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Journal of Pharmaceutical and Biomedical Analysis
35 (2004) 1093–1100
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Microdialysis for evaluating the entrapment and release of a lipophilic drug from nanoparticles

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Received 15 October 2003; received in revised form 7 April 2004; accepted 10 April 2004

Available online 5 June 2004

Abstract

The aim of the study was to evaluate the microdialysis (MD) as a tool to determine entrapment efficiency and drug release of a lipophilic drug model, diclofenac (DIC), from nanocapsules, nanospheres, and nanoemulsions. Factors that could interfere with the MD probe recovery were investigated: perfusion fluid composition, concentration and form of the drug in the perfusate, and recovery method. DIC entrapment efficiency to nanoparticles and the drug release in phosphate buffer pH 7.4 after different dilutions were evaluated by MD and ultrafiltration/centrifugation (UC). DIC recovery for the 5 μL/min flux was concentration and pH dependent. DIC sodium was used for the recoveries determination since it did not differ from the DIC acid recovery for the same media. DIC entrapment efficiency determined applying both techniques were equivalent and close to 100% for all nanoparticles. In pH 7.4 DIC release from the nanoparticles was partial for the dilution rate 1:1 (v/v), around 50–60%. A complete release was observed from 1:10 (v/v) dilution. Only nanocapsules presented a incomplete release for 1:5 (v/v) dilution, around 86%. MD and UC techniques were equivalent for the evaluation of DIC entrapment efficiency and drug release from the nanoparticles.

Keywords: Microdialysis; Ultrafiltration/centrifugation; Diclofenac; Nanoparticles; Entrapment efficiency

1. Introduction

Nanoparticles are colloidal structures below 1 μm, which have been widely studied as drug delivery systems [1,2]. Applying nanoprecipitation technique [3], colloidal systems such as nanoemulsion (NE), nanocapsules (NC) or nanospheres (NS) can be obtained, according to the formulation composition [4,5].

The physicochemical characterization of colloidal dispersions is important for their development. Due to their small size, this evaluation is difficult to achieve [6,7]. For these carriers, the knowledge of the amount of drug associated to the system as well as the characterization of the drug release is of major importance. Many methods have been used to evaluate the drug release profiles from nanostructures. Among these techniques, it can be mentioned the dialysis bag

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doi:10.1016/j.jpba.2004.04.002

diffusion [8,9], bulk-equilibrium reverse dialysis sac [10], centrifugal ultrafiltration [5–11], ultrafiltration at low pressure [12] and centrifugation [13,14]. Drug entrapment can be either determined by ultracentrifugation or by ultrafiltration/centrifugation [6].

Microdialysis (MD) is a sampling technique based on the passive diffusion of compounds down a concentration gradient across a semi-permeable membrane [15]. The microdialysis probe is continuously perfused with a solution devoid of the analyte of interest. In this way, an equilibrium between the concentration outside and inside the probe is never achieved. However, a constant rate of extraction is reached, termed relative recovery (RR). This parameter depends on different factors such as: perfusion flow rate, sampling interval, temperature, characteristics of the analyte and the membrane, analyte diffusion coefficient [15] and, for high flow rates, analyte concentration [16]. The knowledge of the RR allows the calculation of the true concentration of the analyte in the outside medium, and can be determined by the ratio between the concentration of the analyte in the dialysate and in the outside medium [17].

In vivo microdialysis sampling was initially developed to monitor chemical events in brain [18]. Nowadays, its application has been extended to monitor local concentrations of drugs and metabolites in different organs and biological fluids, such as muscle, liver, bile, kidney, blood, skin, tumor and lung in animals and humans [17]. The in vitro microdialysis sampling has been used to determine protein binding of drugs [19,20], partition coefficient [21] and to sample dissolution testing of pharmaceutical formulations [22,23]. Up to now, this technique has not been proposed for studying colloidal systems physicochemical characteristics. Since it is a continuos process, which allows sink condition, it could be suitable for evaluating the release profile of drugs from nanostructures over time as well as the drug entrapment. If this technique proof to be suitable for these determinations, it could allow the differentiation between polymeric nanoparticles from nanocrystals of the drug. The presence of nanocrystals is not desired when preparing aqueous colloidal dispersions and the current techniques for determining drug encapsulation are not capable of differentiating between these two structures. In theory, microdialysis could overcome this limitation because it works at sink condition.

