—Regular Article—

Simultaneous Determination of Ropivacaine, Bupivacaine and Dexamethasone in Biodegradable PLGA Microspheres by High Performance Liquid Chromatography

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A simple and rapid high-performance liquid chromatography method coupled with UV detector was developed and validated for the simultaneous determination of ropivacaine, bupivacaine and dexamethasone in biodegradable poly (lactic-co-glycolic acid) (PLGA) microspheres within 11 min. Chromatographic separation was performed on a XDB-C_{18} column using a mobile phase comprised of acetonitrile-NaH_2PO_4 buffer (pH 3.5, 30 mM) (30 : 70, v/v) with a flow rate gradient program. The method was in good linearity ($r>0.999$) over the range of 0.025–40.0 μg/ml for ropivacaine and bupivacaine, and 0.05–40 μg/ml for dexamethasone. The method was proved to be precise with intra- and inter-day precision less than 3.0% and 6.0% for all drugs and accurate with intra- and inter-day accuracy between −8.0% to 4.5% and between −5.0% to 5.5% for all drugs. The assay was rapid, simple and easy to apply. Therefore, it was very suitable for routine determination and quality control of ropivacaine, bupivacaine and dexamethasone in PLGA microspheres.

Key words——HPLC; simultaneous determination; poly(lactic-co-glycolic acid) (PLGA); ropivacaine; bupivacaine; dexamethasone

INTRODUCTION

Ropivacaine (ROP) and bupivacaine (BUP) were widely used amide-type local anesthetics for surgery and postoperative pain relief in clinic.[1–3] However, the two drugs provide only a limited duration of analgesia after a single dose. To obtain long duration of local anaesthetic action, as well as to minimize the side effects and system toxicity, new methods or regimens for controlled-release local anesthetics are strongly required. In recent, a controlled-release drug delivery system of biodegradable and biocompatible poly(lactic-co-glycolic acid) (PLGA) microspheres containing BUP,[4–6] or ROP,[7,8] or both BUP and dexamethasone (DXM)[9–12] has been largely investigated. DXM, a synthetic steroidal anti-inflammatory drug, is used as an adjuvant to local anesthetics to increase the duration of analgesia.[13–15] Now, interest is increasing in investigation of PLGA microspheres loading both ROP and DXM because ROP has less cardiovascular and nervous toxicity than BUP.[3,16]

During the investigation of PLGA microspheres, many experiments, which can be considered as a very important part job of the investigation, such as encapsulation efficiency, drug loading capacity, and *in vitro* drug release, are needed to be carried out. Many studies are also needed to be performed before the controlled-release drug delivery system was developed. Therefore, to facilitate the pharmaceutical development of PLGA microspheres loading ROP, BUP and DXM, a rapid and specific analytical method is necessary to analyze and quantify the drugs.

Many methods, including HPLC-UV,[17–20] HPLC-MS,[21–24] GC[25] and GC-MS,[26–28] were reported for the analysis of BUP[17,22,25], ROP[17,18,21,22,24,27,28] or DXM[20,23,26,29] in biological matrix (blood, urine) separately. However, these methods are not suitable for the simultaneous analysis of ROP, BUP and DXM in PLGA microspheres because of the limitation of complex sample preparation or long analysis time. In this paper, a simple, rapid, specific and confirmatory HPLC method was described in detail for the simultaneous determination of ROP, BUP and DXM in PLGA microspheres. The developed method involved short running time, wide dynamic linear range and simple sample preparation. These advantages make the method especially suitable for the routine analysis of ROP, BUP and DXM during the investigation of PLGA microspheres. The method was

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also successfully applied to the analysis of ROP, BUP and DXM in PLGA microspheres prepared in house.

EXPERIMENTAL

Chemicals and Reagents Ropivacaine hydrochloride (99% purity), bupivacaine hydrochloride (99% purity) and dexamethasone (99% purity) were purchased from Sigma (St. Louis, MO, USA). Poly (lactic-co-glycolic) (PLGA) (50 : 50, MW : 30000) and polyvinyl alcohol (PVA) (MW : 30000–70000) were purchased from Sigma Inc., USA. Acetonitrile and methanol were HPLC grade, obtained from Fisher Scientific (Fair Lawn, New Jersey). All other reagents were of analytical grade. Deionized water was obtained from a Milli-Q deionization system (Millipore, Bedford, MA).

