In vitro evaluation and intra-articular administration of biodegradable microspheres containing naproxen sodium

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The dispersion of non-steroidal antiinflammatory drugs (NSAIDs) into biodegradable polymeric matrices have been accepted as a good approach for obtaining a therapeutic effect in a predetermined period of time meanwhile minimizing the side effects of NSAIDs. In the present study, it was aimed to prepare Naproxen Sodium (NS), (a NSAID) loaded microsphere formulation using natural Bovine Serum Albumin (BSA) and synthetic biodegradable polymers such as polydactide-co-glycolic acid) (PLGA) (50:50 MW 34000 and 88 000 Da) for intra-articular administration, and to study the retention of the drug at the site of injection in the knee joint. NS incorporated microspheres were evaluated in vitro for particle size (the mean particle size; for BSA microspheres, 10.0±0.3um, for PLGA microspheres, 9.0 ± 0.2 and 5.0±0.1um for MW 34000 and 88 000 Da, respectively), yield value, drug loading, surface morphology and drug release. For in vivo studies, mono-articular arthritis was induced in the left knee joints of rabbits by using ovalbumin and Freund's Complete Adjuvant as antigen and adjuvant. A certain time (4 days) is allowed for the formation of arthritis in the knee joints, then the NS loaded microspheres were injected directly into the articular cavity. At specific time points, gamma scintigrams were obtained to determine the residence time of the microspheres in knee joints, in order to determine the most suitable formulation. This study indicated that PLGA, a synthetic polymer, is more promising than the natural type BSA microspheres for an effective cure of mono-articular arthritis in rabbits.

Keywords: Naproxen sodium, poly(lactide-co-glycolic acid) PLGA, bovine serum albumin, controlled release, experimental arthritis, scintigraphic imaging.

Introduction

In recent years, extensive research studies have been focused on natural and synthetic biodegradable polymeric materials as drug carriers in various drug delivery systems (Muller et al. 1996, Ghaderi and Carlfors 1997). Lactic and glycolic acids and their copolymers (PLGAs) derived from aliphatic polyesters, which are synthetically manufactured, and BSA which has a natural origin are among the most frequently studied biopolymers (Muller et al. 1996, Flandroy et
An increasing number of drugs have been considered for incorporation into the biodegradable microspheres because of the advantages they offer. The reasons for choosing the biodegradable poly(lactide-co-glycolic acid) (PLGA) and BSA polymers as carriers of NS for this investigation are the following: first, PLGA is biocompatible, has regulatory approval, and in vivo degradation occurs by forming the non-toxic monomers, lactic acid and glycolic acid. Furthermore, the release rate of the entrapped drug can be controlled by varying the molecular weight (MW) of the carrier polymer and the copolymer ratio and it might be possible to prolong the retention of the drug at the site of injection (Ghaderi and Carlfs 1997). Secondly, BSA, as a natural biodegradable polymer, has been a favoured carrier for drugs as it is suitable for preparing non-antigenic microparticles, the physicochemical characteristics of which can be modulated by crosslinking methods (Qalis et al. 1998). BSA microspheres are stable and can be removed from the vascular system by phagocytosis (Egbaria and Friedman 1990). In addition, albumin microspheres were described as the best tolerated drug delivery systems by synovial tissue (Ratcliffe et al. 1984, Gupta and Hung 1989, Bogdansky 1990).

Naproxen sodium (NS) has analgesic, antiinflammatory and antipyretic properties and is widely used in rheumatic disorders such as ankylosing spondylitis and osteoarthritis. The antiinflammatory effects of NS and other NSAIDs may be due in part to inhibition of prostaglandin synthesis and release during inflammation. NS has some adverse effects, common with most of the NSAIDs, such as diarrhea, nausea, vomiting, ulcers, abdominal pain and skin rash, and it also has a short half-life.

The treatment of arthritic conditions by direct injection of drugs into the joint cavity is limited by the rapid clearance of the drugs from the joint into the blood stream and the deleterious effects of the drugs on the cartilage. The main requirement for an ideal intra-articular drug carrier system is to retain the active substance in the knee joints until it is taken up by the phagocytic cells (particles themselves must not escape from the synovial cavity into the circulation) (Hunneyball 1986, Rntdiffe et al. 1987, Pavanetto et al. 1994, Burgess and Davis 1998). Incorporation of drugs within biodegradable polymeric particles have been shown to be effective in improving the retention of drugs within the joint cavity (Ratcliffe et al. 1987).

