ELSEVIER

International Journal of Pharmaceutics 234 (2002) 195–203

international journal of pharmaceutics

www.elsevier.com/locate/ijpharm

Preparation and characterization of fentanyl-loaded PLGA microspheres: in vitro release profiles

Hak Soo Choi [a], Sun-Ah Seo [a], Gilson Khang [a], John M. Rhee [a], Hai Bang Lee [b,*]

[a] *Department of Polymer Science and Technology, Chonbuk National University, 664-14 Dukjin, Chonju 561-756, Republic of Korea*

[b] *Biomaterials Laboratory, Korea Research Institute of Chemical Technology, P.O. Box 107, Yusung, Taejon 305-606, Republic of Korea*

Received 12 September 2001; received in revised form 7 November 2001; accepted 23 November 2001

Abstract

We developed several kinds of fentanyl-loaded poly(L-lactide-co-glycolide) (PLGA) microspheres (FMS) for sustained release of fentanyl. FMS were prepared by an emulsion solvent-evaporation method. In this study, the influences of several preparation parameters, such as initial drug loading, polymer concentration, and solvent volume on the release patterns of fentanyl were investigated. Furthermore, it has been well noted that the detection of fentanyl is extremely difficult because its clinical dose level is very low, about 1–3 ng/ml, in cancer-patient treatment. Therefore, we also developed a rapid and sensitive determination method for fentanyl in systemic circulation by employing gas chromatography (GC) system. Fentanyl was slowly released from FMS over 15 days with a quasi-zero order property. From the results, our FMS may be good formulations to deliver the analgesics and suitable for the treatment of severe pain over long periods.

Keywords: Fentanyl; Poly(L-lactide-co-glycolide); Microspheres; Solvent-evaporation method; Gas chromatography

1. Introduction

Fentanyl is a potent synthetic opiate commonly used for surgical analgesia and sedation (Baselt, 1982; Hammargren and Henderson, 1988). It is approximately 200 times more potent than morphine, has a rapid onset (1–2 min), and short duration of action (30–60 min) (Marchall and Longnecker, 1990). Because of its potency and quick onset, even a very small dose of fentanyl can lead to sudden death (Garriott et al., 1984; Pare et al., 1987; Matecjczyk, 1988; Levine et al., 1990). Moreover, the expected concentration range of fentanyl is very low. A concentration of 1–3 ng fentanyl per ml of serum is effective therapeutic range for analgesia and toxicity is reached between 3 and 5 ng/ml when fentanyl is abused. Therefore, the detection of lower levels of the compound from analgesic doses is important.

* Corresponding author. Tel.: +82-42-860-7220; fax: +82-42-861-4151.

E-mail address: hblee@krict.re.kr (H.B. Lee).

0378-5173/02/$ - see front matter
PII: S0378-5173(01)00968-1

In addition, intravenous administration of fentanyl results in a relatively short half-life, about 3.7 h in plasma, and simple parenteral administrations of fentanyl may not be fully effective, so frequent injections and continuous infusions are required to ensure adequate plasma levels (Moffat, 1986). However, these methods have the disadvantage of potentially causing irreversible damage to nerve or surrounding tissues due to fluctuations in concentration and high levels of anesthetic. Additionally, anesthetic delivered in the form of pulse instead of zero-order kinetics may aggravate adverse reactions due to overdosage (Beck et al., 1985; Moffat, 1986). Therefore, a sustained release system is needed to prolong the action of local anesthetic as well as to avoid the inconvenience of patients and to maintain constant therapeutic levels (Ikeda and Pelton, 1990). The development of long-acting local anesthetics is also needed for postoperative analgesia and control of chronic pain of cancer patients (Lee et al., 1999).

Biodegradable polymers produce biocompatible, toxicologically safe by-products that are further eliminated by the normal metabolic pathways. It would deliver the drug at a continuous rate and reduce the administration difficulties (Maria et al., 2000). Poly(L-lactide-co-glycolide) (PLGA) has been widely used as carriers in controlled-release delivery systems due to its biodegradability and relatively good biocompatibility (Cho et al., 2000, 2001; Khang et al., 2000; Lee et al., 2000; Maria et al., 2000). The FDA has also approved it for drug delivery use, so it has been used for the study of a controlled release system over the past decade. The drug release pattern from PLGA microspheres is biphasic, combination of diffusion and biodegradation (Lewis, 1990). Initially, drug is released via diffusion through the polymer matrix as well as through the porous voids of the polymer structure, but biodegradation of PLGA continuously changes the drug release pattern. The second process involves bulk erosion: the polymer matrix uptakes water and the polymer chains are degraded small enough to be soluble, and drug is released during the dissolution of the PLGA matrix. The initial burst is caused by release of drug that is loosely bound to the surface or embedded in a superficial region of the microspheres (Arshady, 1990; Lewis, 1990).

