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Quantitative HPLC determination of itaconic acid residuals in polymer hydrogels

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ABSTRACT

A simple reversed-phase high-performance liquid chromatographic (RP-HPLC) method has been developed and optimized for simultaneous determination of itaconic acid in polymer hydrogels. Separation of itaconic acid was successfully achieved on a C_{18} column utilizing aqueous solution of ortho-phosphoric acid (0.05 %). The detection was achieved using PDA detector at 210nm. The extraction recovery of itaconic acid was >89 % and the calibration curve was linear ($r^2 = 0.9990 \pm 0.06$) over itaconic acid concentrations ranging from 0.1 to 1.0µg/ml (n = 5). Adequate assay for intra- and inter-day precision and accuracy was observed during validation process. An extraction procedure to remove residual itaconic acid was also established. The developed method and the extraction procedure were applied to itaconic acid based hydrogels, tested with or without post polymerization treatment and proved to be accurate and precise for determination of residual monomer content of the material evaluated.

KEYWORDS

Polymer;
Hydrogel;
Residual monomer;
Itaconic acid;
HPLC-DAD.

INTRODUCTION

Hydrogels are three-dimensional, hydrophilic, polymeric networks, with chemical or physical cross-links, capable of imbibing large amounts of water or biological fluids[1,2]. These gels respond to various environmental conditions like temperature, pH and ionic strength, ultrasound irradiation, magnetic and electrical stimulus. Hence they are used in various biomedical applications such as controlled delivery of drugs, peptide, cells within the human body, scaffolds for tissue engineering, medical electrodes, wound dressing, biosensor, contact lenses, moisture sensitive adhesives, diet aid etc. Special hydrogels as superabsorbent materials are widely employed in hygienic uses particularly disposable diapers and female napkins where they can capture secreted fluids, e.g., urine, blood, etc.

Itaconic acid (IA) is originally known as product of pyrolytic distillation of citric acid. It is produced on an industrial scale by the cultivation of *Aspergillus terreus* or *A. itaconicus* using sugar molasses or glucose[3,4]. Its primary application is in the polymer industry where it is employed as a comonomer at a level of 1-5 % for certain products. The methelene group is able to participate in polymerization reactions, which makes itaconic acid a profitable product applied in various type of synthetic polymers. It can be easily incorporated into polymers and may serve as a substitute for petrochemi-

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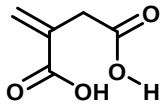

Itaconic acid (IA)

Figure 1 : Structure of itaconic acid

cal-based acrylic or methacrylic acid. Itaconic acid is also important as an ingradiant for the manufacture of synthetic fibres, coatings, adhesives, thickness and binders. The main potential for the utilization of itaconic acid is the manufacture of styrene butadiene copolymers and for lattices and pain emulations to improve adhesion of the polymer. IA is hydrophilic in nature, due to presence of two -COOH groups (Figure 1) and having different pKa values (3.84 and 5.55), so that very small amounts of IA is enough to produce SAP hydrogels with high hydrophilic properties[5,6]. SAP hydrogels are known as good candidates for the controlled release formulations for pharmaceutical applications mostly due to their high biocompatibility. Nowadays these polymeric carriers have been extensively considered in sustained and controlled release devices for the delivery of water-soluble drugs[7,8].

During polymerization the monomers crosslink to create a polymeric matrix. The polymerization is however, not complete, leaving up to 10% of residual monomers capable of leaching out. Residual low molecular weight monomers, like IA are relatively mobile, and may diffuse through the matrix into an immersion medium. To be used as medical materials and sanitarian materials, the amount of the residual monomer must be kept as low as possible. Although a significant body of research exists on environmental and health effects of the presence of low molecular weight extractable or unreacted monomers in polymers, there is still a lack of data regarding the purity of engineered polymeric hydrogels. Reducing the biological and ecological hazards can only be met through tandem testing during the discovery synthesis phase. To be used for bio-medical applications, the amount of the residual monomers must be kept as low as possible.

