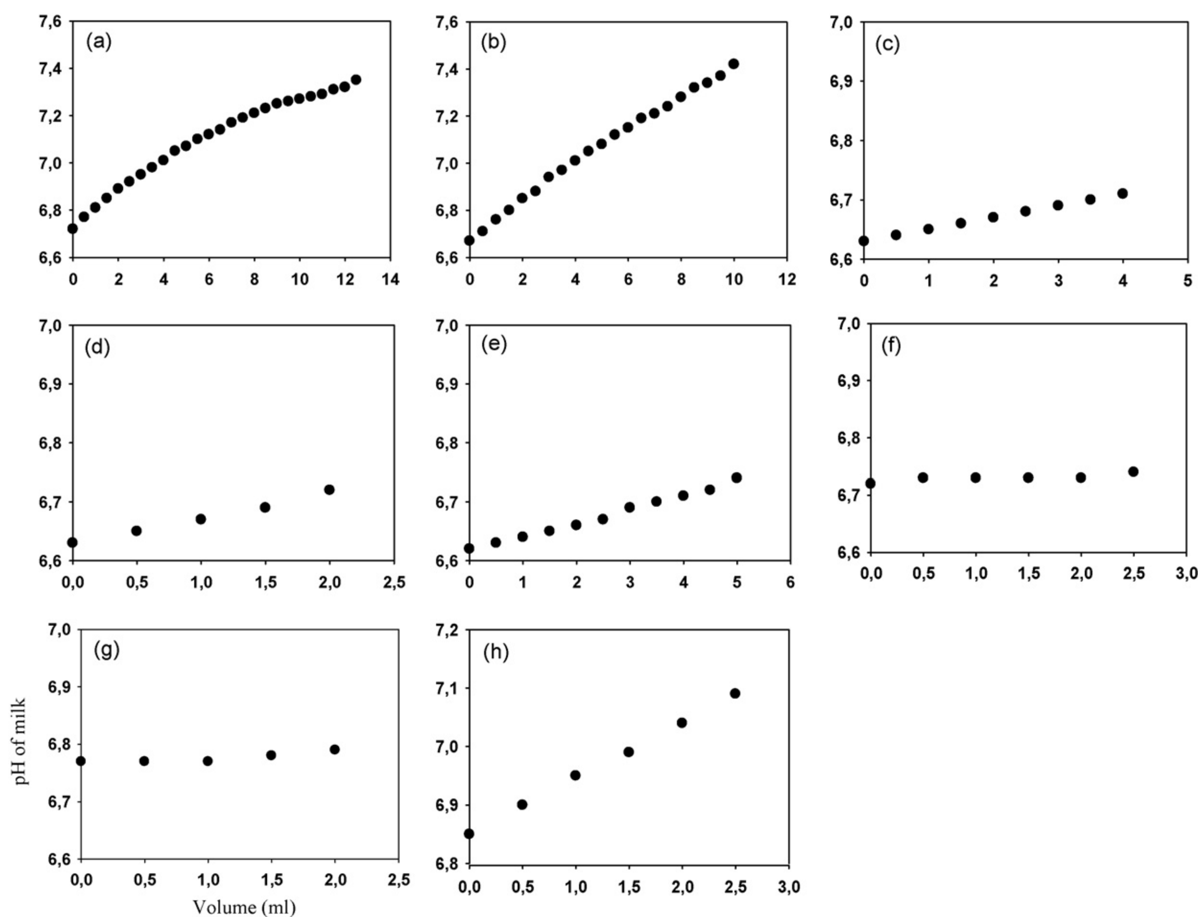

Fig. 4. pH values of 3.5% fat milk after the consecutive addition of different volumes of alkaline buffers containing various doses of NSAIDs. *Key*: (a) 500 mg mefenamic acid in 12.5 ml NaH_2PO_4–NaOH 0.2 M pH 12 buffer, (b) 100 mg tolfenamic acid in 10 ml NaH_2PO_4–NaOH 0.2 M pH 12 buffer, (c and d) 200 and 100 mg of ketoprofen dissolved in 4 and 2 ml NaH_2PO_4–NaOH 0.2 M pH 12 buffer, respectively, (e and f) 7.5 and 15 mg of meloxicam dissolved in 5 and 2.5 ml of glycine–NaOH 0.05 M pH 12 buffer, respectively, (g) 20 mg of temoxicam dissolved in 2 ml of glycine–NaOH 0.05 M pH 12 buffer, (h) 100 mg of nimesulide dissolved in 2.5 ml of glycine–NaOH 0.05 M pH 12 buffer.

