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Absolute quantification of poly(DL-lactide-*co*-glycolide) in microspheres using quantitative ^{1}H NMR spectroscopy

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ABSTRACT

The complex nature and the manufacturing process of poly(DL-lactide-*co*-glycolide) (PLGA), a key component of PLGA-based microspheres, have made the quantification of this copolymer difficult. The main purpose of the current study was to investigate the potential of three different methods for the quantitative analysis of the PLGA content of clinical products. In this regard, leuprorelin acetate microspheres from different vendors were chosen as templates to validate quantitative ^{1}H nuclear magnetic resonance (qHNMR) spectroscopy, size exclusion chromatography (SEC), and high-performance liquid chromatography (HPLC) methods qHNMR proved to be an excellent and rapid PLGA quantification method compared to the other two. The recovery value was 99.12% and the linearity correlation coefficient was 0.9999. The results obtained from the qHNMR method were found to match the data provided by the vendor, suggesting that qHNMR can be utilized as a reliable quality control and inspection tool for PLGA-based clinical products.

1. Introduction

Poly(DL-lactide-*co*-glycolide) (PLGA) is a well-known copolymer that is incorporated in a host of U.S. Food and Drug Administration (FDA)-approved therapeutic devices due to its biodegradability and biocompatibility [1–3]. PLGA is synthesized either by ring-opening polymerization of the cyclic dimers of glycolic acid and lactic acid, or by polycondensation of these monomers [4]. PLGA is a biodegradable polymer that hydrolyzes to produce its monomers in the body [1]. Notably, glycolic acid and lactic acid are by-products of various metabolic pathways in the body [5]; thus, minimal systemic toxicity is associated with PLGA.

PLGA has been widely utilized in medical applications, such as long-acting release products (LARs) [6]. One notable PLGA-based LAR is the leuprorelin microsphere (i.e., Lupron Depot [7]), used for the treatment of advanced prostate and breast cancers. Leuprorelin, the first luteinizing hormone-releasing hormone (LHRH) super agonist, is a gonadotropin-releasing hormone (GnRH) analog. Due to their unique advantages that include improved chemical castration and superior therapeutic effects, sustained-release microsphere products (e.g., leuprorelin acetate encapsulated in PLGA) are very popular for improving patient compliance by reducing dosing frequency [5,7].

Several microsphere preparation methods are reported [8]. The most common technique used for PLGA microsphere preparation is an emulsification-solvent evaporation technique [9], which has been applied to leuprorelin microspheres. This technique facilitates the encapsulation of drugs and involves dissolution of the polymer and compound together in an organic solvent. The oil-in-water emulsion is prepared by adding water and a surfactant to the polymer solution, and droplets are induced by sonication or homogenization. The solvent is then evaporated, and the microspheres are collected after centrifugation. In order to avoid aggregation and reduce initial burst release, various ingredients, such as surfactants, osmolytes, surface-active polymers, pH modifiers, and protein stabilizers can be encapsulated in PLGA-based formulations [10–12]. The PLGA content in the final product may differ from the desired content due to the complexity of the preparation process, and this difference may influence the encapsulation and loading efficiencies, thereby affecting the therapeutic benefits [13]. Since the manufacturing technique and formulation of the microsphere components are complex, it is difficult to quantitatively analyze the PLGA content in these microspheres, which is crucial for quality control and the evaluation of the manufacturing process.

Only a few studies have evaluated methods for the quantification of the polymer in PLGA-based microspheres. High-performance liquid chromatography (HPLC) [14] and HPLC-time

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of flight/mass spectrometry [15] have been used to analyze the monomers following polymer hydrolysis. However, these methods are time-consuming and are sometimes problematic due to hydrolysis and the formation of polylactides caused by self-polymerization [16,17]. Often, only indirect observations can be made using these methods because of a lack of suitable analytical tools. To overcome such difficulties, NMR spectroscopy, which has been used to calculate the lactide/glycolide (L/G) ratio of PLGA [18], is particularly useful because single-pulse experiments give reliable integrations that can be directly related to the studied compounds. NMR spectroscopy has also been used for the quantification of PEG [19,20] and polysorbate 80 [21], with satisfactory sensitivity and excellent recovery. The aims of this study were to investigate the potential of quantitative ^{1}H nuclear magnetic resonance (qHNMR) spectroscopy, size exclusion chromatography (SEC), and high-performance liquid chromatography (HPLC) methods for the quantitative analysis of PLGA. For this purpose, leuprorelin acetate microspheres from different vendors were chosen as templates. Additionally, comparisons of the PLGA content of five commercial leuprorelin acetate microsphere samples suggest that PLGA quantification could be utilized as a reliable quality control tool for the evaluation of the manufacturing process.