In this study, we demonstrated the possibility of the fentanyl-loaded microspheres (FMS) for local anesthesia with the precise and effective control of fentanyl administration, and developed a rapid and sensitive analytic method for fentanyl in the concentration range of 0.5–50 ng/ml by using gas chromatography (GC) with a nitrogen–phosphorous detector (NPD). Also the effects of the preparation conditions on morphology and release profiles were investigated. The patterns of the drug release depend on various factors: initial drug/polymer loading ratio, polymer concentration, and solvent volume.

2. Materials and methods

2.1. Materials

Fentanyl base (Fig. 1) was purchased from McFarland Smith (Edinburgh, UK) and papaverine hydrochloride (Fig. 1), internal standard (IS), from Sigma Chem. Co., Ltd. (Steinheim, Germany). PLGA (Resomer® RG 752, PLGA 75:25 mol ratio by lactide to glycolide, MW 20 000 g/mol) was obtained from Boelinger Ingelheim (GmbH, Germany). Poly(vinyl alcohol) (PVA, MW 30 000–70 000 g/mole), ethyl acetate (EA), *n*-butyl chloride, toluene, and methanol were pur-

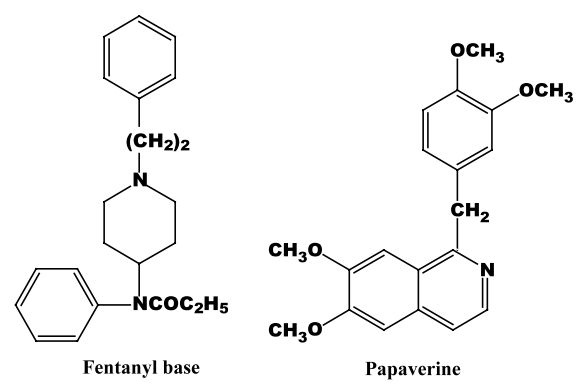

Fig. 1. Chemical structures of fentanyl base and papaverine (IS).

Table 1
The preparation conditions and characteristics of FMS (10 wt/vol% polymer, 3 wt/vol% emulsifier, and 10 ml EA): effect of initial drug loading ratio

Batch	Initial drug ratio (wt%)	FMS mean size ± SD (μm)	Encapsulation efficiency (%)	Yield (%)
A1	5	15.2 ± 6.8	89.7	82.1
A2[a]	10	16.4 ± 8.6	83.5	81.8
A3	20	17.2 ± 7.8	76.2	77.5
A4	30	16.9 ± 7.6	61.5	75.1

[a] Control.

chased from Sigma Chem. Co., Ltd. (St. Louis, MO, USA). Water was obtained by a Milli-Q purification system from Millipore (Molsheim, France). All other chemicals were of analytical grade and used with distilled purification.

2.2. Fabrication method of FMS

The conventional oil-in-water (O/W) emulsion method was applied to fabricate FMS since fentanyl is a slightly water-soluble drug. EA was used as an O phase because of its lower toxicity, ease of removal, and excellent ability to dissolve the polymer (Jalil and Nixon, 1990a). We encapsulated fentanyl in PLGA microspheres using an O/W method that was followed by solvent evaporation. PLGA and fentanyl were dissolved in EA and then emulsified by drop in W phase containing 3% PVA as an emulsifier. EA was removed at 35 °C by evaporation, and monolithic microspheres containing fentanyl were obtained. As the solvent was being removed, the emulsifier continued to maintain the oil droplets in their spherical configuration and prevented from aggregating until the solvent was completely removed, and the microspheres were hardened as discrete particles. Finally, the hardened FMS were washed, centrifuged, and freeze-dried at − 70 °C using freeze drier (FDU-540, EYELA®, Japan). The preparation conditions of FMS are listed in Tables 1–3.

2.3. Morphology observation

Scanning electron microscopy (SEM, S-2250N, Hitachi, Japan) was used to study FMS size and size distribution as well as to reveal the surface quality and porosity of microspheres. Before SEM observation, all samples were mounted on metal stubs and coated with a thin layer of platinum by means of a plasma sputtering apparatus (Emscope, SC 500K, UK) under argon atmosphere. Cross-sectional morphology of microspheres was obtained by embedding the microspheres in an aqueous solution containing 30% gelatin and 5% glycerin. The size distributions of microspheres were determined according to a reference scale. The observations were studied before and after in vitro release test.

