

Development, optimisation, validation and inter-laboratory verification of a reversed phase HPLC method for quantification of human recombinant insulin

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Abstract

HPLC methods for insulin in official monographs require extended runtimes and elevated temperatures. Inter-laboratory reproducibility of HPLC methods obtained from published literature is an on-going challenge, moreso for peptides. This paper serves as a step-by step guide to troubleshoot and establish a validated HPLC method for insulin at room temperature using simple UV detectors with minimal run times. A modified gradient reversed-phase HPLC was developed for the quantification of recombinant human insulin with UV detection at room temperature. An octadecylsilica column was used as the stationary phase while the mobile phase consisted of solution A: 1mmol sodium sulphate and 0.2% triethylamine in water and solution B: acetonitrile. The developed method was then validated using International Conference on Harmonisation (ICH) guidelines. The calibration curve was linear over a concentration range of 10-1000 µg/mL with correlation coefficient of 0.9993, with average recovery percent of $100.89 \pm 1.4\%$ and RSD recovery of 0.01. Insulin retention time was 3.84 ± 0.08 mins, while LOD and LOQ were estimated at 0.63 and 2.0 µg/mL respectively. The developed method conformed to the validation criteria of the ICH guidelines in our laboratories and other independent operator laboratories, and can serve as a rapid and effective method for quantifying insulin from any sample at room temperature using simple detectors.

Keywords : Insulin; HPLC validation; ICH guidelines; inter-laboratory reproducibility; method development; optimisation, biotech products

Introduction

High Performance Liquid Chromatography (HPLC) is an analytical technique used for separation, isolation, and quantification of components of a sample [1]. HPLC is often employed for the quantitative analysis of pharmaceutical products because chemical methods (such as iodometry or colorimetry) and some spectrophotometric methods commonly employed have limited specificity, with chemical methods showing lower precision; while gas chromatography cannot be effectively utilised because of the thermolabile nature of most pharmaceuticals [2, 3]. Moreso, HPLC allows for rapid analysis of different drugs and their metabolites [4].

The biologically active and circulating form of insulin (Figure 1) is a polypeptide comprising 2 amino acid chains linked by two di-sulfide linkages, afforded by the amino acid cysteine. It contains 51 amino acids; 21 amino acids make up the A chain and 30 make up the B chain; with molecular weight of 5800 Da. A di-sulphide linkage exists within the A chain [5]. Insulin is administered subcutaneously for the management of insulin-dependent diabetes in patients whose pancreas does not produce insulin, or those whose cells have low insulin sensitivity. It is also the most important hormone in the regulation of glucose homeostasis in the body [6].

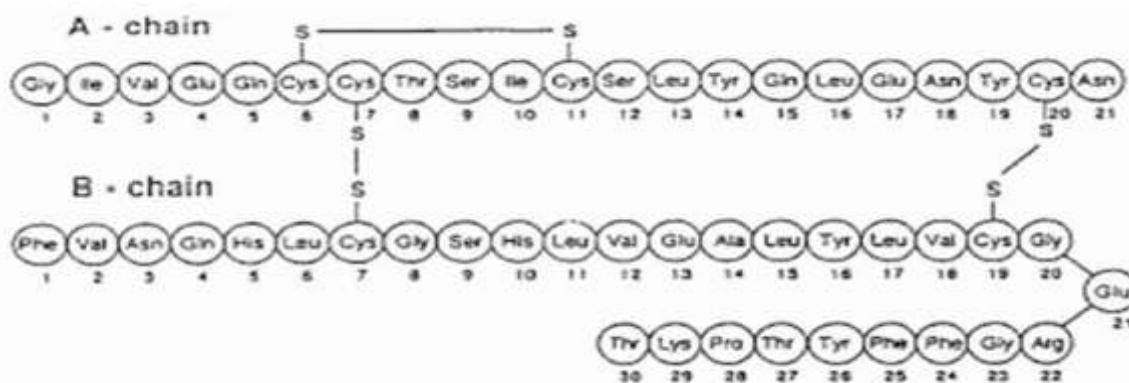


Figure 1. Primary structure of insulin showing A and B polypeptide chains, with inter- and intra-molecular disulphide bridges [7]

HPLC has found increasing applications in the isolation and purification of peptides and proteins in recent years. Proteins like insulin have been quantified using biological assays in the past. These assays, though specific, were limited by their ineffectiveness at detecting very low doses, and could not identify contaminant or decomposition products; hence, the increased use of HPLC [8]. However, the chemical nature and size of proteins makes their HPLC difficult because various factors, such as temperature and pH, need to be optimised [9, 10]. The official monographs (British Pharmacopoeia, United States Pharmacopoeia, European Pharmacopoeia) contain methods for the detection and assay of insulin by HPLC, but these methods require extended runtimes, or the use of elevated temperature [9, 11]. Various researchers have also employed HPLC for quantification of insulin in literature using buffered [9, 10, 12-14] and unbuffered mobile phases [5, 15]. However these methods require either the use of elevated temperatures, specialised detectors or resulted in long run times. More importantly, difficulty in reproducing methods obtained from published literature in different laboratories using different equipment is a constant challenge. The onus therefore is on the researcher to develop and validate a suitable reproducible method for quantification of these proteins. The said method should ideally be carried out at room temperature, with as few variables as possible. The aim of this work therefore was to describe our implementation of a literature derived method and step-by-step progression within our laboratory to establish a simple, rapid and effective method for quantification of insulin by HPLC, which would be validated by ICH guidelines [16]; and can be reproduced in any laboratory using simple UV detectors at room temperature.

Materials and Methods

Materials

Human recombinant insulin powder, phosphate buffered saline tablets, triethylamine and anhydrous sodium sulphate were purchased from Sigma-Aldrich, UK; while o-phosphoric acid and hydrochloric acid were purchased from Fisher Scientific, UK. All water used was double distilled.

Methods

High Performance Liquid Chromatography (HPLC) Assay

Gradient RP-HPLC was employed for quantitative analysis of the insulin in solution and was adapted from Rajan et al [9]. A Dionex 1100 autosampler (AS50) system, with gradient pump (GP50), UV detector (UVD 170U) set at 230nm was used, employing a reversed phase RP-C18 analytical column (Phenomenex 110A, 150x4.6mm, 5µm). The mobile phase consisted of solution A: 1mmol sodium sulphate and 0.2% triethylamine in water, pH adjusted to 3.2 with o-phosphoric acid; and solution B: acetonitrile, both filtered under vacuum and sonicated [9]. A gentle gradient was run from 75:25 v/v to 60:40 v/v compositions of A:B over 5 mins, and

then maintained at 60:40 v/v for 2 minutes. Pump flow rate was 1.5 mL/min, with sample injection volume of 20 µL. The maximum wavelength of absorption of insulin had been pre-determined using a Unicam UV-visible spectrophotometer. Finally a rectilinear (Beer-Lamberts) calibration curve was established for insulin within a concentration range of 10-1000 µg/mL dissolved in 0.01N HCl.