2. Material and methods

2.1. Solvents and reagents

Commercial samples of leuprorelin acetate microspheres were purchased from various vendors (detailed information is available in Table S1 of the Supporting information). $CDCl_3$, mannitol, and gelatin were purchased from Sigma-Aldrich (St. Louis, MO, USA). The PLGA (L:G = 75:25) samples were purchased from Sigma-Aldrich and the Luye Pharma Group (Yantai, China). Tetrahydrofuran (THF) and acetone were purchased from Fisher Scientific (Waltham, MA, USA). The benzoic acid (BA) and sodium lactate reference standards were purchased from the National Institute of Metrology (Beijing, China), and the National Institutes for Food and Drug Control (NIFDC, Beijing, China), respectively.

2.2. NMR sample preparation

2.2.1. Samples for linearity

Approximately 2.5, 5, 7.5, 10, and 12.5 mg of PLGA, and 5 mg of BA (reference standard) were placed in separate tubes (n = 3). Approximately 1 mL of $CDCl_3$ was added, and the mixture was vortexed for 15 min at 25 °C. The clarified solutions were immediately examined by ^{1}H NMR spectroscopy.

2.2.2. Samples for recovery

10 mg of sample 1 (Table S1) with 7 mg of PLGA (from Sigma-Aldrich) and 5 mg of BA as the internal standard were placed in a tube. Approximately 1 mL of $CDCl_3$ was added, and the mixture was vortexed for 15 min at 25 °C.

2.2.3. Samples for accuracy, stability, and reproducibility

Due to the complexities of the commercial samples, two types of simulated samples (i.e., with and without gelatin) were prepared. In analogous preparations using the commercial samples, 30 mg of PLGA, 6 mg of mannitol, 4 mg of leuprorelin, and 5 mg of BA, with/without 10 mg of gelatin were placed in a tube. Approximately 1 mL of $CDCl_3$ was added, and the mixture was vortexed for 1 min at 25 °C.

2.3. NMR experiments

qHNMR spectra were recorded using an Avance III HD 500 NMR spectrometer (Bruker, Billerica, MA, USA) fitted with a 5 mm i.d. BBO probe at 500.15 MHz. The probe temperature was set to 25 °C. ^{1}H NMR spectra were acquired at a spectral width of 7500 Hz (15 ppm), with acquisition times of 4.37 s and relaxation delays (D1) of 20 s. A sufficient S/N ratio was achieved after 128 scans. The total measurement time was approximately 1 h. The ^{1}H 90° pulse widths were calculated prior to collecting the NMR spectra. The ^{1}H longitudinal relaxation time (T_1) values were determined using an inversion recovery pulse sequence available from the pulse sequence library with a D1 value of 60 s and ten different inversion times (τ) ranging from 50 ms to 50 s. The inversion profiles were analyzed using TopSpin 3.2 (Bruker, USA) to determine the T_1 values.

To process the NMR data, the line broadening was set to 1.0 using zero-filled interpolation with 2 × 64 k. After Fourier transformation, the phasing was manually adjusted to zero-order phase correction, and a baseline correction was performed using 5th-order polynomial functions from −1 to 10 ppm. All spectra were manually integrated using TopSpin 3.2. The start and end points for integration of all spectra were consistent throughout the analysis.

2.4. qHNMR analysis of PLGA with reference standards

The BA reference standard and analytical samples were accurately weighed into vials; $CDCl_3$ was added to each vial to completely dissolve the samples. Each solution was then placed into a 5 mm NMR tube and subjected to qHNMR analysis. A quantitative proton signal for BA occurs at 8.04 ppm, that of dehydrated lactic acid (LA) occurs at approximately 5.2 ppm, and that of dehydrated glycolic acid (GA) occurs at approximately 4.8 ppm. None of the signals used for the qHNMR calculations overlapped with signals from mannitol or leuprorelin (Fig. 1). The weights of each PLGA sample included the weight of LA and GA via the following equations:

$$W_{PLGA} = W_{LA} + W_{GA}$$

$$W_{LA} = \frac{W_{BA} \times P_{BA} \times I_{LA} \times H_{LA} \times M_{LA}}{I_{BA} \times H_{BA} \times M_{BA}}$$