Preparation of Microspheres Microspheres were prepared by an oil-in-water (O/W) emulsion/solvent evaporation technique.[30,31] In brief, appropriate amounts of ROP and DXM or BUP and DXM were dissolved in 10.0 ml of acetone. The solution was then emulsified with 20.0 ml of methylene chloride solution containing 400 mg of PLGA. The emulsification was performed by ultrasonication for 1 min at 100 W. The primary emulsion was added into 50 ml of aqueous solution containing 150 mg PVA as a surfactant and was ultrasonicated for 8 min to form a double emulsion. Then, the double emulsion was under moderate magnetic stirring for 3 h at room temperature to remove the organic solvents. The microspheres were collected and cleaned by re-suspending in deionized water and centrifuging at 10000 rpm at room temperature for three times. The final product was freeze-dried for 24 h to obtain a fine white powder. The drug-free microspheres were prepared as the same procedures. The samples were stored in a dryer at 4°C before use.

Chromatography The HPLC system was Shimadzu LC-20AT series (Kyoto, Japan) coupled with a controller (CBM-20A), a quaternary gradient pump, a ultra-violet diode array detector (DAD), an automatic sampler, and an on-line degasser. LCsolution chromatography software was used for operation, data acquisition and analysis. The chromatographic separation was performed on a ZORBAX Eclipse XDB-C_{18} column (4.6 mm×150 mm, 5 μm particle size, Agilent Technologies, USA), protected by a ZORBAX Eclipse XDB-C_{18} analytical guard column (4.6 mm×12.5 mm, 5 μm particle size, Agilent Technologies, USA) at 25°C.

The mobile phase was acetonitrile-NaH_2PO_4 buffer (30 mM, pH 3.5 adjusted with H_3PO_4 (30 : 70, v/v). The mobile phase was filtered by a 0.45 μm nylon membrane and degassed 10 min by ultrasonic equipment before use. The flow rate was 0.8 ml/min from 0 to 7 min and 1.6 ml/min from 8 to 12 min. After 12 min, the flow rate was returned to 0.8 ml/min and kept for 1 min to equilibrate the column. The injection volume was 20 μl. The target compounds were detected by ultra-violet detector in the range of 195～350 nm. The monitoring wavelength was set at 210 nm for ROP, BUP and 240 nm for DXM, respectively.

Stock and Standard Solutions Stock solutions of ROP, BUP and DXM at a concentration of 1.00 mg/ml were prepared in methanol, respectively. All the stock solutions were stored in brown glass bottles at −20°C before use. A standard solution mixture of ROP, BUP and DXM at concentration of 200 μg/ml was prepared by mixing individual stock solution and diluted with methanol. This mixture solution was used to prepare the quality control samples and the calibrators of calibration curve.

Sample Solution Preparation Twenty-five milligrams of microspheres and 3 ml of acetonitrile were transferred into a 5 ml volumetric flask. The solution was diluted to the mark of the flask with acetonitrile after ultrasonicated for 10 min. Then the solution was centrifuged at 10000 rpm for 10 min. To an eppendorf tube, 100 μl of clear supernatant and 900 μl of mobile phase were transferred. Then the solution was centrifuged at 10000 rpm for 5 min after vortex-mixed for 1 min. The clear supernatant of 20 μl was injected into the LC system.

Quality control samples (QCs) used for validation were prepared as follows. Twenty-five milligrams of drug-free microspheres were spiked with ROP, BUP and DXM standard solution. The final microsphere solutions at concentration of low (0.1 μg/ml), medium (2 μg/ml) and high (20 μg/ml) level were obtained after the spiked microspheres were dealt with the same process described as above.

