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Note

Separation of human haemoglobin alkylated at β93 cysteine from its native form by fast protein liquid chromatography

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Exposure to genotoxic chemicals can lead to alkylation of biological macromolecules in the human body. Measurement of the adducts formed by alkylation of haemoglobin (Hb) is currently considered as one of the most promising methods for the monitoring of exposure to carcinogens and mutagens [1,2].

Most methods for the determination of haemoglobin adducts depend on degradation of the protein chain and subsequent determination of the alkylated amino acids. However, the presence of only one type of reactive thiol group, the β93 cysteine, in normal human haemoglobin offers the possibility of using changes in the biochemical and the biophysical nature of this protein after alkylation for a quantitative determination of alkylation.

Recently we described the application of isoelectric focusing (IEF) of carbonmonoxy haemoglobins in ultra-thin polyacrylamide gels with a non-linear pH gradient to separate alkylated from normal HbA. Although this IEF method provides good resolution of the alkylated HbAs from normal HbA, the sensitivity of the method is limited. Owing to the low degrees of alkylation occurring in practice, it is not possible to apply detectable amounts of alkylated haemoglobin to the gels without overloading them with protein. During the past few years there

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has been a rapid development of fast techniques for the chromatography of biological macromolecules by the application of monodisperse polymer particles in liquid chromatography columns with a low back-pressure. These techniques seem promising for the development of a fast preparative separation of cysteine-alkylated from non-alkylated haemoglobin. This type of chromatographic technique, often denoted as fast protein liquid chromatography (FPLC), has been used for the separation of haemoglobin forms and variants [3,4].

This paper describes two different methods by which HbA alkylated with iodoacetamide can be separated from non-alkylated HbA. The first consists of a ion-exchange procedure on a monodisperse quaternary amino column and the second of chromatofocusing, i.e. the development of a pH gradient on an ion-exchange column by elution with a special amphoteric buffer.

EXPERIMENTAL

Chemicals

4,4′-Dithiopyridine (4-PDS), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and iodoacetamide (IAA) were from Janssen Chimica (Beerse, Belgium), Sephadex™ G-25 (medium) and Polybuffer™ 96 were from Pharmacia/LKB (Uppsala, Sweden). All other chemicals were of analytical purity. Only freshly deionized distilled water was used.

Apparatus

The FPLC system consisted of an MV-7 and a MV-8 type motor valve with a 50-μl sample loop and a P-1 pump with prefilter for sample delivery, two P-500 pumps, an LCC-500 liquid chromatography controller, a UV-1 monitor with 10-mm flow-cell, 280-nm filter and flow restrictor, and a pH monitor (all from Pharmacia/LKB).

Haemoglobin samples

Blood from a single human volunteer was collected in a 500-ml plastic bag containing 70 ml of CPD anticoagulans. Purified haemoglobin solutions (3 ml) were incubated with 100 μl of IAA dissolved in saline in closed sterile vials (24 mm I.D.) in a shaking water bath (210 rpm) at 37°C for 1 h. Haemoglobin was isolated as previously described [5]. Briefly, the erythrocytes were washed with phosphate-buffered saline, lysed by centrifugation in 30% sucrose containing 0.5% Triton X-100 as described by Scott [6] and desalted on Sephadex G-25. The final buffer was 1 m*M* phosphate (pH 8.0). The haemoglobin samples were not equilibrated with carbon monoxide. Finally, the solutions were filtered through 2-μm sterile filters.

Chemical determination of free sulphydryl groups in haemoglobin

Free sulphydryl groups in haemoglobin (Hb-SH) were determined by the procedure of Grassetti and Murray [7] as modified by Neis et al. [8]. The assay is based on spectrophotometric determination of the formation of 4-thiopyridine in

the reaction of 4-PDS with Hb-SH. The concentration of heamoglobin was determined with the haemiglobin cyanide procedure of Van Kampen and Zijlstra [9].

Separation by ion-exchange chromatography

Samples of IAA-treated haemoglobin (50 μl) were diluted 30-fold with 1 m*M* phosphate buffer (pH 7.3) and analysed on a prepacked Mono Q HR column (5 cm $\times$ 0.5 cm I.D.). The column was eluted with 20 m*M* Tris–HCl buffer with salt gradient to 0.5 *M* sodium chloride at a flow-rate of 1 ml/min. Buffers of pH 7.8, 7.9 and 8.0 were tested.

