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## Note

# High-performance liquid chromatographic analysis of malonylbis-(methionyl)insulin formed during recombination of insulin chains

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High-performance liquid chromatography (HPLC) has been used for the resolution of amino acids, peptides, proteins and their derivatives both at the analytical and preparative levels[1]. The potential of HPLC for the analysis and purification of several polypeptide hormones including insulin and proteins has been documented[2,3]. Several methods are available for the purification of different insulins and their desamido derivatives[3–10]. The superior resolving power of HPLC over conventional methods of purification in separating insulin, its disulfide isomers and its analogs with D-cysteine has been demonstrated[4]. The natural insulin derived from the pig, cattle and human that differs in one or two amino acid residues has been separated using isocratic conditions[3,5]. Under these conditions the corresponding monodesamido insulins are separated as well thus facilitating the analysis of the proportions of various insulins and their desamido derivatives present in commercially available insulin formulations[5]. The separation of eight different insulins has recently been achieved by HPLC[11].

An HPLC method that enables quantitation of CBM-** or MBM-insulin[12,13] formed during recombination of the A- and B-chain disulfides of insulin (Fig. 1) was desired. The direct application of some of the earlier procedures for the purification of insulin did not help to resolve all the components, especially MBM-insulin, present in physical mixtures of insulin, A- and B-chain disulfides and MBM- or CBM-insulin. Therefore, an HPLC system that resolves the above components was developed.

## EXPERIMENTAL

Zinc-free insulin was prepared from bovine Zn · insulin (Eli Lilly and Co., Lot No. 9SH35AK) by gel filtration on Sephadex G-50 using 10% acetic acid as the solvent[14]. Malonylbis(methionyl)insulin was prepared as described elsewhere[13]. Desamidoinsulin was a gift from Professor F. H. Carpenter. A-chain bis-disulfide and

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** Abbreviations used: CBM = carbonylbis(methionyl); MBM = malonylbis(methionyl); $A(S\text{-}S)_2$ = A-chain bis-disulfide; B(S-S) = B-chain disulfide; ODS = octadecylsilane; ONp = *p*-nitrophenyl ester; $Bu_3P$ = tributylphosphine.

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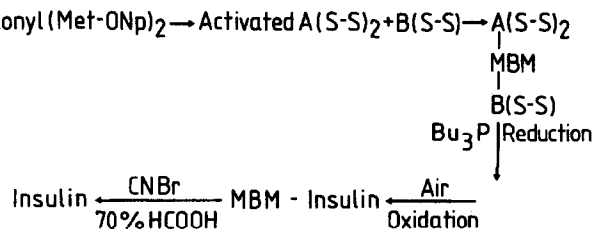


Fig. 1. Scheme for the synthesis of insulin.

B-chain disulfide were prepared from the sulfonates of the corresponding chains by reduction with tributylphosphine and subsequent air oxidation[15]. The commercially available HPLC grade solvents were used for chromatography.

HPLC was performed using an Altex instrument connected to a Beckman automatic integrator system. The ODS ultrasphere column (250 × 46 mm) was provided by Dr. M. W. Riemen. The sample injection was carried out using a 20-$\mu$l loop. During the run the column was maintained at 40°C.

Two different solvents were used for chromatography: A, 0.1 *M* phosphate buffer, pH 3.0, in 20% 2-propanol containing 5% methyl cellosolve; B, 2-propanol. A linear gradient from 0 to 70% of solvent B over 100 min was programmed automatically. The flow-rate was adjusted to 1.0 ml/min and the eluent was scanned at 280 nm. The loading of sample on to the column coincided with the starting of pumps for solvents A and B, which were deaerated before use. After each analysis the column was equilibrated with solvent A for 30 min. The samples were dissolved in solvent A to get a concentration of 2 mg/ml of each component. Unless mentioned otherwise, 20 $\mu$l of sample were used for analysis.

## RESULTS AND DISCUSSION

The two chains of bovine insulin are held together by disulfide bonds between residues $A_7$–$B_7$ and $A_{20}$–$B_{19}$. In addition there is an internal disulfide bond in the A-chain through residues $A_6$ and $A_{11}$. The cross-linking of insulin through residues $A_1$ and $B_{29}$ by a malonylbis(methionyl) group yields malonylbis(methionyl)insulin which on reduction and oxidation forms disulfide bonds as found in native insulin[13]. This investigation led to the possibility of recombining the two chains of insulin using a reversible bifunctional reagent, malonylbis(methionyl) *p*-nitrophenyl ester. The strategy involved is shown in Fig. 1. A method to quantitate the MBM-insulin formed during recombination under different experimental conditions was necessary. HPLC seemed to be the right tool for such exploratory studies, particularly when smaller amounts of reactants were used.

