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Development of RP-HPLC method for simultaneous estimation of lactic acid and glycolic acid

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ABSTRACT

Quantification of the acidic monomers of biodegradable polymers plays an important role in understanding the degradation process. Aim of the present study was to develop a specific and accurate reversed phase high performance liquid chromatographic method (RP-HPLC) for the quantification of lactic acid (LA) and glycolic acid (GA) using conventional C_{18} column with ultra-violet detection. Mobile phase comprised of phosphate buffer (10 mM, pH 3.0) and acetonitrile at a ratio of 95:5 %v/v which resulted in the separation of LA and GA within 5 min run time. The stationary phase was conventional C_{18} column (250 x 4.6 mm, 5 µm). The detection was carried out at UV wavelength of 210 nm and the retention time was found to be 3.11 and 3.69 min for GA and LA respectively. The calibration curve was linear for the selected range with a coefficient of determination 0.9992 ±0.002 1 and 0.9975 ± 0.0015 for GA and LA respectively. The proposed method is sensitive, simple and cost effective compared to the previously reported methods utilizing the special columns. The developed method was validated and the recoveries of GA and LA were 98.51 ±3.46% and 97.20 ±2.84% r espectively. The developed method can be very useful for the estimation of LA and GA during the degradation of polymers based on these acids.

Keywords: Degradation, Glycolic acid, High performance liquid chromatography, Lactic acid, Poly(lactic acid), Poly(lactic-co-glycolic acid).

INTRODUCTION

Biodegradable polymers based on lactic acid [LA] and glycolic acid [GA] are widely used in the preparation of microspheres, nanoparticles, pellets, implants, films and scaffolds for pharmaceutical, biomedical and tissue engineering applications[1-5]. The degradation of these polymers involve hydrolysis of ester bonds leading to the formation of acidic monomers such as LA and GA[6]. The degradation process of these polymers is characterized by monitoring the time dependent changes in polymer molecular weight, physicomechanical properties, mass loss, total carboxyl content and morphology of the polymer. Several analytical methods such as Gel Permeation Chromatography (GPC), Scanning Electron Microscopy, Fourier Transform Infra-Red method, Size Exclusion Chromatography, Nuclear Magnetic Resonance and Gravimetry are used to monitor the degradation process. Most commonly reported technique is the measurement of molecular weight of the degrading polymer with respect to

time using GPC[3,7-8].However, this procedure is expensive, time-consuming and cannot be routinely used for the analysis of large number of samples.

The quantification of degradation products is generally carried out in the incubation medium in which the degradation of the polymeris carried out. Among the various analytical methods for the identification, separation and estimation, reversed phase HPLC (RP-HPLC) coupled with UV detection is widely used. However, the separation of LA and GA and their quantitative estimation is difficult because of their structural similarities, spectral characteristics and similar pKa values[9]. Hence, most HPLC methods reported in the literature utilized columns such as Grom-Sil column, Acclaim OA, Ultra aqueous C_{18}, Hypersil Gold aQ, YMC-Pack ODS-AM, Ultrasphere ODS and Inertsil ODS-3 with varying column lengths[10-14]. For the simultaneous estimation of these acids, use of two ultrasphere-ODS columns in series has been tried previously[15]. However, estimation of these acids using such special columns is expensive and cannot be carried out routinely in most laboratories.

Some of the techniques converted the acidic monomers into easily detectable compoundsbefore the estimation[3,16]. Lactic acid estimation with the use of a Microzym-L (SGI) titrator or other enzymatic kits have been previously reported [17]. A method to estimate the degradation by-products of poly(lactic-co-glycolic acid) [PLGA] using capillary zone electrophoresis has been described previously [18]. Literature also reveals the utilization of electrochemical or potentiometric methods for quantification of smaller molecular weight acids due to poor absorption of UV light [3]. A simple HPLC technique to estimate the degradation products of LA and GA based polymers using routinely available columns has not been widely reported in the literature. Hence, in the present study, a simple, specific and sensitive RP-HPLC method was developed and validated for the simultaneous estimation of LA and GA with conventional C_{18} column.

MATERIALS AND METHODS

Chemicals and reagents

The reference standards for LA and GA were purchased from Sigma Aldrich (Saint Louis, Missouri, USA). Acetonitrile and methanol were of HPLC grade and were procured from Merck specialties Pvt Ltd (Mumbai, India). Ortho-phosphoric acid was purchased from Nice chemicals Pvt Ltd (Cochin, India) and potassium dihydrogen phosphate was purchased from Spectrochem Pvt Ltd (Mumbai, India). Water for the HPLC analysis was generated by “reverse-osmosis” using Milli-Q water (Millipore Co., Bedford, MA, USA). All other chemicals and reagents used in the study were of analytical grade.