2.4. X-ray analysis

Powder X-ray diffraction (XRD, D/MAXIII, Rigaku, Japan) patterns were obtained to study physical state of drug, polymer, physical mixture of drug/polymer (10/90), and drug-loaded microspheres (batch A2). The test was carried out at 40 kV and over a 2θ range of 0–40°. Physical mixture was made of grinding drug and polymer materials.

2.5. In vitro release test

The fentanyl release from the microspheres was established by suspending 20 mg of FMS ($n = 3$) in 50 ml of phosphate-buffered saline (PBS, pH 7.4). The sample tubes were incubated at 37 °C under continuous shaking. The tubes were centrifuged, and the supernatant was withdrawn at a scheduled time. To extract fentanyl from the buffer solution, 10 μl of IS (1 μg/ml) was added to 200 μl of sample in a centrifuge tube. The aqueous phase was extracted with 600 μl of 5% isopropylalcohol in *n*-butyl chloride. The tube was vortex-mixed and the upper organic phase

was transferred to a second centrifuge tube. The sample was evaporated in a vacuum concentration system (Spinvac, Hanil, Korea) at 40 °C. Extraction residue was reconstituted in 50 μl toluene, sonicated, and centrifuged at 12 000 rpm. The solution was injected into the GC system via splitless mode.

2.6. Quantitative analysis of fentanyl

The amount of fentanyl released from the FMS was determined by GC analysis as follows (Choi et al., 2001). Chromatography was performed on a Hewlett-Packard 6890 GC, equipped with an autosampler (HP 7683) and a NPD. High purity helium was used as the carrier gas at a constant pressure of 25 psi. A HP-5 5% phenyl methyl siloxane capillary column (60 m × 0.32 mm I.D. and 0.25 μm film thickness) was used. The initial oven temperature was 150 °C for 1 min. The oven temperature was programmed to 270 °C at 30 °C/min, held 2 min, then to 280 °C at 5 °C/min, and held 9 min (overall run time 18 min). The temperature of the injector and the detector were maintained at 285 and 310 °C, respectively. Flow rates were 2.0 ml/min for the helium gas, 60 ml/min for air, and 3.0 ml/min for hydrogen. Deactivating all glassware, including disposable culture tubes and the injection port liner, with a 5% solution of dimethyldichlorosilane and vapor of hexamehyldisilazane (HMDS) were necessary to avoid adsorption of the drug onto the glassware and optimize recovery.

2.7. Determination of drug content

The encapsulation efficiency of microspheres was measured by dissolving 5 mg of FMS in 10 ml of methylene chloride and reprecipitating the polymer with a known volume of methyl alcohol. After filtration, the solution was injected in GC system operated according to above described method. Fentanyl content in FMS was calculated as the ratio of actual-to-theoretical drug content in the microspheres.

3. Results and discussion

3.1. Morphology of FMS

The morphology of microspheres depends on the rate of polymer precipitation and solvent removal at the interface (Khang et al., 2000). The surface is generally smooth when the polymer precipitates slowly due to slow removal of the organic solvent. Moreover, the presence of PVA in W phase enhances the stability of the emulsion for the O/W system. In this study, morphologies of FMS with different PLGA concentrations were observed (Fig. 2). All FMS from various preparation conditions had a spherical shape and smooth surface with few pores. From the cross-sectional images of the microspheres, it is clear that internal phase of the microspheres are all highly porous and the hollow core are increased with increasing FMS size. In our case, although solvent removal is slow, the internal porosity is generally high since the fabrication temperature is relatively high (35 °C). Subsequently, FMS mean size increases and the particle size distribution widens with in-

Table 2
The preparation conditions and characteristics of FMS (10% drug loading, 3 wt/vol% emulsifier, and 10 ml EA): effect of polymer concentration

Batch	Polymer concentration (wt/vol%)	FMS mean size ± SD (μm)	Encapsulation efficiency (%)	Yield (%)
B1	3	9.2 ± 6.2	62.7	87.6
B2	5	11.6 ± 6.3	77.1	78.4
A2[a]	10	16.4 ± 8.6	83.5	81.8
B3	20	31.6 ± 14.2	99.8	58.9

[a] Control.