The purpose of the present study was to prepare and to evaluate microsphere formulations of NS in vitro and in vivo using PLGA (50:50, MW 34000 and 88000) and BSA as polymeric materials, and to investigate the retention of NS microspheres in inflammed knee joints of rabbits. NS loaded PLGA microspheres were prepared by the conventional solvent evaporation method (Guiziou et al. 1996, Jeyanthi et al. 1996, Bozdag et al. 1998, Tuncay et al. 1998), while the emulsion polymerization method was used for the formulation of BSA microspheres (Tunc-ay et al. 1997, (;alis et al. 1998).

**Materials and methods**

**Materials**

The drug was naproxen sodium (NS) (Syntex Pharm. Int. Ltd., Basle, Switzerland). The synthetic matrix material was different MW grades (34000
and 88000 Da) of PLGA (50:50) (Medisorb, Cincinnati, USA). The natural matrix material was BSA (Armour Pharm. Comp., Collegeville, USA). The crosslinking agent used was glutaraldehyde (25% aqueous solution, Merck, Darmstadt, Germany). Sodium oleate (SO), methanol, and polyvinyl alcohol (PVA) were purchased from Merck, Darmstadt (Germany). Methylene chloride was obtained from Quimon, Madrid (Spain). Technetium-99m (99mTc) as per-technetate was obtained from a generator (Amersham, Buckinghamshire, UK). Polyclonal human immunoglobulin kits were obtained from Mallincrodt, Petten (Holland). White female New Zealand rabbits were raised locally. Ovalbumin and Freund's complete adjuvant were bought from Sigma Chem Co., St. Louis (USA).

**Preparation of microspheres**

PLGA microspheres containing NS were prepared by the solvent evaporation method (Guiziou et al. 1996, Jeyanthi et al. 1996, Bozdag et al. 1998, Tuncay et al. 1998). NS (30 mg) was dissolved in a methanol—methylene chloride mixture (1.5:5.0) containing 300 mg of PLGA copolymer. This organic solution was then added to 100mL of aqueous phase containing 0.4% (w/v) of PVA and 0.1% (w/v) of SO, and stirred continuously (1700rpm) at room temperature for 2h. The microspheres were washed four times with distilled water and dried in a vacuum oven for 24 h.

NS albumin microspheres were prepared according to the emulsion polymerization procedure, in which 0.5 mL of the aqueous solution, containing 25% BSA (w/v) and NS, were mixed with 100mL of cotton-seed oil and homogenized. The resulting homogenate was added with continuously stirring into 100mL of cotton-seed oil at 1400rpm and 25 C. The BSA microspheres was washed with diethyl ether (anhydrous) to remove the oil phase and microspheres were stabilized by glutaraldehyde solution (25%) in different concentrations (0.1 and 0.7mL) using two duration times (15 and 60min). Related values and codes of preparations are shown in table 1.

**Determination of NS content of the microspheres**

For each batch of PLGA microspheres; 50 mg of NS microspheres were weighed accurately and 10mL of pH 7.4 phosphate buffer was added and kept in an ultrasonic bath for 15 min. Then, this solution was filtered through 0.22 urn Millipore niters and the absorbance was assayed at 271 nm spectrophotometrically to determine NS present at the surface of microspheres. Following this procedure,

<table>
<thead>
<tr>
<th>Stabilizing agent</th>
<th>Concentration of the stabilizing agent</th>
<th>Duration of stabilization (min)</th>
<th>Codes</th>
<th>Yield value (%)*</th>
<th>Total drug content (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutaraldehyde</td>
<td>0.1</td>
<td>15</td>
<td>A</td>
<td>70.00 ±0.6</td>
<td>2.00 ±0.02</td>
</tr>
<tr>
<td>25% aqueous solution</td>
<td>0.1</td>
<td>60</td>
<td>B</td>
<td>68.00 ±0.4</td>
<td>2.00 ±0.01</td>
</tr>
<tr>
<td></td>
<td>0.7</td>
<td>15</td>
<td>C</td>
<td>65.00 ±0.4</td>
<td>1.98 ±0.03</td>
</tr>
<tr>
<td></td>
<td>0.7</td>
<td>60</td>
<td>D</td>
<td>60.00 ±0.2</td>
<td>1.82±0.02</td>
</tr>
</tbody>
</table>