The least resolution expected was the separation of malonylbis(methionyl)insulin from $A(S\text{-}S)_2$ and B(S-S). Since insulin was the ultimate product to be purified it was decided to look for a solvent system that resolves insulin, $A(S\text{-}S)_2$, B(S-S) and MBM-insulin. Experience gained through preliminary experiments using combinations of different solvents, based on earlier reports, helped to formulate solvent A. Owing to solubility problems with MBM-insulin in solvents containing no more than 5–10% acetonitrile, the latter could not be used as organic modifier. Isocratic analysis

with solvent A as such did not elute MBM-insulin from the column, although it resolved insulin and A- and B-chain disulfides. When the 2-propanol concentration was raised from 20 to 25 or 30%, the insulin and $A(S\text{-}S)_2$ peaks overlapped. However, MBM-insulin was eluted as a broad peak. Therefore, it was decided to employ linear gradient chromatography using solvents A and B as described in the Experimental section. Fig. 2 shows the elution profile. All the components are separated including desamidoinsulin which was not separated from insulin during isocratic analyses. The different peaks were identified by analysing the individual components under identical conditions. When the 2-propanol concentration in solvent A was raised from 20 to 25 or 30% during linear gradient chromatography poorer resolution of insulin, des-

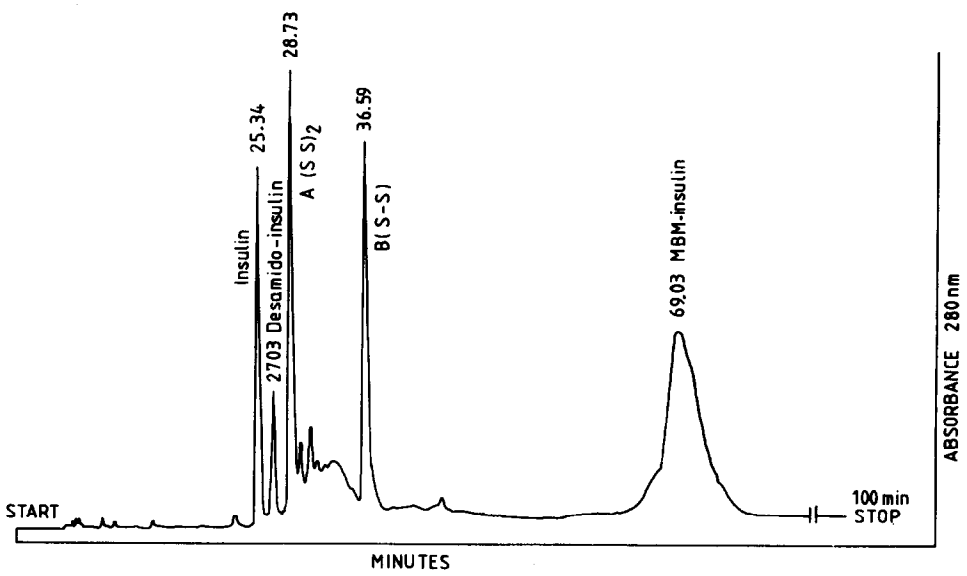


Fig. 2. HPLC of insulin and its derivatives, showing the retention times in minutes. All samples are analysed at the level of 40 μg except for MBM-insulin (80 μg) and desamidoinsulin (10 μg). Analysis conditions as described in the Experimental section.

amidoinsulin and $A(S\text{-}S)_2$ was observed. However, the peak corresponding to MBM-insulin was sharper when 25 or 30% 2-propanol was used in solvent A during linear gradient chromatography.

These observations suggested that the 2-propanol concentration in solvent A is critical for the resolution of insulin, desamidoinsulin and $A(S\text{-}S)_2$. In addition, the linear gradient of 2-propanol helps to resolve B(S-S)as a sharp peak and MBM-insulin. This solvent system could be useful for HPLC of insulin derivatives such as carbonylbis(methionyl)- and/or oxalylbis(methionyl)insulin which would be expected to have properties similar to MBM-insulin.

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