Table 3
The preparation conditions and characteristics of FMS (10% drug loading, 10 wt/vol% polymer, and 3 wt/vol% emulsifier): effect of solvent volume

Batch	Solvent volume (ml)	FMS mean size ± SD (μm)	Encapsulation efficiency (%)	Yield (%)
C1	EA (5)	18.5 ± 11.2	87.2	78.6
A2[a]	EA (10)	16.4 ± 8.6	83.5	81.8
C2	EA (20)	14.2 ± 4.2	79.5	69.3
C3	EA (40)	9.51 ± 5.6	62.1	52.8

[a] Control.

crease in the O phase concentration. Although, all the FMS have a hollow internal structure, FMS fabricated at low concentration of O phase have a uniform size distribution and a very dense layer, while microspheres fabricated at higher concentration of O phase have a thicker but porous cross-section and bigger pores in the middle of the microspheres. As shown in Fig. 3, fentanyl showed several peaks corresponding to the crystalline form while PLGA is mainly amorphous with a poor crystalline part. On the other hand, samples of microspheres (A2) and fentanyl/PLGA mixture after dissolution in EA and evaporation (containing the same quantities of fentanyl and PLGA) showed a halo pattern in which diffraction peaks of drug have decreased indicating that fentanyl in both preparations was in the amorphous state. These results proved that fentanyl and PLGA, after dissolution in EA, produced after solvent evaporation of the solvent a solid dispersion in which the drug was dispersed in a molecular state. Similar results have been reported from high molecular weight PLGA microspheres containing AZT and fentanyl (Lee et al., 1999; Khang et al., 2000).

3.2. Quantitative analysis of fentanyl

There have been lots of efforts for minimizing the complex and time-consuming steps to extract fentanyl (Choi et al., 2001). We obtained an optimal condition of extraction solvents and instrumentals for fentanyl. From this condition, fentanyl was well separated on the GC chromatogram with a run time of 12.4 min (Fig. 4). Fentanyl stock solution (100 μg/ml) was prepared by dissolving fentanyl base in methanol solution. For the preparations of aqueous samples, quality controls, and calibration standards were extracted using silanized centrifuge tubes. The standard curve of fentanyl was linear over the concentration range from 0.5 to 50 ng/ml. The average slope of the standard curves and the average correlation coefficient were 1.6608 and 0.9997, respectively. The limit of detection and limit of quantitation were 0.1 and 0.5 ng/ml, respectively.

3.3. Effect of initial drug loading

To determine an optimal drug loading, various ratios of initial drug loading to the polymer matrix, 5, 10, 20 and 30%, were investigated (Table 1). A decrease in encapsulation efficiency attributed to the increase in the ratio of initial drug loading to the polymer, but did not influence significantly either the mean diameter or size distribution of FMS. The influence of drug loading on the release profiles of fentanyl is shown in Fig. 5. The release rate of A1 (5% drug loading) was slower than that of A4 (30% drug loading) because the crystals of fentanyl in low initial drug loading were finely dispersed in the PLGA matrix and the release pattern of fentanyl was not affected by the biodegradation of PLGA. In contrast, higher initial drug loading entrapped drugs in the polymer matrix like network and drugs were released by simple dissolution and diffusion (Sturesson et al., 1993; Guyot and Fawaz, 1998).

3.4. Effect of polymer concentration

As shown in Table 2, the PLGA concentrations in O phase (10 ml EA, fixed) were 3, 5, 10, and 20 wt/vol%, respectively. The larger size of micro-