* mean ± SD, n = 3.
the polymer was dissolved by adding 5 mL of methylene chloride to the dried and weighed microspheres. Finally, the polymer was precipitated by the addition of methanol and the amount of entrapped active ingredient was also assayed.

For each batch of BSA microspheres, 10 mg of NS microspheres were weighed accurately and washed four times by keeping in an ultrasonic bath for 5 min with 1 mL 0.9% NaCl solution (containing 0.01% Tween 80). After this procedure, they were centrifuged at 3000 rpm for 10 min and supernatant was assayed at 271 nm spectrophotometrically to determine drugs present at the surface of microspheres. For determining the entrapped amount; 5 mL glacial acetic acid was added to the remaining microspheres and stored at 4°C for 24 h followed by centrifugation (5000 rpm) to completely separate the precipitated matrix material. The amount of NS in each sample was determined by measuring the absorbance of the clear supernatant in a spectrophotometer (Shimadzu UV-160 A, Japan) at 272 nm.

**Particle size analysis**

For PLGA and BSA microspheres, the particle size was determined by means of a laser particle sizer (Helos, Sympatec, Germany). The microspheres were suspended in distilled water containing 0.1% Tween 80. After stirring and ultrasonicating for 10s, the particle size distribution was measured.

**Surface morphology of microspheres**

Scanning Electron Microscopy (SEM) of the PLGA and BSA microspheres was carried out to examine the surface morphology. Microspheres were mounted on metal stubs with conductive silver paint and then sputtered with a 150 Å thick layer of gold in a Bio-Rad apparatus. A Scanning Electron Microscope (Jeol-SEM ASID-10 Device in 80 KV) was used to evaluate surface characteristics of the prepared microspheres.

**In vitro drug release**

Drug release from NS loaded PLGA and BSA microspheres was determined at pH7.4 in phosphate buffer. Microspheres (50 mg for PLGA and 100 mg for BSA microspheres) were suspended in 25 mL of dissolution medium in a glass vial placed in a shaker bath at 50 cpm and 37 °C. Samples were collected at various time points (for PLGA microspheres: 1-108 h; for BSA microspheres: 0.25-Sh) and filtered through a Whatman 45 filter and replaced by an equal volume of dissolution medium. The amount of drug released was calculated from the UV absorption measurements of the samples.

**In vivo studies**

Mono-articular arthritis was induced in the left knee joints of 21 white female New Zealand rabbits weighing ~3 kg by a modification of the method which was originally introduced by Ratcliffe *et al.* (1984). It was successfully used by other investigators (Mumper *et al.* 1992, Ercan *et al.* 1993) and also in the authors' laboratory (Vural *et al.* 1990, Nasirideen *et al.* 1998, Tuncay *et al.* 2000a,b). In this method, ovalbumin was used as the antigen and emulsified with Freund's Complete Adjuvant. A physiological solution (0.9% NaCl) of ovalbumin solution at a concentration of 20mg/mL was emulsified with an equal volume of Freund's
Complete Adjuvant. The emulsion was passed through a homogenizer in order to obtain a homogenous dispersion. One millilitre of the emulsion sample was injected into the left knee joints of each rabbit, and the contralateral knees which were injected 0.5 ml of physiological solution were used as the control joints.