Hplc Assay Validation

The HPLC method was validated according to ICH guidelines in terms of specificity, accuracy, precision, linearity, limits of detection/ quantification and stability of insulin solutions [5, 10].

Specificity (Identification and Assay)

20 µL of 200 mg/mL insulin and 0.01N HCl solutions respectively were separately injected into the HPLC system, retention time and resulting chromatograms were recorded. Insulin percentage recovery (the calculated concentration of insulin relative to the actual concentration used) was calculated using the regression equation obtained from linearity experiments below.

Linearity

A Beer-Lamberts calibration curve was developed by plotting mean peak area against insulin concentration. Linearity was estimated by computing the regression line of the calibration curve for insulin concentration from 10-1000 µg/mL; the correlation coefficient (R²) was calculated.

Accuracy

20 µL aliquots of standard solutions were injected into the HPLC system and the protocol described for linearity above followed. The areas under the curve for insulin were recorded and the percentage recovery was calculated using the regression equation.

Precision

Operator precision was determined by developing the insulin calibration curve in triplicates. The area under the curve was calculated by the HPLC software and relative standard deviation (RSD) was calculated using the equation below:

$$RSD = \frac{\text{standard deviation}}{\text{mean}}$$

Calibration curves were repeated on different days (n=6 or 9) using freshly prepared mobile phase and the RSD calculated.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ were determined by the signal-to-noise ratio method [6, 17]. 20 µL of 0.01 N HCl blank was injected into the HPLC system twenty times and the mean peak area recorded. The standard deviation of the recorded peak areas was multiplied by 3 and 10 to calculate the LOD and LOQ respectively. This was repeated twice for two different samples of the blank, in order to validate the obtained results [10].

Inter-laboratory method validation

The validated method was further validated by two independent operators in two different laboratories, using different HPLC systems on different days to establish method reproducibility.

Stability of Insulin Solutions

1000 µg/mL insulin solution was freshly prepared in triplicates and immediately analysed on the HPLC system. The solutions were stored in the fridge (2-8 °C) and quantified after three days (72 hrs). The peak areas and RSD were recorded.

Results and Discussion

HPLC Method Development for quantification of insulin

The HPLC method development began with a method adapted from Rajan et al [9], employing an isocratic elution using a mobile phase comprising 40 volumes of solution A (1mmol sodium sulphate and 0.2% triethylamine (TEA) in water, pH adjusted to 3.2 with o-phosphoric acid), and 60 volumes of solution B (acetonitrile); UV detection at 214nm. This method was employed instead of the USP or BP methods, because unlike these official methods that were run at elevated temperatures, the chosen method could be run at room temperature and had less than half the retention time expected from the BP and USP methods. Thus it minimised the possible variables in the HPLC system. Using this system, insulin eluted almost immediately at about 1 min (Figure 2).

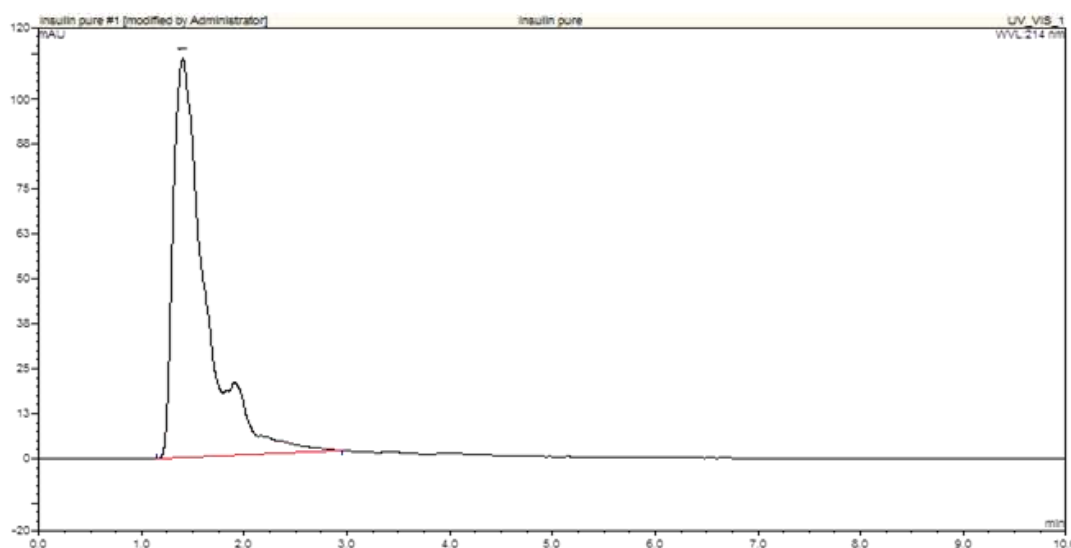


Figure 2. Sample chromatogram of 0.5 mg/mL insulin in 0.01N HCl at 214nm

The resulting peak was poorly resolved with an accompanying hump. Running a blank solution (containing 0.01N HCl) on the HPLC system showed solvent front coming off the column at about the same retention time as the drug (Figure 3). Thus, the hump on the insulin chromatogram resulted from the overlapping of the drug peak with that of the solvent front [6]. RP-HPLC exploits the interactions between the hydrophobic regions of insulin and that of the stationary phase [17]. Fast elution of insulin off the column implied very low affinity of insulin for the non-polar column. Generally in reversed phase HPLC, the interaction of hydrophobic analyte with the n-alkyl chain of the column would lead to longer retention of the analyte on the column. But the presence of steric hindrances in large molecules like insulin may restrict access of the analyte molecules to the pores of the stationary phase where these interactions take place [18]. Also, the presence of polar groups (-NH₂, COO⁻) in the insulin moiety would increase the affinity of insulin for the highly aqueous mobile phase, and result in fast elution of insulin. These factors could account for the fast elution of insulin from the mobile phase [1].

