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Rapid quantitative determination and assessment of insulin in oil formulation by micellar electrokinetic capillary chromatography

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Abstract

A rapid, simple and precise micellar electrokinetic capillary chromatrography (MEKC) with diode array detector had been developed to the determination of insulin in oil formulation. As the micelle-forming ampholytic surfactant, Labrasol was chosen for the separation and analysis of the hydrophobic insulin in oil formulation in this experiment. A buffer containing 10 mmol/ml Tris–HCl plus 10% acetonitrile (ACN) at pH 8.2 was used for running buffer. Samples and tested samples were successfully separated during 8 min with a good linear range($r > 0.99$) and a concentration detection limit of 0.01 mg/ml. Furthermore, quantitative determinations of insulin in oil formulation had been described and assessed after handled with different pre-treatment conditions to reveal the effect of pH, temperature and tryptic digestion.

Keywords: Micellar electrokinetic capillary chromatography; Insulin; Oil formulation

1. Introduction

Insulin, 51-amino acid polypeptide, was an important hormone existed in pancreatic β-cells for regulating glucose metabolism. So far, insulin had been still administered in the form of an injection, a conventional dosage form. Insulin was difficult to orally administer because the proteolytic digestion in gastrointestinal tract (GT) inactivated it. However, the oral route of drug administration remained the most clinically acceptable. So in order to improve the enteral bioavailability of insulin formulation, absorption studies in rats were also carried out with surfactants, fatty acids, proteolysis inhibitors or emulsions [1]. Recently, a novel mode of water-in-oil-in-water (W/O/W) formulation had been proposed to protect insulin against proteolysis and enhance the absorption of insulin [2]. In our assays, the insulin formulations prepared with a special oil (oil A) can slightly but significantly decreased the serum glucose levels compared with the insulin solution in white rats by our previous study. In this formulation, addi-

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tion of Labrasol may lead to a situation where the insulin dissolved in the hydrophobic inner part of the micelle. Therefore, insulin can be fully dispersed in aqueous solution buffer. In addition, Labrasol is not harmful to the human health and widely used in the food industry as a good additive. The experimental results shown that a new and potential oral route for the use of insulin was promising and possible. However, the conventional method such as high-performance liquid chromatography (HPLC) or gel electrophoresis had been testified that it was not suitable for the determination of the insulin in oil formulation by our studies. How to establish a specific reliable method to analysis insulin in oil formulation was an emergency task for oral pharmaceutical research and product process.

As a rapid and high separation efficiency technique, capillary electrophoresis (CE) was widely used in the separation of biological molecular. CE combined beneficial aspects of conventional gel electrophoresis and HPLC but avoid their shortfalls. In addition, separation efficiency and resolution were superior to those offered by HPLC. Moreover, there were various modes of CE to choice for the separation and characterization of proteins and other biological molecules. The method of capillary zone electrophoresis (CZE) had been used to analyze a solution of insulin [3] and the tryptic cleavage of pig insulin [4]. Recently, CE with laser-induced fluorescence (LIF) and LIF competitive immunoassay method has been put forward for separation of the insulin [5,6]. The CE method with LIF detection was very sensitive and had a low concentration detection limit, but it was not as convenience as UV or diode array detector (DAD) UV detection. By our search, no papers had been found on the analysis of hydrophilic insulin dissolved in hydrophobic oil formulation by capillary electrophoresis.

In this paper, we described the development of a simple, timesaving, precise and reproducible electrophoresis method to analysis insulin in oil formulation by MEKC with diode array detector. Furthermore, we carried out quantitative assessment of the insulin concentration change in oil formulation with different pre-treatment handling conditions such as pH, tryptic digestion and temperature.

2. Experimental

2.1. Equipment

Beckman 40 cm length (30 cm to detector) × 75 μm i.d. uncoated fused silica capillary was used in P/ACE™ System MDQ (Fullerton, CA, USA) equipped with a diode array detector. AU capillaries were rinsed for each 30 min with 1 mol/l NaOH and 1 mol/l HCl before the first run, between sample injections, the capillary was rinsed for each 2 min with 0.1 mol/l NaOH and running buffer. Prior to separation, the capillary filled with running buffer was electrophoretically equilibrated for 30 min to reach a stable baseline. After these steps, the capillary was ready to use. The insulin samples with various concentration were injected by pressure at 0.2 psi for 5 s and separated at 25 °C; electropherograms were obtained by continuous monitoring of 214 nm absorbance at the observation window. The data were collected using the computer with the workstation software of P/ACE™ system MDQ.

