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Development and Validation of a Stability-indicating HPLC Method for Determination of Insulin Detemir and Preservatives in their Injection Products

Dua'a GH. Farah[a], Enam A. Khalil[b], Randa SH. Mansour[c] and Imad I. Hamdan[d*]

[a]*Department of Pharmacy, Faculty of Pharmacy, Zarqa University, Zarqa, Jordan*

[b]*Department of Pharmaceutics and Pharmaceutical Technology, School of Pharmacy, The University of Jordan, Amman, Jordan*

[c]*Faculty of Pharmacy, Philadelphia University, 19392 Amman, Jordan*

[d]*Department of Pharmaceutical Sciences, School of Pharmacy, The University of Jordan, Amman, Jordan*

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Insulin detemir (ID) is a long-acting form of insulin that is characterized by the covalent attachment of a lipophilic tail of myristate, and commercially available as Levemir®. No satisfactory simple isocratic stability-indicating HPLC method has been reported for its quality control. A novel simple and isocratic reversed-phase HPLC method was developed and validated for the simultaneous determination of ID along with its dosage form additives in the available commercial preparations. The method employed C4 column (5 µm, 250 × 4.6 mm), a mobile phase consisting of 50 mM phosphate buffer pH 2.7, acetonitrile, triethylamine (62:37:1) and 0.02 g ml^{-1} sodium sulfate, that was delivered isocratically at a flow rate of 1.5 ml min^{-1}, and detection performed at 214 nm. The method was properly validated and was shown linear over the range 80-120% of the assay concentration for ID, phenol, and m-cresol. The method was also selective, specific, precise, accurate and robust. Furthermore, the validated method was applied to separate the major degradation products in those preparations. Forced degradation studies in different pH values, which are the first to be reported for ID, showed that the degradation products were baseline separated from ID itself and/or other formulation additives. Thus, the method has been demonstrated to be a stability-indicating assay.

Keywords: Detemir, Insulin, Stability-indicating, HPLC

INTRODUCTION

Diabetes Mellitus (DM) is a chronic disorder caused by either absolute insulin deficiency (Type I DM), resistance to insulin action (Type II DM), or a combination of both. The peptide hormone, insulin, is considered to be the cornerstone therapy for all type I and many type II DM patients [1]. The goal of insulin therapy is to substitute natural insulin in a manner that mimics the pattern of insulin secretion in healthy individuals. This is usually achieved by administration of a prandial (short-acting insulin) boluses and basal level (long-acting) types of insulin. Classical approaches for protraction of insulin effect relied on the formation of insoluble complexes of insulin with zinc in a suspension format [2]. Attempts have been made to develop insulin analogs that better imitate the physiological basal insulin profile. Insulin Detemir (ID) which is marketed as (Levemir®), is a long-acting form of insulin that was developed in the 1990s and shown to exhibit a favored insulin release profile utilizing a completely new principle of insulin protraction [3]. ID is a chemically modified human insulin which differs chemically from human insulin by the omission of a threonine residue at position B30 and the covalent attachment of myristic acid (14-carbons chain fatty acid) to lysine residue at position B29 [4]. Strong noncovalent binding of ID, through its non-polar myristic acid residue, to human albumin is the core principle of insulin protraction where albumin serves as a

Corresponding author. E-mail: I.hamdan@ju.edu.jo

reservoir for insulin [5].

A limited number of analytical methods were published for the determination of ID in commercially available pharmaceutical formulations. These methods include micellar electrokinetic chromatography [6-8], and high-performance liquid chromatography (HPLC) with tandem mass spectrometry detection (LC-MS) [9-11]. Such methods require expensive instruments that are not always available in laboratories for routine analysis.

Only few HPLC-UV methods have been reported for the determination of ID and its related compounds in pharmaceutical preparations in spite of being attractive alternatives with advantages such as simplicity and potentially high separation efficiency. Theoretically, the separation of ID is expected to be more challenging than ordinary insulin due to the significantly lipophilic myristate tail that is attached to ID together with the hydrophilic amino acids which would result in longer retention times with probably broadened peaks. Preliminary experiments in our labs showed that the official pharmacopeial method (BP) described for the quantification of insulin, while suitable for separation of human and bovine insulin was incapable of eluting ID.

An isocratic HPLC method for the determination of ID has been reported, but the method was unable to separate and quantify the formulation additives (phenol and m-cresol) or the major degradation products, besides it required quite a long run time of approximately 21 min [12]. Shah *et al.* have reported an HPLC method for the determination of the degradation products of ID but still did not show any peaks for the formulation additives [13].

After an extensive literature search, it was realized that no studies have reported a validated stability-indicating HPLC-UV method with isocratic elution that was capable of separating and quantifying ID along with its formulation additives in presence of its major degradation products. No pharmacopeial method was published for the determination of ID. Therefore, the purpose of this study was to develop and validate a simple stability-indicating method for the simultaneous determination of ID in its commercial products to support quality control and stability studies. The method utilized the commonly available HPLC-UV system with a C4 column and an isocratic elution mode.

EXPERIMENTAL

Materials

Levemir® (ID) from Novo Nordisk was purchased from local pharmacy shops (Batch No. HT6J726), m-Cresol and Phenol were obtained from Sigma Aldrich (USA). All HPLC grade solvents were from (TEDIA®, USA). Laboratory reagent-grade sodium phosphate dibasic anhydrous, sodium sulfate anhydrous (SS), orthophosphoric acid, and hydrogen peroxide were obtained from (Thermo Fisher Scientific (U.K).

Instruments

A Shimadzu HPLC unit (LC-2010C, HT Japan) equipped with a pump, auto-sampler, and a UV detector was employed. Separation and quantitation were performed on Hypersil™ C4 column (250 mm L × 4.6 mm i.d. and 5 µm particle size) from Thermo Scientific (USA). The output signal was monitored through LC solution® software.

Methods

HPLC method development. Chromatographic separation was achieved on Hypersil™ C4 column (250 mm × 4.6 mm i.d. and 5 µm particle size), using a mobile phase consisting of an isocratic system of 62% of 50 mM sodium phosphate buffer, pH 2.7, and 37% acetonitrile (ACN) containing 1% v/v triethylamine. The separation was performed isocratically with a flow rate of 1.5 ml min^{-1} at 34 °C. The injection volume was 20 µl and chromatograms were monitored at 214 nm. All mobile phases were filtered under vacuum through a 0.45 µm nylon filter and degassed using an ultrasonic bath prior to use.

Standard preparation. Standard stock solutions of ID (25 IU ml^{-1}) were prepared by adding 1 ml of the 100 IU ml^{-1} solution (Levemir®) to 3 ml of 2% acetic acid solution. The solution was vigorously mixed so that the solution became clear. Prepared stock solutions were kept in the refrigerator for not more than 3 days. Standard stock solutions of m-cresol (515 µg ml^{-1}) and phenol (450 µg ml^{-1}) were prepared by dissolving 103 mg and 90 mg of them respectively, in 200 ml of 5% methanol.