$$W_{GA} = \frac{W_{BA} \times P_{BA} \times I_{GA} \times H_{GA} \times M_{GA}}{I_{BA} \times H_{BA} \times M_{BA}}$$

where I_{BA}, I_{LA}, and I_{GA} are the integrated values of the BA, LA, and GA signal peaks, respectively; H_{BA}, H_{LA}, and H_{GA} are the number of protons corresponding to the BA, LA, and GA signals, respectively; M_{BA}, M_{LA}, and M_{GA} are the molecular weights of BA, LA, and GA, respectively; W_{BA}, W_{LA}, and W_{GA} are the weights of BA, LA, and GA, respectively; and P_{BA} is the purity of BA. The PLGA content of the commercial sample was calculated using the following equation:

$$content_{PLGA} = \frac{W_{LA} + W_{GA}}{W_{sample}}$$

where W_{sample} is the weight of the commercial leuprorelin acetate microsphere sample.

2.5. Linearity of the qHNMR method

The ^{1}H NMR spectra of five linearity samples (see *Samples for linearity*) were recorded and processed. PLGA calibration graphs were obtained by plotting the ratio between the gravimetric mass and experimental mass derived by qHNMR spectroscopy.

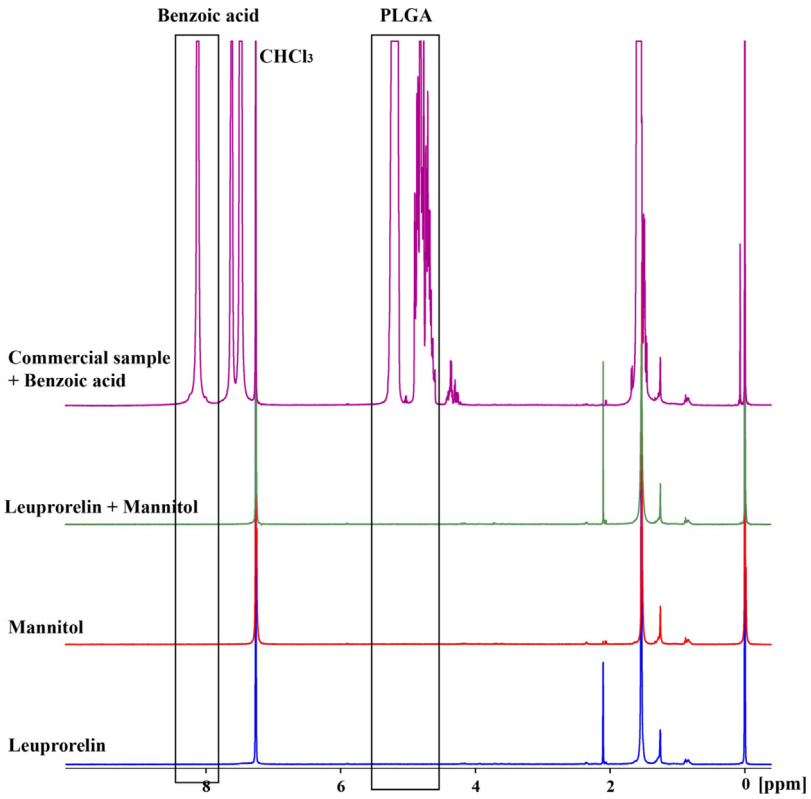

Fig. 1. ^{1}H NMR spectra ($CDCl_3$) of leuprorelin, mannitol, leuprorelin + mannitol, and a commercial leuprorelin acetate microsphere sample + benzoic. None of the signals used for the qHNMR calculations overlap with signals from mannitol or leuprorelin.

2.6. Recovery of the qHNMR method

Recoveries were determined via triplicate measurements of the processed samples dissolved in $CDCl_3$. After solvent extraction, each sample was examined by qHNMR spectroscopy. The recovery was calculated using the following equation:

$$Recovery = \frac{W_{PLGA} - W_{sample} \times content_{sample}}{W_{PLGAstandard} \times content_{standard}} \times 100\%$$

where W_{PLGA} is the weight of PLGA calculated by the qHNMR method, W_{sample} is the weight of the commercial leuprorelin acetate microsphere sample, $content_{sample}$ is the PLGA content of the commercial sample, $W_{PLGAstandard}$ is the weight of the PLGA standard provided by Sigma-Aldrich, and $content_{standard}$ is the PLGA content of the PLGA standard provided by Sigma-Aldrich.