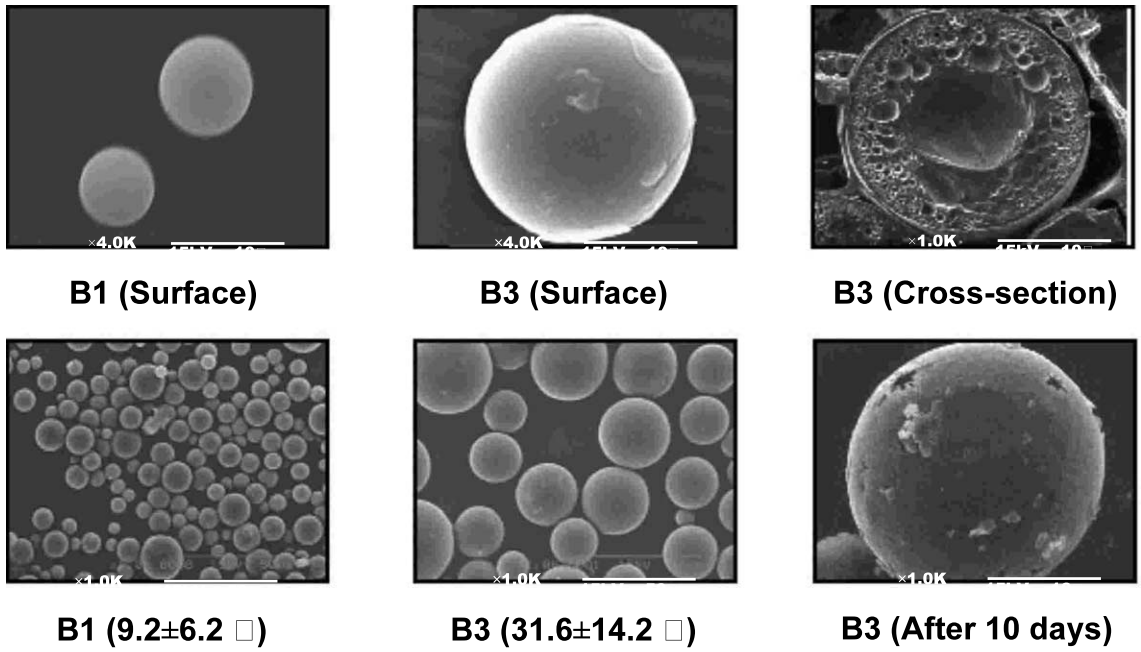

Fig. 2. SEM morphology of FMS with different preparation conditions.

spheres was obtained from a higher PLGA concentration than a lower, also lower solvent volume came to the same results: batch B3 (20% PLGA) exhibited the highest average diameter, 31.6 μm. It is explained that increasing of PLGA concentration resulted in high solution viscosity and increasing microspheres size (Guyot and Fawaz, 1998; Khang et al., 1999, 2000; Lee et al., 1999, 2000). The cumulative drug release profiles of FMS from different PLGA concentrations are shown in Fig. 6. Whatever the types of FMS, no burst effect has been observed and all FMS exhibited a S-shaped release pattern. The slowest release rate was observed in B1 (3% PLGA). From results of the SEM graphs and Table 2, we observe that microspheres of a larger mean size have a lower density. The density relationship with formation of hollow structure microspheres in general translates into a faster drug release. Thus, microspheres fabricated at high concentration of PLGA are characterized by a lower density, fully porous internal structure (Fig. 2), larger mean size and broader size distribution (Table 2).

3.5. Effect of solvent volume

The influence of solvent volume at a constant W phase volume (100 ml) on the FMS characteristics was studied in Table 3. The O phase volume had a significant effect on the size of FMS: the

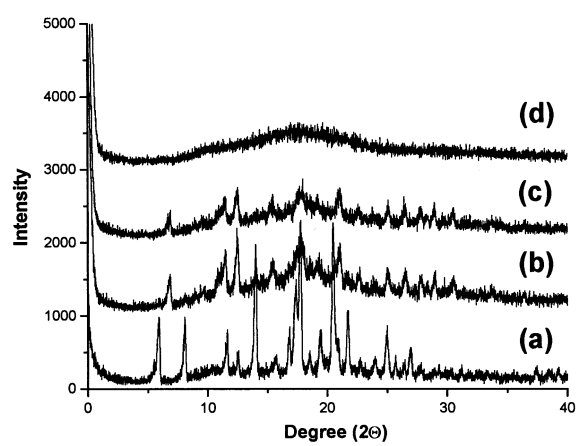

Fig. 3. X-ray diffraction patterns of: (a) fentanyl base; (b) physical mixture of fentanyl/PLGA (10/90); (c) 10% drug-loaded microspheres; and (d) PLGA.