HPLC method validation. The method was validated in accordance with the international conference on harmonization (ICH) guidelines Q2R1 for specificity,

linearity, accuracy, the limit of detection, the limit of quantification, intra-day precision, robustness, ruggedness, and system suitability parameters of the measurement [14].

Specificity: Using the described conditions, the chromatogram for Levemir® gives three well-resolved peaks, which are supposed to be for insulin and its formulation additives (m-cresol, and phenol). To confirm the specificity of the suggested method, the spectra of m-cresol and phenol were monitored separately using a standard solution for each. In addition, method specificity was assured by assaying ID in the presence of its degradation products under various forced degradation conditions.

Linearity: The linearity of the method was evaluated by processing six-point calibration curves ranging from 2-12 IU ml^{-1} for insulin, 41.2-247.2 µg ml^{-1} for m-cresol, and 36-216 µg ml^{-1} for phenol on three different days, and each concentration was carried out in triplicate under the described conditions. The calibration curves were constructed by plotting the peak area against the concentrations of each component. Linear regression analysis was applied to calculate the slope, intercept, and linear correlation coefficient (r^2).

Precision: The precision study was carried out to determine the intra-day (repeatability) by analyzing six standard solutions of ID, m-cresol, and phenol at 100% target concentration (8 IU ml^{-1} ID, 164.82 µg ml^{-1} m-cresol, and 144 µg ml^{-1} phenol) in the same day under the same experimental conditions. Results of the precision test were expressed as relative standard deviation (RSD%).

Accuracy and recovery: For ID, the accuracy of the method was assessed by using nine determinations covering the specified range. Three different standard solutions with a known amount of insulin (3, 7, and 11 IU ml^{-1}) in triplicates were subjected to the proposed HPLC method. Accuracy in terms of recovery (%) was reported. Relative standard deviation (RSD%) for the nine recovery percentages was also calculated.

For phenol and m-Cresol, the same nine solutions prepared for the accuracy test of ID were also subjected to quantification of m-cresol and phenol using the proposed method. Accuracy was determined as the percentage recovery.

Limit of detection (LOD) and limit of quantitation (LOQ): Limits of detection (LOD) and quantification (LOQ) were calculated based on the standard deviation of the response and the slope of the calibration curve, according to the following equations:

$$LOD = 3.3\ \sigma/S \quad (1)$$

$$LOQ = 10\ \sigma/S \quad (2)$$

where σ is the standard deviation of responses (Y-intercept) and S is the slope of the calibration curve.

Robustness: Robustness was determined by studying the effect of small variations in chromatographic conditions of the proposed method at three different levels on peak area and retention time. The chromatographic conditions selected for the study were percent of acetonitrile in mobile phase (37% ± 1%), pH (2.6, 2.7, and 2.9), and column temperature (34 ºC ± 2 ºC). At each different condition, standard solutions containing ID, m-cresol, and phenol at 100% target concentration were injected in triplicate and analyzed, and relative standard deviation (RSD%) of peak area and retention time were reported.

Ruggedness: The ruggedness of the assay method was evaluated by comparing the inter-day assay results for ID, m-Cresol, and phenol. A standard solution containing ID, m-cresol, and phenol at 100% target concentration was injected over six days and relative standard deviation (RSD%) of peak response and retention time were calculated.

System suitability parameters: System suitability parameters were monitored to make sure that the system was working correctly during the analysis. A standard solution at 100% target concentration was injected and analyzed as six replicates. The method performance data including capacity factor (K′), injection repeatability (RSD%), United States Pharmacopoeia (USP) tailing factor (T), resolution (Rs), and USP theoretical plate number (N) were measured.

Forced degradation studies. In order to evaluate if the developed method was selective enough to separate the active drug from its degradation products, forced degradation studies of ID under various stress conditions

were carried out. The sample at time = zero min served as control. Recoveries of ID in all the reaction solutions were calculated against the known concentration of ID. The stock solution was prepared in the same way mentioned above.

Degradation under acidic conditions at room temperature: The sample was prepared by dissolving 640 µl of ID stock solution in 1360 µl of 2% acetic acid solution (pH = 3.11) at room temperature for 4 h. Aliquots were taken at 0, 0.5, 1, 2, and 4 h for analysis using the proposed method.

Thermal degradation under neutral condition: The sample was prepared by dissolving 640 µl of ID stock solution in 1360 µl of phosphate buffer saline (pH = 7.4). The sample was then kept in a water bath at 60 °C for 4 h, and aliquots were taken at 0, 0.5, 1, 2, and 4 h for analysis using the proposed method.

Thermal degradation under acidic condition: The sample was prepared by dissolving 640 µl of ID stock solution in 1360 µl of 2% acetic acid solution (pH = 3.11). The sample was then kept in a water bath at 60 °C for 4 h, and aliquots were taken at 0, 0.5, 1, 2, and 4 h for analysis using the proposed method.

Thermal degradation under alkali condition: The sample was prepared by dissolving 640 µl of ID stock solution in 1360 µl of 50 mM ethanolamine solution (pH = 11). The sample was then kept in a water bath at 60 °C for 4 h, and aliquots were taken at 0, 0.5, 1, 2, and 4 h for analysis using the proposed method.

Degradation under oxidative condition: The sample was prepared by dissolving 640 µl of ID stock solution in 1360 µl of 3% hydrogen peroxide (H_2O_2) at room temperature for 4 h. Aliquots were taken at 0, 0.5, 1, 2, and 4 h for analysis using the proposed method.

Photolytic degradation: The sample was prepared by dissolving 640 µl of ID stock solution in 1360 µl of phosphate buffer saline (pH = 7.4). The sample was exposed to direct sunlight for 4 hours, and aliquots were taken at 0, 0.5, 1, 2, and 4 h for analysis using the proposed method.

Application to pharmaceutical formulation. The developed analytical method was applied for the simultaneous determination of ID in the commercially available pharmaceutical formulation, along with the additives phenol and m-cresol. Three different concentrations (80, 100, 120% of the target concentration) were prepared from Levemir® flexpen (Batch No. HT6J726) dosage form and then analyzed using the proposed method. To assess the accuracy of the suggested method, mean % recovery of each component was calculated.

RESULTS AND DISCUSSION

HPLC Method Development and Validation

Parameters affecting chromatographic behavior were studied thoroughly and optimized in order to attain the most suitable chromatographic separation of ID, m-cresol, and phenol. The reported HPLC method described by USP monograph [15] for human insulin injection (26% phosphate buffer containing SS: 74% ACN, C18 column) was tried at the beginning but failed to elute ID up to 30 min run time.

Method development was then started employing a C4 column and mobile phases containing increasing percentages of ACN (25-70%) in a phosphate buffer (50 mM, pH = 3.7) at room temperature. Three distinct peaks were observed using 25% ACN, however; none of them was for ID. The observed peaks were for solvent, phenol, and m-cresol, and this was confirmed by injecting standard solutions of each. Increasing the percentage of ACN (25-70%) resulted only in an overlapping of the peaks without elution of ID peak. Therefore, the percentage of ACN was kept at a minimum (25%) and the detection wavelength was optimized to ensure that the absence of insulin's peak was not due to detection problems.