The aim of this study was to evaluate microdialysis as a technique for the determination of diclofenac (DIC) entrapment efficiency and its release from colloidal systems (NC, NE and NS) prepared by nanoprecipitation. Diclofenac was used as a model compound and ultrafiltration/centrifugation, as reference technique to determine the amount of drug associated to the nanoparticles (NP) as well as to characterize the drug release.

2. Materials and methods

2.1. Preparation of diclofenac free acid

A diclofenac sodium salt (1.045 g, 3.28 mmol) aqueous solution (100 mL) was treated with HCl aqueous solution (5 M) (pH 2.0). The aqueous phase was extracted with chloroform (3 × 100 mL). The organic phase was dried with Na_2SO_4 anhydrous, then filtered and evaporated under vacuum. The residue (diclofenac free acid) was recrystallized from ethanol–water (1:1, v/v) yielding white crystals (78%) with m.p. 158–160 °C. DIC free acid physicochemical properties are: pK_a 3.8 ate 25 °C, MW of 296.1 and practically insoluble in water at room temperature.

2.2. Preparation of NC, NS and NE dispersions

NC were prepared by interfacial deposition of poly(ε-caprolactone) [3]. Briefly, the lipophilic solution consisted of Miglyol 810® (3.3 mL), diclofenac (free acid) (0.150 g), sorbitan monostearate (0.766 g), the poly-ε-caprolactone (1.000 g) and acetone (267.0 mL). This organic phase was added under moderate magnetic stirring into an aqueous solution containing polysorbate 80 (0.766 g in 533.0 mL of water) at room temperature. Acetone was removed by evaporation under reduced pressure and the final concentration of the dispersion was adjusted to 1.5 mg/mL of diclofenac. NS and NE (DIC 1.5 mg/mL) were also prepared as described for NC, omitting the oil and the polymer, respectively.

2.3. Characterization of the dispersions

The particle size was measured by laser light scattering (Nanosizer®, Andilly, France). The ζ potential

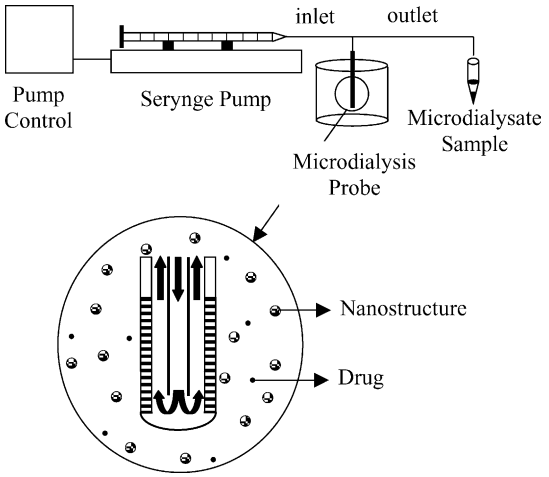

Fig. 1. Schematic representation of the microdialysis equipment and probe.

was measured using a Zetasizer® 4 and correlated with the Malvern® standard solution (Malvern, UK), presenting value of $\zeta = -46.5 \pm 5.9$ mV. Samples of NC, NS and NE were added to 8 mL of NaCl aqueous solution (1 mM). After stirring, the mixtures were injected and measurements were made in triplicate (25 °C).

2.4. Microdialysis equipment

The MD system consisted of a BAS MD-1020 Pump, BAS Bee Hive Controller, a BAS MD-1001 Baby Syringe Drive and a CMA/20 microdialysis probe of polycarbonate, MW cut-off of 20 kDa, 4 mm membrane length (CMA Microdialysis AB, Sweden) (Fig. 1).