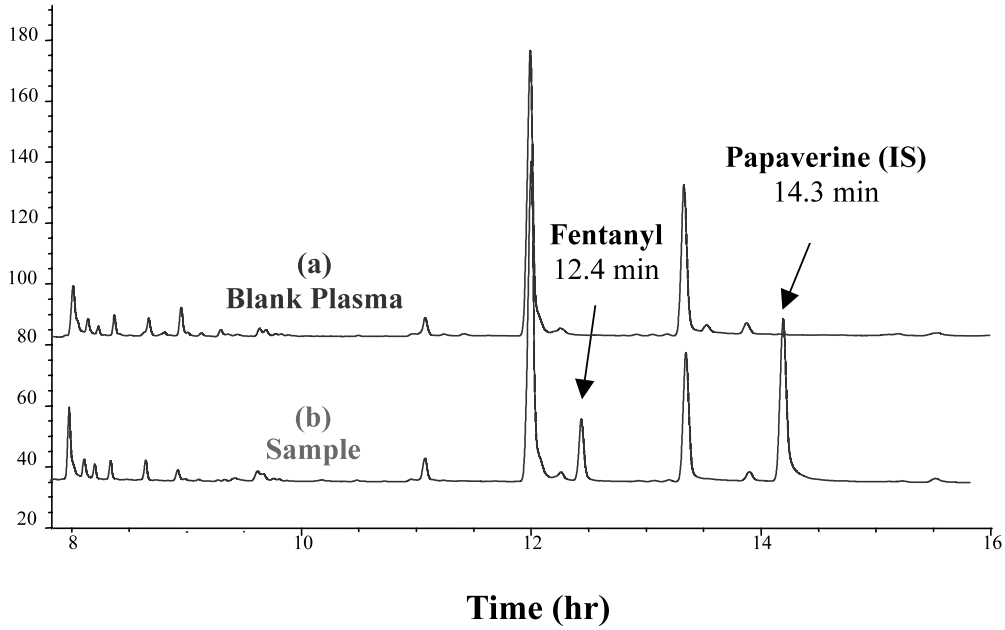

Fig. 4. Chromatograms of: (a) blank plasma sample; and (b) extracted standard sample containing 10 ng/ml fentanyl.

FMS fabricated at 5 ml of EA were bigger (C1, 18.5 ± 11.2 μm) than those fabricated at 40 ml of EA (C3, 9.51 ± 5.6 μm). The viscosity of the O phase was increased with decreasing solvent volume, as the polymer weight and the volume of water were fixed. Consequently, high solvent volume yielded a matrix characterized by more numerous and smaller size (Spenlehauer et al., 1986; Schlicher et al., 1997; Yang et al., 2001). Moreover, it could be observed that the FMS from a high solvent volume showed further long release profiles than those from a low volume, which was the same result of low polymer concentration (Fig. 7).

4. Conclusion

Biodegradable microspheres for controlled analgesics release were manufactured and their release patterns were investigated successfully. To obtain the sustained fentanyl delivery with effective and precise control, FMS were fabricated using the conventional O/W emulsion solvent-evaporation method. From X-ray and SEM results, it appeared that experimental conditions used in this work allowed the formation of microspheres having a matrix structure in which fentanyl was uniformly dispersed in molecular state. From the results of preparation conditions, the encapsulation efficiencies of drug were between

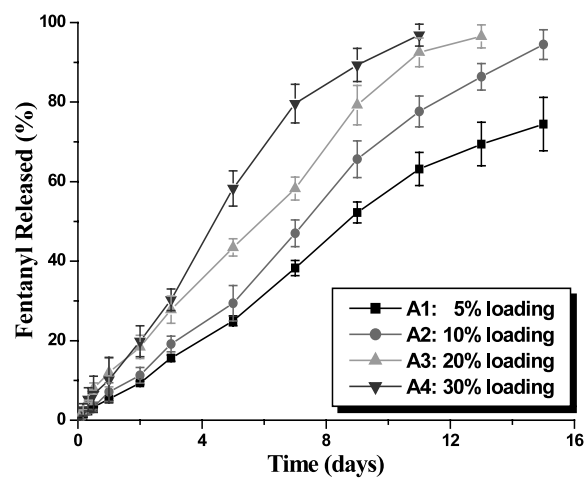

Fig. 5. Effect of initial drug loading ratio on the fentanyl release pattern ($n = 3$, 10 wt/vol% polymer, 3 wt/vol% emulsifier, and 10 ml EA).

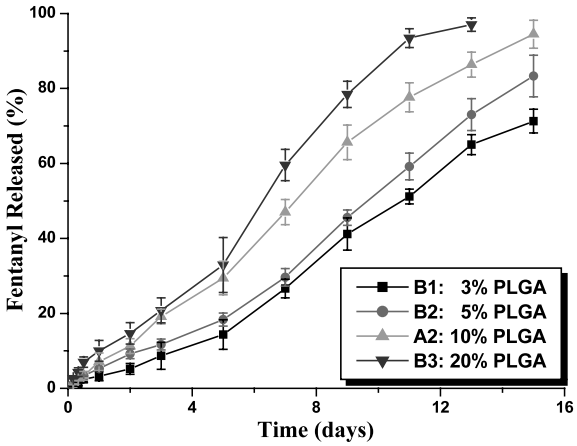

Fig. 6. Effect of polymer concentration on the fentanyl release pattern ($n = 3$, 10% drug loading, 3 wt/vol% emulsifier, and 10 ml EA).

