

Quantitation of Humalog Insulin by Reversed-Phase High-Performance Liquid Chromatography

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Abstract

Introduction:

The methodology for *in vitro* testing of insulin delivery systems for long-term infusion of lispro insulin requires insulin flow studies over time, with the measurement of lispro concentrations in wells or other laboratory fluid collection systems. We postulated that the efficiency of the insulin assay could be improved if the insulin collected in the wells could be measured without having to perform dilutions of insulin samples. The manufacturer's method for the identification of Humalog[®] insulin by high-performance liquid chromatography (HPLC) does not provide a detailed method for the quantitation of the clinical formulation. No reports could be found in the literature describing a method to quantitate lispro insulin's entire concentration range, nor could we find detailed information on pH effects. The purpose of this study was to investigate the use of a reversed-phase HPLC method to quantitate Humalog insulin at three levels of pH.

Methods:

Serial dilutions of Humalog insulin stock solution were prepared at pH levels of 2.6 and 7.4 (concentrations 0.20–100 IU/ml) to construct calibration curves. Samples were also prepared at lower concentrations (0.23–15.0 IU/ml) at pH levels of 4.0 and 7.4. Areas under curve data were plotted against known concentrations, and the limit of quantitation and precision of the assay were determined.

Results:

Calibration curves for the 0.20–100 IU/ml concentration (pH 7.4 and 2.6) revealed no significant differences ($p < 0.01$) between those curves. pH and concentration did not have an effect on the overall precision of the method. Linearity was not conserved past the 15-IU/ml concentration. Coefficient of variation (CV) values were generally <15% except at the lower concentrations where the largest CVs were observed. Mean known concentrations plotted on a logarithmic scale demonstrated linearity from 12.5 to 100 IU/ml.

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Abbreviations: (AUC) area under the curve, (IP) isoelectric point, (LOQ) limit of quantitation, (PBS) phosphate-buffered saline, (RP-HPLC) reversed-phase high-performance liquid chromatography, (TFA) trifluoroacetic acid

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Abstract cont.**Conclusions:**

Humalog insulin clinical formulation can be quantitated over the entire concentration range by a reversed-phase HPLC method using nonlinear regression analysis. The method is reproducible at lower (<15 IU/ml) and higher insulin concentrations. Linear regression analysis may be used when the concentrations of interest are in the 0- to 15-IU range. Preparation of insulin solutions at pH 2.6, 4.0, and 7.4 did not significantly affect the reproducibility of the assay.

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Introduction

Our interest in the quantitation of Humalog® (lispro insulin, Eli Lilly and Co., Indianapolis, IN) stems from the *in vitro* testing of insulin delivery systems. A major concern with using lispro insulin for long-term infusions, as would be the case with an implantable drug delivery system, is the overall stability of the insulin molecule.¹⁻⁴ It is possible for the insulin to polymerize and/or precipitate in the pump reservoir, tubing, or catheter,^{5,6} therefore altering the amount of drug delivered to the patient, or even result in complete occlusion of the system.^{7,8} Insulin lispro has also been shown to precipitate in the cannulas of pumps and to cause metabolic decompensation in a few cases.^{9,10}

Some *in vitro* testing methodologies for long-term insulin delivery systems used in our laboratory, and by others,¹¹ require insulin flow studies over time, with the measurement of lispro concentrations in wells or other laboratory fluid collection systems. In our experience we often find it necessary to measure the full insulin concentration to determine if the device is delivering the correct amount of insulin. We believe that the efficiency of the insulin assays could be improved if the insulin concentrations in the wells could be measured without having to perform dilutions of insulin samples, as was advised by the manufacturer (personal communications). The manufacturer has published a method for the identification of Humalog insulin using reversed-phase high-performance liquid chromatography (RP-HPLC), but does not provide a method for the quantitation of the full concentration range without dilution of the commercial lispro formulation. The manufacturer's method is more qualitative and looks specifically at the identification of the insulin lispro molecule and the excipients and preservatives in the clinical formulation (des-amido lispro insulin, zinc, etc.).¹¹ Their upper assay

limit for Humalog insulin concentration is approximately 23 IU/ml (0.8 mg) with no other information available on analysis of the full concentration. No reports could be found in the literature describing a method to quantitate lispro insulin full concentration, nor could we find detailed information on pH effects. Therefore, the purpose of this study was to investigate the use of a RP-HPLC method to quantitate the clinical formulation of Humalog insulin at three levels of pH.