A sample of ID was injected at different wavelengths in the range (200-250 nm), and the chromatograms at employed wavelengths were obtained, but still, no additional peaks were observed (Fig. 1). Based on the spectral data and previous literature, the detection wavelengths were set at 214 nm. Accordingly, it was concluded that under all of the examined conditions, insulin was strongly retained on the stationary phase and never eluted.

SS is usually employed at relatively large concentrations (28.4 mg ml^{-1}) in the mobile phases recommended by pharmacopeial methods for the determination of ordinary human insulin [15]. SS is thought to act through some kind

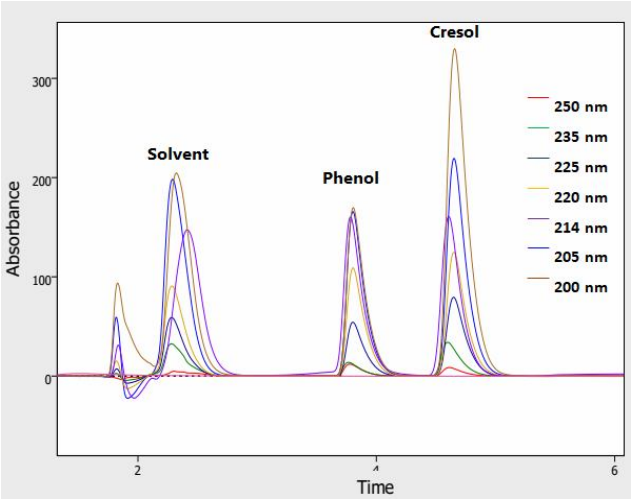

Fig. 1. HPLC chromatogram of insulin detemir pharmaceutical preparation at different wavelength using C4 column and a mobile phase containing 25% acetonitrile: 75% phosphate buffer (pH = 3.7) at room temperature and flow rate = 1ml/min.

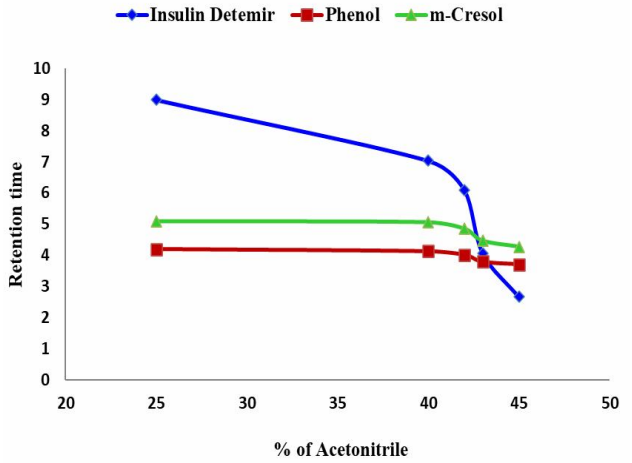

Fig. 2. The effect of changing relative content of acetonitrile in mobile phase on retention time of insulin detemir, using phosphate buffer (pH = 3.7) at 38 °C and flow rate = 1.5 ml min^{-1}.

of salting-out mechanism by competing on the residual free silanol groups in addition to the potential effect on secondary structure of insulin, thus encouraging their interaction with the lipophilic hydrocarbon side chain instead of silanol groups [16]. Thus, SS was added to the mobile phase containing 25% ACN at a concentration of (20 mg ml^{-1}). This relatively low concentration was chosen to decrease the risk of salt precipitation during method optimization. At this condition, ID peak was observed at 9 min albeit with peak broadening and tailing.

After observation of the ID peak, the next challenge was to improve the peak shape and resolution. As a starting point, the temperature was set at 38 °C instead of room temperature in an attempt to attain a better peak shape for ID. Increasing column temperature resulted in a noticeable increase in the peak height and a decrease in the peak width of ID. Accordingly, a temperature of 38 °C was used thereafter in optimization experiments.

Further optimization to achieve better separation and peak shape for ID, was carried out by varying organic phase content from (25%-50%) while using controlled column temperature (38 °C) and SS salt in the mobile phase at pH (3.7). Results are summarized in (Fig. 2). It was found that increasing ACN percentage was inversely proportional to the retention time of ID particularly within the narrow range of 40-45%. However, the effect on the peaks of preservatives was only minimal. At 40 and 42% of ACN; good resolution and lower retention times for ID were achieved (7 and 6 min, respectively). Because the retention time of ID was decreasing, more seriously than the peaks of the excipients, increasing the percentage of ACN to 43% resulted in overlapped peaks for insulin and the excipients. Further increase in ACN up to 45% led ID to be eluted before the preservatives with good separation and suitable run time (5 min). Nevertheless, this condition (45%) was not chosen for further optimization since SS tended to precipitate, and the mobile phase had to be kept warm throughout the whole experiment, which may generate technical problems. In addition to that, a lower concentration of SS (1.5 mg ml^{-1}) was tried in an attempt to use such a percentage of ACN, but it had an adverse impact on the peak shape of insulin. When 50% ACN was prepared, mobile phase separation occurred even with warming the mobile phase at (38 °C). Accordingly; the next best condition would be 40% of ACN, and thus further optimizations were carried out at that percentage.

As the peak of insulin was still suboptimum, efforts

were made to minimize the tailing of insulin peaks by using an ion-pairing reagent. Each of trifluoroacetic acid (TFA), diethylamine (DEA), and triethylamine (TEA) was tested, but the best peak shape improvement was achieved using (1%) triethylamine. The presence of TEA did not only result in a symmetric peak of ID but also shifted the retention time from (~7 min to 3.85 min), which supported the belief that the interaction of ID with residual silanol groups contributes significantly to its retention behavior. However, since this effect is by far more serious for insulin than simple phenol compounds, the peak of insulin crossed that of phenol resulting in some overlapping.

At this point, the effect of varying pH of the mobile phase (3-5.5) was studied (Fig. 3). Unlike the phenolic preservatives, which were not expected to change their ionization state within the examined range of pH, the retention time of insulin tended to decrease as the pH was increased from pH 3 to 4.5. Because the studied range of pH was quite away from the expected pKa values of basic amino acids within the insulin molecule, the observed behavior could be explained through changes in the degree of ionization of the carboxylic acid groups of the acidic amino acids whose pKa values lie well within the studied range of pH. At lower pH values, carboxylic acid groups of amino acids as aspartic (pKa = 2.1, 3.9) and glutamic acid (pKa = 2.1, 4.3) would be mostly protonated [17], and thus more lipophilic and having relatively higher retention. Increasing the pH to 5.5 would cause the carboxylic acid groups to ionize, making ID molecules less hydrophobic, and hence having lower retention times. Accordingly, the working mechanism of retention for ID under these conditions was obviously reversed-phase mechanism rather than normal phase. Moreover, the pH of the mobile phase appeared to influence the shape of insulin's peak, where higher pH values (4.5 and 5.5) resulted in sharper and higher peaks; consistent with having more uniformed species (ionized) at those pH values. According to Fig. 3, pH values of 3 and 3.7 gave suboptimal separation of insulin and other excipients, and best separation conditions were observed while using pH 4.5. At pH 4.7 and 5.5 insulin peak overlapped with the solvent peak. Then pH was kept at 4.5 in subsequent optimization experiments, and the percentage of ACN was varied in a range of (30%-45%). The results are summarized in Fig. 4. Although the data