Instrumentation

The chromatographic estimation of LA and GA was carried out using Shimadzu LC 2010CHT (Shimadzu Corporation, Kyoto, Japan) equipped with low pressure quaternary gradient pump along with the dual wavelength UV-Visible detector, column oven and auto sampler. The chromatographic data were recorded and processed using LC solution 1.24SP1 software. The column oven temperature was maintained at 25 ^{o}C and the chromatographic separation was achieved usingSupelco C_{18} (250 x 4.6 mm, 5 µm) column (Supelco, USA). The isocratic elution was performed with phosphate buffer (10 mM, pH 3.0) and acetonitrile at 95:5 %v/v ratio as mobile phase. The flow rate was maintained at 1 ml/min and the injection volume was 50 µl. The effluent was monitored at a wavelength of 210 nm for both LA and GA.

Preparation of solutions

10.0 mg of GA was dissolved in minimum quantity of milli-Q water. Based on the density of LA, 8.2 µl was pipetted (equivalent to 10.0 mg by weight) and added to milli-Q water containing GA and the solution was made up to 10 ml to get a concentration of 1.0 mg/ml of LA and GA. The calibration standards were prepared by serial dilution method to get concentrations of 2, 5, 10, 20, 50 and 100 µg/ml of LA and GA using the mobile phase.

The mobile phase was prepared by dissolving 1.36 g of potassium dihydrogen phosphate in 980 ml of milli-Q water to which approximately 130 µl of concentrated ortho phosphoric acid was added to adjust the pH to 3.0. The remaining volume was made up to 1000 ml with milli-Q water to prepare 10 mM phosphate buffer solution. The resultant buffer solution was filtered through 0.22 µm filter membrane and sonicated(Equitron®, Medica Instrument Mfg Company, Mumbai, India)for 10 min before use. Similarly, acetonitrile was sonicated for 10 min before use. The diluent was prepared byadding 2.5 ml of acetonitrile to 47.5 ml of 10 mM phosphate buffer solution of pH 3.0.

Sample preparation

For the preparation of linearity curve, calibration standards were added with the required quantity of mobile phase to make up the volume to 1.0 ml to get the linearity range of 2, 5, 10, 20, 50 and 100 µg/ml from the stock solution. An aliquot of 50.0 µl of this solution was injected for the HPLC analysis.

Method validation

System suitability

The system suitability was evaluated by injecting six replicates of solution containing 50.0 µg/ml of LA and GA solution. The acceptance criterion is ±2.0% percent coefficient variation (%CV) for the peak area and the retention time for both LA and GA.

Limit of detection (LOD) and Limit of quantification (LOQ) [Sensitivity]

LOD is the ability of an analytical method to detect the lowest concentration of the analyte and is defined as the lowest concentration level resulting in a peak area of three times the baseline noise. LOQ is the lowest concentration of the analyte which can be quantitatively analysed with acceptable precision and is defined as the lowest concentration that provides a peak area with signal-to-noise ratio higher than 10, with precision (%CV) and accuracy (%bias) within ±10%. Both LOD and LOQ were calculated based on the slope and response from the calibration curve as per ICH guidelines.

Linearity (Calibration curve)

The linearity of an analytical procedure is its ability to obtain the test results which are directly proportional to the concentration of the analyte. A series of solutions containing 2, 5, 10, 20, 50 and 100 µg/ml of LA and GA were prepared and injected into the HPLC to record the chromatograms. The peak area of LA and GA were plotted against the concentration to get the regression equation and coefficient of determination.

Accuracy and precision

The accuracy of an analytical procedure expresses the closeness of agreement between the value found and the value which is accepted either as a conventional true value or an accepted reference value. It is generally performed by recovery studies. It was determined for both intra-day and inter-day variations using the triplicate analysis of LA and GA samples of known concentration. Precision of the assay was determined by repeatability (intra-day) and intermediate precision (inter-day). The repeatability was determined by injecting 20.0 µg/ml of LA and GA solution (n = 6) on the same day and the percent coefficient of variation (% CV) was calculated. The intermediate precision was assessed by comparing the assays on different days.

Robustness

The robustness is the capacity of a method to remain unaffected by small deliberate variations in the method parameters. In the present study, variation in pH of the mobile phase (± 0.2), mobile phase composition (± 2.0 %), wavelength of detection (± 5.0 nm) and flow rate (± 10.0 %) were evaluated.

Stability

The stability of LA and GA solution was determined to check the short-term stability. Stock solution (20 µg/ml) was kept at room temperature for 12 h and then analysed. The long-term stability of the acids was analysed by storing the samples at 4 ^{o}C for 30 days. Auto-sampler stability was determined by storing the samples for 24 h in the auto-sampler.

