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Research Article

A SIMPLE AND SENSITIVE HPLC METHOD FOR THE DETERMINATION OF INSULIN IN RAT PLASMA AND ITS APPLICATION IN PHARMACOKINETIC STUDY

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ABSTRACT

Objectives: The objectives of this research were to develop and validate a reverse phase-high performance liquid chromatography (RP-HPLC) method for determination of insulin in rat plasma and demonstrate its application in pharmacokinetic study.

Methods: Plasma sample was treated with deproteinizing agent which consisted of acetonitrile and propanol (1:1). The mobile phase comprised of water and acetonitrile mixture and both components contained 0.1% trifloroacetic acid (TFA). The analysis involved using gradient elution by increasing the proportion of mobile phase from 15% to 40% of acetonitrile within 20 min at a flow rate of 0.6 ml/min at 210 nm. A Phenomenex C18 column (5µm 4.6 x 250 mm) was used for the chromatographic separation.

Results: The method produced linear response over the concentration range of 0.39 to 50 µg/ml. The mean extraction recovery was 99.6%, while the coefficient of variation of within-day and between-day measurements was less than 8%. The stability percent ratios of insulin over 30 days were 97.5% to 100.41%. The limit of quantification (LOQ) and limit of detection (LOD) of the method were 0.39 µg/ml and 0.13 µg/ml, respectively. Pharmacokinetic profiles of insulin in rat plasma shows AUC values at 192.47± 10.9 µg/ml·min.

Conclusion: The speed, specificity, sensitivity and reproducibility of this method are particularly suitable for routine determination of insulin in rat plasma.

Keywords: RP-HPLC, Insulin, Deproteinization, Gradient elution, Pharmacokinetic profiles.

INTRODUCTION

Diabetes Mellitus is a group of metabolic disease which is characterized by insufficient production of insulin (type I) or failure of the body to utilize the insulin produced (type II). Currently the number of patients that suffer from diabetes mellitus is reaching 285 million adult worldwide, and by the year 2030 the numbers are expected to reach 439 million adults [1]. Insulin treatment is the main therapy for diabetes type I [2], while type II diabetes patients require insulin administration when oral hypoglycemic agents become inadequate [3,4].

Insulin is important for regulating glucose homeostasis, glycogen breakdown, gluconeogenesis, lipolysis and ketogenesis [5]. It consists of 51 amino acids shared between two intramolecular chains, A-chain and B-chain [6,7]. A-chain consists of 21 amino acids and the B-chain consists of 30 amino acid residues, both of these chains were linked by two disulphide bridges [8]. Insulin is a water-soluble, unstable protein. It is impermeable through the intestinal mucosa causing poor absorption via oral administration. Furthermore, insulin is extensively degraded by proteases in the gastrointestinal tracts [9].

Various studies have been developed on the quantitative method to determine the level of insulin in a plasma sample. These methods include employing capillary electrophoresis [10] and high performance liquid chromatography (HPLC) [11,12]. HPLC provides simplicity while allowing adequate sensitivity, selectivity, precision and accuracy in the determination of various compounds in human, rabbit and rat plasma analysis [13,14,15,16]. However, plasma-insulin determination using isocratic HPLC has proven to be time consuming and involves tedious sample preparations. Thus reversed-phase gradient HPLC is an excellent method for peptide and small protein analysis [17]. This method engages a simple sample preparation procedure which yields specific and accurate results.

In this study we focused on the development and validation of reverse phase gradient HPLC in determination of insulin in rat plasma.We also demonstrated the implementation of this method in pharmacokinetic study.

MATERIALS AND METHODS

Materials

Bovine insulin and phosphate buffered saline were purchased from Sigma-Aldrich (USA). Acetonitrile (ACN) was obtained from Merck (Germany). Trifluoroacetic acid was obtained from Scharlau (Spain). Deionized water was obtained from Reservoir® Elga water system (United Kingdom). All other chemicals used were of analytical grade unless otherwise stated.

Preparation of standard solution

A standard stock solution was prepared by dissolving bovine insulin in 0.01 M hydrochloric acid [12,18] at a concentration of 1000 µg/ml. The standard solutions (n=8) were constructed by spiking insulin-free rat plasma with known amount of insulin ranging between 0.39 - 50 µg/ml. Three insulin concentrations were prepared as in-house quality control, which were 50 µg/ml, 6.25 µg/ml and 0.78 µg/ml, respectively.