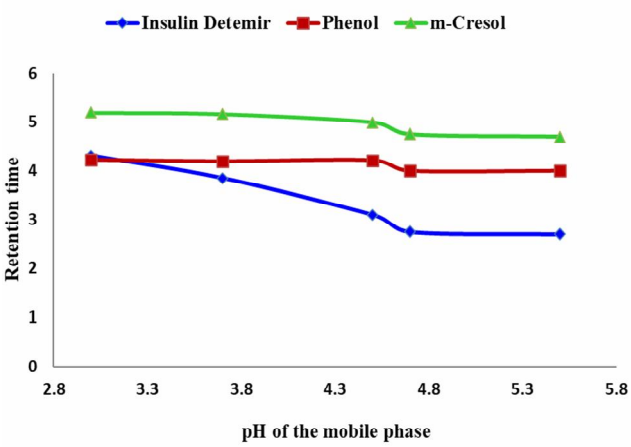

Fig. 3. The effect of changing pH of mobile phase on retention time of insulin detemir, using 40% acetonitrile: 1% TEA: 59% phosphate buffer containing 20 mg ml^{-1} sodium sulphate at 38 °C and flow rate = 1.5 ml min^{-1}.

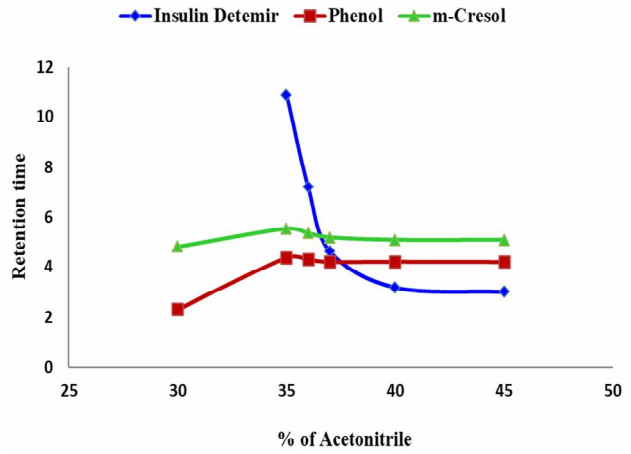

Fig. 4. Effect of percentage content of acetonitrile in mobile phase on retention time of insulin detemir and phenolic preservatives. Mobile phase contained 1% TEA and phosphate buffer containing 20 mg ml^{-1} sodium sulphate at 38 °C and flow rate = 1.5 ml min^{-1}.

in Fig. 4 support the reversed-phase mechanism for retention of ID, it was interesting to note that a dramatic fall in the retention of ID took place as the percentage organic was increased within only a narrow range (30-35%). At 30% ACN, the peak of insulin did not appear up to 30 min

run time, and then increasing ACN by only 5% (35%) eluted insulin at 11 min. A further 1% increase in the percentage of ACN resulted in a significant drop in retention time to 7.2 min, where best separation was achieved. In comparison, the retention times of the small molecules (phenolic preservatives) did not appear to noticeably change. Therefore, additional mechanisms most likely have contributed to the observed behavior of ID *e.g.,* changes in the folding of insulin secondary structure that requires a threshold of ACN concentration in order to transform. That accords very well with previous studies that demonstrated significant dependence of structural transitions of insulin on percentages of coexisting organic solvents [18].

Despite the beneficial impact in achieving the desired separation, the observed high rate of decrease in retention time was not commendable for the robustness of the method.

Perfect separation and symmetric peak shape of ID were achieved using (36% ACN, 1% TEA, 63% buffer pH = 4.7, and 20 mg ml^{-1} SS) as mobile phase. However, buffer precipitation frequently occurred at that condition, which led to an increase in pressure and associated problems. Therefore, it was decided to seek optimum separation conditions while having a more acidic pH value using 36% ACN, 1% TEA, 63% buffer (pH = 2.7), and 20 mg ml^{-1} SS at 38 °C retarded insulin peak to 11 min and resulted in good separation.

Finally, the effect of the column's temperature was investigated over the range (34-44 °C), and the obtained results are presented in Fig. 5. Retention times for phenol and m-cresol decreased with increasing temperatures which is typical chromatographic behavior for small molecules. However, an interesting chromatographic behavior of ID has been observed; where its retention time increased with increasing the temperature. These findings were consistent with previous reports which ascertained that the retention of peptides on HPLC stationary phases, in general, is determined to a good extent by the conformation of the molecule [19-20]. A particularly interesting report concluded that different types of insulin could be separated on reversed-phase columns by manipulating temperature below 45 °C [21]. Purcell *et al.* demonstrated that at

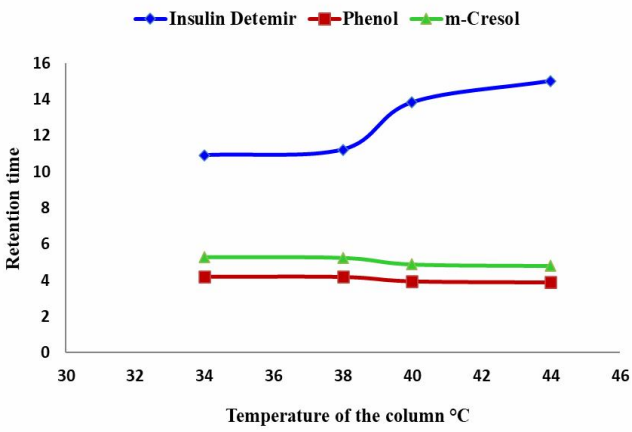

Fig. 5. Effect of temperature of the column on retention time of insulin detemir. Mobile phase: 36% acetonitrile: 1% TEA: 63% phosphate buffer containing 20 mg ml^{-1} SS with a flow rate = 1.5 ml min^{-1}.

temperatures higher than 45 °C almost all variants of insulin behave similarly, suggesting the adoption of a uniform conformation. Additionally, they showed that C4 stationary phase rather than C18 has a particular ability to differentiate between the different conformers of insulin, which aligns with our results. Therefore, our findings accord with previous studies in the sense that C4 stationary phase has a particular ability to discriminate between insulin conformers which interconvert as a result of temperature changes. However, our findings were distinguished in that they showed an abrupt and clear change in retention behavior of ID within a narrower range of temperature (34-44 °C), suggesting interconversion between two conformers. To the best of our knowledge, this is the first time that interconversion was demonstrated for ID on HPLC stationary phases. If confirmed, these findings would not only be useful in optimizing a method for the quantitative determination of ID, but also as a method to assess the existing conformers of ID at various conditions.