Method Validation

Specificity The specificity of the method was evaluated by all the possible interference of impurities, degradation products or related components from PLGA microspheres. Three different batches of drug-free microspheres were analyzed by the HPLC

Table 1. Results of the Stress Condition Experiments

Stress conditions	Retention time of degradation products (min)		Peak purities		
	210 nm	240 nm	ROP	BUP	DXM
Basic (0.1 mol/l NaOH, room temperature, 4 h)	4.096, 4.576, 7.413	4.096, 4.576, 7.413, 7.744, 8.801, 10.592, 11.563	0.9999	0.9997	1.000
Acid (0.1 mol/l HCl, room temperature, 24 h)	4.334, 6.820	3.904, 4.334, 7.01, 8.149	0.9999	1.000	1.000
Oxidative (3% H_2O_2, room temperature, 24 h)	1.899, 2.336, 5.535, 8.106, 9.173	1.899, 9.173	1.000	0.9998	1.000
Sunlight (sunlight, room temperature, 24 h)	4.221, 5.839, 8.998	2.825, 3.936, 6.053, 9.211, 11.461	0.9997	1.000	1.000
Temperature (60°C, 24 h)	N[a]	4.081, 6.083, 8.291, 8.831	1.000	1.000	1.000

N[a], no degradation peak was observed.

procedure. Each blank PLGA microsphere solution was consecutive injected three times.

Within the study of specificity, a series of degradation studies were carried out, where the blank PLGA microsphere solutions spiked with 20 μg/ml of ROP, BUP and DXM were subjected to different degrees of stress as shown in Table 1, by following the ICH guidelines.[32] After HPLC-UV analysis, peak resolutions and peak purities of all drugs were tested.

Linearity, LOD and LLOQ Drug-free microsphere solutions were spiked with appropriate amount of standard working solution to obtain the calibrators: 0.025, 0.05, 0.2, 0.5, 1.0, 5, 10, 20, 40 μg /ml for ROP and BUP, and 0.05, 0.2, 0.5, 1.0, 5, 10, 20, 40 μg/ml for DXM. The curves were obtained by plotting the peak area *versus* the concentration of the drugs.

The limit of detection (LOD) and the lower limit of quantification (LLOQ) were defined as the concentration with a signal-to-noise (S/N) of at least 3 and 10.

Precision and Accuracy To measure the system precision, a standard solution mixture of ROP, BUP and DXM at concentration of 2 μg/ml was consecutively injected in six times.

The precision and accuracy of the method was determined by repeated analysis of low, medium and high levels of QC samples in six replicates on six different days. Precision was reported as relative standard deviation (RSD, %) and accuracy as relative error (RE, %).

Stability The short-term stability and long-term stability of standard solution of the analytes were evaluated under different storage conditions. The long-term stability was evaluated after the stock solutions stored at −20°C for 3 months. For short-term stability, working standard solution and microsphere solution were assayed after stored at room temperature (25°C) for 48 h and in a refrigerator at 4 °C for 4 days. Each determination was performed in triplicate. The results were evaluated by comparing peak area with those of freshly prepared standard solutions.

RESULTS AND DISCUSSION

Selection of UV Detection Wavelength After DAD detection, the wavelength of 210 nm was selected for the quantification of ROP, BUP and 240 nm for DXM because absorbance of ROP and BUP at 210 nm was sensitive enough and DXM has the maximum UV absorbance at 240 nm.

Optimization of Chromatographic Conditions To obtain good separation of the analytes in a short running time, the composition of mobile phase, pH (range 2.0～5.0) and phosphate concentration (range 5～50 mM) of buffer and the elution model were investigated. All the experiments were carried on a C_{18} column.

Since the microspheres were easily dissolved in acetonitrile, acetonitrile was selected for the mobile phase. Different ratios of acetonitrile in mobile phase were tested. After a number of experiments, acetonitrile and buffer solution in volume ratio of 30 : 70 was chosen for the best resolution.

The pH of buffer influenced the retention time of ROP and BUP greatly but did little to DXM. With the pH increasing, the retention time of ROP and BUP increased and the peak shapes of ROP and BUP became asymmetric. When pH was 3.5, appropriate retention time, good resolutions and symmetric peak was obtained.

The phosphate concentration had similar effects on

the analytes as the pH did. When 30 mM phosphate was in buffer, suitable retention time and sharp peaks were obtained. Therefore, 30 mM phosphate was chosen for further experiments.

In the course of HPLC method development, isocratic elution and gradient elution at 1 ml/min flow rate were also tested. By using isocratic elution, ROP and BUP were eluted fast while DXM was very slow because the different polarity of the drugs. So, it was too long for isocratic elution to elute all the analytes. Although shorter time for the gradient elution to elute the three compounds, the baselines were drifted severely and at least 5 min was needed for column equilibrium before next injection. At last, a flow rate gradient model was selected (*see* **Chromatography** section). Under the flow rate gradient model, the system equilibration was easy to finish because of no change in the composition of the mobile phase during the running time.