61.5 and 99.8%, depending on the particular formulation. The total microspheres recovered amount varied between 52.8 and 87.6%. It was observed that the encapsulation efficiency and the yield decreased as a function of the increase in initial drug loading amount (Table 1). As increasing of PLGA concentration from 3 to 20%, encapsulation efficiency was increased from 62.7 to 99.8% (Table 2). Similar results were also obtained when decreasing the solvent volume (Table 3). We assumed that the dominating loss of fentanyl must be due to transport of droplets of the O phase to the W phase. The increased viscosity in the O phase caused by the increased PLGA concentration or the decreased solvent volume will decrease the loss transport of fentanyl and contribute to the enhanced entrapment efficiencies (Raouf et al., 1996). Moreover, the O droplets containing fentanyl formed from the W phase were very small and the diffusion amount of fentanyl to the external phase during the solvent evaporation was relatively small, explaining the high encapsulation efficiency obtained. The pattern of drug release depends on various factors, such as initial drug loading ratio, polymer concentration, and solvent volume in W phase. Generally, small size and fast drug release are attributed to more water uptake, swelling ratio, and polymer degradation (Jalil and Nixon, 1990a,b,c; Wada et al., 1991). In contrast, in our case, fentanyl release rate from all batches decreased with decreasing microsphere size (Figs. 5–7). It might be suggested that the drug release profiles would be affected by the morphology of the microspheres; increased concentration of O phase made porous stable microspheres and resulted in denser microspheres. From the results, this sustained, besides, constant localized release system can potentially provide anesthesia for a longer period than injection or topical administration. Studies on the animal experiments are in progress.

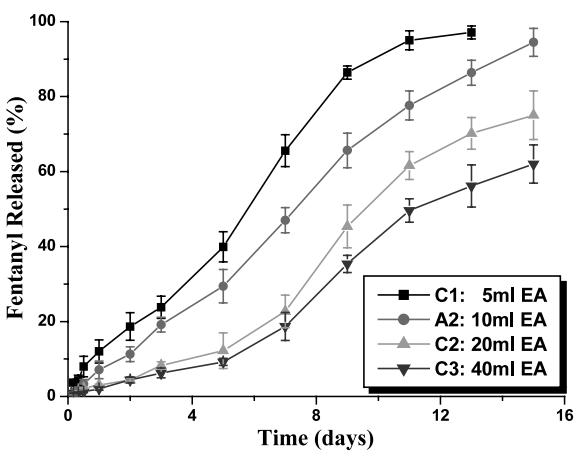

Fig. 7. Effect of solvent volume on the fentanyl release pattern ($n = 3$, 10% drug loading, 10 wt/vol% polymer, and 3 wt/vol% emulsifier).

Acknowledgements

This work was supported by KMOHW (Grant No.; HMP-98-G-2-050-A).

References

Arshady, R., 1990. Biodegradable microcapsular drug delivery systems: manufacturing methodology, release control and targeting prospects. J. Bioact. Compatible Polym. 5, 315–342.

Baselt, R.C., 1982. Disposition of Toxic Drugs & Chemicals in Man, 2nd ed. Biomedical Publications, Davis, CA, pp. 325–326.

Beck, L.R., Pope, V.Z., Tice, T.R., Gilley, R.M., 1985. Long-acting injectable microsphere formulation for the par-

enteral administration of levonorgestrel. Adv. Contracept. 1, 19–129.

Cho, J.C., Khang, G., Choi, H.S., Rhee, J.M., Lee, H.B., 2000. Preparation of biodegradable PLGA microspheres for sustained local anesthesia and their in vitro release behavior. Polymer (Korea) 24, 728–735.

Cho, J.C., Khang, G., Rhee, J.M., Lee, H.B., 2001. Influence of processing variables on fentanyl citrated-loaded PLGA microspheres by novel W*/O/O solvent evaporation method. Korea Polymer J., in press.

Choi, H.S., Shin, H.C., Khang, G., Rhee, J.M., Lee, H.B., 2001. Quantitative analysis of fentanyl in rat plasma by gas chromatography with nitrogen phosphorous detection. J. Chromatogr. B. 765, 63–69.

Garriott, J.C., Rodriquez, R., Di Mario, V.J.M., 1984. A death from fentanyl overdose. J. Anal. Toxicol. 8, 288–289.

Guyot, M., Fawaz, F., 1998. Nifedipine loaded-polymeric microspheres: preparation and physical characteristics. Int. J. Pharm. 175, 61–74.

Hammargren, W.R., Henderson, G.L., 1988. Analyzing normetabolites of the fentanyls by gas chromatography/electron capture detection. J. Anal. Toxicol. 12, 183–191.

Ikeda, R., Pelton, C., 1990. Diversion programs for impaired physicians. West. J. Med. 152, 617–621.