2.2. Chemicals and sample preparation

Unless stated otherwise, all chemicals used in our experiments were purchased from Sigma Chemical (St. Louis, MO, USA). Tris (hydroxymethyl)-aminomethane was purchased from Bio-Rad Pacific LTD (Kowloon, Hong Kong) and urea was obtained from Aldrich Co (Milwaukee, WI, USA). Acetonitrile (ACN), high performance liquid chromatography (HPLC) grade, was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Labrasol was kindly donated by Gattefossè Company (France). All solutions were prepared using water purified by a Millipore system and further filtered with a 0.22 μm membrane (Millipore, Bedford, MA, USA).

The running buffer for MKEC consisted of 10 mmol/ml Tris–HCl, 10% Labrasol, and 10% ACN (pH 8.2); sample buffer consisted of 5

mmol/ml Tris–HCl, 10% Labrasol, and 10% ACN (pH 8.2). Samples of insulin oil formulation and control oil formulation were prepared by dissolving appropriate amount of oil A droplets 5% v/v in the sample buffer and mixing with 0.1% v/v dimethyl sulfoxide (DMSO) solution to given a final volume of 1.0 ml. Standard insulin solutions for the calibration curve were prepared by dissolving a series concentrations ranged from 0.02 to 0.8 g in sample buffer to given a final volume of 1.0 ml.

2.3. Pre-treatment sample preparation

2.3.1. Influence of pH

Insulin oil formulation was dissolved in a serious of pH sample buffer range from 0 to 5 to observe whether insulin in oil formulation could be cleavaged by such a low pH buffer. The concentration of 0.8 mg/ml insulin samples had been put into a series pH sample buffer and kept with 2 h at 37 °C and then injected immediately to separate. As a contrast test, insulin in aqueous solution had the same handling method with these in oil formulation.

2.3.2. Trypsin digestion

Trypsin was a widely used proteolytic enzyme, commonly used for protein cleavage because of its high specificity and ability to digest protein. With a protein-to-enzyme ratio of 25:1, insulin (0.50 mg/ml) was cubed with trypsin at 37 °C [7] for 15, 30, 60, 120 min period to observe the effect of trypsin for insulin in oil formulation and in aqueous solutions, respectively. These enzyme reactions had been stopped by using trypsin inhibitor and stored at 4 °C before injected into capillary.

2.3.3. Temperature stability

Oil formulations, which contained 0.8 mg/ml insulin, were put into a temperature incubator at 50 °C for 0.5, 1.0, 2.0, 4.0 and 8.0 h period, respectively. Then samples were injected into capillary to separate immediately. In contrast to oil formulation, the same amount of insulin in aqueous was handled with the same conditions.

3. Results and discussion

3.1. Separation optimization

UV absorption detection remained the most common and the most frequently used detection mode in CE of proteins and peptides. In general, the UV absorption of the peptide zone in the spectral region 200–220 nm characterizes the peptide bond quantity and a longer peptide provides a higher absorbance signal response at equal molar concentration [8]. In our experiment, insulin was observed to have an absorption maximum at 214 nm and chosen for the detection wavelength.

Fig. 1 described the separation of insulin at 10 mmol/ml Tris–HCl 10% Labrasol (pH 8.2) running buffer and Fig. 1A represented the separation of insulin in aqueous solution. A single peak was found in this electrophorogram. However, when insulin was mixed with oil A, many clutter and spike peaks had been observed (Fig. 1B). And the peak intensity of insulin was small than this in aqueous solution. Labrasol, which was negative in charge, would form a layer surrounding the tagged peptides and similar to a micelle to eliminate interactions of the positively charged groups of insulin with the capillary. So the insulin separation efficiency could be greatly improved. Meanwhile, insulin oil formulation with Labrasol was easily dissolved in the running buffer. Therefore the pre-treatment of samples before injected directly into capillary was not necessary. Furthermore, as the pseudostationary phase, Labrasol also allowed the use of relatively high amounts of organic modifier [9,10]. ACN was essentially to use in the sample and running buffer on the analysis of insulin in oil formulation. As a organic modifier, the use of ACN resulted in better peak shape and resolution for many hydrophilic proteins[11]. With joule heat decreasing, the separation efficiency of insulin could be promoted greatly and emigration time reduced in this experiment. In addition, the use of ACN can improve the solubility of running buffer components to avoid the air bubble peaks occurred. After adding ACN in the rung buffer (10 mmol/ml Tris–HCl, 10% Labrasol, pH 8.2), the peak performance of insulin in oil formulation was improved greatly