Methods

To construct calibration curves, serial dilutions of Humalog insulin stock solution were prepared at pH levels of 2.6 and 7.4, with concentrations ranging from 0.20 to 100 IU/ml. The commercial preparation (insulin lispro U-100, Eli Lilly and Co.) was obtained from the hospital pharmacy. The pH 2.6 samples were prepared by initially acidifying the Humalog insulin stock solution with a 6.25 N HCl solution (Fisher, Fort Lawn, NJ) and serially diluting the acidified stock solution with 0.01 N HCl (Fisher). The pH 7.4 solutions were prepared by serially diluting the stock solution with phosphate-buffered saline (PBS, pH 7.4 (Sigma, St. Louis, MO).

Humalog insulin samples were also prepared at lower concentrations (linear region) ranging from 0.23 to 15.0 IU/ml at pH levels of 4.0 and 7.4. The pH 4.0 solutions were prepared by initially acidifying the stock Humalog insulin with a 0.1 N HCl (Fisher) to a pH of 4.0, diluting the insulin in a ratio of 1:5.6 to a concentration of 15.0 IU/ml with PBS (pH 4.0), and serially diluting this solution with PBS, pH 4.0 (Sigma). The pH 7.4 samples were prepared in the same manner as the previous samples, but without acidification. All samples were refrigerated and analyzed on the same day as prepared.

Solutions were analyzed in triplicate on a Waters Symmetry 300 C18 column (5 μm , 250 \times 4.6 mm) (Waters Corporation, Milford, MA) with an ultraviolet detector of 214 nm. A gradient method was used with mobile phases of 0.10% trifluoroacetic acid (TFA) in water and 0.10% TFA in acetonitrile. Millennium[®] 32 software was utilized to process peak areas and retention times.

Area under the curve (AUC) data were plotted against known concentrations to construct calibration curves. The assay limit of quantitation (LOQ) and precision were determined. Data for the LOQ were obtained in a series of previous experiments using Novolin insulin (Novo Nordisk Pharmaceutical, Clayton, NC) and are not reported here.

Data Analysis

Standard curves were plotted at each pH and the linear region was identified visually. The mean \pm SD was determined for each lispro concentration and pH level. Linear and polynomial regressions were constructed for quantitation of the lower and full concentration ranges, respectively. A two-way ANOVA with repeated measures was used to determine significant differences between the curves at each pH. The precision of the method was determined by calculating the mean coefficient of variation (CV) for each concentration. A p value <0.05 was used to determine statistical significance.

Results

This RP-HPLC analysis method resulted in a chromatogram with two distinct peaks (**Figure 1**): one being the lispro insulin peak and the other being *m*-cresol, the primary preservative found in clinical formulation. The lispro insulin and *m*-cresol eluted from the column with approximate retention times of 6.7 and 8.2 minutes, respectively, with the entire injection lasting 18 minutes to ensure column equilibration. Inspection of the insulin peaks revealed no peak tailing or baseline drifting.

Figure 2 illustrates calibration curves for the full lispro insulin concentration range (0.20–100 IU/ml) at pH levels of 7.4 and 2.6. There were no significant differences ($p < 0.01$) between the curves as determined by two-way ANOVA with repeated measures. Therefore, pH and concentration did not have an effect on the overall precision of the method. Visual inspection of the curves showed linearity at the lower insulin concentrations (up to 15 IU/ml).

