On-line Trace Enrichment for the Determination of Insulin in Biological Samples Using Reversed-phase High Performance Liquid Chromatography with Column Switching

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Column-switching technique with a reversed-phase high performance liquid chromatographic method has been developed for the routine analysis of radioiodinated insulin and its degradation products in biological fluids. The diluted biological samples were loaded onto a precolumn packed with LiChrosorb RP-8 (25-40 μm) using 0.1% trifluoroacetic acid (TFA) in water as a washing solvent. After valve switching, the concentrated insulins were eluted in the back-flush mode and separated by a W-Porex C_{18} column with a gradient of 0.1% TFA in water and 0.1% TFA in acetonitrile as the mobile phase. The method showed good precision, accuracy and speed with the detection limit of 20 pg/ml. Total analysis time per sample was about 40 min and the coefficients of variation were less than 8.2%.

Key words : Column-switching technique, RP-HPLC, Insulin, Biological samples

INTRODUCTION

There are several methods for the determination of insulin in biological samples. Among them are radio-immunoassay (RIA) (Jorensen, 1969; Velasco et al., 1974), enzyme immunoassay (Hinsberg et al., 1981) and HPLC (Benzi et al., 1990; Duckworth et al., 1988; Hamel et al, 1988; Kitabchi and Stentz, 1985; Marchetti et al., 1986; Pell et al., 1986). Because of its sensitivity RIA is at the present the approach of choice for routine insulin assay in plasma; however, the accuracy of the results depends on the specificity of the antibody used, the detectability and the presence of its degradation products and analytical artefacts.

Reversed-phase (RP) HPLC is to be preferred for the separation of insulin and its degradation products in biological samples because of advantages such as short turnaround time, method reliability, efficiency and specificity (Benzi et al., 1990; Duckworth et al., 1988; Hamel et al, 1988; Kitabchi and Stentz, 1985;

Marchetti et al., 1986; Pell et al., 1986). Those methods usually involved a work-up procedure based on gel-permeation chromatography (Duckworth et al., 1988; Hamel et al, 1988; Kitabchi and Stentz, 1985; Marchetti et al., 1986; Pell et al., 1986) or solid-phase extraction steps (Benzi et al., 1990) that involve multiple steps and concentration process. All the processes are time consuming and prone to errors and formation of artefacts. These problems have severely hampered the development of new formulations and have therefore proved to be solved by the use of a column-switching technique which allows on-line purification with no extraction step and which provides a precolumn enrichment (Jang and Lee, 1994; Jung et al., 1993; Lee et al., 1990; Lee et al., 1992).

The objective of this paper was to develop an automated HPLC method for the simultaneous determination of radioiodinated insulin and its degradation products in biological samples that is more sensitive, selective, reproducible and convenient method in terms of sample handling and speed of analysis. The present paper describes precolumn-switching technique which allows on-line sample loading, rapid elution of the analytes from a precolumn and direct analysis on the analytical column.

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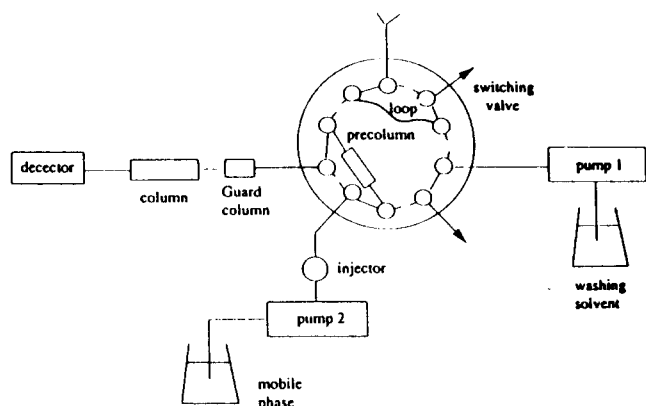
Fig. 1. Schematic diagram of ten-port switching system.
--- load; — inject

MATERIALS AND METHODS

Materials

Porcine insulin was obtained from Sigma Chemical Co. (St. Louis, MO, USA). A14-[^{125}I] Insulin (porcine) was obtained from NEN (Dupont, USA) and the specific activity was 2200 Ci/mmol. All other reagents were of HPLC grade.