2.5. Determination of microdialysis recovery conditions

The influence of different parameters on the MD probe recovery was evaluated: DIC form (sodium salt or free acid) in presence or absence of hydrophilic surfactant (polysorbate 80), dissolution media (water, phosphate buffer pH 4.6 and pH 7.4), and drug concentration in phosphate buffer pH 7.4 (15, 30, 75, 150, 300 and 750 μg/mL). For all the experiments, the microdialysis probe was inserted into glass vials containing the media and DIC. The flow rate was set at 5 μL/min and the temperature was kept at 37 ± 0.5 °C. The 5 μL/min flow rate was chosen due to the sensitivity of the analytical method. The experiments were conducted under moderate stirring. The influence of DIC form, surfactant and dissolution medium on the probes recovery was determined according to the conditions described in Table 1. DIC and polysorbate 80 concentrations were equivalent to those used for the preparation of the colloidal dispersions. The probes, flushed with the respective media without the drug, were allowed 30 min equilibration into the system before three samples were collected at 20 min intervals (100 μL) and analyzed by HPLC. The relative recoveries were determined by gain [17]. The microdialysis recoveries were used to determine the free levels of DIC in the respective media and concentrations.

For the determination of the relative recovery by gain, when the drug was added to the external media and the perfusate was drug free, the following equation was used [18]:

$$\mathrm{RR} = \left(\frac{C_{\mathrm{DIAL}}}{C_{\mathrm{EXT}}}\right) \times 100 \quad (1)$$

where RR is the relative recovery, C_{EXT} is the drug concentration in the external medium, and C_{DIAL} is the drug concentration in the dialysate.

The relative recovery by lost was determined in an experiment where unloaded nanoparticles dispersion were used to evaluate the influence of the nanostructure on the microdialysis probe recovery. In these cases, the drug was added to the perfusate solution

Table 1
Composition of the media (*M*) for the evaluation of microdialysis probe recoveries

Composition	M1	M2	M3	M4	M5	M6	M7	M8
Polysorbate 80[a]	No	Yes	No	Yes	No	No	Yes	No
Buffer (pH)	4.6	4.6	4.6	–	–	7.4	7.4	7.4
Water (pH)	–	–	–	5.5	5.5	–	–	–
DIC[b]	Free acid	Free acid	Na salt	Free acid	Na salt	Free acid	Free acid	Na salt

[a] Polysorbate 80 concentration = 7.66 mg/mL.
[b] DIC concentration = 1.5 mg/mL.

(20 μg/mL) and it was absent in the external medium (unloaded NC, NS or NE dispersion) around the probe. In this scenario, the recovery was calculated by Eq. (2) [17]:

$$RR = \left(\frac{C_{PERF} - C_{DIAL}}{C_{PERF}}\right) \times 100 \quad (2)$$

where C_{PERF} is the drug concentration in the perfusate solution.

2.6. Entrapment efficiency

Free diclofenac (non-associated to the nanostructures) was determined in the ultrafiltrate after separation of the nanoparticles by ultrafiltration/centrifugation technique (Ultrafree-MC 10,000 MW, Millipore) or by MD. For UC determination, 400 μL of DIC dispersions was added to the Ultrafree-MC and centrifuged for 10 min at 5000 rpm. Total DIC concentration was measured using HPLC after dissolution of the colloidal dispersions by acetonitrile. The concentration of diclofenac associated to nanostructures was calculated from the difference between the total and the free drug concentrations, measured in the dispersions and in the ultrafiltrate or microdialysate, respectively [24]. An aqueous solution containing 15 μg/mL of DIC sodium was used for microdialysis probe calibration.

2.7. Drug release

The release of DIC associated to NC, NS and NE were evaluated in phosphate buffer pH 7.4 using two different conditions: over time for the same dilution and at a fixed time for different dilutions. For the first set of experiments, the nanoparticles were diluted 1:1 (v/v) with buffer and the microdialysis probe was inserted into the system which was kept at 37 °C under moderate stirring. Microdialysate samples were collected every 5 up to 30 min. For the second set of experiments, the formulations were diluted in different proportions (v/v): 1:1; 1:5; 1:10; 1:20 and 1:50. These dilutions correspond to 750, 300, 150, 75 and 30 μg/mL of DIC. Right after dilution, the probes were inserted into the systems which were kept at the same conditions described above and three samples were harvested at 20 min intervals and analyzed by HPLC. These experiments were repeated for the 1:1, 1:5 and 1:10 (v/v) dilutions and, after 5 min at 37 °C under moderate stirring, samples were collected and submitted to ultrafiltration/centrifugation in triplicate (Ultrafree-MC 10,000 MW, Millipore).