Several dilutions were also prepared at the lower concentrations (0.23–15 IU/ml) to examine the linear

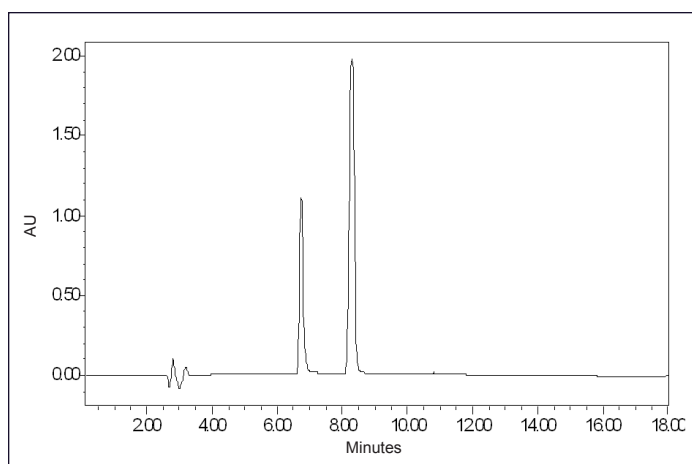


Figure 1. Sample chromatogram for lispro (Humalog) insulin. Peaks represent lispro insulin and *m*-cresol (preservative), respectively.

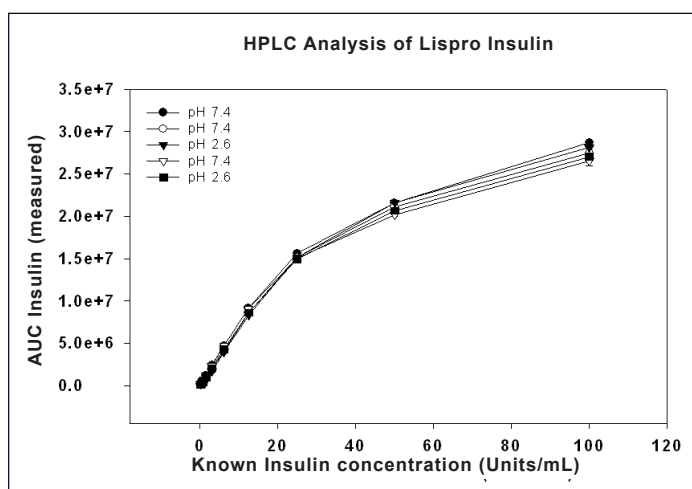


Figure 2. Plot of RP-HPLC results (AUC of HPLC insulin peak) of lispro insulin versus known injected concentration at pH levels of 2.6 (two runs) and 7.4 (three runs). Circles represent one run, triangles one run, and square one run (total of three runs).

region more closely (**Figure 3**). Linearity was observed up to 15 IU/ml at pH values of 7.4 and 4.0, with no significant differences between curves. Linearity is not conserved past the 15-IU/ml concentration.

The precision of this analytical method was also determined. The coefficients of variation for each concentration were determined for both the full concentration range (**Table 1A**) and the lower linear range (**Table 1B**). Coefficient of variation values were generally below 15%, except for some of the very low concentrations (three points in **Table 1A**).

Finally, mean known concentrations were plotted on a logarithmic scale (**Figure 4**) and demonstrated linearity from 12.5 to 100 IU/ml.

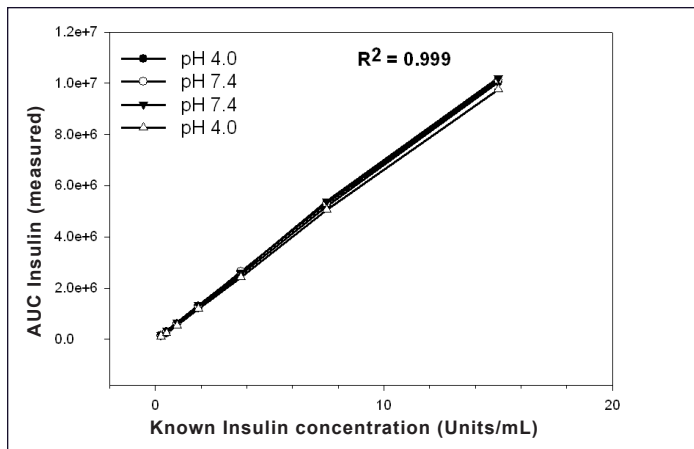


Figure 3. Plot of linear region (0.23–15 IU/ml) of RP-HPLC results (AUC of HPLC insulin peak) of lispro insulin versus known injected concentration at pH levels of 4 (two runs) and 7.4 (two runs). Circles represent one run and triangles represent a second run.