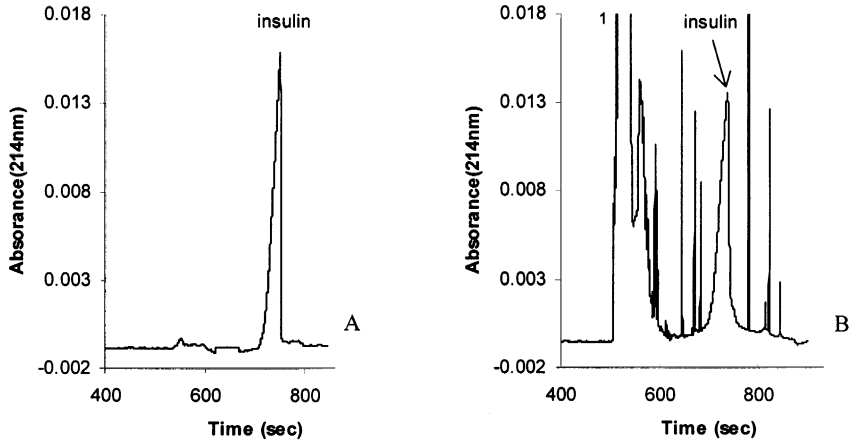

Fig. 1. Electropherograms of insulin. Insulin preparation; the amount of 0.8 mg insulin standard was dissolved in (A) 5 mmol/ml Tris–HCL, 10% Labrasol (pH 8.2) sample buffer to give a volume of 1 ml and separated at 10 mmol/ml Tris–HCl, 10% Labrasol (pH 8.2 running buffer; In (B) 10 mmol/ml Tris–HCl, 10% Labrosol (pH 8.2) mixed with 50 μl oil A to give a volume of 1 ml and separated at 10 mmol/ml Tris–HCl, 10% Labrasol (pH 8.2) running buffer; Other conditions were as follows: capillary, 40 cm (30 cm to detector) × 75 μm; injection time, 0.2 psi for 5 s (pressure mode); Run Voltage, 15 kV; Detector UV, 214 nm; separation temperature, 25 °C.

and sharp, narrow peak with high intensity at 5.7 min (peak 3) could be observed (Fig. 2). In this electrophoregram, peak 1 was DMSO and peak 2 represented the absorbance of oil A at 214 nm. Fig. 3 indicated the separation of insulin in oil formulation at a running buffer (10 mmol/ml, Tris–HCl 10% Labrasol, pH 8.2) containing oil A, the oil peak became small and not to be found nearly in the electrophoregram.

3.2. Calibration curves and validation

Numerous studies have shown that the use of an internal standard is crucial for reproducibility in CE in order to compensate for injection errors and minor fluctuations of the migration times. The internal standard (I.S.) method was used to carry out quantitative analysis. DMSO was chosen as the reference peak, as well as represent electrophoregram flow (EOF) in the separation. The assay was validated with respect to linearity, range of quantitation, detection, reproducibility, selectivity, precision and robustness.