2.7. HPLC analysis of PLGA after alkali hydrolysis [14]

The microspheres (5 mg) were dissolved in 2 mL of acetone. A solution of ethanol and KOH (0.05 N KOH-EtOH, 2.5 mL) was added to the microsphere suspension, which was incubated at 20 °C for 1 h and evaporated under a stream of nitrogen at 50 °C over 15 min. After evaporation, the residue was dissolved in an aqueous solution of phosphoric acid (pH 2.1). The hydroxy acids in the solution were assayed using an Agilent 1260 HPLC instrument (Agilent, Santa Clara, CA, USA) with the following operating conditions: isocratic elution; column, Nucleodur C18 Gravity 4.6 × 250 mm, 5 μm (Macherey-Nagel, Düren, Germany); mobile phase, aqueous solution of phosphoric acid (pH 2.3); flow rate, 1 mL/min; detection, 210 nm; column temperature, 30 °C. The analytes were quantified from their corresponding peak areas using sodium lactate as the external standard method.

2.8. Size exclusion chromatography (SEC) analysis [18,22]

PLGA quantification by SEC was performed on a Waters e2695 system (Waters, Milford, MA, USA) equipped with an auto-sampler, thermostated column compartment, and refractive index detector. To separate the injected polymers, elution was performed at a flow rate of 1 mL/min with THF. Two Agilent PLgel columns (300 mm × 7.5 mm, 5 μm, with 500 Å and 10,000 Å pores) were connected in series. The injection volume was 100 μL, the sample temperature was set to 6 °C, and the column temperature was set to 35 °C. For sample preparation, 5 mg of the microspheres were mixed with 1 mL of THF at 25 °C for 12 h to fully disperse the microsphere matrix. A calibration curve was recorded using the PLGA solutions (concentration range from 1 to 20 mg/mL), with each calibration solution corresponding to a microsphere sample. Due to the lack of a suitable PLGA reference standard, two commercial PLGA samples from different vendors with similar molecular weights and molecular weight ranges were used for calibration.

3. Results and discussion

3.1. qHNMR formulation of PLGA

The chemical formula of PLGA is shown in Fig. 2 along with the ^{1}H NMR spectrum of the commercial leuprorelin acetate microsphere sample with benzoic acid in $CDCl_3$. The TMS reference signal is indicated at 0.00 ppm. Due to the high molecular weight of this polymer, ^{1}H signals from the methine and methylene protons are not distinct; however, the mobile chains provide a clear and identifiable ^{1}H NMR spectrum. There are three well-resolved signals that correspond to PLGA [22] (Table 1): the methyl group of LA at ~1.5 ppm, the CH group of LA at ~5.2 ppm, and the CH_2 group of GA at ~4.8 ppm.

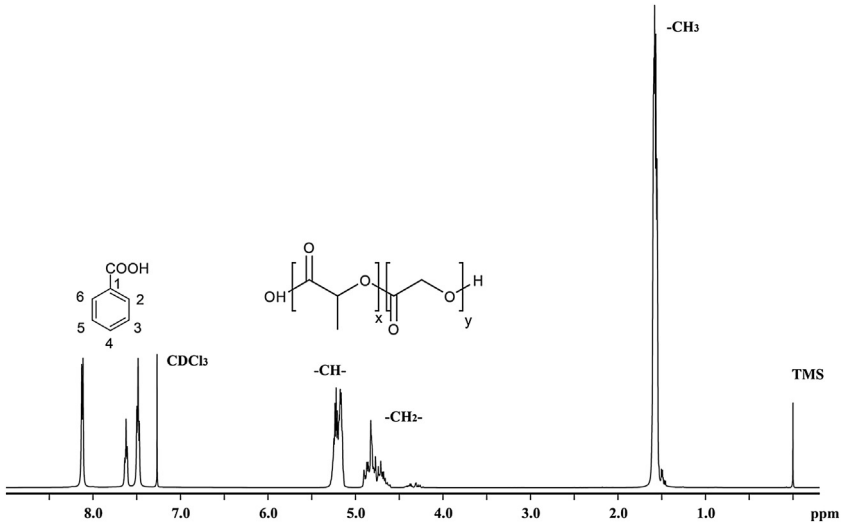

Fig. 2. Component structures and ^{1}H NMR peak assignments of the commercial leuprorelin acetate microsphere sample with benzoic acid in $CDCl_3$.