The literature survey revealed that there is no reported procedure to highly quantify the residual itaconic acid in polymer hydrogels. Therefore it was our goal to develop a fast and simple analysis protocol enabling the simultaneous and unambiguous detection of itaconic acid in hydrogels. We aimed to achieve an optimized practical method to easily determine the residual monomer in hydrogels. So HPLC, as one of the most common analytical method for quantitative measurement of organic acids, was used for the purpose. The developed method was validated as per ICH guidelines for specificity, linearity, accuracy and precision. LOD and LOQ were determined by calibration curve method[9,10].

EXPERIMENTAL

Chemicals

Itaconic acid monomer was purchased from fluka and used as received. Orthophosphoric acid from E-MERK, Germany. All other chemicals used in this study were of analytical grade quality, with deionized water (milli Q, Millipore (India) Pvt Ltd, Bagalore) being used for preparing the solutions.

Synthesis of collagen-g-poly (acrylamide-co-itaconic acid), collagen-g-P(AM-co-IA)

A pre-weighed amount of hydrolyzed collagen (1.0g) was added to 50 ml deionized and filtered to remove its insoluble phosphate salt. Then the solution was added to a three-neck 500ml reactor equipped with a mechanical stirrer (RZR 2021, a three-blade propeller (Heidolph, Schwabach, Germany) and stirred (250rpm) for 10 min. The reactor was placed in a thermostated water bath to control the reaction temperature at 80°C. After dissolving and homogenizing the mixture, the monomers AM, IA and the crosslinker, MBA, were simultaneously added and the reaction mixture was stirred for 15 min. Then the initiator APS (oxidant) and TMED (reductant) was added (TABLE 1). The solution was stirred at 400–500 rpm while maintaining the temperature and inert atmosphere. The temperature was maintained at 80°C and the reaction mixture was stirred continuously for 24 h. The low molecular weight substances remaining in the samples after polymerization were extracted with boiling ethanol for 24 h. The product was collected by centrifugation and dried in the oven under vacuum at 60°C for 24 h. The dried graft polymer was added to 300ml deionized water. It was allowed to swell during agitation in a water bath at the constant temperature of 60°C for 24 h. Then it was extracted with ethanol in a soxhlet for 6 h followed by water at 100°C

TABLE 1 : Composition of the feed mixture

Polymer code(ml)	IA (mg)(ml)	MBA 1% (w/v)(ml)	TMED 1%(w/v)	APS 1% (w/v)	% Seq
H1	0	0.4	0.3	0.2	489
H2	20	0.4	0.3	0.2	517
H3	40	0.4	0.3	0.2	589
H4	60	0.4	0.3	0.2	1890
H5	80	0.4	0.3	0.2	1851
H6	60	0.2	0.3	0.2	1611
H7	60	0.6	0.3	0.2	1419
H8	60	0.8	0.3	0.2	1111
H9	60	0.4	0.1	0.2	1621
H10	60	0.4	0.2	0.2	1901
H11	60	0.4	0.4	0.2	1800
H12	60	0.4	0.2	0.1	1616
H13	60	0.4	0.2	0.3	1813
H14	60	0.4	0.2	0.4	1867

Reaction conditions: Hydrolyzed collagen: (1.0g); acrylamide: 1.0g, H2O: 30ml, temperature: 80°C

for 72 h. The precipitate was filtered and dried under vacuum at 60°C. After grinding, the resulting powder was stored away from moisture, heat and light. Homopolymer, PAM and copolymer and copolymer P (AM-co-IA) was also synthesized using the same method and the polymerization have been confirmed by FT-IR studies (Figure 2 and TABLE 2).