Sample preparation

The blood plasma was treated with deproteinizing agent (DA) which consisted of acetonitrile: propanol (1:1), at the ratio of 2:1 (DA: samples/standards). The mixtures were vortexed for 1 minute and were then centrifuged at 10000 rpm for 10 minutes. After centrifugation, 50 µL of the supernatant was filtered and injected into the HPLC system for chromatographic separation.

Chromatographic system and condition

The UPLC system from Waters (USA) coupled with Photo Diode Array (PDA). Chromatographic separation was operated on a Phenomenex reversed-phase (Jupiter 5u C18 300A) column with particle size of 5 µm (150 mm × 4.6 mm i.d.). The mobile phase comprised of a mixture of water and acetonitrile and both solutions contained 0.1% trifloroacetic acid. Gradient was applied by increasing acetonitrile concentration from 15% to 40% within 20 min. The eluent was later monitored with a PDA detector set at 210 nm with a flow rate of 0.6 ml/min. The experiments were conducted at ambient temperature and the total area of peak was used to quantify the insulin.

Linearity

Calibration plots were constructed for bovine insulin standard solutions by plotting the concentration of compounds versus peak area response. Standard solutions containing 0.39-50 µg/m1 of bovine insulin in plasma were prepared and 50 µl was injected into the HPLC column. The calibration equation (y = mx + b) were calculated from the calibration curves. The regression equations were calculated from the calibration graphs with the standard deviations of the slope and value interception [19].

Accuracy and Precision

Accuracy of the assay method was determined for both within-day and between-day variations using the six times analysis of the samples. Precision of the assay was determined by repeatability (within-day) and intermediate precision (between-day) [20]. Intermediate precision was assessed by comparing the assays on different days (6 days). According to United States Food and Drug Administration (USFDA) [21], all data was expressed as %CV (coefficient of variation). The acceptance value for precision and accuracy for each concentration should not exceed 15% of percentage of coefficient of variation (%CV) from the theoretical value.

Detection and Quantitation Limits (sensitivity)

Limit of detection (LOD) and quantitation (LOQ) were estimated from the signal-to-noise ratio. The detection limit was defined as the lowest concentration level resulting in a peak height of three times the baseline noise. The quantitation limit was defined as the lowest concentration level that provided a peak height with a signal-to-noise ratio higher than 10, with precision (% RSD) and accuracy (% bias) within ± 10% [22].

Stability

Stability of insulin was measured by preparing three replicates (n=3) of insulin samples at three different concentrations of 0.78 µg/ml, 6.25 µg/ml and 50 µg/ml, respectively. The stability of the samples was assessed by percentage recovery based on criteria's recommended by USFDA [20]. The procedures were designed as follow; (a) freeze-thaw stability, which was determined after three consecutive freeze-thaw cycles. (freezing temperature -20°C for 24 hours and thawing unassisted at room temperature); (b) short term stability of samples was determined by exposing sample to room temperature for 24 hours; (c) long term stability of samples was determined after keeping the samples frozen at -20°C for 30 days; (d) post-preparative/ auto sampler stability studies were determined after keeping the sample in an auto sampler at 4°C for 24 hours and (e) the working solution stability was determined by exposing working solution at room temperature for 6 hours.

RESULTS AND DISCUSSION

Chromatogram of insulin

Sample preparation was one of the essential factors in achieving good results. In this study, we developed a simple sample preparation procedure by using acetonitrile and propanol at 1:1 ratio as deproteinizing agent. The deproteinizing agent to sample ratio were set at 2:1. According to previous studies [23,24,25], acetonitrile or methanol was added to plasma under certain condition to terminate the reaction and to precipitate protein. Moreover, acetonitrile and propanol have insulin solubility, thus insulin can still be detected after centrifugation process. The analysis of blank plasma (Figure 1a) shows no interference in the chromatogram. The overall analysis time was 20 min and insulin retention time was approximately at 15.8 min (Figure 1b).