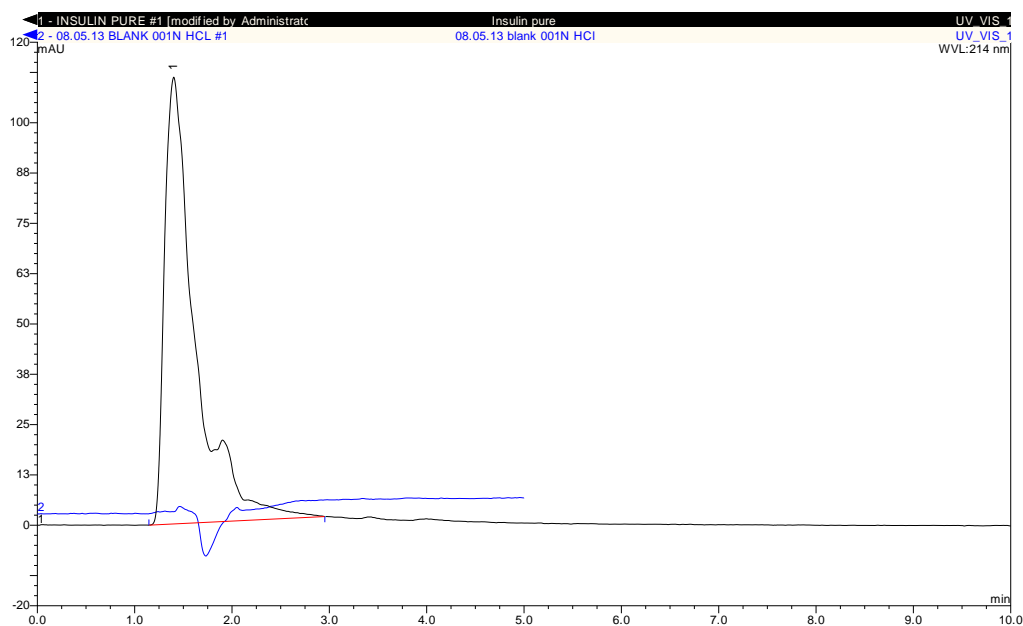


Figure 3. Chromatogram showing elution of the solvent front (blue line) at about the same time as insulin (black line) resulting in poor resolution of the insulin peak.

According to the BP 2013, "insulin is practically insoluble in water" (despite the presence of $-OH$ and $-NH$ bonds in the comprising amino acids), however, some authors have reported insulin as highly water soluble [6]. It was expected that hydrophobic insulin would interact with the non-polar column (reverse-phase HPLC) and would therefore have a longer retention time on the column [1]. As this was not the case as discussed above, reducing the non-polar organic modifier in the mobile phase would reduce the interaction of insulin with the mobile phase (increased mobile phase polarity), thereby increasing insulin's interaction with the non-polar column; ultimately resulting in an increase in insulin retention time [19]. Thus various mobile phase compositions (from 50:50 v/v to 75:25 v/v) were employed in an attempt to increase insulin's retention time, and thereby separate the drug peak from that of the solvent front.

A mobile phase composition of solution A:B 70:30 v/v resulted in an increase in insulin retention time from 1 to 2 minutes. However, this minimal shift was not sufficient to completely separate the insulin peak from that of the solvent front (Figure 4). This minimal shift in insulin retention time despite a 10% change in mobile phase composition could be attributed to the large size of insulin molecules, as large molecules may not easily enter the pores of the column to increase column retention [18].

A further reduction in the amount of the organic modifier used would be expected to further increase the retention time of insulin for reasons discussed above. However, the final composition of the mobile phase would be highly aqueous and would not effect a complete separation of other impurities or degradation products when utilising this method for quantification of insulin from samples. Moreso, highly polar aqueous phases may result in poor retention and separation due to the collapse of the octadecyl alkyl side chains in the stationary column [20]. Xu et al [6] reported an increase in insulin retention time with increasing acetonitrile content of the mobile phase.



Figure 4. Insulin chromatograms at various mobile phase compositions. None of the combinations tried were able to effectively increase insulin retention time to >2 minutes.

At this point, it became necessary to run a blind HPLC to determine the exact mobile phase composition where insulin elutes off the column. Thus, a gradient HPLC was run from 80 – 20% v/v for solution A and 20 – 80% v/v for solution B. From Figure 5, it was observed that insulin eluted at about 6 minutes which was equivalent to mobile phase composition of 60:40 v/v for solution A:B. Thus, insulin was eluted off the column immediately the mobile phase composition turned to 60:40, and using an isocratic system would always produce a poorly resolved peak because of interference from the solvent front. Also the resultant peak caused an upward shift in the baseline, probably due to the changing gradient.

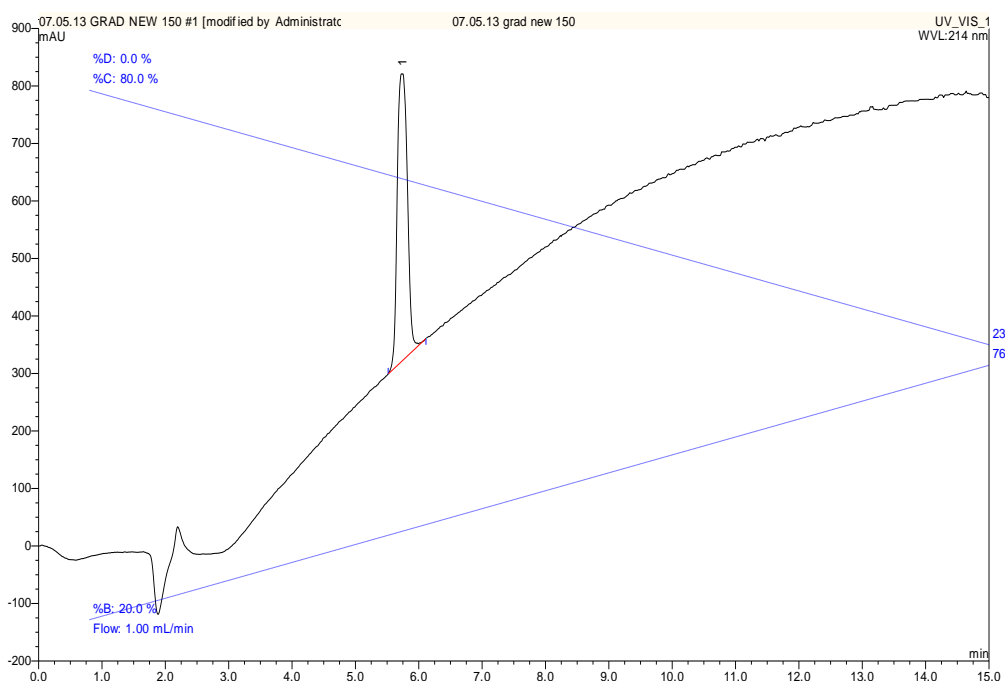


Figure 5. HPLC chromatogram showing insulin elution at 6minutes upon gradient elution from solution A: 80:20 TO 20:80. An upward shift in the baseline may be due to the changing concentration gradient.