Chromatographic System

The HPLC system consisted of a Beckman 126 pump (Fullerton, CA, USA), a Beckman pump, a Rheodyne 7125 injector (Cotati, CA, USA), a ten-port multifunction valve (Valco, Houston, TX, USA), Beckman 166 UV detector and Beckman 171 Radioisotope detector. Data handling was performed by System Gold using Beckman 406 Analog Interface module. The instrumental arrangement for ten-port column-switching system is shown in Fig. 1.

The precolumn (20×3.9 mm i.d.) was tap-filled with LiChrosorb RP-8 (25-40 μm, Merck, Darmstardt, Germany) and was changed after injection of 20 samples. A guard column was Nova-Pak C$_8$ guard column (4.0× 10 mm i.d., Waters Assoc., MA, USA) and the analytical column was W-Porex 5 C$_{18}$ packed column (250× 4.6 mm i.d., 300 Å, Phenomenex, CA, USA).

The washing solvent was 0.1% TFA in water and the flow rate was 0.5 ml/min. The mobile phase consisted of two components: (A) 0.1% TFA in water and (B) 0.1% TFA in acetonitrile. A linear gradient was used as the mobile phase: from 20% B in A to 50% B in A (0-20 min) and 100% B (20-25 min). The flow rate of the mobile phase was 1.0 ml/min. The radioactivity of the effluent was monitored using flowthrough radioisotope detector equipped with solid cell. The column temperature was ambient.

Analytical Procedure

The sequence of sample analysis using column swi-

ching technique included the following steps and required about 40 min for completion. The plasma samples were diluted three times with 0.1% TFA in water. The prepared samples were kept at ice before injection.

Step I (0-10 min): The aliquot (1 ml) of diluted plasma samples was injected onto the precolumn. Polar interfering plasma components were washed out to waste while the guard column and analytical column were equilibrated with the mobile phase.

Step II (10-35 min): The washing solvent passed directly to waste by switching the valve to inject. The retained components were eluted in the back-flush mode from the precolumn to guard column/analytical column by the gradient mobile phase. The eluted drugs were separated in the analytical column. The analytical column and precolumn were washed with 100% B to prevent any memory effects during the next injection.

Step III (35-40 min): After valve switching to load, the precolumn and analytical column were reequilibrated with the washing solvent and the mobile phase, respectively.

RESULTS AND DISCUSSION

Most RP-HPLC of insulin reported so far are based on one of two principles: (a) control of the degree of ionization of the insulin and (b) use of ion pair reagents (Vigh et al., 1982). The usefulness of RP-HPLC in the analysis of intact and degraded insulin has been reported in fibroblasts (Kitabchi and Stentz, 1985), human monocytes (Marchetti et al., 1986), hepatocytes (Duckworth et al., 1988; Hamel et al., 1988; Pell et al., 1986) and human plasma (Benzi et al., 1990).

The chromatographic separation of A14-[^{125}I] insulin on commercially available octadecyl-silica packings with different carbon contents has been evaluated using various buffer systems. Optimum recovery and efficiency of A14-[^{125}I] insulin were noted with W-Porex 5 C$_{18}$ column and a gradient of 0.1% TFA in water and 0.1% TFA in acetonitrile. A gradient was necessary for the simultaneous determination of A14-[^{125}I] insulin and its degradation products without the loss of efficiency and recovery. A gradient was useful for purging nonpolar interference components from analytical column.

Typical chromatograms illustrating the separation of A14-[^{125}I] insulin and its degradation products are shown in Fig. 2. The retention time of A14-[^{125}I] insulin was 12.8 min. The analytical column showed no decrease in efficiency after more than 300 injections of biological samples.