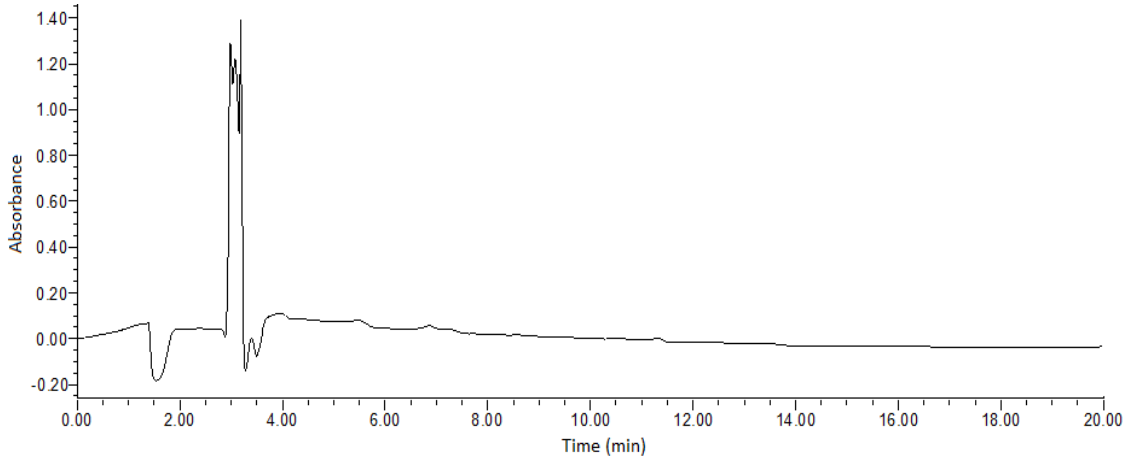

Fig. 1a: Chromatogram of blank rat plasma, no interference was found in the chromatogram.

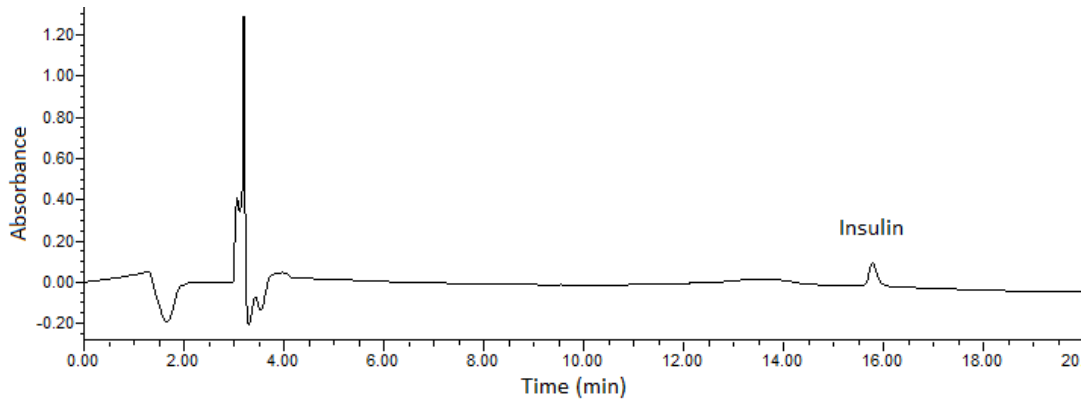

Fig. 1b: Chromatogram of rat plasma spiked with 100 µg/ml insulin. Insulin peak was eluted at 15.8 min.