In order to obtain a better resolved insulin peak, we further investigated the role of the mobile phase additives to the HPLC quantification. Triethylamine (TEA) was used in the mobile phase to facilitate end-capping of the free silanol groups on the reversed phase column, resulting in the prevention of band tailing in HPLC [21, 22]. However, TEA is easily degraded and has been implicated to interfere with UV absorption because it has a high UV-cut off above 190nm. This means that TEA can be picked up by the detector and would interfere with the resolution of obtained peaks. Because the column had been described as 'end-capped' by the manufacturers, it would be irrelevant to retain TEA in the mobile phase if it was interfering with the results. Thus, TEA was removed from solution A, and a gradient HPLC repeated with solution A (1mmol sodium sulphate in water, pH adjusted to 3.2 with o-phosphoric acid) 80 – 20% and solution B (acetonitrile) 20 – 80%. The resulting chromatogram produced no peak for insulin even after 30 minutes, signifying the importance of TEA for insulin elution. Thus, although a column may be claimed to be end-capped by the manufacturers, this process might not completely mop up the free silanol groups. This, as well as column use and age, would necessitate the addition of a moiety like TEA to compete with the analyte for the free silanol groups on the column.

Following the unsuccessful reproduction of the HPLC method adapted from the journal article, a return to first principles was necessary, and a UV scan was run for 0.5 mg/mL insulin in 0.01N HCl. The spectrum displayed two maximum wavelength peaks – a major peak at 230nm and a smaller peak at 275nm (data not shown). Thus a HPLC gradient (80:20 to 20:80) was run at 214, 230 and 275 nm respectively. From Figure 6 it can be seen that at 214nm, there was an upward shift in the baseline, at 275nm, the insulin peak was highly reduced; while at 230nm, a well resolved peak with a relatively stable baseline was achieved. Thus, 230nm was chosen as the wavelength for recombinant insulin detection.

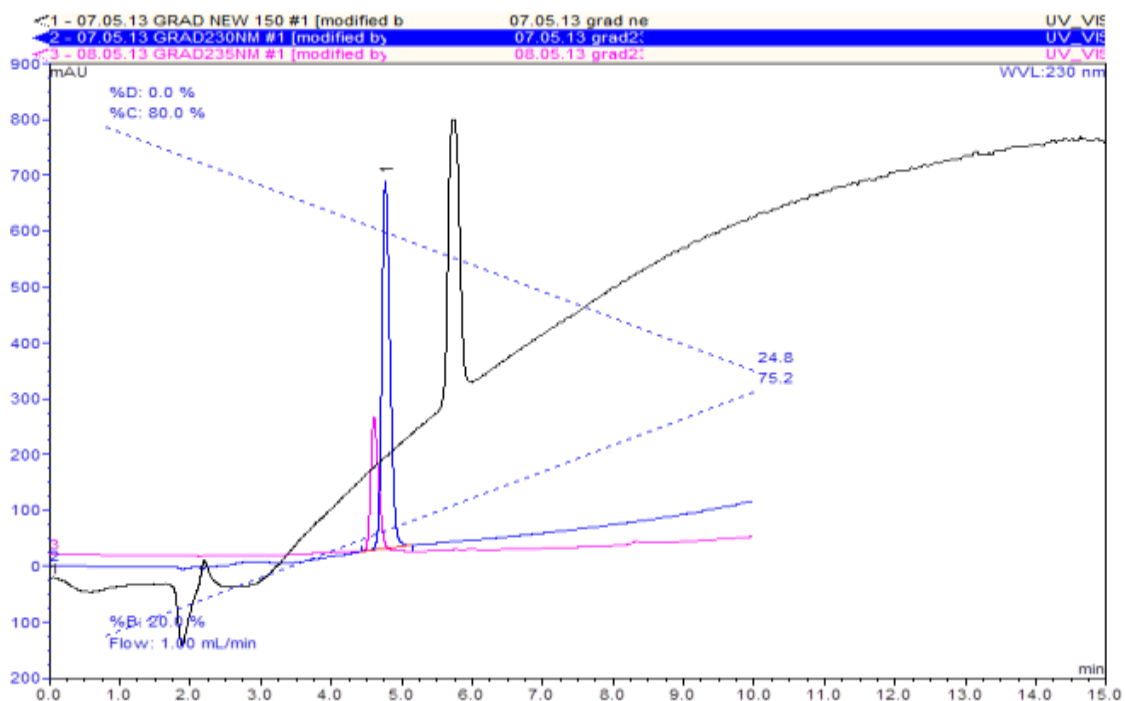


Figure 6. Chromatograms showing insulin elution at various wavelength: 214 nm (black), 230 nm (blue) and 275nm (pink).

Because insulin eluted off the column immediately the mobile phase composition was 60:40, and this early elution resulted in a poorly resolved peak due to the concurrent appearance of the solvent front, isocratic HPLC could not be applied to this system. A gradient (80:20 to 20:80 v/v) HPLC for insulin was run at 230nm and resulted in a well resolved peak with a relatively stable baseline, and no interference from the solvent front, and which eluted after about 4 minutes (Figure 7). Because it had been established that insulin eluted off the column at 60:40 mobile phase composition, it was not necessary to continue the steep 80:20 to 20:80

gradient, it seemed necessary to employ a gentle gradient. To determine the optimum gradient for separation, the following gradients (Table 1) were run:

Table 1. Mobile phase compositions employed to optimise insulin elution off the analytical column

Option 1	Option 2
0-5 mins: 75:25 - 50:50	0-5 mins: 70:30 - 45:55
5-7 mins: 50:50 - 50:50	5-7 mins: 50:50 - 50:50
7-10 mins: 50:50 - 75:25	7-10 mins: 50:50 - 75:25

The resulting chromatogram for option 1 showed a well resolved peak, completely separated from the solvent front, with a slight shifting of the baseline. Modification of the gradient to option 2 resulted in a poorly resolved peak which eluted off the column very fast and showed solvent front interference. Thus the first protocol was maintained.