3.2.1. Linearity, range, limit of quantitation and detection

After each run the electropherogram showed axes labeled migration time (s) and UV absorbance at 214 nm. The linearity of the calibration curves was obtained by a series of

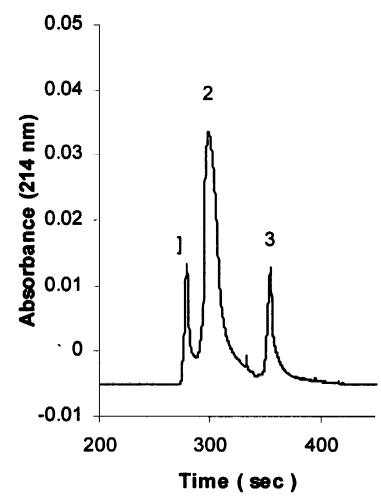

Fig. 2. Electropherograms of insulin in oil formulation. Running buffer, 10 mmol/ml Tris–HCl, 10% Labrasol (pH 8.2), 5% oil A and 10% CAN. The insulin of 0.8 mg dissolved in 5 mmol/ml Tris–HCl, 10% Labrasol (pH 8.2) 5% oil A solution and 0.1% DMSO (v/v) to give a volume of 1 ml. (1) DMSO; (2) oil A peak; (3) insulin. See Fig. 1 for other separation conditions.

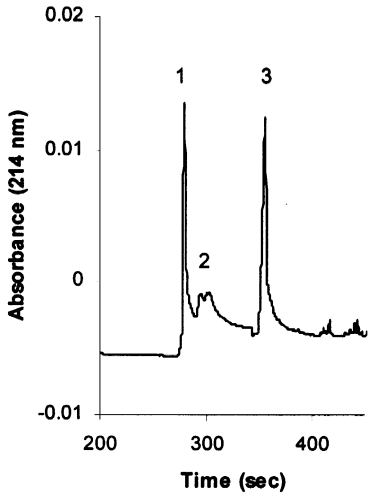

Fig. 3. Electropherograms of insulin in aqueous solution. The amount of 0.8 mg insulin dissolved in 10 mmol/ml Tris–HCl, 10% Labrasol (pH 8.2) and 0.1% DMSO (v/v) to given a volume of 1 ml; (1) DMSO; (2) oil A peak; (3) insulin. See Fig. 2 for other separation conditions.

concentration standards (0.02–0.8 mg/ml) both in aqueous solution and oil formulation. Linearity was shown using a regression analysis of calibration curves. The regression analysis of the calibration curves showed good linearity (insulin in aqueous solution; $y = 2.1837x - 0.0057$, $r = 0.997$; oil insulin formulation; $y = 1.9917x - 0.1108$, $r = 0.993$) and confirmed to us that the running buffer was suitable for the separation of insulin in oil formulation shown in Table 1. Thus, the response of insulin standard and the insulin formulation samples was found to be linear in the range 0.02–0.8 mg/ml. The limit of the detection was 0.01 mg/ml and the fact that 0.02 mg/ml to be the limit of quantification (LOQ) required for reliable analysis in oil formulation. Although the LOQ obtained by CE is higher it should be considered that the amounts of sample injected in CE are much smaller compared with HPLC. The volume of sample injected by pressure at 0.2 psi for 5 s was calculated to be approximately 15 nl. However, the typical HPLC injection volumes is at the range of 1–20 ul. So the sensitivity limitations of the UV detection using CE is in fact due to the very small amounts injected.

Table 1
Calibration data and R.S.D.s of migration times and peak areas for the insulin

(a) R.S.D.s based on six measurements with replicate injections of each species at the conditions as in Fig. 1
(b) Correct area (sample peak area/I.S. peak area
(c) The line equation; $y = 2.1837x - 0.0057$
(d) The line equation; $y = 1.9917x - 0.1108$, where y was the peak area and x the final concentration

3.2.2. Reproducibility and recovery

The repeatability of migration times and peak areas were evaluated by considering six successive experiments (Table 1). Results expressed in R.S.D. range of insulin in oil formulation are 0.3–0.7% (mean 0.45%) and 3.0–8.0% (mean 2.7%) for migration times and corrected peak areas (with respect to DMSO), respectively. Obviously, the insulin absorbance in oil formulation would appear larger or smaller depending on the total protein content of the test sample. For practical reasons, the recoveries of insulin oil formulation ranged from 0.02 to 0.8 mg/ml are varied from 90 to 96% and averaged about 92% ($n = 6$). As compared with HPLC, the average recovery ratio with the same control concentration is only about 40% (data not shown). Sample and product losses were maybe occurred at handling process.