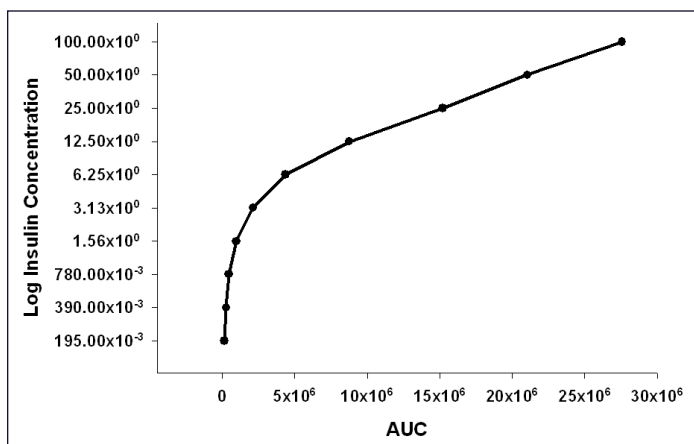


Figure 4. Mean plot of RP-HPLC results (AUC of HPLC insulin peak) of lispro insulin versus the log of the known injected concentration at a pH level of 7.4.

Discussion

These results demonstrate that the clinical formulation of Humalog insulin can be quantitated over a concentration range of 0.20 to 100 IU/ml using nonlinear regression with curves that showed good reproducibility. We have also demonstrated that linear regression analysis can be used to quantitate concentrations up to 15 IU/ml. This observation was expected as per our past communications with the manufacturer, although no specific concentration ranges had been specified. For such an analysis, a 10-fold dilution is recommended when concentrations are expected to be in the 15- to 100-IU/ml range.

Varying the pH of the solutions showed no significant effect on the quantitative measurement of lispro insulin.

Table 1A. Mean Coefficients of Variation for Lispro Concentrations (0.20–100 IU/ml) at pH 7.4 and pH 2.6^a

Known insulin concentration (IU/ml)	CV (%) pH 7.4	CV (%) pH 2.6
0.20	6.00	11.00
0.39	25.00	21.10
0.78	49.00	14.00
1.56	25.00	11.00
3.13	14.00	6.70
6.25	8.00	4.50
12.50	4.00	2.90
25.00	1.00	2.00
50.00	2.00	3.30
100.00	3.00	4.00

^aMean concentrations were obtained from RP-HPLC runs at pH 7.4 and pH 2.6.

Table 1B. Mean Coefficients of Variation for Lispro Insulin Concentrations with Dilutions for Lower Range Assay (0.23–15 IU/ml)^a

Known insulin concentration (IU/ml)	CV (%) pH 7.4	CV (%) pH 4.0
0.23	0.13	0.08
0.49	0.19	0.30
0.94	0.19	0.39
1.88	0.46	0.40
3.75	1.40	1.59
7.50	3.07	3.43
15.00	6.76	15.37
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—	—	—
—	—	—

^aMean concentrations were obtained from RP-HPLC runs at pH 7.4 and pH 4.0.

When initially acidifying the insulin solution, it is very important to be cognizant of the isoelectric point (IP) of the insulin (lispro insulin IP is 5.4). If this pH is reached, the insulin will precipitate out of solution and the concentration expected will be higher than what is observed. Although no significant differences were found between curves with changes in pH, we observed higher CVs for three of the lowest concentrations; however, this

did not occur in the lower range assay (**Table 1B**). This observation may be explained by an increased relative error at the low concentrations and/or precipitation of small amounts of lispro from acidification. For preparation of the pH 2.6 samples, the lispro stock solution was acidified from a starting pH of ~7.0 to a pH of ~2.6. This solution was then diluted with additional HCl, but at a lower normality. During the initial acidification procedure, if the solution was not mixed completely during the addition of the HCl, some of the lispro may have precipitated as the solution reached the isoelectric point. This precipitation was more evident at the lower concentrations due to the increased relative error. Because we were dealing with very low concentrations (e.g., 0.23 IU/ml), any amount of precipitation at these low concentrations would be significant when compared with the initial total concentration.