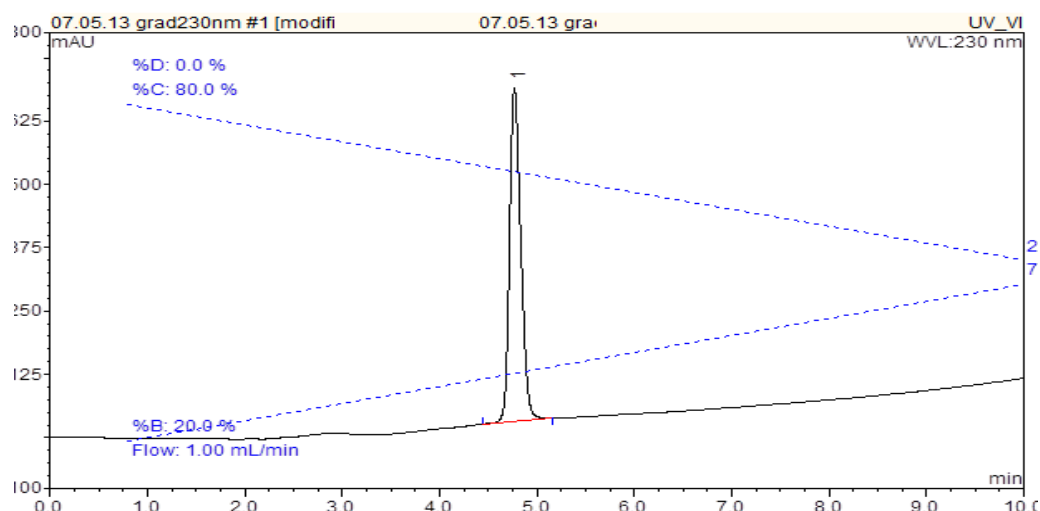


Figure 7. Gradient HPLC for insulin at 230 nm

Finally, to achieve a sharper peak, the flow rate was increased to 2 mL/min. A sharper insulin peak was achieved but it eluted off the column at 2 minutes. Gerber [18] reported that increased flow rate could reduce retention times, increase baseline noise and produce narrower peaks. Therefore, flowrate was maintained at an average of 1.5 mL/min. The chromatogram for the final method is shown in Figure 8 below:

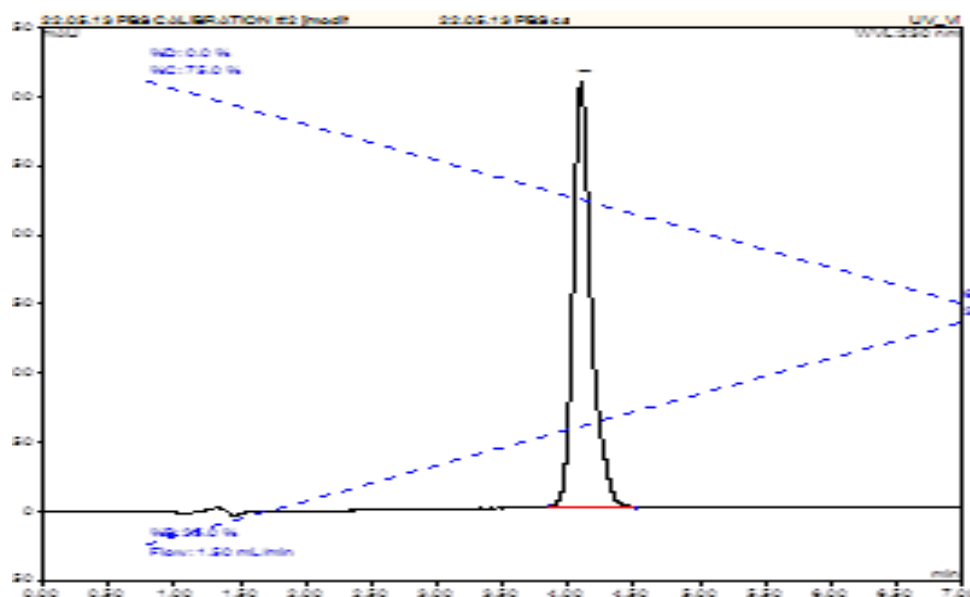


Figure 8. Representative chromatogram showing the developed method for insulin elution employing a gentle gradient at 230 nm detection wavelength and 1.5 mL/min flow rate

HPLC Assay Validation

Specificity

To investigate the specificity of the HPLC method, 20 μ L each of insulin in 0.01N HCl and the blank (0.01N HCl) were separately injected into the HPLC and chromatograms developed. Figure 9 shows the chromatograms of insulin and the blank run in triplicate. From this chromatogram, the insulin peak was well resolved with retention time of 3.84 ± 0.08 , there was no interference from the solvent front which eluted at 1.12 ± 0.04 mins (Table 2). For use with insulin formulations, it would be necessary to test all excipients of the formulation to ensure specificity of the method for insulin quantification in the presence of contaminants. According to Gupta [23], an assay shows specificity when the signal measured from the test compound shows no interference with signals from other excipients at the test wavelength. As an evaluation of accuracy, insulin concentrations ranging from 50 – 500 μ g/mL were used to calculate the percentage recovery of insulin using the described method. The mean absolute recovery value for insulin was $100.89 \pm 1.40\%$ (Table). These results connote that the developed method can quantify insulin effectively. However, the effect of dosage form excipients on the recovery of insulin using this method will need to be investigated [6].

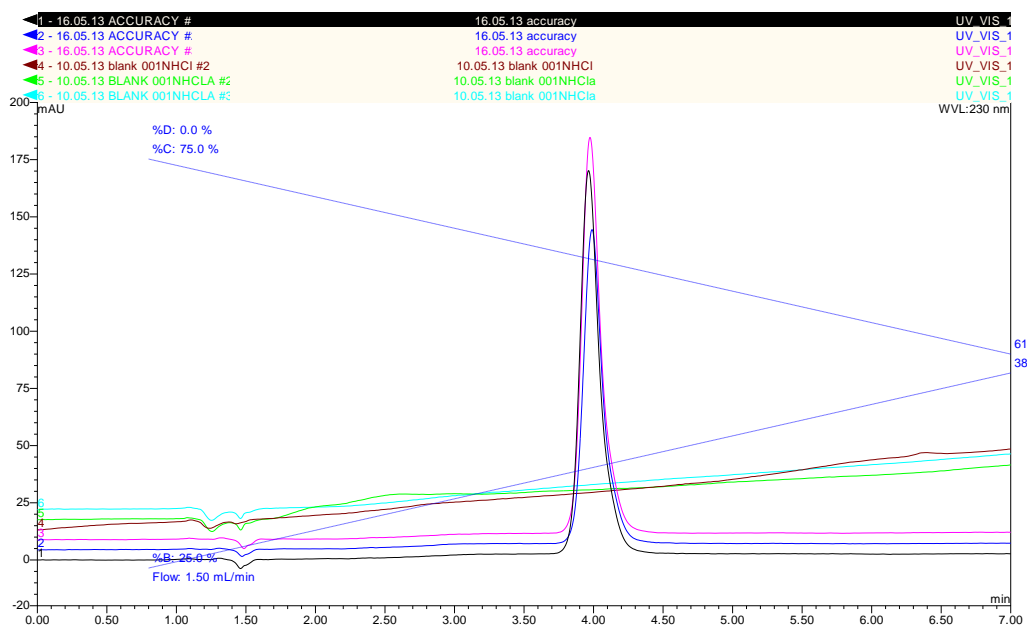


Figure 9. Representative chromatograms showing selectivity and instrument precision of HPLC method. Triplicate measurements of 500 µg/mL insulin solution produced identical peaks that were well resolved from that of the blank.