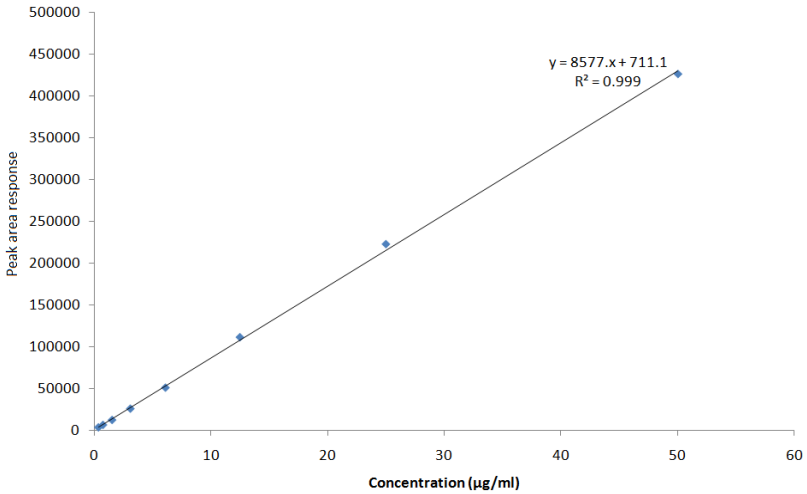

Fig. 2: Mean standard curve of insulin standard spiked rat plasma ranges from 0.39 – 50.0 µg/mL under gradient condition; Mobile phase- acetonitrile: water (15:75) to (40:60) in 20 min; column- 5 µm×150 mm×4.6 mm; flow rate 0.6 ml/min; detection-210 nm

Linearity

Calibration curves of insulin standard in rat plasma showed a linear line over the range of 0.39 – 50 µg/ml. The data was consistent throughout the experiment which showed a consistency of coefficient, intercept and slope. The calibration curve gives R^2 of 0.999 and y-interception was 711.1 as shown in Figure 2.

Accuracy and precision

The accuracy of this method was assessed by the determination of the percentage recovery of insulin in rat plasma for both between-day and within-day variations. Eight different standard solutions of concentration in the range of 0.39 to 50 µg/ml were analyzed. Then the results obtained were summarized in Table 1 and Table 2. The results described the accuracy of this method with a mean recovery of 97.4% for within-day samples and 98.9% for between-day samples, respectively.

We then assessed the precision of this method from the repeatability of eight different standard solutions for six times in the same day (within-day). The intermediate precision used the same eight standard solutions six times on different days (between-day). The precision is described by the percentage relative standard deviation (RSD). In Table 1, the relative standard deviation for within-day samples ranged from 2.88% to 9.69% and between-day samples ranged from 0.59% to 9.18% as stated in Table 2. The proposed method is proven precise and accurate since the results obtained is within the acceptance criteria by USFDA guidelines for precision and accuracy determined at each concentration level. The guidelines dictate that the values should not exceed 15% of the RSD except for the lowest limit of quantitation, where it should not exceed 20% of the RSD.

Table 1: Within day results expressed in mean ± standard deviation (S.D), accuracy in percentage of recovery (%) and precision in coefficient of variation (%CV). Each data represents a set of triplicates.

Within-day			
Concentration (µg/ml)	**Mean ± S.D (µg/ml)**	**% Accuracy (Recovery)**	**Precision (Repeatability) % CV**
50	48.79 ± 1.41	98.10	2.88
25	25.49 ± 2.35	103.00	9.21
12.5	12.73 ± 0.61	102.30	4.78
6.25	5.82 ± 0.16	96.40	2.72
3.125	2.94 ± 0.28	95.50	9.69
1.56	1.39 ± 0.09	91.30	6.46
0.78	0.71 ± 0.03	93.80	4.11
0.39	0.37 ± 0.02	98.40	6.46

Table 2: Between day results expressed in mean ± standard deviation (S.D), accuracy in percentage of recovery (%) and precision in coefficient of variation (%CV). Each data represents a set of triplicates.

Between-day			
Concentration (µg/ml)	**Mean ± S.D (µg/ml)**	**Accuracy (Recovery %)**	**Precision (Intermediate) % CV**
50	50.34 ± 0.30	100.70	0.59
25	24.86 ± 0.38	99.40	1.53
12.5	12.83 ± 0.76	102.60	5.93
6.25	6.32 ± 0.20	101.20	3.17
3.125	3.08 ± 0.19	98.60	6.21
1.56	1.55 ± 0.09	99.60	5.62
0.78	0.74 ± 0.06	94.80	7.57
0.39	0.37 ± 0.03	93.80	9.18

Sensitivity

Eight different concentration of insulin were assayed to calculate the limit of detection (LOD) and limit of quantitation (LOQ). LOD represent the lowest concentration that can be detected by the method, while LOQ is the lowest concentration that can be determined at an acceptable precision and accuracy. In this study, the LOD and LOQ for insulin analysis were 0.13 µg/ml and 0.39 µg/ml, respectively.

Stability

Sample pre-treated with deproteinizing agent shows consistent stability in all conditions that were set by USFDA [21]. The recovery data was obtained by comparing the samples to the freshly prepared sample. The stability percent ratios ranged from 97.05% to 100.41%. The results of stability studies were within the acceptable range as shown in Table 3.