Interestingly, these findings accord very well with a recent report that studied structural changes of ID in response to changing temperature within this range [22]. Nevertheless, in order to get optimum separation of the commercially available ID preparations, with suitable

retention time, the temperature was decreased to 34°C, and ACN content increased to 37%. A perfect separation for all components with very good peak shapes was achieved using those conditions; a typical HPLC chromatogram of insulin formulation is shown in Fig. 6. The advantages of this method include the ability to be used for the analysis of ID in commercial formulations without interference from any excipients, suitable run time, and the ability to simultaneously quantify phenolic preservatives in the formulation.

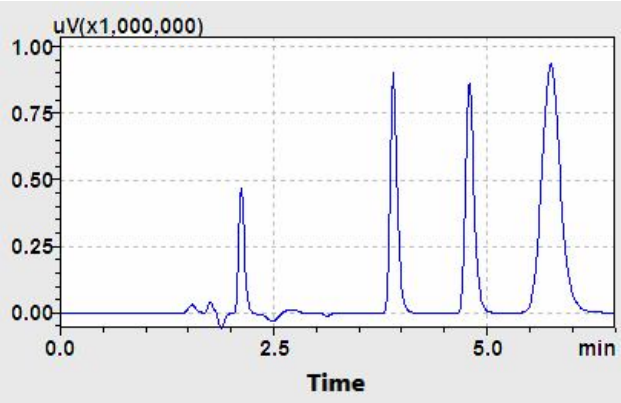

Fig. 6. Typical HPLC chromatogram of insulin detemir formulation. Peak identification: levemir is the last and broader peak. Peaks at 3-5 min belong to phenol and creasol preservatives.

HPLC Method Validation

Method validation was performed according to the international council on harmonization (ICH) guidelines [14]. Below is a detailed discussion about the major validation criteria examined:

Specificity and selectivity. ID, phenol, m-cresol, and the degradation products were well-resolved under the applied conditions. The chromatograms for the solvent and formulation additives were recorded separately under the described conditions, and the identity of each peak was confirmed by the retention time (Fig. 7). Specificity of the method was confirmed by the absence of any interference from the solvent, standard solution of phenol, and standard solution of m-cresol at the retention times of peaks of interest. Additionally, method specificity was assessed through forced degradation studies, as will be discussed in a later section. Although characteristic peaks for the degradation products were observed, no co-eluting peaks with ID or its formulation additives could be detected, which showed that the proposed HPLC method was adequately specific.

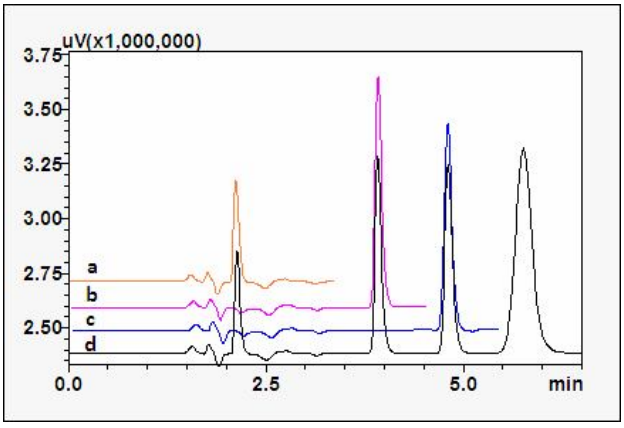

Fig. 7. Typical overlaid HPLC chromatograms of (a) sample solvent (b) phenol standard solution (c) m-cresol standard solution (d) insulin detemir formulation (Levemir®).

Calibration curves, linearity and ranges. Calibration curves were constructed by plotting the peak area against concentrations of each component. Three calibration curves were constructed and all components showed good linearity ($R^2 > 0.99$) over the specified range (80-120%) of the test concentration. The results are listed in Table 1. Typical calibration curve equations were y = 2022450.5881x + 456018.633, y = 40632722.22x + 343912.5 and y = 39789928.92x + 252459.167 for ID, phenol and m-cresol respectively.

Precision, accuracy and recoveries. According to ICH guidelines recommendations, data for precision (repeatability) was collected for six replicates of the test concentration and represented as RSD in Table 2, while accuracy was examined over three concentration levels covering the specified range (low, medium, and high level; each in triplicate). The concentrations were calculated using the resulted regression equation for each component. The results for recoveries of ID, phenol, and m-cresol were obtained according to formula (1). The method showed good precision with RSD less than 0.6%. The recoveries

Table 1. Linearity Data for the Quantification of Insulin Detemir, Phenol and m-Cresol in Levemir® Formulation Using the Proposed Method (n = 3)

Component	R^2		Linearity range	LOQ	LOD
	Average	RSD%			
Insulin detemir	0.9978	0.0782	2-12 IU ml^{-1}	1.996 IU ml^{-1}	0.598 IU ml^{-1}
Phenol	0.9982	0.007	41.2-247.2 µg ml^{-1}	30.005 µg ml^{-1}	9.001 µg ml^{-1}
m-cresol	0.99865	0.007	36-216 µg ml^{-1}	30.2 µg ml^{-1}	9.07 µg ml^{-1}

Table 2. Precision and Accuracy Data for the Quantification of Insulin Detemir, Phenol, and m-Cresol in Levemir® Formulation Using the Proposed Method

Component	Precision			Accuracy			
	Test concentration	Measured concentration (n = 6) Average ± SD	RSD%	Nominal concentration	Measured concentration (n = 3) Average ± SD	Recovery% (n = 3)	RSD
Insulin Detemir	8 IU ml^{-1}	8.194 ± 0.047	0.58	3 IU ml^{-1}	2.98 ± 0.008	99.35	0.272
				7 IU ml^{-1}	7.096 ± 0.087	101.37	1.23
				11 IU ml^{-1}	11.09 ± 0.091	100.82	0.82
Phenol	0.144 µg ml^{-1}	0.1469 ± 0.0003	0.209	0.054 µg ml^{-1}	0.0538 ± 0.0001	99.77	0.182
				0.126 µg ml^{-1}	0.1258 ± 0.00048	99.87	0.388
				0.198 µg ml^{-1}	0.1977 ± 0.0001	99.88	0.055
m-cresol	0.1648 µg ml^{-1}	0.1678 ± 0.0004	0.267	0.0618 µg ml^{-1}	0.0621 ± 0.0001	100.55	0.217
				0.1442 µg ml^{-1}	0.146 ± 0.0002	101.519	0.171
				0.2266 µg ml^{-1}	0.2285 ± 0.0002	100.87	0.113

were in the range of (99-102.8%) with overall RSD less than 1.2%. These results demonstrated that the developed method is reproducible and highly accurate.

LOQ and LOD. Limits of detection (LOD) and quantification (LOQ) for each component are listed in (Table 1). LOQ for ID, phenol and cresol were 1.996 IU ml^{-1}, 30 µg ml^{-1}, and 30.2 µg ml^{-1}, representing 2, 1.6, and 1.4% of the original concentration respectively. This indicated that the method is sufficiently sensitive for the quantification of ID and its formulation additives.