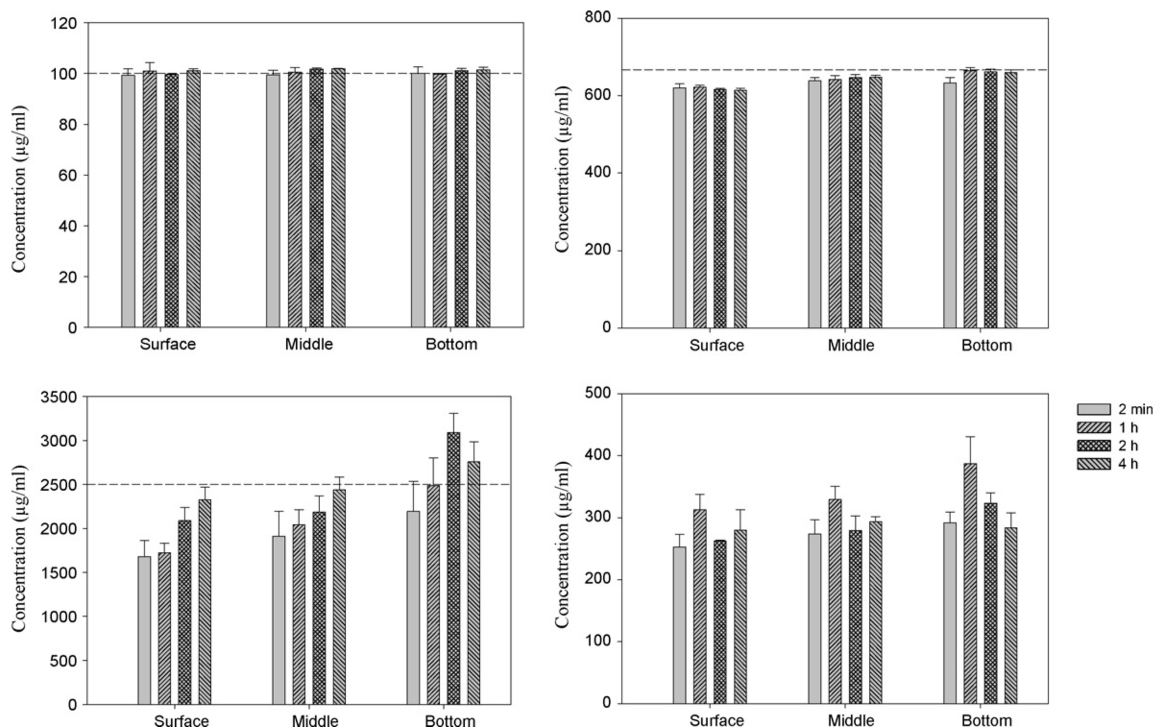

Fig. 5. Concentration of meloxicam (top left), cyclosporine A (top right) aspirin (bottom left) and salicylic acid (bottom right) measured in milk, after the addition of the drugs dissolved in the appropriate medium. Samples were taken from surface, middle and bottom of the milk volume at 2 min, 1, 2 and 4 h. The dashed horizontal line indicates the theoretical content (100, 666.67 and 2500 µg/ml for meloxicam, cyclosporine A and aspirin, respectively). For details about the preparation of the formulations see Section 2.