RESULTS

Method development and optimization

GA is freely soluble in the water whereas LA is miscible in the water. The solutions containing LA and GA were(100.0 µg/ml) scanned separately at a wavelength range of 400–200 nm using ultraviolet spectrophotometer (UV-1601PC, Shimadzu Corporation, Japan) to determine the maximum wavelength of LA and GA. The maximum wavelength (λ_{max}) was found to be 210 nm. The diluted concentration of 100 µg/ml of LA and GA was prepared and injected into the HPLC and the chromatograms were recorded using Supelco C_{18} column. However, both LA and GA were eluted at void volume (< 2.5 min) with no separation. In order to achieve the separation, mobile phase was modified and the chromatographic separation was optimized. Phosphate buffer of pH 3.0 of varying strengths such as 10, 20, 30 and 40 mM were used as mobile phase. The retention time of LA and GA did not alter significantly with the increasing buffer strength and hence, in the present study, 10 mM strength was used. The effect of phosphate buffer (10 mM) pH (3.0, 3.5, 6.0 and 7.0) on the retention times of LA and GA was also investigated. The results showed that as the pH of buffer increased, retention times of LA and GA were decreased which may be attributed to protonated form of acidic group. [13]The mobile phase ratio was optimized using varying ratios of the buffer and acetonitrile at a flow rate of 1.0 ml/min with Supelco C_{18} column as stationary phase. It was observed that 10 mM phosphate buffer pH 3.0 and acetonitrile at a ratio of 95:5 %v/v provided the optimum retention time of 3.11 and 3.69 min for GA and LA respectively. In this optimized chromatographic condition, sharp peak with an asymmetric factor of<1.5 with good column efficiency, baseline separation, high theoretical plates was obtained with Supelco C_{18}column (250 x 4.6 mm, 5 µm) for both GA and LA. Flow rates of 0.9, 1.0 and 1.1 ml/min were

used and the chromatograms were recorded. All the flow rates showed symmetrical peaks with acceptable capacity factor. For the present study, 1.0 ml/min was selected although 0.9 and 1.1ml/min can also be used. Based on the optimization procedure, it was observed that Supelco C_{18} column as stationary phase, 10 mM phosphate buffer of pH 3.0 and acetonitrile (95:5 %v/v) as mobile phase at a flow rate of 1.0 ml/min were suitable for the estimation of LA and GA.

Method Validation

Validation is a documented program that provides a high degree of assurance that the method will consistently produce the products meeting the predetermined specifications and quality. In the present study, the developed method was validated as per ICH (Q2R1) guidelines.In the present study, the method was developed by taking into consideration the solvent's UV cut-off. The solvent absorption was not found to interfere with the absorption of LA and GA. The method was found to be specific with no interferences from the polymer degradation samples. The typical standard chromatogram of LA and GA is shown in Figure 1.

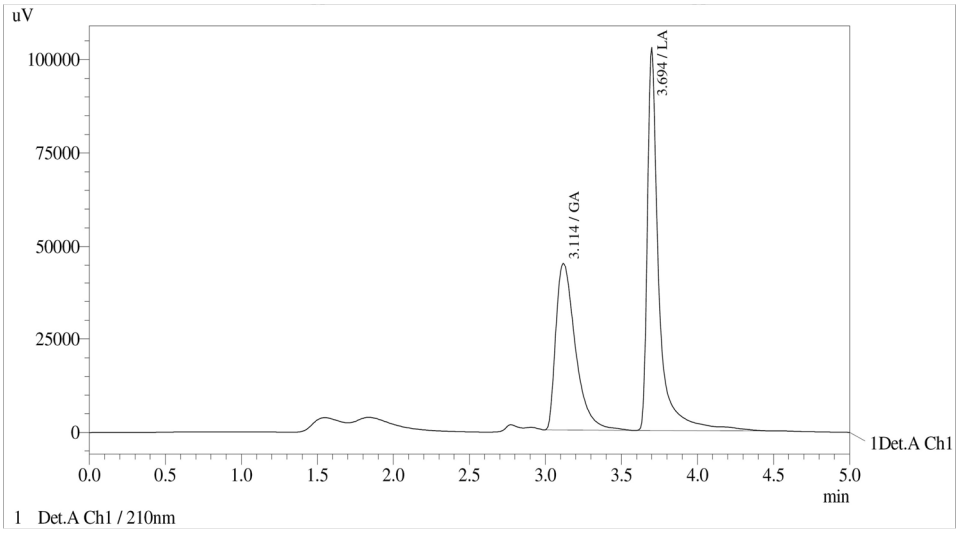

Figure 1: Typical standard chromatogram of LA and GA

System suitability

The 10.0 % asymmetry of LA and GA was close to 1.50 which indicated that the peak shape is symmetrical. The high counts of theoretical plates/meter (>2000) revealed that the column efficiency and the resolution between GA and LA was 1.50.