Robustness and ruggedness. Robustness was tested by using 'one factor at a time' method. The factors evaluated were percent of acetonitrile in mobile phase, pH, and column temperature (Table 3). No significant changes in the RT or area of ID, m-Cresol, or phenol were observed when these conditions were varied as described in the experimental section. The low value of the RSD (<2%) indicates that the method is robust. The RSD of retention times and peak areas for ID, m-cresol, and phenol samples repeated on different days was <2%, this assures the ruggedness of the proposed method (Table 4).

System suitability test. To ensure that HPLC system and the proposed method are capable of providing data of acceptable quality, system suitability tests such as capacity factor (K′), injection repeatability (RSD%), USP tailing factor (T), resolution (Rs), and USP theoretical plate number (N) from six consecutive injections were evaluated

Table 3. Results of Robustness Test for the Proposed Method for ID, m-Cresol and Phenol (n = 3)

Condition	Modification	ID				Phenol				m-Cresol			
		Mean area ± SD	RSD	Mean RT ± SD	RSD	Mean area ± SD	RSD	Mean RT ± SD	RSD	Mean area ± SD	RSD	Mean RT ± SD	RSD
Percent of acetonitrile in mobile phase	36%	17789106 ± 149559	0.84	7.2 ± 0.01	0.15	6739927 ± 36600	0.54	3.97 ± 0.02	0.54	7569978 ± 47226	0.62	4.92 ± 0.04	0.85
	37%	17903463 ± 45625	0.25	6.32 ± 0.012	0.18	6703446 ± 3865	0.057	4.01 ± 0.0005	0.014	7512867 ± 6651	0.08	4.97 ± 0.0005	0.011
	38%	17817478 ± 61378	0.34	5.8 ± 0.002	0.04	6692885 ± 31620	0.47	4.011 ± 0	0	7545811 ± 49876	0.66	4.95 ± 0.0005	0.011
pH of the mobile phase	2.6	18042813 ± 58178	0.32	6.45 ± 0.02	0.31	6693151 ± 6328	0.094	4.1 ± 0.001	0.024	7535394 ± 51499	0.68	5.1 ± 0.0005	0.011
	2.7	17903463 ± 45625	0.25	6.32 ± 0.012	0.18	6703446 ± 3865	0.057	4.01 ± 0.0005	0.014	7512867 ± 6651	0.08	4.97 ± 0.0005	0.011
	2.9	17765185 ± 12289	0.07	6.09 ± 0.001	0.025	6773419 ± 6438	0.095	4.01 ± 0.0005	0.014	7586673 ± 11233	0.14	4.9 ± 0.001	0.02
Column temperature	32 ºC	17858302 ± 31692	0.18	6.06 ± 0.012	0.2	6690160 ± 8948	0.13	4.05 ± 0.003	0.075	7494366 ± 8114	0.1	5.02 ± 0.002	0.045
	34 ºC	17903463 ± 45625	0.25	6.32 ± 0.012	0.18	6703446 ± 3865	0.057	4.01 ± 0.0005	0.014	7512867 ± 6651	0.08	4.97 ± 0.0005	0.011
	36 ºC	17834092 ± 11052	0.06	6.45 ± 0.003	0.054	6679378 ± 1477	0.02	3.97 ± 0.02	0.038	7486051 ± 10685	0.14	4.92 ± 0.002	0.05

Table 4. Results of Ruggedness Test for the Proposed Method for ID, m-Cresol and Phenol (n = 3)

Component	Test concentration	Inter-day measured concentration (n = 6) Average ± SD	RSD%	Inter-day retention time (n = 6) Average ± SD	RSD%
Insulin detemir	8 IU ml^{-1}	8.12 ± 0.05	0.6	6.3 ± 0.015	0.247
Phenol	0.144 µg ml^{-1}	0.147 ± 0.001	1.1	3.977 ± 0.018	0.466
m-Cresol	0.1648 µg ml^{-1}	0.167 ± 0.0004	0.267	4.98 ± 0.07	1.43

and presented in Table 5. All values were within the limits established by ICH guidelines**.** Therefore, we can recommend that a resolution > 4.3 with an RSD of ID peak area < 1.5 must be achieved to ensure system suitability.

Forced Degradation Studies

None of the previously reported HPLC methods for determination of ID has been proven as stability-indicating quality control assays. The capability of the proposed method as a stability-indicating one was assessed by stress testing. Samples of ID were subjected to harsh conditions to enforce accelerated degradation of ID so that the potential degradation products could be generated and consequently utilized to prove the suitability of the method

Table 5. Results of Parameters Related to System Suitability Test for the Proposed Method

System suitability test	Phenol	m-Cresol	ID
Tailing factor (USP)	1.12	1.1	1.05
Theoretical plates (USP)	9801	9216	3745
%RSD[a] (n = 6)	1.08	1.04	1.3
Capacity factor (USP)	2.26	3	4.1
Resolution[b]	4.67	4.67	4.4

[a]For peak area. [b]Represents resolution of phenol from cresol, cresol from phenol and ID from cresol respectively.

as a stability-indicating assay. The employed conditions were neutral, acidic, basic, oxidative, thermal, and photolytic degradation. Representative chromatograms of the stress testing experiments are shown in (Figs. 8-9). Accordingly, there were no signs of serious overlapping between the newly emerging degradation product peaks and any of the peaks of the initial components of ID. Interestingly, the chromatograms of Fig. 8 show clearly different degradation profiles according to the pH of the medium. While neutral and acidic pH values were associated with the emergence of only one new peak immediately to the right of ID peak, alkaline pH led to the