2.8. HPLC assay

A Waters HPLC system was used: TM 600 pump, 717 plus autosampler, TM 486 Tunable Absorbance detector and a Nova-Pak® C18-3.9 mm × 300 mm column. The mobile phase consisted of acetonitrile-phosphate buffer pH 5 (50:50, v/v). The flow rate was kept at 1 mL/min. DIC was detected at 280 nm. The analytical method, validated according to USP 24, showed a linear response at the concentration range evaluated (6–60 μg/mL), with a correlation coefficient higher than 0.991 and LOQ of 6 μg/mL. The MD and UC samples were injected directly into the system, without previous treatment.

2.9. Statistical analysis

The data obtained from the different experiments were compared by Student "t" test when comparing MD and UC results and by ANOVA when comparing the formulations. A significant difference was assumed for $P < 0.05$. All the experiments were conducted in triplicate.

3. Results and discussion

3.1. Physicochemical characterization

Table 2 shows the results concerning the physicochemical characteristics of the dispersions. All formulations presented acid pH due to the characteristics of their components, mainly the polymer and the free DIC. The particles size were bellow 300 nm

Table 2
Colloidal systems properties

Sample	pH	Size (nm)	ζ potentials (mV)
NC	4.7 ± 0.2	240 ± 35	−32.1 ± 0.2
NS	4.4 ± 0.0	221 ± 16	−30.8 ± 0.1
NE	4.4 ± 0.0	206 ± 29	−29.5 ± 0.2

in all cases in agreement with the characteristics of the colloidal dispersions obtained by the nanoprecipitation technique using poly(ε-caprolactone) as polymer and/or Miglyol 810® as oily phase [5]. The ζ potential presented negative values around −30 mV, enough to assure the physical stability of the systems [4].

3.2. Microdialysis recoveries

Microdialysis relative recovery is dependent of several factors: temperature, flow rate, physicochemical characteristics of the drug and the probe membrane, among others [15]. In order to investigate whether this technique is adequate to evaluate the drug association to nanoparticulate systems it was necessary to determine its relative recovery at different conditions.

The microdialysis probe recoveries were determined for DIC free acid as well as DIC sodium salt. The free acid form was evaluated because it is the form used to prepare the colloidal systems. On the other hand, the sodium salt is the form of DIC released from the nanostructures. The different media investigated intended to simulate the in vivo conditions observed after the oral administration of the drug: phosphate buffer at pH 4.6 simulated gastric medium and at pH 7.4 simulated enteric medium. The recoveries were also determined in water, which is the external phase of the colloidal dispersions.

For DIC free acid, it was not possible to determine MD probe recovery in water and phosphate buffer pH 4.6 in the absence of polysorbate 80. Due to the insolubility of the drug in these media, the surfactant was necessary to disperse it. The microdialysis recoveries for DIC free acid were: 0.27 ± 0.17% in phosphate buffer pH 4.6 with surfactant, 6.12 ± 0.97% in water (pH 5.5) with surfactant, 15.23±0.43% in phosphate buffer pH 7.4 and 13.41 ± 0.45% in phosphate buffer pH 7.4 with surfactant. The results showed a significant increase in probe recoveries as a function of pH increase due to the higher solubility of the drug, which presents a pK_a of 4.0 [25]. In pH 7.4, at which the drug is totally solubilized in the media, the presence of surfactant did not interfere with the probe recovery.

Concerning DIC sodium, a similar behavior was observed regarding the pH increase. The relative recoveries were 9.84 ± 1.98% in water and 18.56 ± 2.06% in phosphate buffer pH 7.4. For these two media, the recoveries of DIC free acid and sodium salt were not significantly different ($P < 0.5$). Taking into consideration the set of results and assuming that the low recovery observed for the drug in pH 4.6 would not be influenced by the presence of surfactant because it is only pH dependent, this experiment was not carried out.

Based on the results that showed similar recoveries for both forms of DIC (free acid and sodium salt), the salt form was selected for the calibration of microdialysis probes in the following experiments: entrapment efficiency and drug release.