Equipment and chromatography conditions

The chromatographic system consisted of a computer-controlled pump (model LC 20AT), autosampler (model SIL-10AF) equipped with a 200µl sample loop, photodiode array (PDA) detector (model SPD-M20A). Shimadzu LC Solution software was used for the system and data management. The separation was performed in isocratic mode at a flow rate of 1.0ml /min and a temperature of 40°C on an analytical column Gemini 5µ C_{18}, 150×4.6mm (Phenomenex, USA). An RP C_{18} Security guard (4×3mm, Phenomenex) was employed to protect the analytical column. The mobile phase was aqueous 0.05% orthophosphoric acid and the injection volume was 50µl. The observer backpressure values were in the range from 1400 to 1450 psi. Data was acquired and processed by LC solution software (Shimadzu, Japan).

Standard solution preparations

A stock solution containing 100µg/ml IA was prepared in a solution of methanol: 0.05% orthophosphoric acid (10:90) and it was stored in amber colored volumetric flask at 4-6°C. Working standard solution (1mg/ml) was also prepared in the same medium. Calibration standards were prepared by appropriate quantitative dilution from the stock solution.

TABLE 2 : Main FTIR Peaks of PAM, P (AM-co-IA), Collagen-g-P (AM-co-IA)

Wavenumbers of absorption bands of original groups (cm^{-1})	Wavenumbers of absorption bands (cm^{-1}) of		
	PAM P	(AM-co-IA)	Collagen-g-P (AM-co-IA)
Amide			
Free-NH_2 stretching (amide II) 3400, 3500	3422	3417	3401
Bonded-NH_2 stretching (amide II) 3180, 3350	3207	3145	3126
NH_2 bending (amide II) 1620-1650	1621	1600	1600
-C = O stretching (amide I) 1650	1663	1650	1649
C-N stretching 1410	1414	1396	1390
-NH out of plane bending 600-700	608	600	599
Carboxyl			
Bonded –O-H stretching			
3200-3400		3217	3215
2500-2700		2608	2606
-C = O stretching 1735-1750		1713	1711
-C-O stretching 1210-1320		1200	1199

Solution state stability at working pH

Stability testing was carried out to evaluate the stability and extend of degradation of the stock solution containing IA. Fresh stock solution of IA (100µg/ml) was prepared, working solutions at three concentration levels were made from this standard solution and kept at 4-6°C. Sampling was done at regular time intervals for a period of 7 days in triplicate. Each sample was run in HPLC after filtering through 0.45µm filter. The peak area of the standard IA solution at different concentration was compared at different time point to determine the stability as a function of time. Percent residual and % relative standard deviation (R.S.D) were found to be within statical limits. Hence, the solution remains stable over a period of 7 days under 4-6°C (TABLE 3).

Wavelength optimization

The PDA absorbance over the 205-500nm range was recorded (Figure 1) and the wavelength used for quantification was 210nm.

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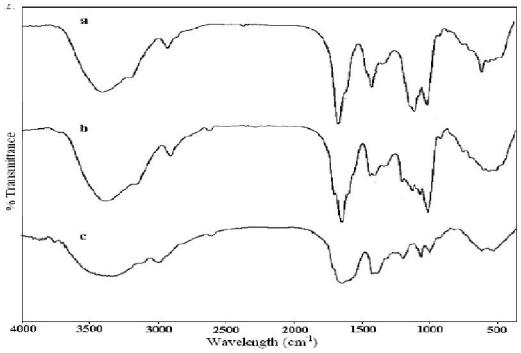

Figure 2 : FTIR spectra of PAM (a), P (AM-co-IA) (b), Collagen-g-P (AM-co-IA)

System suitability

Having optimized the efficiency of chromatographic separation and the quality of the chromatography was monitored by applying the system suitability tests like capacity factor, tailing factor and theoretical plates. The system suitability method acceptance criteria set in each validation run were: Injection precision RSD < 1% (n > 5), capacity factor k' > 2.0, tailing factor T = 2.0, theoretical plates N > 2000. The HPLC system was equilibrated with the mobile phase, followed by five injections of the IA standard. In all cases the RSD for five consecutive injections was < 0.3 %. All other parameters were found to be satisfactory [capacity factor (4.2), tailing factor (0.025), and theoretical plates of the column (3497)].