Table 1
NMR assignments for PLGA and benzoic acid in $CDCl_3$.

Position no.	^{1}H (δ_H) (ppm)	T_1 (s)
—CH_3 in PLGA	1.52–1.62	0.64
—CH_2 in PLGA	4.59–4.92	0.83
—CH in PLGA	5.06–5.35	2.34
H-3,5 in Benzoic acid	7.48	2.90
H-4 in Benzoic acid	7.63	3.67
H-2,6 in Benzoic acid	8.12	3.36

Table 2
Recovery and linearity data quantified by qHNMR, HPLC, and SEC.

	^{1}H NMR	HPLC	SEC1[a]	SEC2[b]
Recovery (%) n = 3	99.12	94.72	90.34	88.27
RSD (%)	0.75	0.27	1.40	1.11
R^2	0.9999	0.9984	0.9958	0.9937

[a] PLGA reference standard from Sigma-Aldrich.
[b] PLGA reference standard from the Luye Pharma Group.

3.2. Linearity, accuracy, stability, and reproducibility of qHNMR with a benzoic acid reference standard

Benzoic acid was selected as the internal standard for the quantitative NMR experiments because its proton signals are well separated from those of PLGA; moreover, it can be easily dispersed in $CDCl_3$ with PLGA. Benzoic acid is also a high purity standard reference material that can be obtained easily from NIST or the National Institute of Metrology.

To determine the linearity of the sample signals, PLGA standard solutions at five different concentrations (approximately 2.5, 5, 7.5, 10, and 12.5 mg/mL) with approximately 5 mg of BA standard were prepared. The calibration curve for PLGA (Fig. S1) shows the correlation between the gravimetric mass and the experimental mass determined by qHNMR. The regression equation for the curve was $y = 1.0011x + 0.024$. The slope (1.0011) and r^2 value (0.9999) in the linearity calculation were demonstrated for concentrations ranging from 2.5 to 12.5 mg/mL. In this concentration range, the relative error between the experimental value determined by qHNMR analysis and the gravimetric value of each sample was less than 1%.

The accuracy of the qHNMR method was assessed by analyzing two types of simulated samples prepared with and without gelatin (described in Section 2.2). The ratios of the qHNMR results versus the gravimetric values were determined to be 1.0019 (0.17% RSD, n = 5) with gelatin and 0.9993 (0.32% RSD, n = 5) without gelatin, indicating that the commercial sample matrix did not affect the qHNMR results for PLGA and BA in the deuterated solvent.

PLGA is reported to be quite stable in $CDCl_3$. Therefore, the repeatability of the qHNMR method was investigated over 24 h and 6 d. Our experiments showed that, in the fresh test samples (i.e., with/without gelatin), the change in the integral value ratios of GA/LA versus BA were <0.5% over 24 h when stored at 25 °C in $CDCl_3$, and approximately 4% over 6 d when stored at 25 °C (Figs. S2–S5). These results indicate that the samples did not change significantly over 24 h at 25 °C with the BA reference standard in the solvent, and they changed very little over 6 d. Overall, the instrument and samples were stable over the test periods and the developed method demonstrated sufficient linearity, accuracy, and repeatability.

3.3. Evaluation of PLGA quantification methods

The major goal of the present work was to compare PLGA quantification using qHNMR, HPLC, and SEC. In order to ensure the reliability of these methods, three individual calibration curves were obtained for each method. Individual calibration curves and the master calibration curve for each quantification method were linear over the tested concentration range (Table 2). Recovery experiments were also performed in triplicate for each method. The qHNMR method demonstrated superior PLGA recovery as compared to the other two methods.

Table 3 shows the analysis results of five samples of leuprorelin acetate microspheres determined by qHNMR, HPLC, and SEC (with two different PLGA reference standards). Note that the data in Table 3 correspond to those of PLGA polymers isolated from formulations that are currently in clinical use. All three methods exhibited comparable quantification results, indicating their applicability for PLGA quantification. However, the results were not identical. Notably, only sample 1 had PLGA content data provided by the vendor. For this sample, only the qHNMR results matched the data provided by the vendor; large differences were observed between the HPLC and SEC results (with the two reference standards) and the vendor data for sample 1. The qHNMR method was the most sensitive of all the methods investigated in this work. Notably, PLGA and BA are highly soluble in $CDCl_3$, and the signals required for the calculations were not obscured by other signals. The linearity, accuracy, stability, reproducibility, and recovery of the qHNMR method were excellent for PLGA quantification.