Jalil, R., Nixon, J.R., 1990a. Biodegradable poly(lactic acid) and poly(lactide-co-glycolide) microcapsules: problems associated with preparative techniques and release properties. J. Microencapsul. 7, 297–325.

Jalil, R., Nixon, J.R., 1990b. Microencapsulation using poly(D,L-lactic acid). II. Effect of polymer molecular weight on the microcapsule properties. J. Microencapsul. 7, 245–254.

Jalil, R., Nixon, J.R., 1990c. Microencapsulation using poly(L-lactic acid). III. Effect of polymer molecular weight on the microcapsule properties. J. Microencapsul. 7, 41–52.

Khang, G., Cho, J.C., Lee, J.W., Rhee, J.M., Lee, H.B., 1999. Preparation and characterization of Japanese encephalitis virus vaccine loaded PLGA microspheres for oral immunization. Bio-Med. Mater. Eng. 9, 49–59.

Khang, G., Lee, J.H., Lee, J.W., Cho, J.C., Lee, H.B., 2000. Preparation and characterization of PLGA microspheres for the sustained release of AZT. Korea Polymer J. 8, 80–88.

Lee, H.B., Khang, G., Cho, J.C., Rhee, J.M., Lee, J.S., 1999. Fentanyl-loaded PLGA microspheres for local anesthesia. Polymer Preprints 40, 288–289.

Lee, H.B., Khang, G., Cho, J.C., Rhee, J.M., Lee, J.S., 2000. Fentanyl-loaded PLGA microspheres for long acting anesthesia. In: Park, K., Mrsny, R.J. (Eds.), Controlled Drug Delivery: Designing Technologies for the Future. ACS, Washington DC, pp. 385–394.

Levine, B., Goodin, J.C., Caplan, Y.H., 1990. A fentanyl fatality involving midazolam. Forensic Sci. Int. 45, 247–251.

Lewis, D.H., 1990. Controlled release of bioactive agents from lactide/glycolide polymers. In: Chasin, M., Langer, R. (Eds.), Biodegradable Polymers as Drug Delivery Systems. Dekker, New York, pp. 1–41.

Marchall, B.E., Longnecker, D.E., 1990. General anesthetics. In: Gilman, A.G., Rall, T.W., Nies, A.S., Taylor, P. (Eds.), The Pharmacological Basis of Therapeutics, 8th ed. Pergamon Press, New York, pp. 305–307.

Maria, J., Kamel, B., Oliver, Z., Dani, A., Piero, O., Frederic, H., Hans, P.M., Bruno, G., 2000. In vitro and in vivo evaluation of a somatostatin analogue released from PLGA microspheres. J. Control Rel. 7, 19–28.

Matecjczyk, R.J., 1988. Fentanyl related overdose. J. Anal. Toxicol. 12, 236–238.

Moffat, A.C., 1986. Clarke's isolation, identification of drugs in pharmaceuticals. In: Body Fluids and Post-Mortem Materials, 2nd ed. Pharmaceutical Press, London, pp. 617–618.

Pare, E.M., Monforte, J.R., Gaul, R., Mirchandani, H., 1987. A death involving fentanyl. J. Anal. Toxicol. 11, 272–275.

Raouf, G., Cecilia, S., Johan, C., 1996. Effect of preparative parameters on the characteristics of poly(D,L-lactide-co-glycolide) microspheres made by the double emulsion method. Int. J. Pharm. 141, 205–216.

Schlicher, E.J., Postma, N.S., Zuidema, J., Talsma, H., Hennink, W.E., 1997. Preparation and characterization of poly(D,L-lactic-co-glycolic acid) microspheres containing desferrioxamine. Int. J. Pharm. 153, 235–245.

Spenlehauer, G., Veillard, M., Benoit, J.P., 1986. Formation and characterization of cisplatin loaded poly(D,L-lactic-co-glycolic acid) microspheres for chemoembolization. J. Pharm. Sci. 75 (8), 750–755.

Sturesson, C., Carlfores, J., Edman, K., Andersson, M., 1993. Preparation of biodegradable poly(lactic-co-glycolic) acid microspheres and their in vitro release of timolol maleate. Int. J. Pharm. 89, 235–244.

Wada, F., Hyon, S., Ikada, Y., 1991. Salt formation of lactic acid oligomers as matrix for sustained release of drugs. J. Pharm. Pharmacol. 43, 605–608.

Yang, Y.Y., Chung, T.S., Ng, N.P., 2001. Morphology, drug distribution, and in vitro release profiles of biodegradable polymeric microspheres containing protein fabricated by double-emulsion solvent extraction/evaporation method. Biomaterials 22, 231–241.