3.2.2. Meloxicam–milk formulation

The concentration of the drug was constant in all milk samples taken from surface, middle and bottom of the milk formulation at 2 min, 1, 2 and 4 h, Fig. 5. The concentration range of meloxicam, for the pooled (position and time) data, was 99.2–101.9 µg/ml

3.2.3. Cyclosporine–milk formulation

The addition of 5 ml cyclosporine containing ethanolic solution to 145 ml of full fat milk did not cause any visible coagulation. The pH of milk remained unchanged. The emulsion remained unchanged for the duration of the experiment (4 h). The concentration measurements showed that the resulting liquid is homogenous for the duration of the experiment and the drug is stable, Fig. 5. There were small variations between the concentrations observed in surface, middle and bottom of the formulation which can be attributed to the nature of the emulsion. Larger species, such as large fat globules, tend to position closer to the bottom. A lipophilic compound is more likely to solubilize inside the lipophilic interior of such species, therefore the concentration of cyclosporine is relatively higher at the bottom. In any case the differences are small and within accepted range (613.29–664.70 µg/ml) for a theoretical content 666.67 µg/ml.

3.2.4. Aspirin–milk formulation

The pH of the aspirin–milk formulation (6.10 ± 0.02) was more acidic than the pH of the milk used (6.60 ± 0.02) but it was more alkaline than the pH of the 20 ml tap water (5.12 ± 0.06), containing the dissolved aspirin. The emulsion remained unchanged for the duration of the experiment (4 h). The aspirin concentration measurements showed that the resulting liquid is not homogeneous (Fig. 5). While the acetylsalicylic acid's concentrations at the surface and the middle of the aspirin–milk formulation were similar and lower than the theoretical content, the concentration of acetylsalicylic acid at the bottom was higher, possibly due to incomplete dissolution of the effervescent tablet. This is more pronounced at the later time points (1–4 h), Fig. 5. The concentration values of salicylic acid (the product of acetylsalicylic acid's hydrolysis) exhibit a similar fluctuation as a function of time at all positions measured. These values are roughly 10% of the corresponding acetylsalicylic acid concentration (Fig. 5).

3.3. In vivo studies

3.3.1. Meloxicam

The plasma meloxicam concentration–time profile is shown in Fig. 6. C_{max} was observed at 45 min, while in all other meloxicam published in literature pharmacokinetic studies with commercial formulations (solid formulations), t_{max} is about 4.5–5 h (Marcelín-

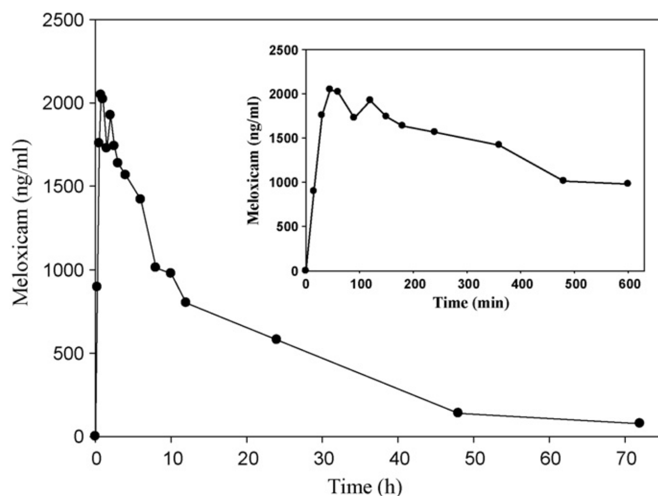

Fig. 6. Plasma concentration–time profile for meloxicam after a single oral administration of meloxicam–milk formulation containing 15 mg of meloxicam. The insert shows the plasma concentration–time profile for meloxicam for the first 10 h.