The relative recovery of MD probe is, in general, not dependent on the drug concentration. However, it has been reported in the literature that, for high flow rates, a recovery dependency could be observed [16]. Because the flow rate used in this study is in the upper limit of the range used for MD technique [26], it was necessary to determine the influence of drug concentration on the relative recovery. The results showed that the relative recovery is inversely proportional to drug concentration (Table 3). The relative recovery for the lower DIC concentrations investigated (15 and 30 μg/mL) were statistically different from the recovery determined for the higher concentrations (150, 300 and 750 μg/mL). Due to these results, for each drug release experiment after dilution or drug entrapment measurements, the prove recovery determined for the same media and concentration was used to calculate the free DIC levels.

The relative recovery of DIC sodium was also measured at a concentration of 15 μg/mL in water (21.22± 1.66%). This experiment was necessary in order to

Table 3
Microdialysis recoveries of DIC sodium in phosphate buffer pH 7.4

DIC (μg/ml)	Recovery (%)
15	24.98 ± 1.25
30	22.65 ± 2.09
75	22.38 ± 1.84
150[a,b]	15.89 ± 2.61
300[a,b,c]	14.68 ± 0.92
750[a,b,c]	14.31 ± 1.73

[a] Significantly different from 15 μg/mL.
[b] Significantly different from 30 μg/mL.
[c] Significantly different from 75 μg/mL.

allow the determination of DIC entrapment using MD. The 15 μg/mL concentration was selected based on the entrapment efficiency results obtained by UC.

Besides the experiments described in this paper, additional studies were conducted to assure the adequacy of MD as a sampling technique for nanoparticles characterization. Dynamic light scattering of microdialysate samples from different experiments was performed and the results showed the absence of nanostructures confirming that they do not cross the probes membrane and proving that the concentrations measured in the dialysates refers to free levels of the drug in the respective system. The relative recovery was also determined using a solution of the drug (DIC sodium salt in water) as the perfusion medium and, as external medium, water or a dispersion of unloaded nanoparticles to confirm that the presence of these structures in the medium did not interfere with the diffusion of the drug and, consequently, with the recovery. This experiment was performed using the drug in the perfusate (recovery by lost) instead of in the medium (recovery by gain). If the drug was added to the unloaded colloidal dispersions it could adsorb to the nanoparticles, decreasing the free drug concentration in the system not allowing the calculation of the relative recovery. According to the literature, drug adsorption onto unloaded polymeric nanoparticles is possible and this phenomena was confirmed by Lopes et al. [5] using ethionamide. In the present study, the relative recovery determined by lost using DIC as drug model was 21.27 ± 5.32% in water and for nanostructures it was 26.20 ± 8.25, 25.08 ± 9.82 and 22.53 ± 9.50% for NC, NS and NE, respectively, showing no significant difference among the results. Taking together all these results is possible to conclude that the nanostructures did not interfere with the relative recoveries of DIC determined by MD.

3.3. Entrapment efficiency

In order to evaluate microdialysis, generally used to measure free levels of compounds in different media [27], as a technique to determine drug association to colloidal systems, ultrafiltration/centrifugation, widely used with this purpose, was used as reference. Table 4 shows the free DIC concentration and entrapment efficiency of NC, NS and NE determined using both techniques. The results showed that the free and entrapped DIC concentration are statistically different depending on the method used. Microdialysis results showed higher levels of free DIC for all nanostructures and, consequently, lower entrapped drug concentration. These results could be attributed to the difference between the methods used. The main disadvantage of ultrafiltration/centrifugation is that this method does not allow for the differentiation between nanostructures (NC, NS and NE) and drug nanocrystals of the same size range eventually present in the external phase of the dispersions [24]. Because microdialysis works under sink condition, it could promote nanocrystal dissolution resulting in a lower and more accurate entrapment efficiency. However, at the conditions used in this experiment, microdialysis showed a disadvantage in relation to the current technique. Since the recovery was concentration dependent, due to the high flow rate used, a previous estimation of the free DIC concentration expected in the colloidal dispersions was necessary in order to pre-determine the recovery of the probe at a similar condition.