Table 3: Stability data quality control sample (n=3); 0.78 µg/ml, 6.25 µg/ml and 50 µg/ml. Each sample was assigned into several predetermined condition.

Recovery (%) ± S.E.			
	Concentration (µg/ml)		
	50	6.25	0.78
(a) Freshly prepared insulin	100±0.0	100.28±00.1	98.04±0.01
(b) Room temperature for 6hr	100±0.0	100.19±0.02	98.67±0.02
(c) Room temperature for 24hr	100±0.00	100.18±0.02	98.70±0.02
(c) Room temperature for 24hr	100±0.0	100.18±0.01	98.72±0.01
(e) Freeze thaw at -20°C for 24hr	100±0.01	100.23±0.03	98.33±0.03
(f) Frozen at -20°C for 30days	99.99±0.00	100.41±0.02	97.05±0.02

Pharmacokinetic profiles

Absorption experiments were performed by an *in-situ* closed loop method, as reported previously [26,27]. In the pharmacokinetic profiles, the maximum concentration (C_{max}) achieved was 0.99±0.03 µg/ml at maximum time (T_{max}) of 100 min (Figure 3). Area under the curve (AUC) of intestinal absorption was calculated using trapezoidal method from zero to final sampling time [28]. The AUC value from triplicate experiment was 192.47±10.9 µg/ml·min. At early stages (0-15 min), insulin was absorbed through the intestinal tract and entered hepatic portal vein. After passing through the liver, insulin was transported throughout the body in the plasma [29]. Insulin reached its maximum concentration before it was metabolized by tissues. Finally, insulin concentration decreased due to metabolism and elimination process by muscle, adipocytes and kidney tissue [30].

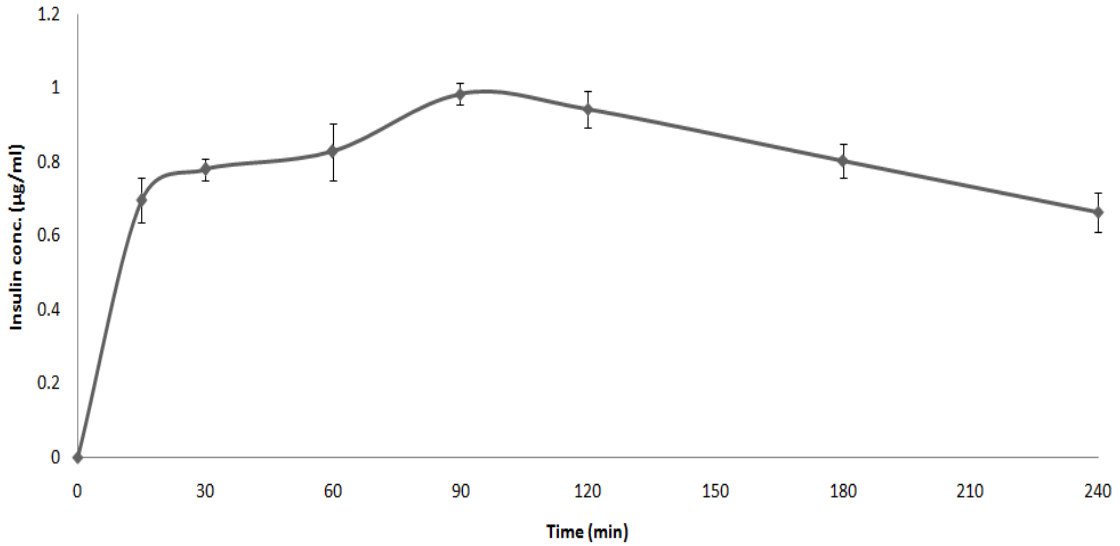

Fig. 3: Insulin concentration profile using *in situ* closed loop method. Administration of insulin (20 IU/250 g) with optimized HPLC condition. The results are expressed as the mean ± S.E. of at least 3 experiments.

CONCLUSION

In conclusion, a rapid and reliable gradient RP-HPLC method for determination of insulin in rat plasma was developed and validated. This method implements simple and effective sample preparation using deproteinizing agents providing a sensitive and specific analysis of insulin with short analysis time. In addition, the method is successfully applied in the pharmacokinetic study of insulin.

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