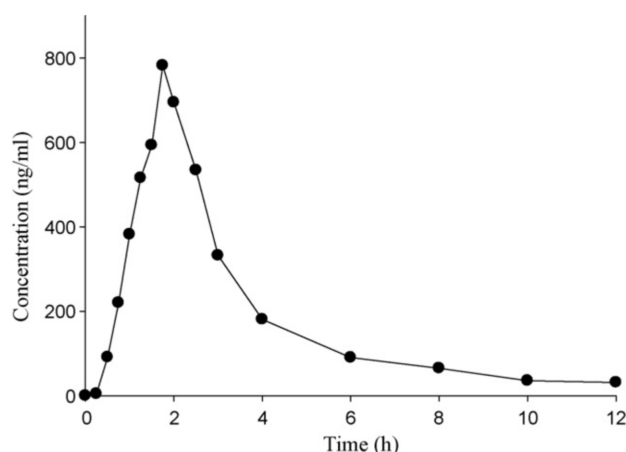

Fig. 7. Plasma concentration–time profile for cyclosporine A after a single oral administration of cyclosporine–milk formulation, containing 100 mg of cyclosporine.

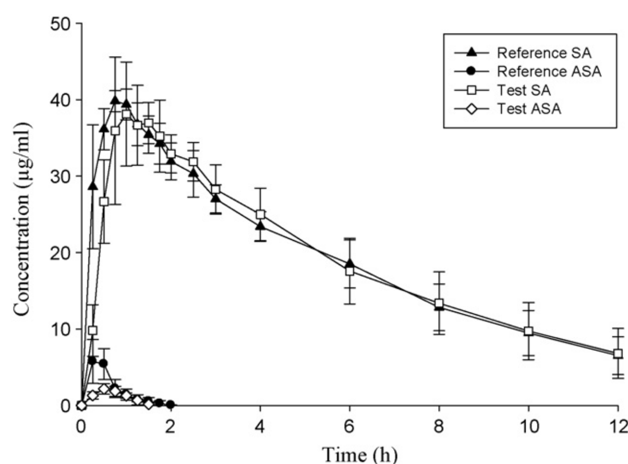

Fig. 8. Mean plasma concentrations–time profiles [mean ± S.D. ($n = 4$)] for salicylic acid and acetylsalicylic acid after oral administration, following a replicate design, to two healthy volunteers of aspirin–milk formulation (test) and reference formulation of 500 mg aspirin.

Jiménez et al., 2005; Bae et al., 2007; Gschwend et al., 2007; Mobic®, Boehringer Ingelheim, n.d.).

3.3.2. Cyclosporine

The plasma cyclosporine concentration–time profile is shown in Fig. 7. The pharmacokinetic parameters obtained are presented in Table 3. For comparative purposes the pharmacokinetic parameters of commercial cyclosporine formulations are also listed in Table 3.

3.3.3. Aspirin

The salicylic acid and acetylsalicylic acid's plasma concentration–time profiles are shown in Fig. 8. Both the concentrations for salicylic acid and acetylsalicylic acid are presented. The pharmacokinetic parameters obtained for both formulations examined are presented in Table 4.

4. Discussion

In the present study, non-steroidal anti-inflammatory drugs were used as model ionized lipophilic compounds. The solubility of NSAIDs in acidic and biorelevant media has been the subject of a large number of studies (Aunins et al., 1985; Galia et al., 1998; Avdeef et al., 2000; Levis et al., 2003; Sheng et al., 2006; Stephenson et al., 2006) because of their relevance with physiological conditions. For our purposes, however, the NSAIDs solubility studies were carried out in alkaline solutions i.e. phosphate and glycine buffers. All non-steroidal anti-inflammatory drugs exhibited increased solubility compared to their solubility in acidic or neutral pH, Fig. 1. In fact, the solubility values determined allow the preparation of an alkaline solution containing the therapeutic dose in a buffer volume suitable for oral administration (third column, Table 1). In addition, the lower actual pH values (determined after the establishment of the solubility equilibrium) than the nominal pH of the buffer solutions, induce a slight or a moderate change (Fig. 4) of the initial milk pH (6.67 ± 0.076) in the final NSAIDs–milk formulations, (fourth and fifth columns, Table 1). For example, the pH of tolfenamic acid–milk formulations ranges from 7.03 to 7.42 for drug doses 100–300 mg and volumes added of buffer solutions ranging from 5 to 12 ml.