To demonstrate arthritic lesions by gamma scintigraphy before therapy (Rubin et al. 1989, Nasirideen et al. 1998), Technetium-99m labelled polyclonal human immunogammaglobulin (\(^{99m}\)Tc-HIG) was used as the radiopharmaceutical. The labelling efficiency of \(^{99m}\)Tc labelled HIG was determined by impregnated thin layer chromatography (ITLC) using ITLC-SG (Gelman Scientific Inst., USA) mini strips and saline as solvent. Four days after the induction of arthritis, 1 mCi (0.2mL) of \(^{99m}\)Tc-HIG in saline solution was administered intravenously through the ear vein of each rabbit. Four hours after the administration of the radiopharmaceutical, static images were obtained from the posterior position with a gamma camera (Toshiba GCA 60TE, Japan) using a LEAP collimator. To obtain an image, 300—500 kilocounts were collected. Regions of interest were drawn over both arthritic (target) and contralateral (non-target) knee joints. To determine the A/C ratios, the radioactive count ratios obtained from the equal regions of interest of arthritic (A) and the control areas (C) were used.

As the test sample, NS containing microspheres, and as control samples, blank microspheres and NS solution in buffered saline, were used. One day after the demonstration of arthritic lesions, samples were injected intra-articularly to rabbits. A dose of 7mg/0.5 mL (calculated on a kg basis, taking into consideration the adult dose of NS, 275mg/70kg orally) NS containing preparations were injected into each rabbit in their respective groups. Blank microspheres and NS solution in buffered saline were administered as stated above (0.5 mL) to each rabbit in their respective groups.

On days 3,17 and 30, evaluation of arthritis was performed by the i.v. injection of 1 mCi of \(^{99m}\)Tc-HIG as stated above. Four hours after the administration of the radiopharmaceutical, scintigraphic images of the rabbits were obtained as described previously. In the target and non-target areas, regions of interest were created, and in each group, the radioactivity count ratios (A/C) were determined. The average values of each group and their standard deviations are presented in table 2.

**Results and discussion**

**Formulation studies**

**PLGA microspheres.** Microencapsulation by the solvent evaporation method, involves two major steps; the formation of stable droplets of the drug-containing polymer solution and the subsequent removal of solvent from the droplets (Hermann and Bodmeier 1998). A mixture of methanol and methylene chloride (1.5:5.0) solution was used as the organic phase to increase the solubility of the drug. Stirring time was optimized at 2h. During the preparation procedure, PVA and SO was used in a mixture of (4:1) ratio as stabilizers. This was found to be a critical parameter in providing the separation of microparticles (Arshady 1990, Watts et al. 1990).
Table 2. Measurements of average radioactivity count ratios (mean ± SD) at 4h in ratios of target to non-target areas before and after therapy.

<table>
<thead>
<tr>
<th>Code*</th>
<th>n</th>
<th>Pre-treated ± SD</th>
<th>Post treated ±SD</th>
<th>Post treated ± SD</th>
<th>Post treated ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>3rd day</td>
<td>17th day</td>
<td>30th day</td>
</tr>
<tr>
<td>A</td>
<td>5</td>
<td>3.278±1.437</td>
<td>2.544±0.559</td>
<td>2.352±0.998</td>
<td>1.684±0.359</td>
</tr>
<tr>
<td>B</td>
<td>5</td>
<td>3.008±0.432</td>
<td>2.866±1.330</td>
<td>2.330±1.362</td>
<td>2.028±0.799</td>
</tr>
<tr>
<td>C</td>
<td>5</td>
<td>2.700±1.204</td>
<td>1.913±0.703</td>
<td>1.465±0.478</td>
<td>1.300±0.353</td>
</tr>
<tr>
<td>D</td>
<td>5</td>
<td>1.900±0.341</td>
<td>1.830±0.935</td>
<td>1.980±0.666</td>
<td>1.800±0.532</td>
</tr>
<tr>
<td>E</td>
<td>5</td>
<td>2.708±0.866</td>
<td>2.090±0.902</td>
<td>1.998±0.604</td>
<td>2.045±0.585</td>
</tr>
</tbody>
</table>

* Rabbit treated with; A: NS containing BSA microspheres, B: NS containing PLGA microspheres, C: Empty BSA microspheres, D: Empty PLGA microspheres, E: NS solutions in buffer solution.

Table 3. Characterization of NS loaded PLGA (50:50) microspheres.