3.2.3. Precision and stability

To compare migration time reproducibility for runs performed on different days, a total of six insulins (0.02–0.8 mg/ml) in oil formulation were observed on the same day (intra-day precision) and over two consecutive days. Within each series every sample was injected six times. In the R.S.D.s of oil insulin formulation, intraday precision is better than that of day-to-day precision. The R.S.D.s of intraday relative migration times and correct peak areas variation were 0.61 and 6.1%, respectively; day-to-day relative migration times and correct peak areas variation on a given day/capillary were approximately 2.2 and 6.5% in agreement with the standards of that day.

3.2.4. Selectivity and sensitivity

In our hands, no signal peak was observed at the migration time of insulin both in aqueous

solution and oil formulation, when performing the assay with insulin-free samples. This method was also applied to the analysis of oral insulin samples from capsule participating to the effect experiment of white rat. As the only protein existed in the oral capsule, insulin had a almost fixed emigration time in the chromatogram. No other peaks would be observed at this emigration time. In addition, the available concentration of insulin in capsule was within the linearity range, so the selectivity and sensitivity of the proposed method is sufficient to detect the insulin come from real oral capsule.

3.2.5. Interferences and robustness

Deliberate small changes in the operating parameters with regard to buffer pH (pH 8.10–8.30), buffer molarity (15–25 mM), applied voltage (13–17 kV), and detection wavelength (200–230 nm) had been designed to test the assay for robustness. Only one parameter was changed in the experiments at a time. Except for changes of the detection wavelength, variation of the other parameters did not significantly alter the corrected area ratios. Results shown the deviation of the correct peak areas never exceeded 6% under these conditions. However, any small change of detection wavelength would significantly affect the area as the large variations of the absorbance coefficients may occur within a few nm. The maximum deviation would exceed 20% when changed detection wavelength from 214 to 200 nm or 230 nm.

3.3. Influence of pH on insulin

Fig. 4A illustrated the influence of pH on insulin in aqueous solution and B in oil formulation. Within the pH value ranged from 0 to 4, the influence tendencies for insulin both in aqueous solution and oil formulation were conformable. After pH 4.0, the descending ratios of insulin in aqueous solutions speeded as compared with those in oil formulation. And when the pH value was closing to 5, insulin in aqueous (not oily) hardly dissolved in the sample buffer and detected by DAD detector. This was due to the isoelectric point (pI) of insulin was 5.35. On the other hand, insulin both in aqueous solution and oil formulation was beginning to denature and theirs concentration decreased quickly if pH value was lower than 2 and at pH 0, there had only 40% insulin remained. Within, the pH value ranged from 3 to 4, insulin had a maximum concentration both in aqueous solution and oil formulation.

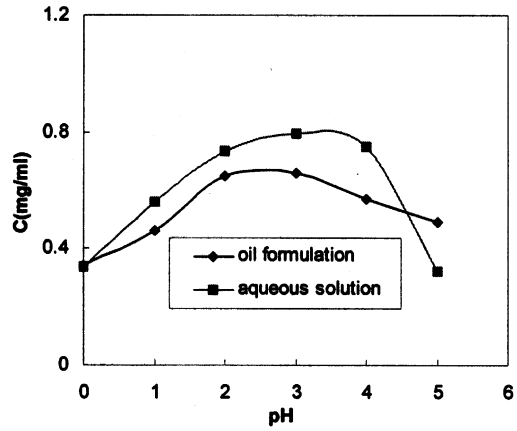

Fig. 4. The pH influence for the insulin (see text for description).

3.4. Tryptic digestion for insulin

Fig. 5A was the trypsin digestion for the insulin standard at 37 °C for different times. To the insulin aqueous solution, it was very easily to be digested by trypsin at a short time. Within 1 h, the concentrations of insulin decreased sharply from 0.50 to 0 mg/ml. Fig. 5B represented the

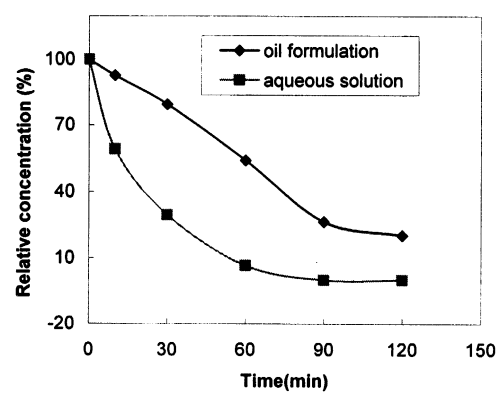

Fig. 5. Temperature stability for insulin (see text for description).