Table 2. Specificity of insulin HPLC method

S.N.	Sample	Retention time (min)	% RSD
1	Insulin	3.84 ± 0.08	2.08
2	Blank (0.01N HCl)	1.12 ± 0.04	3.66

Linearity

A Beer-Lamberts calibration curve (Figure 10Figure) was established for insulin over a concentration range of 10-1000 µg/mL by plotting the peak area from the chromatograms against the concentration. The regression analysis gave the equation of the line as $y = 0.0543 - 1.0026x$, with $R^2 = 0.9993$. Parameters of the regression coefficient are presented in Table 3. The high correlation coefficient connotes a linear relationship between peak area and insulin concentration within the proposed range [5, 24]. Therefore, the concentration of insulin in any sample can be calculated from this curve using this method.

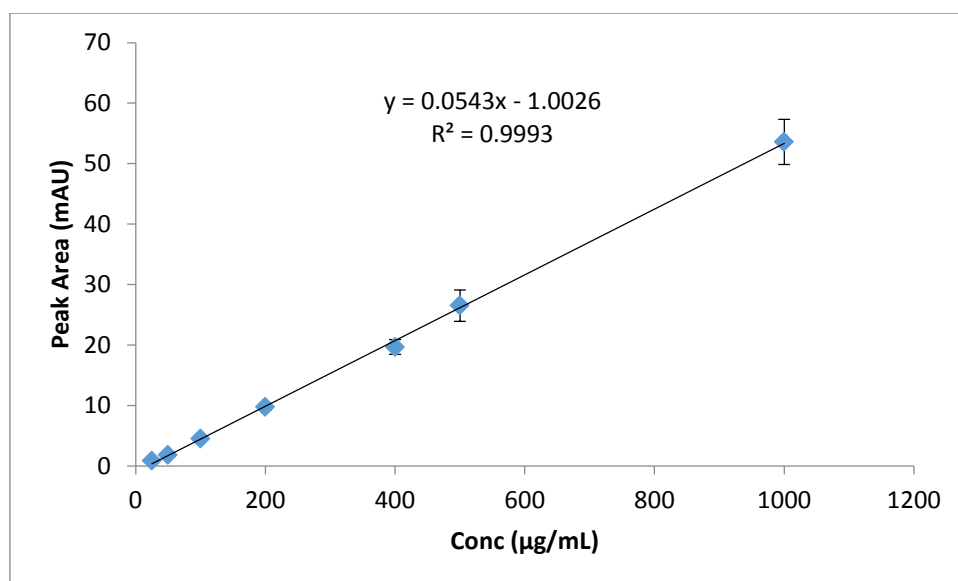


Figure 10. Beer-Lamberts calibration curve for insulin

Table 3. Factors of the regression equation linking insulin concentration to the analytical response (area under the peak)

S.N.	Parameter	Value
	Linearity range, µg/mL	10-1000
	LOD, µg /mL	0.63
	LOQ, µg /mL	2.00
	Slope of regression equation	0.0543
	Intercept	-1.0026
	Correlation coefficient	0.9993
	Standard error	0.8099

Repeatability (instrument and operator precision)

Instrument precision (accuracy) was determined by injecting the same concentration of insulin solution into the HPLC three times and calculating the %RSD. Figure 9 shows the overlapping of the three IND chromatograms (lines 1-3), indicating the precision of the instrument. From Table 2, the RSD for insulin and the blank were relatively low at 2.01 and 3.66% respectively. Similar results were obtained during validation of a HPLC method for insulin reported by Moussa et al [10]. Operator precision was determined by establishing the calibration curve in triplicates. %RSD values calculated ranged from 7.7 – 9.76% (Table 4). These values were within the limits for precision as reported by Epshtein [24].

Inter-day precision was determined by establishing calibration curves on different days – results are shown in Table 5. The overall %RSD values ranged from 3.3 – 9.3%. These values for %RSD are within the limits for

precision. Ephstein [24] reported that for precision in analysis of pharmaceuticals, %RSD should range between 0-15%.

Table 4. Table highlighting intra-day accuracy and precision parameters of the HPLC method due to high %recovery and low RSD (n=3)

S.N.	Insulin conc. actual ($\mu\text{g/mL}$)	Peak Area (mAU)	RSD	Insulin conc. calculated ($\mu\text{g/mL}$)	Recovery (%)
1	50	1.78 ± 0.14	0.07	51.31 ± 2.55	100.56 ± 2.42
2	100	4.49 ± 0.43	0.10	101.11 ± 7.85	101.38 ± 4.89
3	200	9.27 ± 0.06	0.06	197.60 ± 10.14	98.80 ± 4.71
5	500	26.52 ± 2.59	0.10	506.90 ± 47.67	101.11 ± 3.99
6	1000	53.60 ± 3.72	0.07	1005.57 ± 68.56	102.63 ± 3.30
Mean % recovery = $100.89 \pm 1.40\%$					
RSD % Recovery = 0.01					
LOD = $0.63 \mu\text{g/mL}$					
LOQ = $2.00 \mu\text{g/mL}$					
Correlation coefficient			0.9993		

Table 5. Inter-day precision and accuracy parameters for HPLC method (n=6)

Insulin conc. actual ($\mu\text{g/mL}$)	Amount measured on day 1 ($\mu\text{g/mL}$)	RSD	Amount measured on day 2 ($\mu\text{g/mL}$)	RSD	Overall RSD
50	51.31 ± 2.55	0.05	56.03 ± 4.37	0.08	0.08
200	197.60 ± 10.14	0.05	202.43 ± 3.71	0.02	0.04
400	380.55 ± 22.20	0.06	397.27 ± 15.18	0.04	0.05
500	506.90 ± 47.67	0.09	457.48 ± 30.44	0.07	0.09
1000	1005.57 ± 68.56	0.06	1012.82 ± 55.61	0.05	0.06

Limit of Detection & Limit of Quantification

The LOD and LOQ were calculated from the signal-to-noise ratio, as well as from the standard deviation of the response and the slope and were found to be 0.63 and $2.00 \mu\text{g/mL}$ respectively (Table 4). Thus the lowest

amount of insulin that can be detected and quantified respectively, within the limits of accuracy and precision, using this method are 0.63 and 2.0 µg/mL [5, 17].