Limit of detection (LOD) and Limit of quantification (LOQ) [Sensitivity]

In the present method, LOD and LOQ were calculated based on the standard deviation of the response and slope.

$LOD = 3.3 \times SD/S$ and $LOQ = 10 \times SD/S$

SD: Standard deviation of blank response; S: Slope of the calibration curve.

LOD and LOQ of LA was found to be 0.50 and 2.00 µg/ml respectively whereas for GA, it was found to be 0.80 and 2.00 µg/ml respectively. Results indicate that the developed method was sensitive for the quantification of LA and GA.

Linearity

The proposed method was linear in the range from 2.0 to 100.0 µg/ml for both LA and GA. The slope and intercepts can be used to determine the unknown concentration. Linearity is generally reported by coefficient of determination (r^2) and in the developed method it was found to be > 0.995 which indicates that the proposed method was linear for both LA and GA.

Accuracy and precision

A known amount of standard GA and LA were spiked (80, 100 and 120 %) in triplicate in a sample containing known concentration of LA and GA. The recovery of LA and GA was calculated from these samples. At three

different concentrations, recoveries were found to be within the range of 90 to 110 %.The mean % recovery (Mean±SD) was found to be 97.20 ± 2.84 and 98.51 ± 3.46 for LA and GA, respectively.

The Precision was measured by repeatability and intermediate precision. The repeatability and intermediate precision of LA was found to be 0.56 and 0.85 % CV respectively whereas for GA, it was found to be 0.72 and 1.45 % CV respectively. The acceptance criteria for the repeatability and intermediate precision is 1.0 and 2.0 % CV respectively. The results indicate that the method is precise and reproducible.

Robustness

The robustness was evaluated by varying method parameters such as percent organic solvent, pH of the buffer, ionic strength of buffer etc., and its effect, if any, on the results of the optimized conditions was evaluated. The overall % RSD in various parameters was found to be less than 2.0 % which is within the acceptable limit. The results indicated that the method was robust.

Stability

The stability of LA and GA solution was found to be within the acceptable limits of 90-110 % when stored at room temperature as well as at 4 ^{o}C for 30 days. The summary of analytical parameters is shown in Table 1.

Table 1: Summary of analytical method validation of LA and GA

Validation parameters	Validation results		Acceptance criteria
	LA	GA	
Specificity	No interferences at retention time of LA and GA		No interference at RT of analytes
Linearity (r^2) (2 – 100 µg mL^{-1})	0.9975±0.0015	0.9992±0.0021	> 0.99
Repeatability precision (% CV)	0.56	0.72	< 1.00
Intermediate precision (% CV)	0.85	1.45	< 2.00
Accuracy (% Mean±SD)	97.20±2.84	98.51±3.46	90-110
LOD (µg mL^{-1})	0.50	0.80	S/N ratio should be 3:1
LOQ (µg mL^{-1})	2.0	2.0	S/N ratio should be 10:1
Robustness (% RSD)	1.35	1.52	< 2.00

DISCUSSION

Both LA and GA are low molecular weight compounds with polar functionalities and exhibit poor chromophore nature. LA contains a hydroxyl group adjacent to the carboxyl group making it α-hydroxy acid. It has a molecular weight of 90 and pKa of 3.86 [19-20]. GA is the smallest α-hydroxy acid. It is a hygroscopic crystalline solid with a molecular weight of 76 and pKa of 3.83 [21]. These properties of LA and GA indicate that they have similar spectral structure, pKa values and hence is difficult to separate them using HPLC. Hence, 100% aqueous buffer is the choice of mobile phase for quantification of both LA and GA. To ensure complete protonation of acidic groups, a low pH buffer is generally used for best interaction between organic acid and C_{18} stationary phase. However, with 100% aqueous mobile phase the C_{18} chain collapses with resultant loss of retention [22]. To restore the chain structure and column efficiency, the column must be flushed with organic mobile phase for longer periods of time. For these reasons, such acids are generally estimated with the use of special columns. However, such columns are expensive and are not routinely used.

In the present study, a simultaneous HPLC method was developed and validated for the quantification of LA and GA using a conventional column by modifying the chromatographic conditions. The results of the present study indicate that the developed method was found to be accurate, precise and specific with no interferences at the retention time of LA or GA meeting the acceptance criteria as per the guidelines. The reported method is suitable for the quantification of both LA and GA with wide range of concentrations. This is important as the concentration of degradation products is expected to be less during the initial stages whereas higher concentrations of these acids are released during the later stages of the degradation. The present method is also less expensive as it uses conventional C_{18} column.

CONCLUSION

From the results of the present study, it can be concluded that the developed method can be successfully used for the quantification of LA and GA to estimate the degradation products of PLA and PLGA in their degradation studies. However, as the developed method is specific for the acidic monomers such as LA and GA, intermediate products of degradation such as oligomers of LA and GA cannot be measured.

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