Baseline separation of ROP, BUP and DXM was achieved within 11 min under the optimum chromatographic conditions. Representative chromatograms at 210 nm and 240 nm of drug-free microsphere solution, drug-free microsphere solution spiked with 2 μg /ml of the analytes and PLGA sample were shown in Fig. 1. The retention times of ROP, BUP and DXM were 3.49, 4.92 and 9.94 min, respectively. Although largely different polarity between ROP, BUP and DXM, the total running time, including the column equilibration time, for each sample was only 13 min. It was a rapid and economic method for the simultaneous determination of ROP, BUP and DXM in the pharmaceutical study and quality control.

Specificity The chromatogram of blank PLGA

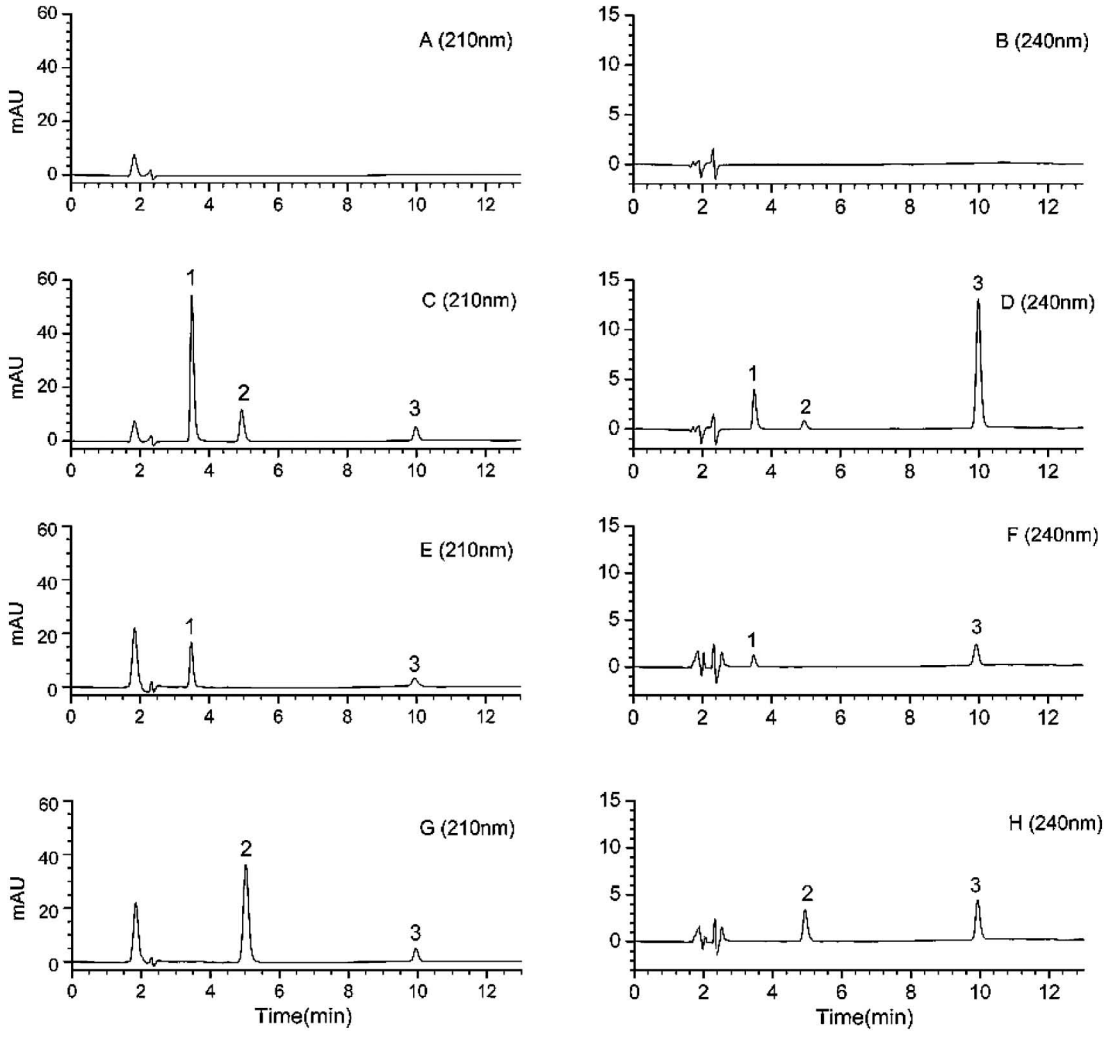

Fig. 1. Chromatograms of ROP, BUP and DXM in PLGA

(A, B) drug-free PLGA microsphere solution, (C, D) drug-free PLGA microsphere solution spiked with 2.0 μg/ml of analytes, (E, F) PLGA microspheres loading ROP and DXM, (G, H) PLGA microspheres loading BUP and DXM (1-ROP, 2-BUP and 3-DXM).

microspheres (Fig. 1(A) and (B)) showed no peaks appear at the retention time of ROP, BUP and DXM. When blank PLGA microspheres spiked with standard solution, the peaks of target analytes were pure (purity index >0.9998). It indicated that the developed method was specific and selective against the interferences from PLGA microspheres.

Degradation tests were also carried out to evaluate the specificity of the method. The degradation products obtained from different degradation stress were listed in Table 1 and the representative chromatograms corresponding to the degradation stress conditions were shown in Fig. 2. Under different stress conditions, several major degradation products (peaks at 4.096, 4.576 and 7.413 min of basic stress, peaks at 3.904 and 7.010 min of acid stress, peaks at