Table 4
Entrapment efficiency and free DIC in nanoparticles determined by microdialysis and ultrafiltration/centrifugation (mean ± S.D.)

Formulations	NC	NS	NE
Free DIC (%)/microdialysis	1.5 (0.6)	1.7 (0.7)	2.9 (0.7)
Free DIC (%)/ultrafiltration[a]	0.1 (0.0)	0.1 (0.0)	1.4 (0.1)
Entrapped (%)/microdialysis	98.5 (0.6)	98.3 (0.7)	97.1 (0.7)
Entrapped (%)/ultrafiltration[a]	99.9 (0.0)	99.9 (0.0)	98.6 (0.1)

[a] Significantly different from microdialysis determination.

3.4. Drug release

In general, the release of drugs from matrixes is described as a function of time. However, it was previously shown that the release profile of nanocapsules prepared using preformed polymers and loaded with DIC is a function of dilution of the colloidal system in the media rather than a function of time [24]. In this study, both parameters were evaluated. The results showed that, for all three formulations, the drug release was independent of time because the amount of drug release after 5 min was not statistically different from the amount released after 30 min (Table 5).

Fig. 2 shows the release of DIC from NC, NS and NE in phosphate buffer pH 7.4 as a function

Table 5
Percentage released as a function of time determined by microdialysis

Interval (min)	NC (%)	NS (%)	NE (%)
0–5	57.1 ± 6.5	62.5 ± 3.5	51.0 ± 9.0
5–10	60.2 ± 4.3	66.5 ± 0.6	57.0 ± 7.3
10–15	56.4 ± 3.5	67.5 ± 8.5	62.1 ± 3.2
15–20	59.5 ± 2.0	66.5 ± 1.0	60.6 ± 4.3
20–25	60.9 ± 5.3	64.9 ± 6.0	58.4 ± 4.7
25–30	60.7 ± 2.7	66.7 ± 5.1	57.9 ± 5.4

of dilution, obtained by microdialysis and ultrafiltration/centrifugation. Regarding the comparison of MD and UC, both techniques resulted in similar release profiles for all three formulations investigated. The experiments using higher dilution (1:20 and 1:50 v/v) were not performed for ultrafiltration because 100 ± 10% of drug release was already achieved for lower dilutions.

The data also allows the comparison of drug release among the formulations. For 1:1 v/v dilution all colloidal systems presented similar DIC release, around 50–60%. For the 1:5 v/v dilution, NS and NE released the total amount of drug entrapped while NC showed only a partial release of the drug (86.6 ± 6.4%). This result suggests that, in this dilution, around 14% of the total amount of drug present in the formulation was not available for dissolution by the medium. For higher dilutions, the total amount of DIC was released from the nanoparticles.

In conclusion, the results presented in this study showed that microdialysis is an equivalent technique to ultrafiltration/centrifugation to evaluate lipophilic drugs such as DIC entrapment efficiency and the drug release from polymeric nanoparticulated dispersions.

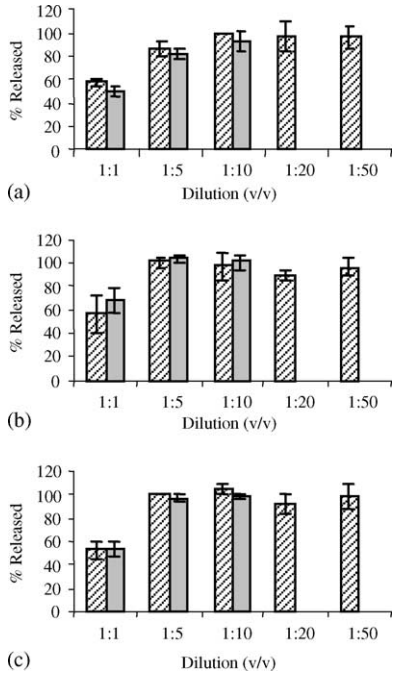

Fig. 2. Release profiles of DIC from (a) NC, (b) NS and (c) NE in phosphate buffer pH 7.4 by microdialysis (striped box) and ultrafiltration (gray box) as a function of dilution ($n = 3$).

Acknowledgements

The researchers acknowledge the individual financial support received from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq/Brasília/Brazil).

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