Inter-laboratory Method Validation

The HPLC method was validated according to ICH guidelines by independent operators in different laboratories, using different HPLC systems; on the same day and on three different days, to assess the method's reproducibility. For the first site, retention time was 3.70 ± 0.03 mins, with a peak well resolved from the solvent front (Figure). Regression analysis yielded equation of the line as $y = 0.0851x - 1.2928$ and $R^2 = 0.9998$. Intra-day and inter-day results are presented in Table 6 and Table 7 respectively. From here, it can be seen that the method was reproducible with %RSD of 1-3%. Similar results obtained from the second site are depicted in Table 8. Thus this method could be reproduced in any laboratory and employed for the quantification of insulin from samples.

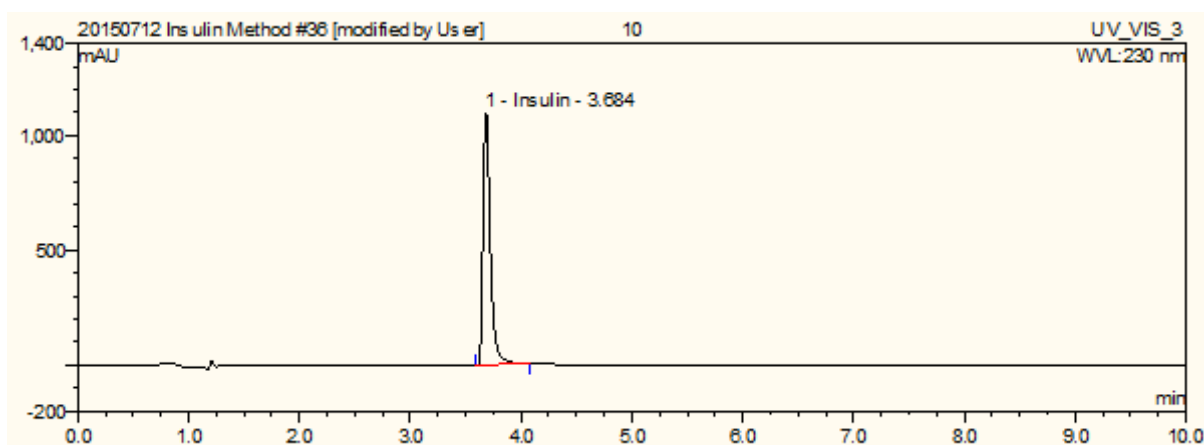


Figure 11. Representative chromatogram of insulin elution during inter-laboratory validation of HPLC method at Liverpool John Moores University, UK. A well separated insulin peak with no interference from the mobile phase was seen.

Table 6. Intra-day HPLC validation parameters for insulin method carried out by an independent operator at Liverpool John Moores University, using a different HPLC system on the same day, to assess method reproducibility (n=3)

S.N.	Insulin conc. actual (µg/mL)	Insulin conc. calculated (µg/mL)	RSD	Recovery (%)	RSD
1	62.5	55.60 ± 1.10	0.02	88.95 ± 1.76	0.02
2	125.0	120.01 ± 1.30	0.01	96.01 ± 1.04	0.01
3	250.0	254.47 ± 6.07	0.02	101.83 ± 2.43	0.02
4	500.0	513.29 ± 13.14	0.03	102.66 ± 2.63	0.03
5	1000.0	1003.48 ± 23.84	0.03	100.35 ± 2.38	0.02
Mean % recovery		= 97.96 ± 5.65			
RSD % Recovery		= 0.02			

LOD = 0.53 µg /mL

LOQ = 1.78 µg /mL

Correlation coefficient 0.9998

Table 7. Inter-day precision and accuracy parameters carried out by an independent operator at Liverpool John Moores University, using a different HPLC system on three different days, to assess method reproducibility (n=9)

Insulin conc. actual (µg/mL)	Amount measured on day 1 (µg/mL)	Amount measured on day 2 (µg/mL)	Amount measured on day 3 (µg/mL)	Overall amount measured (µg/mL)	Overall RSD
62.5	55.60 ± 1.10	57.51 ± 2.98	59.43 ± 3.00	57.51 ± 1.91	0.03
125.0	120.01 ± 1.30	123.40 ± 2.79	125.28 ± 6.81	122.90 ± 2.67	0.02
250.0	254.47 ± 6.07	259.08 ± 3.28	257.82 ± 10.57	257.16 ± 2.33	0.01
500.0	513.29 ± 13.14	521.10 ± 4.14	516.62 ± 22.13	517.00 ± 3.92	0.01
1000.0	1003.48 ± 23.84	985.23 ± 10.22	985.94 ± 4.07	991.54 ± 10.34	0.01

Mean % recovery = 96.06 ± 8.29%

RSD % Recovery = 0.09

Table 8. Intra-day HPLC validation parameters for insulin method carried out by an independent operator at the University of Wolverhampton, using a different HPLC system on the same day, to assess method reproducibility (n=3)

S.N.	Insulin conc. actual (µg/mL)	Insulin conc. calculated (µg/mL)	RSD	Recovery (%)	RSD
1	62.5	62.11 ± 2.19	0.04	98.38 ± 3.50	0.04
2	125.0	123.82 ± 2.43	0.02	99.06 ± 1.95	0.02
3	250.0	246.95 ± 11.10	0.04	98.78 ± 4.44	0.04
4	500.0	506.26 ± 16.73	0.03	101.25 ± 3.35	0.03
5	1000.0	997.81 ± 47.11	0.05	99.78 ± 4.71	0.05

Mean % recovery = 99.65 ± 0.97

RSD % Recovery = 0.01

LOD = 0.74 µg /mL

LOQ = 2.47 µg /mL

Correlation coefficient 0.9999

Stability of Insulin Solutions

Results of stability of 1000 µg/mL insulin solutions stored in the fridge for 72 hours are shown in Table 9. The percent recovery was calculated using the regression equation of the calibration curve. ANOVA for the peak areas and amount of insulin measured on both days showed no significant difference ($p > 0.05$). The overall RSD of 7.94 – 8.07% shows that insulin solutions can be stored in the fridge for up to three days without deterioration. This is in line with results obtained by Moussa et al [10], who reported that insulin solutions remained stable in the fridge for up to 5 days.