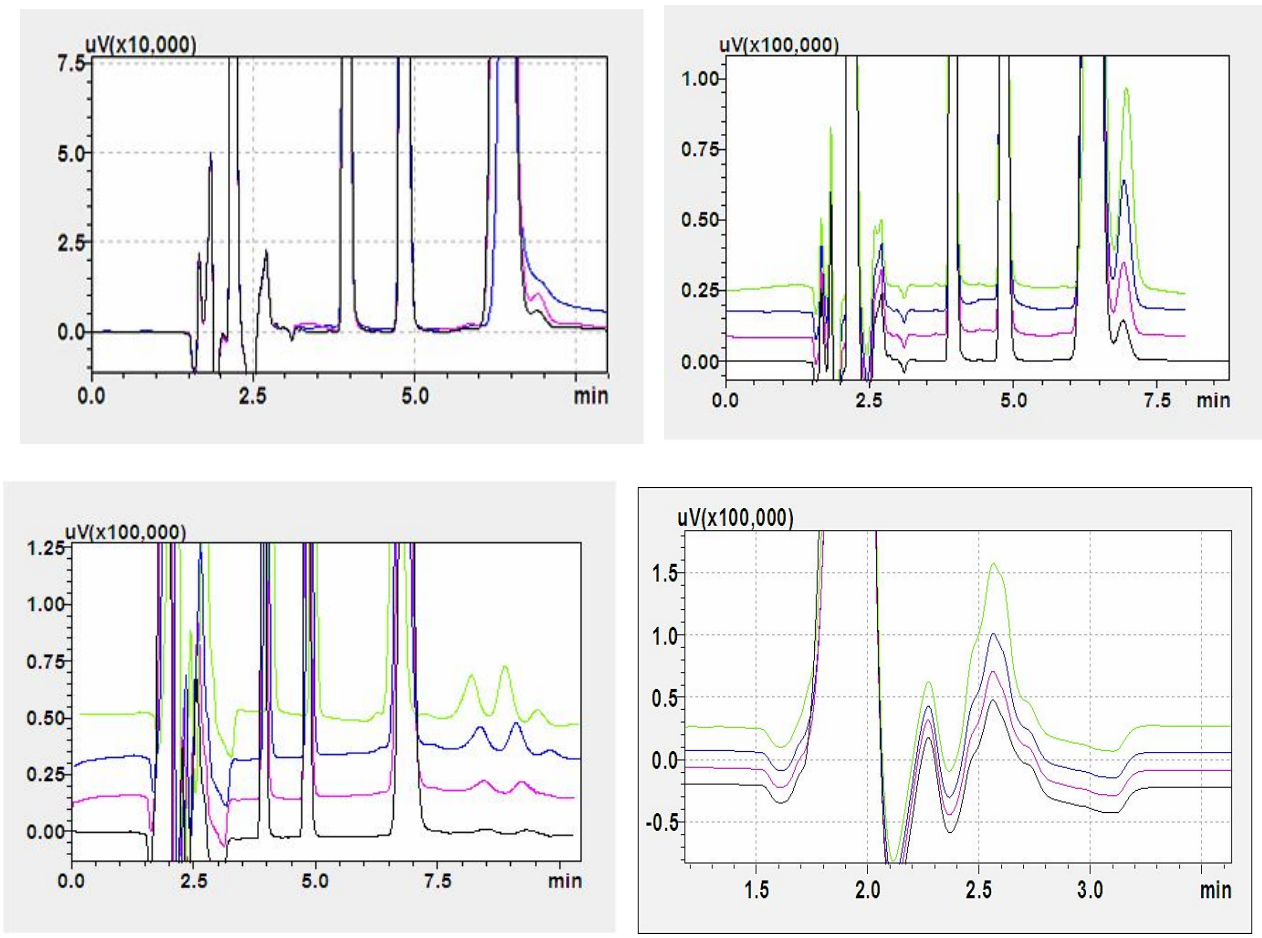

Fig. 8. Typical overlaid HPLC chromatograms of Insulin detemir degradation products under neutral (a) acidic (b) and basic (c) conditions at (60 °C). In (d), a chromatogram for degradation under basic conditions is zoomed out to show the emerging degradation product peak at 2.5 min. In each case, the overlaid chromatograms represent sampling at 0.5, 1, 2, and 4 h. Arrows directions indicate increasing time of sampling.

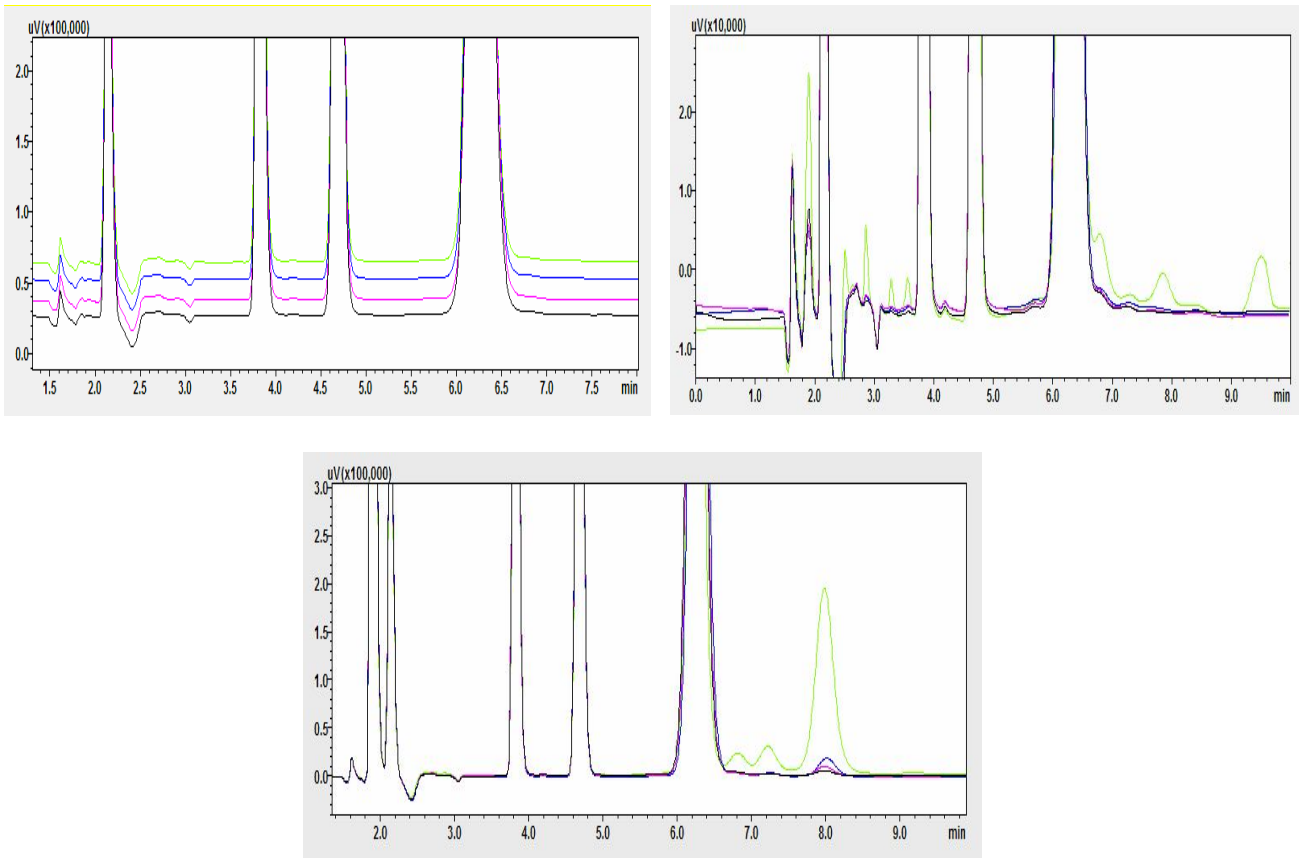

Fig. 9. Typical overlaid HPLC chromatograms of Insulin detemir degradation products under (a) acidic, (b) oxidative and (c) photolytic conditions at room temperature. In each case, the overlaid chromatograms represent sampling at 0.5, 1, 2, and 4 h. Arrows directions indicate increasing time of sampling.

appearance of one early eluting peak ~ 2.6 min and at least three well-resolved peaks with fairly longer times than the peak of ID. Indeed there have been no published reports about the degradation profiles of ID as a function of pH of the medium, however, our data accords very well with the corresponding studies reported for the commonly used human insulin [23-24]. In their studies, they confirmed that the degradation pathways for insulin are so different at acidic and basic pH values. In acidic and neutral pH values, the predominant degradation product was that of the deamidation reaction. On the other hand, at basic pH disulfide cleavage leading to smaller size alpha and beta-strands of insulin together with various size insulin polymerization products would be predominant. Therefore, the early eluting peak at about 2.6 min observed in the alkaline degradation medium most likely corresponds to the shorter insulin strands and the late eluting multiple peaks correspond to the larger size polymerization products of ID. Although ID was found to be susceptible to degradation when the sample was heated in acidic conditions, it was stable with no prominent degradation peaks under the same acidic solution at room temperature (Fig 9a). Upon exposing ID to 3% H_2O_2 at room temperature for 4 h, three late eluting peaks were emerged at 6.9, 7.2, and 8 mins.