Based on the content uniformity results of meloxicam–milk formulation (Fig. 5), the NSAIDs seem to be homogeneously dispersed in the aqueous phase of milk. Moreover, current work indicates that the NSAIDs–milk formulations maintain all the physicochemical characteristics of milk, such as ζ-potential, conductivity and size distribution of the fat globules and micelles (unpublished data). Although the design of milk-based formulations requires the *in situ* mixing of the drug- containing alkaline buffer solution with the milk, just prior to oral administration, the results of Fig. 5 demonstrate that the meloxicam–milk formulation is stable and homogeneous for up to 4 h. In full agreement with the above observations are the *in vivo* results of meloxicam–milk formulation (Fig. 6). These results clearly demonstrate that the very rapid rate of meloxicam absorption (t_{max} = 45 min), compared to 4.5–5 h for commercial tablets (solid formulations) (Marcelín-Jiménez et al., 2005; Bae et al., 2007; Gschwend et al., 2007; Mobic®, Boehringer Ingelheim, n.d.), is associated with the fact that meloxicam is in solution in the novel formulation. Milk serves as the dispersing agent of the meloxicam/buffer solution and the drug is uniformly dispersed in the volume of milk. No discoloration or phase separation were observed for up to 4 h. Due to the buffering capacity of milk, the pH value of the final drug/milk formulation remained unaltered (pH 6.73), suitable for oral administration.

In this work, danazol and cyclosporine were used as model lipophilic unionized drugs to study their solubilities in ethanol–water mixtures and further development of the corresponding drug–milk formulations. Both drugs exhibited high solubilities: danazol 27.15 ± 2.08 mg/ml in absolute ethanol at 25 °C (Fig. 2), while cyclosporine >60 mg/ml in ethanol–water 60:40 mix-

Table 3
Pharmacokinetic parameters of cyclosporine A after single oral administration of the novel formulation.

Pharmacokinetic parameters	Novel formulation	Neoral® 100 mg[a]	Cicloral® 100 mg[a]	Sandimmune® 100 mg[a]
C_{max} (ng/ml)	782.3	581 ± 118	475 ± 88	376 ± 146
$AUC_{0\to t}$ (μg h/ml)	2.11	–	–	–
$AUC_{0\to\infty}$ (μg h/ml)	2.28	1.83 ± 0.46	1.56 ± 0.25	1.30 ± 0.49
t_{max} (h)	1.75	1.38 ± 0.43	1.25 ± 0.26	1.88 ± 0.64
k_{el} (h^{-1})	0.182	–	–	–

[a] Kees et al. (2006).

Table 4
Pharmacokinetic parameters (mean ± S.D.) of the aspirin *in vivo* study for the test and reference formulations.

Pharmacokinetic parameters	Reference		Test	
	Salicylic acid	Acetylsalicylic acid	Salicylic acid	Acetylsalicylic acid
C_{max} (μg/ml)	39.81 (±1.38)	5.75 (±2.87)	38.12 (±6.79)	2.16 (±0.65)
$AUC_{0\to t}$ (μg h/ml)	233.19 (±27.13)	4.01 (±1.45)	229.98 (±44.73)	1.78 (±0.51)
$AUC_{0\to\infty}$ (μg h/ml)	273.99 (±51.29)	4.32 (±1.61)	273.18 (±72.92)	2.19 (±0.28)
t_{max} (h)	0.75	0.25	1.00	0.50
k_{el} (h^{-1})	0.18 (±0.04)	2.15 (±0.75)	0.18 (±0.06)	2.00 (±1.11)

tures at all temperatures studied. These values are much higher than their aqueous solubilities (Bakatselou et al., 1991; Ismailos et al., 1991) and allow the incorporation of therapeutic doses in ethanolic solutions for both drugs. The results of Fig. 5 for the content uniformity of cyclosporine–milk formulation as a function of time provide explicit evidence for the homogenous dispersion of cyclosporine in the milk-based formulation. Moreover, current work indicates that the cyclosporine–milk formulation maintains all the physicochemical characteristics of milk, such as ζ-potential, conductivity and size distribution of the fat globules and micelles (unpublished data). In parallel, Fig. 3 underlines the importance of milk fat content for the solubility of the highly lipophilic danazol in milk (Macheras et al., 1990). The role of fat content for cyclosporine solubility in milk has been also observed in previous studies in our laboratory (Ismailos, 1999). Finally, the *in vivo* results of cyclosporine (Fig. 7) demonstrate the rapid and extensive absorption of cyclosporine, if one compares the pharmacokinetic parameters of the novel cyclosporine formulation with these of the commercially available formulations (Kees et al., 2006). Our preliminary *in vivo* work demonstrates that both exposure parameters (peak, C_{max} and exposure, AUC_{∞}) of the novel formulation are much higher than the corresponding mean ± S.D. values of the formulations in the market, Table 3.