In column-switching technique, the precolumn packing material, washing solvent, washing time and flow rate should be selected to obtain the quantitative ad-
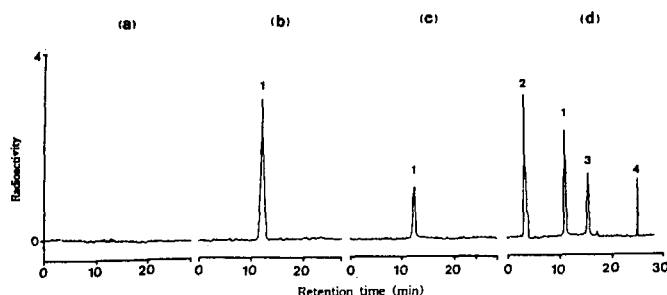
Fig. 2. Chromatograms of (a) a blank plasma, (b) a blank plasma spiked with A14-[^{125}I] insulin, (c) a plasma sample from a rat 5 min after an intravenous injection of 3 µg/kg (10^7 cpm) A14-[^{125}I] insulin and (d) a mouse liver homogenate incubate with A14-[^{125}I] insulin for 10 min. Peaks: 1=A 14-[^{125}I] insulin; 2, 3, 4=degradation products of A14-[^{125}I] insulin.

Table I. Reproducibility of A14-[^{125}I] insulin assay in plasma samples (n=4)

Concentration added (pg/ml)	Concentration found (pg/ml)	C.V. (%)
20	17.0	6.8
50	46.0	7.3
100	94.3	8.2
200	177.1	7.7

sorption of insulin from biological samples and remove the interference components from the precolumn.

LiChrosorb RP-8 (25-40 µm), a nonpolar octylsilane bonded phase adsorbent, is a suitable precolumn packing because of its good recovery for insulin, stability at pH 1-7 and easy availability.

In using 0.1% TFA in water as washing solvent, the majority of the plasma components is not selectively adsorbed while insulin exhibit the retention on LiChrosorb RP-8 precolumn. The recovery of insulin exhibited a high dependence on the flow rate of washing solvent. That is, a longer residence time was required during adsorption step to ensure the quantitative uptake. To obtain good recovery and clean chromatograms, the clean-up process was completed in less than 10 min by washing the precolumn at a flow rate of 0.5 ml/min with 0.1% TFA in water.

The correlation of peak-height ratios with the concentrations of A14-[^{125}I] insulin in plasma was linear in the range of 20-200 pg/ml. The correlation coefficients were better than 0.99.

Detection limit was determined as the concentration of compound giving a signal-to-noise ratio greater than 3 : 1. The limit of detection of A14-[^{125}I] insulin was 20 pg/ml after an injection of 1000 µl of diluted plasma (equivalent to 330 µl of plasma).

The recovery of A14-[^{125}I] insulin from plasma was determined by the analysis of fixed amount of A14-[^{125}I] insulin in plasma, followed by replicate injection of the same amount of a standard in 100 µl buffer directly onto the analytical column, providing the 100% value. Mean recovery of A14-[^{125}I] insulin in plasma was 90.0± 4.6%.

The precision (defined as the coefficient of variation of replicate analysis) and the accuracy (defined as the deviation between added and found concentration) of the assay for insulin were evaluated over the plasma concentration range of 20-200 pg/ml. The results are

shown in Table I. The coefficient of variation varied from 6.8% to 8.2% of the added amount in the spiked plasma samples.

The present method has been successfully applied to the analysis of the samples spiked with rat plasma or incubated with mouse liver homogenate. The chromatograms of A14-[^{125}I] insulin spiked with rat plasma and incubated with mouse liver homogenate are shown in Fig. 2c and 2d, respectively.

CONCLUSION

For the determination of A14-[^{125}I] insulin and its degradation products in biological samples, a new HPLC method with direct injection of diluted biological samples was developed using the column-switching technique. This method is readily applicable to the therapeutic drug monitoring of radioiodinated insulin in plasma because of its excellent precision, sensitivity, specificity and speed.

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