We selected a measurement range of 0.23–15 IU for examination of the linear region of the assay based on some preliminary work in this laboratory (data not published). Our goal was to determine if linearity would be affected at the lower concentrations with large changes in pH. No significant changes were observed between the curves for those runs. We would once again caution when acidifying to be aware of the IP to avoid any insulin precipitation. With regard to reproducibility of results by others, it should be pointed out that our samples were refrigerated and analyzed on the same day as prepared, which should have resulted in negligible degradation. Perhaps some minor changes or shifts from the linear to the nonlinear region might occur but we believe that the predominantly nonlinear behavior of the calibration curves would be preserved as demonstrated by multiple runs.

In conclusion, the Humalog insulin (lispro) clinical formulation can be quantitated over the entire concentration range by a reversed-phase HPLC method using nonlinear regression analysis. The method was reproducible in the lower (0.23–15 IU/ml) and the upper (0.20–100 IU/ml) ranges of insulin concentrations examined. Linear regression analysis may be used when the concentrations of interest are in the 0- to 15-IU range. Preparation of insulin solutions at pH 2.6, 4.0, and 7.4 does not significantly affect the reproducibility of the assay.

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References:

1. Gin H, Renard E, Melki V, Boivin S, Schaepepelynck-Belicar P, Guerci B, Selam JL, Brun JM, Riveline JP, Estour B, Catargi B; EVADIAC Study Group. Combined improvements in implantable pump technology and insulin stability allow safe and effective long term intraperitoneal insulin delivery in type 1 diabetic patients: the EVADIAC experience. *Diabetes Metab.* 2003 Dec;29(6):602-7.
2. Renard E, Souche C, Jacques-Apostol D, Lauton D, Gibert-Boulet F, Costalat G, Bringer J, Jaffiol C. Improved stability of insulin delivery from implanted pumps using a new preparation process for infused insulin. *Diabetes Care.* 1999 Aug;22(8):1371-2.
3. Boivin S, Belicar P, Melki V. Assessment of in vivo stability of a new insulin preparation for implantable insulin pumps. A randomized multicenter prospective trial. EVADIAC Group. Evaluation Dans le diabete du Traitement par Implants Actifs. *Diabetes Care.* 1999 Dec;22(12):2089-90.
4. Demirdjian S, Bardin C, Savin S, Zirinis P, Chast F, Slama G, Selam JL. Lispro insulin is suitable for external pumps but not for implantable pumps. *Diabetes Care.* 1998 May;21(5):867-8.
5. Scavini M, Hammarberg B, Dosio F, Torri M, Petrella G, Galimberti G, Vai S, Fazio F, Pozza G. A method for assessing catheter patency in implanted pumps for long-term intraperitoneal insulin delivery. *Artif Organs.* 1997 May;21(5):405-8.
6. Poulsen C, Langkjaer L, Worsoe C. Precipitation of insulin products used for continuous subcutaneous insulin infusion. *Diabetes Technology & Therapeutics.* 7(1):142-50, 2005.
7. Loughheed WD, Woulfe-Flanagan H, Clement JR, Albisser AM. Insulin aggregation in artificial delivery systems. *Diabetologia.* 1980 Jul;19(1):1-9.
8. Loughheed WD, Zinman B, Strack TR, Janis LJ, Weymouth AB, Bernstein EA, Korbas AM, Frank BH. Stability of insulin lispro in insulin infusion systems. *Diabetes Care.* 1997 Jul;20(7):1061-5.
9. Wright AWD, Little AJ. Cannula occlusion of insulin lispro and insulin infusion systems. *Diabetes Care.* 1998 May;21(5):874-5.
10. Wolpert HA, Faradji RN, Bonner-Weir S, Lipes MA. Metabolic decompensation in pump users due to lispro insulin. *BMJ.* 2002 May 25;324(7348):1253.
11. DeFelippis MR, Bell MA, Heyob JA, Storms SM. *In vitro* stability of insulin lispro in continuous subcutaneous insulin infusion. *Diabetes Technol Ther.* 2006 Jun;8(3):358-68.