Table 9. Stability of 1000 µg/mL insulin solutions after storage at 2-8 °C for 72 hours (n=3). Results showed that insulin solutions were stable for 3 days when stored in the fridge.

	Day 0	Day 3	Overall RSD
Peak Area	62.32	62.32	8.07
(mAU)	67.80	61.37	
	54.15	56.14	
Amount	1054.78	1052.54	7.94
measured	1145.52	1036.66	
	919.45	949.26	

Conclusions

The aim of this work was to describe the steps involved in our optimisation and validation of a simple, rapid and efficient method for insulin quantification by HPLC, which could be reproduced in any laboratory at room temperature. Insulin was effectively eluted at 3.8 mins with no interference from the solvent front. The method was validated in our laboratories and independent operator laboratories with similar results that complied with the ICH guidelines for HPLC method validation. Thus, the developed method is rapid with short runtime, at room temperature with simple UV detectors, and low detection and quantification limits, which can be employed for the HPLC quantification of insulin from any sample with high reproducibility from other laboratories.

Data Availability

Email corresponding author for supplementary data.

Conflicts of Interest

The authors declare no conflict of interest in this work.

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References

1. Aguilar, M.-I., *HPLC of Peptides and Proteins*. 2004: Springer.
2. Nachtmann, F., *Automated high-performance liquid chromatography as a means of monitoring the production of penicillins and 6-aminopenicillanic acid*. *Chromatographia*, 1979. **12**(6): p. 380-385.
3. Roškar, R. and T.T. Lušin, *Analytical Methods for Quantification of Drug Metabolites in Biological Samples*. 2012: INTECH Open Access Publisher.
4. Chan, C., K. Chan, and G. French, *Rapid high performance liquid chromatographic assay of cephalosporins in biological fluids*. *Journal of Antimicrobial Chemotherapy*, 1986. **18**(4): p. 537-545.
5. Sarmiento, B., et al., *Development and validation of a rapid reversed-phase HPLC method for the determination of insulin from nanoparticulate systems*. *Biomedical Chromatography*, 2006. **20**(9): p. 898-903.
6. Xu, X., et al., *Quantitative determination of insulin entrapment efficiency in triblock copolymeric nanoparticles by high-performance liquid chromatography*. *Journal of Pharmaceutical and Biomedical Analysis*, 2006. **41**(1): p. 266-273.
7. Snyder, L.R., J.J. Kirkland, and J.L. Glajch, *Practical HPLC method development*. 2012: Wiley-Interscience.
8. Grego, B. and M. Hearn, *Role of the organic solvent modifier in the reversed phase high-performance liquid chromatography of polypeptides*. *Chromatographia*, 1981. **14**(10): p. 589-592.
9. Rajan, D.S., et al., *Development of RP-HPLC for analysis of human insulin*. *Indian journal of pharmaceutical sciences*, 2006. **68**(5): p. 662.
10. Moussa, B., F. Farouk, and H. Azzazy, *A validated RP-HPLC method for the determination of recombinant human insulin in bulk and pharmaceutical dosage form*. *Journal of Chemistry*, 2010. **7**(S1): p. S449-S457.
11. Sarmiento, B., et al., *Alginate/chitosan nanoparticles are effective for oral insulin delivery*. *Pharmaceutical research*, 2007. **24**(12): p. 2198-2206.
12. Makhlof, A., Y. Tozuka, and H. Takeuchi, *Design and evaluation of novel pH-sensitive chitosan nanoparticles for oral insulin delivery*. *Eur J Pharm Sci*, 2011. **42**(5): p. 445-51.
13. Oliva, A., J. Fariña, and M.a. Llabrés, *Development of two high-performance liquid chromatographic methods for the analysis and characterization of insulin and its degradation products in pharmaceutical preparations*. *Journal of Chromatography B: Biomedical Sciences and Applications*, 2000. **749**(1): p. 25-34.
14. Yilmaz, B., Y. Kadioglu, and I. Capoglu, *Determination of Insulin in Humans with Insulin-Dependent Diabetes Mellitus Patients by HPLC with Diode Array Detection*. *Journal of chromatographic science*, 2012. **50**(7): p. 586-590.
15. Amidi, M., et al., *Preparation and physicochemical characterization of supercritically dried insulin-loaded microparticles for pulmonary delivery*. *European Journal of Pharmaceutics and Biopharmaceutics*, 2008. **68**(2): p. 191-200.
16. Geneva, I., *Switzerland: ICH; 1996*. ICH Q1B, Photostability testing of new drug substances and products.

17. Moslemi, P., A.R. Najafabadi, and H. Tajerzadeh, *A rapid and sensitive method for simultaneous determination of insulin and A21-desamido insulin by high-performance liquid chromatography*. Journal of Pharmaceutical and Biomedical Analysis, 2003. **33**(1): p. 45-51.
18. Gerber, F., et al., *Practical aspects of fast reversed-phase high-performance liquid chromatography using 3µm particle packed columns and monolithic columns in pharmaceutical development and production working under current good manufacturing practice*. Journal of Chromatography A, 2004. **1036**(2): p. 127-133.
19. Lindsay, S. and D. Kealey, *High performance liquid chromatography*. Other Information: From review by Jon F. Parcher, Univ. of Mississippi, in J. Am. Chem. Soc., Vol. 110, No. 11 (1988). 1987. Medium: X; Size: Pages: 264.
20. Olsen, B.A., *Hydrophilic interaction chromatography using amino and silica columns for the determination of polar pharmaceuticals and impurities*. Journal of Chromatography A, 2001. **913**(1–2): p. 113-122.
21. Li, H., R.I. Krieger, and Q.X. Li, *Improved HPLC method for analysis of 1-hydroxypyrene in human urine specimens of cigarette smokers*. Science of The Total Environment, 2000. **257**(2–3): p. 147-153.
22. Dolan, J.W. and L.R. Snyder, *Troubleshooting LC systems: a comprehensive approach to troubleshooting LC equipment and separations*. 1989: Springer Science & Business Media.
23. Gupta, V., et al., *Development and validation of HPLC method-a review*. International Research Journal of Pharmaceutical and Applied Sciences, 2012. **2**(4): p. 17-25.
24. Epshtein, N., *Validation of HPLC techniques for pharmaceutical analysis*. Pharmaceutical Chemistry Journal, 2004. **38**(4): p. 212-228.