ID was found to be unstable upon exposure to sunlight for 4 h. many peaks were emerged between 1.5-4 min, and after 7 min. This was also consistent with the previous report that, in native insulin, the A and B chains are held together by two disulfide bridges. Disruption of either of these bonds is likely to affect insulin's structure. UV-

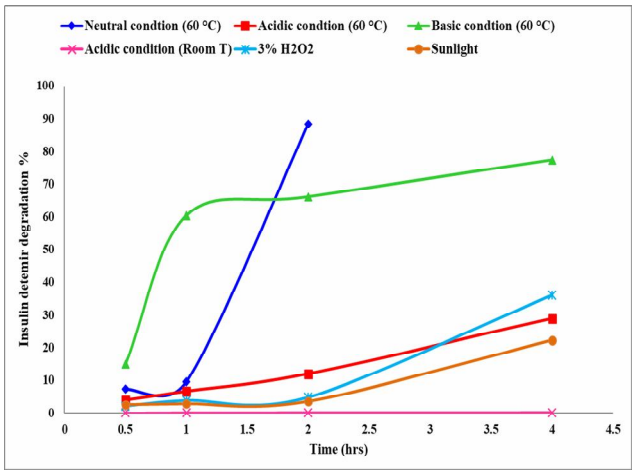

Fig. 10. Degradation behavior of Insulin detemir under different degradation conditions over 4 h.

excitation of insulin was shown to induce disulfide bridge breakage which results in secondary and tertiary structure loss. Furthermore, Continuous UV-excitation of the peptide hormone in solution leads to the progressive formation of tyrosine photo-product dityrosine, leading to covalent insulin dimerization [25].

The satisfactory separation of ID from thermal degradation products enabled the determination of the percentage of degraded ID as a function of exposure time (Fig. 10). ID was highly degraded when subjected to basic conditions. More than 60% of ID was degraded, showing four degradation products, during the first 2 h.

Upon exposing ID to neutral conditions (PBS pH 7.4) at 60 °C for 4 h, about 88% of ID has undergone degradation in the first 2 h, and the major degradation product showed an extra well-separated peak at 6.9 min. Although there appears a lag time for the degradation of ID in neutral conditions, after about 2 h the percentage of degraded ID exceeded the corresponding value in the basic medium. At 4 h, a jelly-like precipitate was formed that did not enable sample analysis.

ID was found to be most stable under acidic conditions at room temperature, where less than 0.1% of ID has undergone degradation throughout 4 h.

After 4 h of ID exposure to oxidative stress and sun light, intermediate degradation occurred with a degradation percentage of 36% and 22% respectively. This observation sheds the light on the importance of protecting ID formulation from UV light during production, packaging, storage, or administration processes.

Most importantly, to this work, was the observation that ID peak was well- resolved in all degradation studies (Figs. 8-9) which indicates that the method is sufficiently selective to be utilized as a stability-indicating assay.

Application to Pharmaceutical Formulation

The developed method was used to simultaneously determine the content of phenol, m-cresol, and ID in marketed Levemir® flexpen dosage form. The mean % recoveries at (80,100, and 120% of the target concentration) ±RSD were all satisfactory and indicated the accuracy of the proposed method (Table 7).

CONCLUSIONS

To the best of our knowledge, no stability-indicating assay for ID has been published. This work represents the first optimized and validated stability-indicating assay for ID. Moreover, the developed method is the first simple isocratic HPLC method that enables simultaneous quantification of ID and the formulation additives *i.e.*, phenol and m-cresol using UV detection. Through the course of method development, the importance of stationary phase chemistry in achieving the optimum retention of ID was evident. Using the less commonly utilized stationary phase (C4) complete peak separation and resolution were achieved in less than 8 min. The proposed method was specific, sensitive, accurate, linear, and precise. The validated method has been demonstrated to be stability-indicating because it could separate the degradation products from the parent ID. The degradation profiles for ID in acidic and basic media seem to differ significantly but, in a manner, resembling that of commonly used human insulin.

ID was appeared to exhibit some structural transitions within a narrow range of temperature so that it elutes at longer retention times with higher temperatures, thus consistent and supplementing previous findings. Overall, the suggested method can be used for routine analysis, quality control, and for checking quality during stability

Table 6. Results of Forced Degradation Studies

Forced degradation study	Condition	% of ID recovered after 4 h
Acid degradation	2% Acetic acid solution, pH 3.1, room T, 4 h	99.9
Thermal neutral degradation	PBS, pH 7.4, 60 °C, 4 h	Not analyzed*
Thermal acidic degradation	2% Acetic acid solution, pH 3.1, 60 °C, 4 h	71.01
Thermal basic degradation	TEA, pH 11, 60 °C, 4 h	22.58
Oxidative degradation	3% H_2O_2, room T, 4 h	63.97
Photolytic degradation	Sunlight, 4 h	77.7

*A jelly-like precipitate was formed that did not enable sample analysis.

Table 7. Application of the Proposed Method to the Marketed Levemir® Flexpen Dosage Form

Component	Theoretical concentration	Mean % recovery	RSD
	3 IU ml^{-1}	100.3	0.28
Insulin detemir	7 IU ml^{-1}	99.8	1.01
	11 IU ml^{-1}	100.6	0.83
	0.054 µg ml^{-1}	101.65	0.76
Phenol	0.126 µg ml^{-1}	100.04	0.33
	0.198 µg ml^{-1}	99.76	0.28
	0.0618 µg ml^{-1}	100.65	1.03
m-Cresol	0.1442 µg ml^{-1}	101.8	0.28
	0.2266 µg ml^{-11}	100.3	0.35
Component	Theoretical concentration	Mean % recovery	RSD
Insulin detemir	3 IU ml^{-1}	100.3	0.28
	7 IU ml^{-1}	99.8	1.01
	11 IU ml^{-1}	100.6	0.83
Phenol	0.054 µg ml^{-1}	101.65	0.76
	0.126 µg ml^{-1}	100.04	0.33
	0.198 µg ml^{-1}	99.76	0.28
m-Cresol	0.0618 µg ml^{-1}	100.65	1.03
	0.1442 µg ml^{-1}	101.8	0.28
	0.2266 µg ml^{-1}	100.3	0.35

studies of pharmaceutical formulations of ID.

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