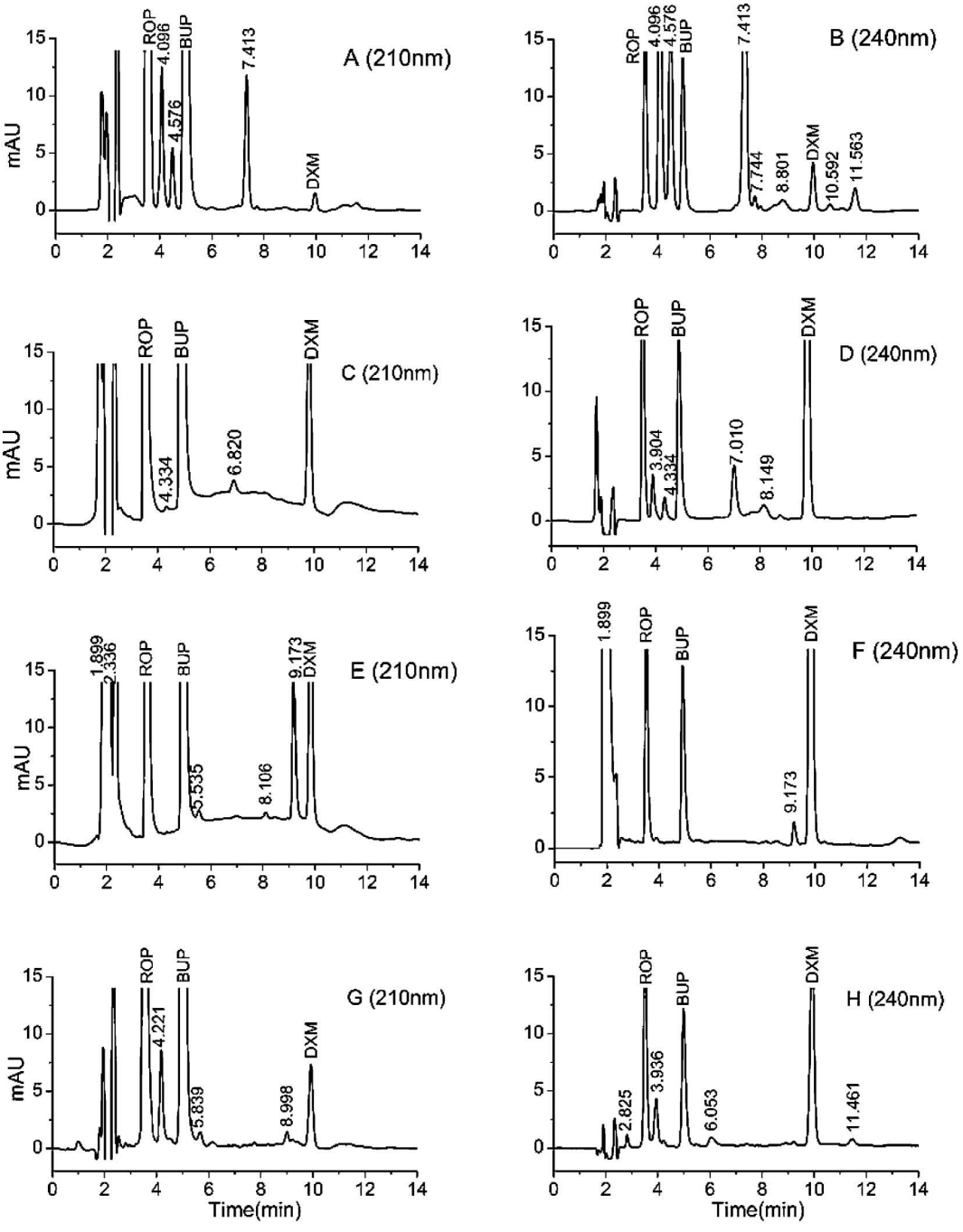

Fig. 2. Chromatograms of Degradation Tests

(A, B) basic stress (0.1 mol/l NaOH, room temperature, 4 h), (C, D) acid stress (0.1 mol/l HCl, room temperature, 24 h), (E, F) oxidative stress (3% H_2O_2, room temperature, 24 h), (G, H) sunlight stress (sunlight, room temperature, 24 h).

1.899 and 2.336 min of oxidative stress and peaks at 4.221 and 3.936 min of sunlight stress) and different small degradation peaks were performed. No obvious degradation products were observed under 60℃ for 24 h except several small peaks at 240 nm (Chromatogram was not shown). It is not clear about what the degradation products are and more studies should be carried out to uncover how the degradation products are produced, but the target analytes were baseline separated and had good resolutions ($R>2$) to the adjacent peaks (Fig. 2). The peaks of target analytes under different stress conditions were pure (purity index ≥ 0.9996) (Table 1). The purity indexes indicated that there was no degradation products co-eluted with the target analytes.

According to the results, it can be concluded that the proposed method was specific.

Linearity, LOD and LLOQ Calibration curves were calculated by the peak area (y) *versus* the concentration (x) of the analytes using weighted least squares linear regression analysis (the weighting factor was $1/C$). Good linearity was achieved over the range 0.025−40 μg/ml for ROP and BUP, and 0.05−40 μg/ml for DXM. The regression equations of ROP, BUP and DXM were $y=104700.67x-604.81$ ($r=0.9998$, $n=6$), $y=56578.88x-245.99$ ($r=0.9995$, $n=6$) and $y=32233.52x-133.16$ ($r=0.9996$, $n=6$), respectively.