Analytical procedure

Powdered hydrogel sample (0.300g) was accurately weighed and added to 10ml methanolic orthophosphoric acid (10:90, pH = 2.3) in a polypropylene tube. After vortex (Cyclo mixer, CM 101, Remi Instruments, India) for 10 minutes the sample hydrogels were placed in an ultrasonicator bath (Toshcon, SW-7, India) for half an hour followed by placing in an orbital shaker (Labline instruments, India) at 37°C with constant agitation (200 rpm) for 12 h. Then it was centrifuged (Eppendorf, 510R, Germany) for 10 minutes at 3500 rpm at 4°C. The supernatant was taken by means of a syringe, then filtered through a 0.45µm syringe filter (Millipore millex-HV, Hydrophillic PVDF) and finally put in a sample vial (Waters, USA).

Validation of analytical method

The developed method was validated as per ICH guidelines for linearity, accuracy and precision, specificity, LOD and LOQ.

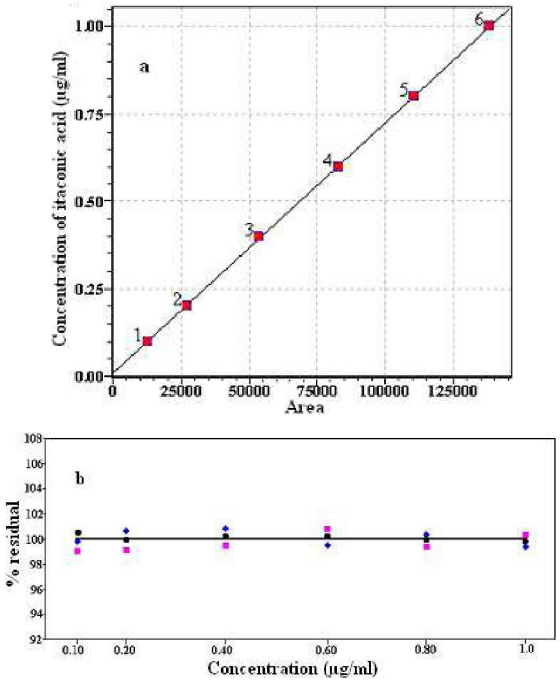

Figure 3 : Linearity curve of itaconic acid (a), Residual plot: Assessment of linearity (for a better view only three readings are shown for each calibration point)

Range of linearity

Standard solutions (0.1, 0.2, 0.4, 0.6, 0.8 and 1.0mg/ml), each in five replicates were injected into the system. Linearity of the analytical procedure was evaluated by plotting detector response (peak area) against analyte concentration (Figure 3(a)). The regression equation was Y = a X + b, where Y denotes peak area and X is the concentration of IA (µg/ml). The % residuals (Figure 3(b)) were plotted in order to detect and access any systematic deviations.

$$\% \text{ Residuals} = \frac{\text{Predicted} \times 100}{\text{True value}}$$

Residual plot is obtained by calculating the percent residual of each calibration standard and plotting it against concentration. Figure 3b shows that, the data points are evenly scattered on both sides of the 100% line and exhibit a decreasing variance with increasing calibration level.

Accuracy and precision

Accuracy and precision of the analytical method was determined by analyzing quality control samples (QC) at three different concentrations within the cali-