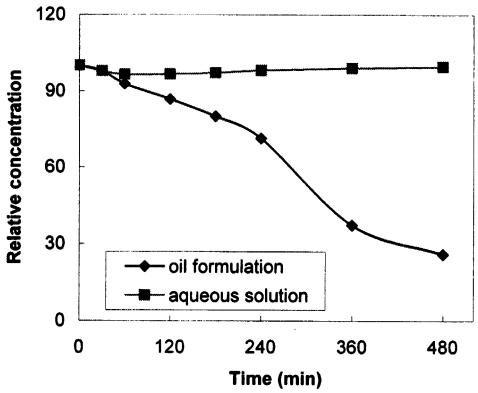

Fig. 6. Tryptic digestion for insulin (see text for description).

tryptic digestion for the insulin (0.50 mg/ml) in oil formulation obtained from insulin oral capsule. Protected by oils, insulin was degraded slowly. After 1 h, more than half amount of insulin (0.27 mg/ml) had been remained yet. And at the end of 2 h, more than 20% insulin still existed in tryptic digestion reaction solutions. So, this special oil had the function to minimize degradation of insulin in oil formulation.

3.5. Temperature stability test for insulin

Increasing test time at 50 °C had a significant effect on the degradation of insulin in oil formulation (Fig. 6A) in contrast to this in aqueous solution (Fig. 6B). After keeping at 50 °C for 8 h, 72% insulin in oil formulation had been degraded. Contrary to the oil formulation, insulin had almost not any changes in aqueous solution after handled at 50 °C for 2 to 8 h shown in Fig. 6B. However, when insulin oil formulation stored at 4 °C, the concentration of insulin also kept unchanged (data not shown). The loss of insulin may be caused by the rancidification of oil at 50 °C. This result also explained why the oil insulin formulation was recommend to store at 4 °C.

4. Conclusions

Micellar electrokinetic capillary chromatrography with a diode array detector was developed to separate and analysis insulin in oral pharmaceutical proteins in this experiment. In the separation of insulin in oil formulation, Labrasol as co-solvents to dissolve oil droplets in aqueous buffer was very important to obtain good separation and high reproducibility of analysis. ACN was also very useful for the better separation and peak shape. As compared with other methods such as gel electrophoresis and HPLC, CE offered separation without the time-consuming requirements for gel preparation and staining procedures. On-line detection can save times and was easily to optimize the separation conditions. Meanwhile, the automation and high separation efficiency were superior to those offered by HPLC. All of these explained the MEKC approach could offer rapid, simple, accurate and precise information to separate and quantity insulin. A good linearity ($r = 0.996$) of the insulin calibration curve was observed in oil formulation and LOQ was 0.02 mg/ml. The pH experiments indicated that insulin both in aqueous solutions and oil formulations suffered a minor effect caused by an extreme low pH acid solution. Considering the absorption of proteins in GT was finished within 1 h, this advantages was very useful for the insulin to resistant the degradation of the stomach acid pH $\pm$ 2.0). Protected by oil, also the tryptic digestion had a lesser influence on the insulin in oil formulation than in aqueous solutions. In this experiment, more than 55% insulin in oil formulation was still remained after 1 h tryptic digestion. Therefore, most insulin in oil formulation could be absorbed by GT. It was clear that the temperature had far most influence on the stability of insulin in oil formulation than in aqueous solution. As a consequence, some anti-oxidation additives would be necessary to reduce the degradation of insulin oil formulation acting as an oral route in clinical use. The coherence of the real samples come from oral capsule with their effect for the white rat have proved the selectivity and sensitivity of the proposed method were sufficient for real-life applications in our animal experiments. The future work including the on-line monitoring of insulin changes in the special tissues such as liver of white rats have been designed and prepared.

Acknowledgements

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