In the present work, aspirin was used as a model for moderately soluble drugs. The effervescent aspirin tablets were utilized in order to avoid problems related to aspirin's instability in aqueous media. Since aspirin has similar aqueous solubility in water (USP 30-NF 25, 2007) and milk (Gourniezaki, 2008), roughly 3 mg/ml, the main advantages of aspirin–milk formulation is the presentation of aspirin in soluble form in the gastrointestinal tract in a medium (milk) which is suggested universally for gastrointestinal tract protective purposes (Dial et al., 1995). The content uniformity results of the aspirin–milk formulation (Fig. 5) indicate incomplete dissolution of aspirin since (i) the measurements at the surface and middle of the milk volume are below the theoretical content and (ii) the measurements at the bottom of the milk volume are progressively becoming higher than the theoretical content. This is probably related to the inadequate volume (20 ml) used for the initial disintegration of the effervescent tablet. The similarity of the salicylic acid measurements over time (close to 10% of the corresponding acetylsalicylic acid value) at all positions (surface, middle and bottom) of the milk volume (Fig. 5) is indicative of the stability of acetylsalicylic acid in milk as well as of the homogeneous dispersion of salicylic acid in milk. The *in vivo* results of the comparative bioavailability study, presented in Fig. 8 and Table 4, are indicative of the similarity of salicylic acid concentration–time profiles of the two formulations studied. The slightly inferior bioavailability of the acetylsalicylic acid in the novel milk-based formulation could be the result of either the effect of the caloric content of milk on the gastric emptying and/or the higher hydrolysis of acetylsalicylic acid to salicylic acid during the initial disintergration of the effervescent tablet.

Due to the strong buffering capacity of milk, alkaline buffers of non-steroidal anti-inflammatory drugs can be used to accommodate the therapeutic doses of NSAIDs and develop the corresponding drug–milk formulations. This approach can be considered universal for ionized lipophilic drugs. Drug–milk formulations can be also prepared for unionized lipophilic drugs exhibiting adequate solubility in ethanol or water–ethanol mixtures. Although more *in vivo* studies are required to assess the *in vivo* performance of milk-based formulations, our preliminary results with meloxicam, cyclosporine and aspirin, showed satisfactory pharmacokinetic properties. More specifically, by incorporating milk in the formulation, the drug is in a dissolved form, resulting in either enhanced (meloxicam, cyclosporine) or similar (aspirin) pharmacokinetic properties compared to marketed formulations. However, the aspirin–milk formulation has the advantage of the concurrent administration of aspirin with milk in a liquid form, which is recommended for the solid dosage forms of aspirin.

Milk is a basic component of the formulation as it provides the proper environment to preserve the drug in a dissolved form, the final pH in physiologically accepted values and moreover, thanks to its gastroprotective characteristics, stomach irritation could be prevented. Overall, the milk-based formulations rely on a novel, simple and physiologically friendly approach for the delivery of ionized and unionized lipophilic drugs. Further *in vitro* studies need to be performed in order to elucidate the physicochemical properties of the final drug/milk formulations, such as the colloidal stability of the emulsion, the particle size and distribution of milk components. Moreover, the stability of alkaline and ethanolic solutions needs to be studied in long term and accelerated conditions at various temperatures. Also, lactose-free milk-based formulations should be prepared for patients with lactose intolerance. This work opens a new avenue for research for the use of milk, a natural and abundant medium, as a dispersing agent and drug carrier.

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