<table>
<thead>
<tr>
<th>Codes</th>
<th>MW of the PLGA (50:50) (Da)</th>
<th>Yield value (%)*</th>
<th>Particle size (50%, urn)*</th>
<th>Total drug content (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>34 000</td>
<td>79.00 ±0.5</td>
<td>9.0 ±0.2</td>
<td>2.00 ±0.01</td>
</tr>
<tr>
<td>B</td>
<td>88 000</td>
<td>80.00±0.6</td>
<td>5.0±0.1</td>
<td>10.00±0.02</td>
</tr>
</tbody>
</table>

*mean ± SD, n=3.

PLGA was used in two different MWs (34000 and 88 000 Da) (table 3). Particle size of each batch was determined to be ~9.0±0.2 and 5.0 ±0.1 urn (volume distribution) for 34000 and 80 000 MW PLGA microspheres, respectively (table 3). SEM studies revealed that microspheres were spherical in shape, a uniform size distribution was obtained and there were no drug crystals present on the surface for both of the MWs used. Microspheres which were prepared from 34000 MW of PLGA showed porosity on their surfaces, while microspheres prepared from 88 000 MW had smooth surfaces (figure l(a,b)). In a number of studies (Hermann and Bodmeier et al. 1998), it was demonstrated that the type and polarity of organic solvent, amongst several other factors such as the solvent removal technijiiif and polymer type, had a significant influence on the morphology of microspheres. The authors found that the size and the number of pores were increased with the progressive reduction in MW (Song et al. 1997). Other characterization data concerning PLGA microspheres are shown in table 3.

Surface morphology of NS loaded PLGA microspheres were also investigated following the in vitro release experiments in which an expansion in the pore sizes were noticed in MW of 34 000 Da PLGA (figure l(a)).

Molecular weight did not seem to have a significant effect on the yield (table 3). When determining drug loading, it was taken into consideration that NS might be present both on the surfaces and entrapped in microspheres. Hence, it was concluded th.it there was a variation in drug loading related to the MW of PLGA (for 34000 and 88000 MW; 2% and 10%, respectively). Song et al. (1997) found that high efficiency of BSA entrapment was accomplished with high MW PLGA, whilst lower MW PLGA under the same conditions produced much lower values. The high MW PLGA might form a stronger film which would
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Figure 1. Surface morphology of NS containing PLGA microspheres (SEM photograph). (a) A₁, A₂: MW of 34000 Da; before and after in vitro release, respectively, (b) B₁, B₂: MW of 88000 Da; before and after in vitro release, respectively.

Figure 2. In vitro release profiles of NS containing PLGA microspheres.

not easily be disrupted, thus could provide a better barrier against drug leakage. Similar results were obtained in previous literature (Çiftci et al. 1996, Tuncay et al. 2000b).

The drug release from microspheres is mainly dependent on the parameters as drug (position of drug in the microsphere, MW, concentration of drug), micro-
spheres (size and density of microspheres, the extent and nature of crosslinking) and release medium (pH, polarity) characteristics (Tomlinson et al. 1984). As shown in figure 2 the NS release from the low MW PLGA microspheres was faster. In 34000 MW PLGA microspheres, in vitro release of the active substance was completed in 72 h, whilst 88 000 MW PLGA microspheres released only 70% of the active substance in the same period. These results were in good agreement with other studies reported concerning drug release from microspheres of PLGA having different MWs (Cohen et al. 1991, Song et al. 1997). Previously, a number of researchers have confirmed that low MW PLGA degraded much faster than the high MW PLGA (Cohen et al. 1991, Song et al. 1997). A weight loss was usually observed during the degradation of low MW PLGA, but not for high MW (Kenley et al. 1987). Shah et al. (1992) further confirmed that weight loss was not significant until MW decreased by several hundred fold. Along with the weight loss, the polymer matrix became more hydrophilic and more porous, which inevitably enhanced the drug release.

BSA microspheres. Due to the fact that BSA and the active substance NS were both soluble in aqueous medium, the emulsion polymerization method was selected as the preparation procedure. In this method, in order to increase the drug loading and yield value of microspheres, anhydrous ether was used to prevent the dissolution of NS by water which might be already present in the ether. Microspheres were stabilized by the glutaraldehyde solution in different concentrations using various duration times (table 1) during the preparation process.