The LOD of ROP, BUP and DXM for this method were 0.0036, 0.0061 and 0.0085 μg/ml at a signal-to-noise ratio of 3. The LLOQ of ROP, BUP and DXM were 0.012, 0.020 and 0.028 μg/ml at a signal-to-noise ratio of 10, respectively. The LODs and LLOQs are low enough for the determination of ROP, BUP and DXM in the researches and quality control of pharmaceutical formulations since the contents of the drugs should be high enough to make sure the drug action.

Precision and Accuracy The system precision was evaluated by six consecutive injections of standard solution containing 2μg/ml of ROP, BUP and DXM. The results (Table 2) of retention time, resolution, peak area, peak tailing and theoretical plates showed that the repeatability of the system was good enough for the simultaneous analysis of the ROP, BUP and DXM.

The precision (RSD, %) of intra- and inter-day precision were smaller than 3% and 6% for ROP, BUP and DXM, respectively. The accuracy (RE, %) of intra- and inter-day ranged from −6.0% to 4.5% and from −2.0% to 3.0% for ROP, ranged from −5.0% to 1.0% and from −3.2% to 5.5% for BUP, and ranged from −8.0% to 4.0% and from −5.0% to 2.5% for DXM, respectively. All the results proved that the method was precise and accurate (Table 3).