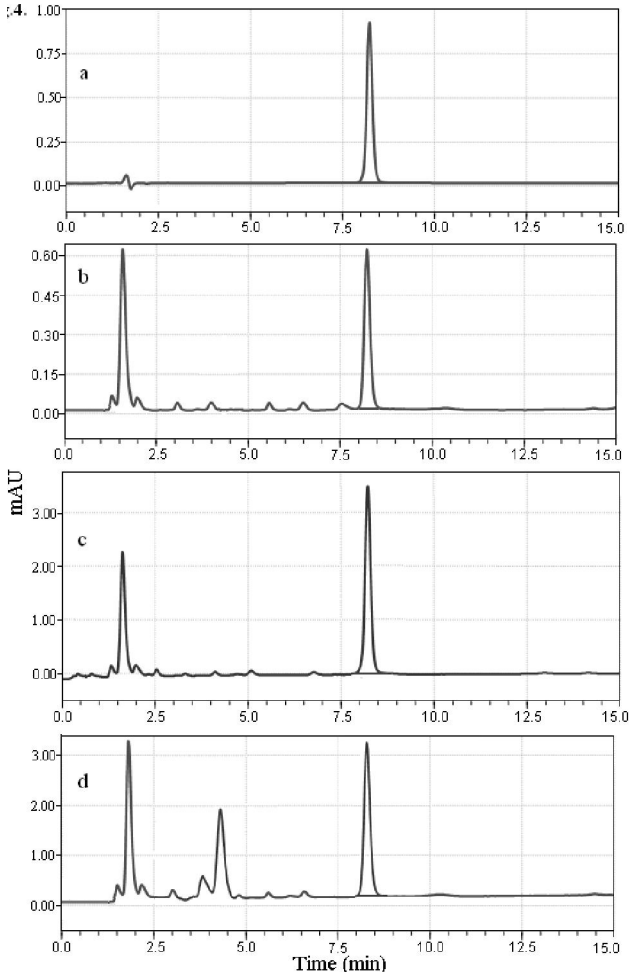

Figure 4 : Representative HPLC chromatograms of standard itaconic acid (0.2mg/ml) (a), fortified in PAM gel (4 µg/ml) (b), residual itaconic acid detected in P (AM-co-IA) (c) and in collagen-g-p (AM-co-IA) hydrogel (d)

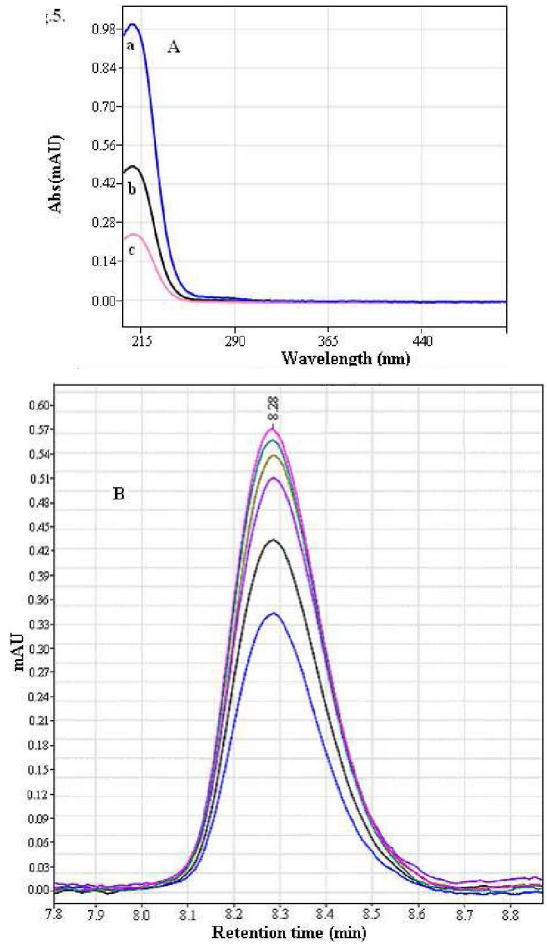

Figure 5 : (A) Absorption spectrum of itaconic acid (0.2mg/ml) in the mobile phase (measured at height h (a), h/2 (b) and h/4 (c) reapectively. No changes in spectral pattern was observed). (B) Itaconic acid fortified (4.0mg/ml) was detected at the wavelengths of 210, 200, 212, 215, 220 and 222 nm simultaneously

bration range in triplicate (n = 3). QC standards were prepared in the same media and are dilutions from weightings independent from those used for preparation of calibration curves.