The particle size of each batch was 10.0 ±0.3 μm (volume distribution) for BSA microspheres (for A, B, C and D coded formulations). Light and SEM studies showed that they were spherical in shape, had a smooth surface and homogeneous distribution (figure 3(a)—(c)). BSA microspheres did not show a significant change in morphology after the release experiments (figure 3(h,c)). Other characterization data concerning BSA microspheres is shown in table 1.

It was observed that an increase in crosslinking agent and crosslinking degree caused a decrease in yield value, similar to the results of previous researchers (Vural et al. 1990, Tuncay et al. 2000a). A variation in drug loading related to the concentration of crosslinking agent and duration of stabilization was obtained for BSA microspheres. The decrease in drug loading for microspheres which were formulated with 0.7 mL glutaraldehyde (25%) and 60 min stabilization time can be considered as an indication of this effect (table 1).

The release profiles of NS from BSA microspheres were as shown in figure 4. BSA microspheres showed a biphasic release pattern with a fast release (burst effect) followed by a slower release. An initial burst observed was due to the release of the drug present on the surface and solubility of NS in dissolution medium. The release profile was less affected by the duration of crosslinking. For the formulations A and B prepared with 0.1 mL glutaraldehyde; a formulation with a crosslinking duration time of 15 min released 100% of the drug in 15 min, while in formulation B, in which duration time is 60 min, the release was completed in 3 h (figure 4). For C and D coded formulations which were prepared with 0.7 mL glutaraldehyde; having crosslinking duration times of 15 and 60 min, respectively, nearly released 100% of the drug in 8 h (figure 4). In the light of these results, it might be concluded that as the stabilization degree increased, time for drug release
Figure 3. Surface morphology of NS loaded BSA microspheres. (a) Light microscopy photograph, (b) Scanning electron microscopy (SEM) photograph before *in vitro* release, (c) SEM photograph after *in vitro* release.
was extended. Previous studies in the literature supported these results (Çaş et al. 1998, Tuncay et al. 2000a).

**In vivo studies**

The labelling efficiency of $^{99m}$Tc-HIG complex was 99% as determined by ITLC. Arthritic lesions were demonstrated well by $^{99m}$Tc-HIG (figure 5). Regions of interest (ROI) were drawn both in the arthritic and normal contralateral knee joints and, radioactivity count ratios of target to non-target areas were calculated with standard deviations of mean values in the *in vivo* studies.

When the release results of microspheres were evaluated, microspheres prepared by 34000 MW PLGA and B coded BSA formulation (prepared with 0.1 mL glutaraldehyde with a crosslinking duration time of 60min) seemed to be the appropriate candidates for *in vivo* trials. For PLGA microspheres, according to the results of the experiments a statistical difference was observed at pre-treatment, 3rd, 17th and 30th days between the NS containing and the blank PLGA microspheres (results compared by means of Mann-Whitney U Test, $U=16$, $p < 0.05$) (table 2). For BSA microspheres, NS containing BSA microspheres and blank microspheres (used as control group) were compared after the treatment, at 3rd, 17th and 30th days no statistical difference was observed (results compared by means of Mann-Whitney U Test, $U=12$, $p > 0.05$). Subsequently, the most effective results were obtained with NS containing PLGA microspheres. The reason why there was not a statistical difference between the NS loaded BSA microspheres and the blank microspheres may be explained by the antigenic effects of BSA (Bogdansky 1990).

Consequently, preparation of NS microspheres by using biodegradable polymers, PLGA and BSA as a carrier matrix seems to be a good approach so that retention of active substance in the knee joints might be provided. PLGA seems to be more promising than BSA for intra-articular administration of a NSAID, NS. MW of PLGA (50:50) and increase in crosslinking agent and crosslinking degree of BSA appeared to be the critical parameters for release characteristics from NS loaded microspheres.

![Figure 4](image-url)  
*Figure 4. In vitro release profiles of NS loaded BSA microspheres.*

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**Note:** The image has been replaced with a placeholder as the actual figure is not provided in the text. The description in the text is based on the context provided.
Figure 5. Scintigrams of arthritic rabbits taken 4h post-administration of $^{99}$Tc-HIG. (A) NS containing BSA microspheres, (B) NS containing PLGA microspheres, (C) Empty BSA microspheres, (D) Empty PLGA microspheres, (E) NS solutions in buffer solution.
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References


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