Table 3
Quantification of commercial samples of PLGA using qHNMR, HPLC, and SEC.

Sample No.	Contents from vendor (%)	^{1}H NMR (%)		HPLC (%)		SEC1[a] (%)		SEC2[b] (%)	
		contents	RSD	Contents	RSD	contents	RSD	contents	RSD
1	75%	75.15	1.06	71.35	3.29	67.38	3.58	65.09	3.54
2	n/a	72.25	1.21	74.24	2.72	67.10	3.50	64.86	2.63
3	n/a	74.99	0.96	73.84	0.47	66.17	4.18	64.17	4.19
4	n/a	87.23	0.24	82.87	1.7	76.41	1.76	74.66	2.67
5	n/a	88.72	0.40	83.19	0.24	77.61	1.59	73.99	0.43

n/a – not available.
[a] PLGA reference standard from Sigma-Aldrich.
[b] PLGA reference standard from the Luye Pharma Group.

Table 3 summarizes the PLGA contents of five commercial samples determined by SEC with two PLGA standards. Typically, SEC is used to obtain a polymer's molecular weight [22]. In this case, SEC was used for PLGA quantification. Since the molecular weights of commercially manufactured PLGA usually have wide distributions, it is difficult to obtain a PLGA reference standard with the same properties (i.e., similar molecular weight, molecular weight range, and 3D structure) [23–25]. For this reason, two commercial PLGAs (from Sigma-Aldrich and the Luye Pharma Group) were used as reference standards for SEC. As shown in Table 3, the recovery and content data from the two SEC methods were lower than the others. In addition, Table 3 shows that the PLGA contents of the same sample using the SEC method with two different PLGA standards systematically differ by approximately 2%. However, it is not possible to obtain a PLGA reference standard that is identical to a commercial sample. Despite the general convenience of this quantification method, the use of an additional PLGA analytical standard gives rise to an internal QC method for manufacturers, but it is not ideal for market surveillance testing.

The five commercial samples of PLGA were also analyzed by HPLC following alkali hydrolysis. Conventional quantification by HPLC appears to be the most sensitive technique in most cases, but it requires PLGA alkali hydrolysis. Moreover, several issues affect the results of this method, including the extent of alkali hydrolysis, calibration of the hydroxy acids, calibration of the polyesters, composition of the polyesters, and purity of the standards [14]. Furthermore, copolymerization of lactic acid [16] during analysis also needs to be considered, which may result in large RSD values. Another limit of HPLC is the requirement of reference standards for quantitative analysis. In this work, the only commercially available high-purity standard was sodium lactate.

3.4. Environmental and time considerations

Environmental protection is of paramount importance in this era. For this reason, a fundamental concern for sustainable growth of the pharmaceutical industry is the further development of green chemistry. Organic solvents, which are indispensable in this industry, are gaining significant attention because of their adverse effects on the environment and ecosystems. The concept of green chemistry has been adopted for organic solvents in order to meet the essential challenges of protecting human health and the environment from chemical hazards. To this end, the HPLC and SEC methods include many well-known drawbacks in terms of: (i) toxic organic solvents (i.e., THF and acetone) and large amounts of mobile-phase waste; (ii) expensive columns that need to be replaced frequently due to contamination; and (iii) long sample preparation and measurement times (at least one day). An alternative option is to utilize NMR spectroscopy, which has become a popular and convenient tool for the quantitative determination of various compounds. Overall, the experiment requires just one 1-h session and less than 10 min for sample preparation. The only reagent required is 1.0 mL of $CDCl_3$, and this volume of organic solvent can be easily handled.

4. Conclusions

The processing of microspheres involves many steps, including exposure of PLGA to water, drying, and centrifugation—all of which may affect polymer assays. In this study, qHNMR, SEC, and HPLC were used to quantify five commercially available samples of leuprorelin acetate microspheres from different vendors. Additionally, PLGA contents were compared and the methods were validated. qHNMR was found to be the most sensitive method investigated in this work. The linearity, accuracy, stability, reproducibility, recovery, and time efficiency of this method are excellent for PLGA quantification, which suggests that qHNMR can be utilized as a reliable quality control and inspection method for PLGA quantification to evaluate the performance of manufacturing techniques.

Author contributions

All authors have given approval to the final version of the manuscript.

Competing financial interests

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jpba.2017.08.046.

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