The precision (% RSD) of the analytical procedure was evaluated by determining the intra- and inter-day coefficient of variation and reported as % RSD for a statically significant number of replicate measurements. The intra-day precision of the selected method was estimated by the analysis of three different concentrations of IA in triplicate and three times on the same day. The inter day precision was accessed by analyzing samples in the same way as for intra-day precision assay, and was repeated for three consecutive days.

Specificity

Specificity was determined by comparing the response of IA in the sample matrix with the response of same component in a solution containing pure analyte. Baseline separation was obtained between IA, sample matrix and no interference with the main peak was observed. Confirmation of peak identity was effected by co-chromatography. The peak purity index is a measure of spectral heterogeneity of a peak based on the comparison of spectra over the entire peak. The non-ideal effects are quantified and provided as a value of peak purity index. When the peak is pure, the peak purity index is greater than 0.990.

Limit of detection and quantitation

LOD and LOQ decide about the sensitivity of the method. LOD is the lowest detectable concentration of the analyte while LOQ is the lowest amount of the

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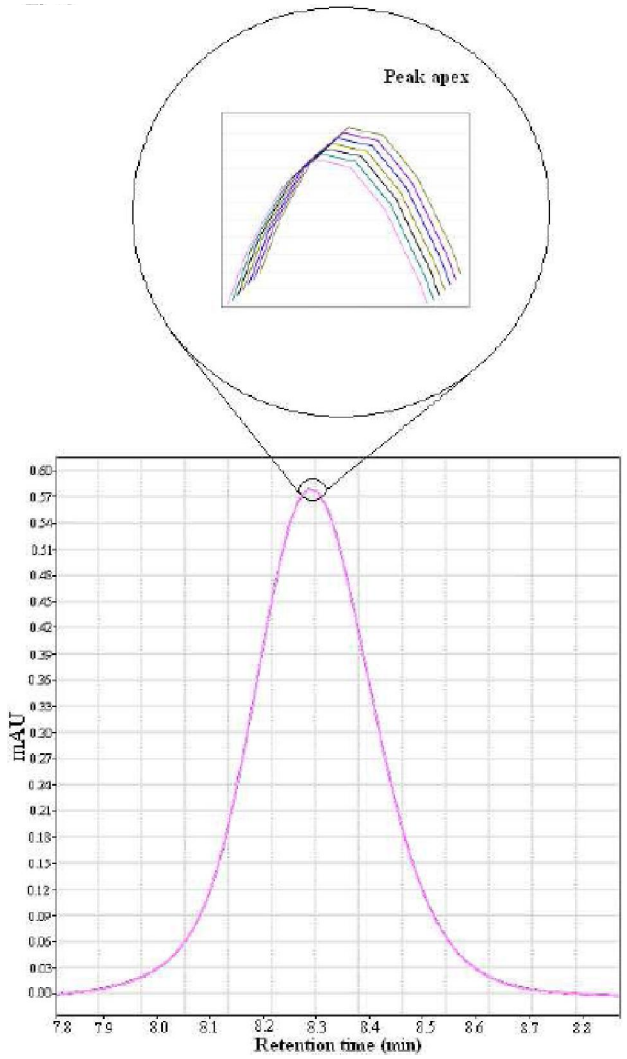

Figure 6 : Overlay of signals of itaconic acid fortified (4.0mg/ml) at wanelengths 210, 200, 212, 215, 220, and 222 nm after normalized to equal areas

analyte in a sample, which could be quantitatively determined with suitable precision and accuracy. The standard deviation of y-intercept of regression lines was determined and kept in following equation for the determination of detection limit and quantitation limit. Detection limit = 3.3 s/s; quantitation limit = 10 s/s, where s is the standard deviation of y–intercept and s is the slope of the calibration curve.