Stability The stability results (data not shown) demonstrated that all the stock solution were stable at least for 3 months stored at −20℃. The working standard solutions and microshpere solutions were stable at least 48 h at room temperature and at least 4 days at 4℃.

Application to Microspheres Study The method was applied to the simultaneous quantification of ROP, BUP and DXM from two PLGA microsphere samples. Sample#1 loading ROP and DXM and sample#2 loading BUP and DXM. Each sample was measured in five times. The encapsulation efficiency (EE) was calculated as (drugs loaded in microspheres

Table 2. System Precision Results ($n=6$)

	ROP	BUP	DXM
Retention time (min)	$x=3.49\pm0.03$	$x=4.92\pm0.04$	$x=9.94\pm0.02$
	R.S.D.=0.86%	R.S.D.=0.81%	R.S.D.=0.20%
Resolution	$x=9.42\pm0.075$	$x=6.32\pm0.03$	$x=12.89\pm0.07$
	R.S.D.=0.80%	R.S.D.=0.47%	R.S.D.=0.54%
Peak area	$x=392001\pm1236$	$x=105326\pm162$	$x=120418\pm1175$
	R.S.D.=0.32%	R.S.D.=0.15%	R.S.D.=0.98%
Peak tailing	$x=1.23\pm0.01$	$x=1.16\pm0.01$	$x=1.15\pm0.005$
	R.S.D.=0.81%	R.S.D.=0.86%	R.S.D.=0.87%
Theoretical plates	$x=30449\pm256$	$x=42499\pm221$	$x=157495\pm1404$
	R.S.D.=0.84%	R.S.D.=0.52%	R.S.D.=0.89%

x=mean±S.D.; S.D., standard deviation; R.S.D., relative standard deviation.

Table 3. Intra- and Inter-day Precision and Accuracy Results

Added (μg/ml)	Intra-day (n=6)			Inter-day (n=6)		
	Found (μg/ml) mean±S.D.	Precision (R.S.D., %)	Accuracy (R.E., %)	Found (μg/ml) mean±S.D.	Precision (R.S.D., %)	Accuracy (R.E., %)
ROP						
0.10	0.094±0.0016	1.7	−6.0	0.098±0.0048	4.9	−2.0
2.0	2.09±0.01	0.5	4.5	2.06±0.05	2.4	3.0
10.0	9.67±0.14	1.4	−3.3	9.84±0.24	2.4	−1.6
BUP						
0.10	0.095±0.0012	1.3	−5.0	0.0968±0.0056	5.8	−3.2
2.0	2.02±0.03	1.5	1.0	2.11±0.043	2.0	5.5
10.0	9.85±0.19	1.9	−1.5	9.90±0.21	2.1	−1.0
DXM						
0.10	0.092±0.002	2.2	−8.0	0.095±0.005	5.3	−5.0
2.0	2.08±0.01	0.5	4.0	2.05±0.04	2.0	2.5
10.0	9.73±0.02	0.2	−2.7	9.85±0.25	2.5	−1.5

/theoretical drug loading) ×100% and the drug loading capacity (DLC) was calculated as (mass of drug loaded in microspheres/mass of microspheres) ×100 %. The EE (mean±S.D.) of sample#1 was (70.79±2.33)% and (61.67±1.36)% with a DLC (mean±S.D.) of (7.17±0.14)% and (1.86±0.058)% for ROP and DXM, respectively. Sample#2 has a EE (mean±S.D.) of (67.92±1.24)% for BUP and (67.06±2.02)% for DXM corresponding a DLC (mean±S.D.) of (8.08±0.21)% and (1.66±0.048)%, respectively.

CONCLUSION

In this paper, an HPLC method was first developed and validated for the simultaneous determination of ROP, BUP and DXM using UV detection. The simultaneous determination of the three drugs was achieved in a short running time (about 10 min), although there was a great difference in polarity between the drugs. The method was proved to have a good performance of sensitivity, specificity, precision, accuracy and widely dynamic linear range. It involves simple sample preparation and shows good chromatographic performances (e.g., good peak shape, short column equilibration time and simple mobile phase). All the advantages make the method very suitable for the routine analysis of ROP, BUP and DXM in PLGA microspheres during the investigation of the microspheres. Furthermore, it could be also used for the simultaneous determination of the three drugs in pharmaceutical formulations and for routine analysis in quality control.

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