RESULTS AND DISCUSSION

Extraction of itaconic acid from hydrogel samples

Methanolic orthophosphoric acid (10:90) was used as extraction solvent. The coiled and packed chains of hydrogel matrix unfold and make rooms or voids for solvent molecules as it was allowed to swell and the total residual monomer in form of either acid or its salt diffuses from gel network to the extracting solution. Representative HPLC chromatograms of itaconic acid in different hydrogel matrix are shown in figure 4.

TABLE 3

Concentration level(μg/ml)	Day 3	Day 7
0.10	98.75±0.83(0.84)	97.14±0.85(0.88)
0.60	99.19±0.45(0.45)	98.99±0.58(0.59)
1.00	100.09±0.20(0.20)	99.85±0.26(0.26)

Chromatograms obtained by running three concentrations on 3rd and 7th day from the preparations of stock solution have been compared with those obtained initially. Values given under day 3 and day 7 denote peak area ± SD (% RSD) calculated with Respect to the average peak area of the respective concentrations as obtained initially

Stability of stock itaconic acid solution

TABLE 3 shows stability data of the stock solution containing IA. The stock solution was found to be stable for 1 week as % recovery was within the statistical limits. Further, no appreciable change was observed in the measured concentration of the IA in the presence of hydrogel matrix during the period.

Validation of the method

The methods developed for analysis of IA in hydrogel matrix was validated for linearity, accuracy, precision, specificity, and quantification limits as per ICH guidelines. Linear regression analysis confirms that the r^2 values for IA was found to be >0.9990, confirming the linear relationship between the concentration of the IA and area under the curve. Validation parameters have been highlighted in TABLE 4. Peak purity evaluation was performed with objective of obtaining additional supportive information during selection of appropriate analytical conditions that allowed specific determination of IA in gel matrix. For a pure peak, the spectral pattern at any point in time is similar because they show a pattern unique to the compound (Figure 5). However, if the peak belongs to the unseparated compound peaks (interfering compounds from matrix), the spectrum pattern at the top of the peak and the time before and after the peak are different due to the difference in retention times of each compounds. The 'peak purity' which is an indicator used to verify whether the peak is

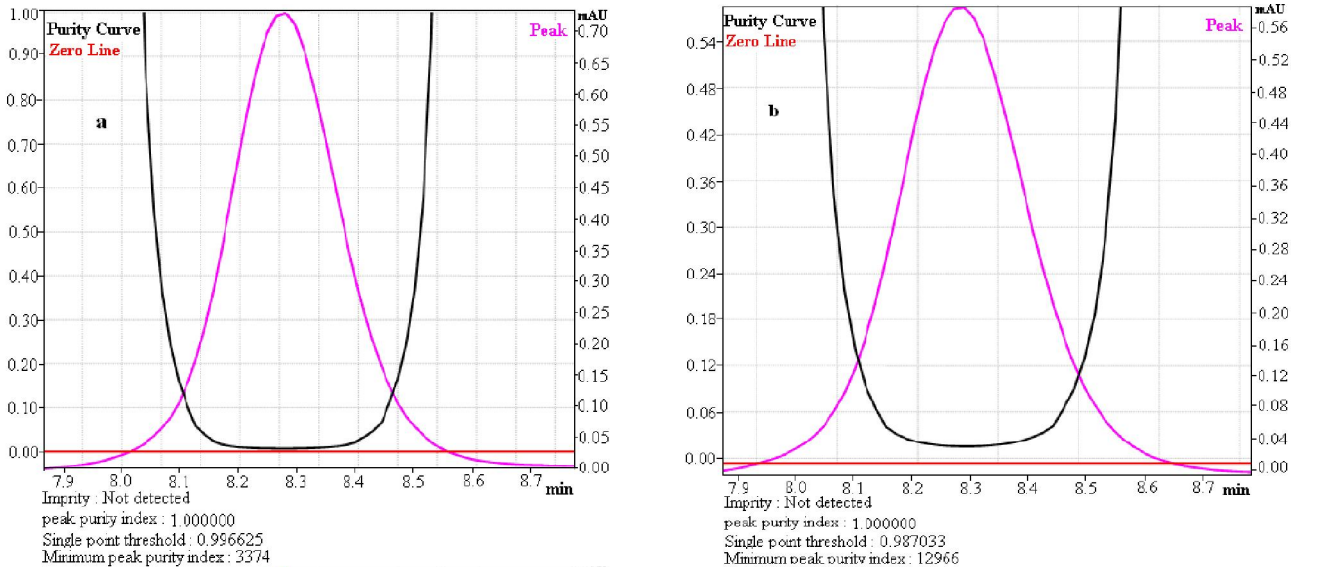

Figure 7 : Representative peak purity curve of itaconic acid in mobile phase (a) and in hydrogel matrix (b) each of the peak has a positive value of minimum peak purity index confirming the purity of the peaks and specificity

TABLE 4

Itaconic (µg/ml)acid	Intraday		Interday	
Fortified	Mean±S.D, % RSD	Recovery (%)	Mean±S.D, % RSD	Recovery (%)
4.00	3.63±0.20, 5.50	90.75	3.59±0.22, 6.12	89.75
6.00	5.56±0.17, 3.06	92.67	5.42±0.20, 3.69	90.33
8.00	7.69±0.19, 2.47	96.12	7.59±0.26, 3.42	94.88

n = 3

TABLE 5

Parameters	Values
Analytical wavelength	210nm
Calibration range (µg/ml)	0.1-1.0µg/ml
Slope(a)	$0.007\pm7.01e^{-7}$
Intercept(b)	9.78±0.074
Correlation coefficient (r^2)	0.9990±0.06
LOD(µg/ml)	0.034µg/ml
LOQ(µg/ml)	0.105µg/ml

comprised of a single compound can be calculated using this mechanism. In addition to the spectral pattern comparison, signals of fortified IA (4.0mg/ml) at different wanelengths after normalized to equal areas and the total peak purity of the standard IA and in fortified sample was also determined. Good overlap, where the peak shape and retention time match indicates the specificity of the analysis (Figure 6). The peak purity index value for each one was 1.000000 and 0.999998 respectively (Figure 7). Accuracy and precision data showed that the recoveries ranged from 89 to 96 %. Both intra- and inter-day precision (% R.S.D.) of QC standards were less than 2% over the selected range (TABLE 4). The calculated LOD and LOQ concentrations confirmed that the methods were sufficiently sensitive (TABLE 5).

CONCLUSIONS

Healthcare is an essential aspect of human survival. Polymeric materials in different forms with specific characteristics have generated significant interest in a number of biomedical applications including drug delivery. Polymers used as drug delivery systems for pharmaceuticals need to exhibit biocompatible characteristics in terms of both the polymer effect on the organism receiving the drug delivery system and, the polymer effect on the drug to be delivered. Several aspect of polymeric systems ultimately contribute to its overall biocompatibility or lack of thereof. A key factor that influences the biocompatibility of an implant polymer is the presence of low molecular weight extractable or unreacted monomers and polymerization initiators. In this report, we implemented and optimized an HPLC method to accurately and precisely quantify residual itaconic acid monomer in hydrogels. This method can fulfill the requirements of a synthetic chemist or quality control labs for practically quantifying the residual itaconic acid in hydrogels. The developed method is very simple and results obtained confirm suitable accuracy,

specificity and precision. The mobile phase was easily prepared and gave reproducible results. With the use of non-buffered mobile phases, problems associated with buffers *viz*. time required in its preparation, pH adjustments, chocking of tubings and proper washing of the system after its use has been avoided. In RP-HPLC organic solvents are generally used as components of mobile phase. Since most organic solvents are not environment friendly and are potentially toxic to the operators, minimizing the amount of organic solvents is of great importance. So we used dilute acidic solution (0.05% orthophosphoric acid) as both extracting media and mobile phase. The applied RP-HPLC method seems well suited for analysis of residual itaconic acid in other types of hydogels and composites also.

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