Separation by chromatofocusing

The haemoglobin samples were diluted 60-fold with starting buffer (see below) and 50 μl were injected on a prepacked Mono P HR column (20 cm $\times$ 0.5 cm I.D.), which was equilibrated with a 25 m*M* triethylamine–acetic acid starting buffer (pH 8.6). The column was eluted at a flow-rate of 0.5 ml/min with Polybuffer 96 diluted twenty-fold. The pH was adjusted to 6.6 with acetic acid. Both buffer solutions contained 1 m*M* sodium nitrite and 1 m*M* potassium cyanide.

RESULTS

Separation of HbA alkylated at the β93 cysteine with IAA from normal HbA with ion-exchange chromatography on a Mono Q column at pH values near 8 was readily attained with shallow pH gradients. At pH values in this range, which is only ca. 1 pH unit above the isoelectric points of the haemoglobins, the retention of both HbA and its IAA adduct was low. Better separations were obtained at pH 7.8 than at pH 7.9 and 8.0, and the retention was lowered. At pH values below 7.8 the remaining net charge of the proteins was too low to allow separations at the buffer strength needed to equilibrate the proteins. Fig. 1 illustrates the separation obtained at pH 7.8. Elution was started with 20 m*M* Tris–HCl buffer of this pH. After 1.5 ml a linear salt gradient was started, which reached 50 m*M* sodium chloride after 15 ml. Thereafter the column was washed with higher salt concentrations (maximum 0.5 *M*). The retention volume of HbA was 8.4 ml and that of the alkylated HbA 10.8 ml. The alkylated HbA peak was identified from the good correspondence between the amount of alkylation calculated from the peak areas and the results of the spectrophotometric determinations (Table I). Even without special chromatographic optimization the ferri forms of alkylated and non-alkylated HbA were also separated and eluted during the washing procedure (see Fig. 1). Table I gives the relative thiol alkylation determined by separation on Mono Q and by treating the remaining free thiol groups with 4-PDS.

Preliminary chromatofocusing experiments indicated that ferrihaemoglobin hampered the determination of alkylated HbA. To prevent simultaneous presence of reduced and oxidized haemoglobins, all haemoglobins were converted into their ferricyano form by the addition of 1 m*M* sodium nitrite and 1 m*M* potassium cyanide to all solutions. Separation of alkylated from non-alkylated HbA with chromatofocusing proved to be difficult. A very shallow pH gradient was needed,

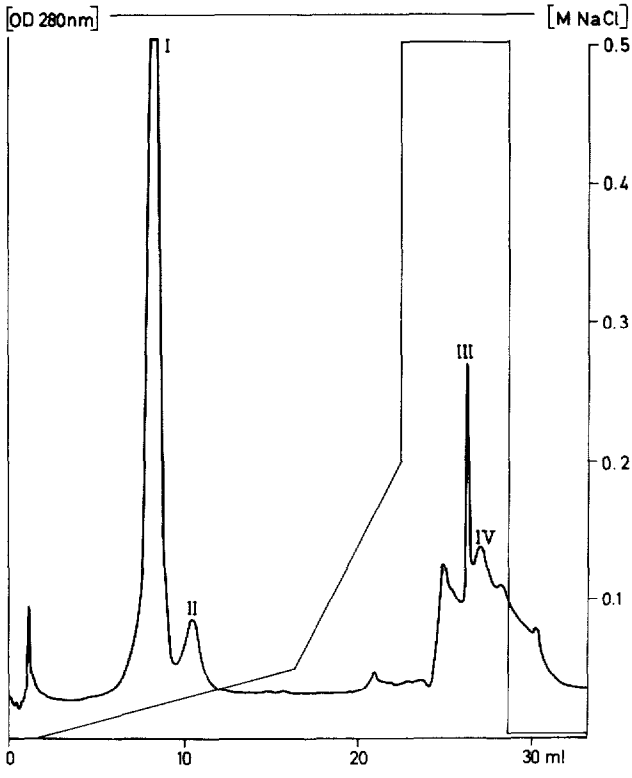

Fig. 1. Separation of HbA (I) from HbA alkylated with IAA (II) on a quaternary amino (Mono Q) at pH 7.8. The ferrihaemoglobin forms of HbA (III) and alkylated HbA (IV) were eluted during the washing procedure.

TABLE I

ALKYLATION OF HbA BY IAA; COMPARISON OF FPLC AND SPECTROPHOTOMETRIC RESULTS

Concentration of IAA added to purified Hb (m*M*)	Percentage of SH left	
	Determined by FPLC on Mono Q	Determined with the 4-PDS assays
0.0	100	100
1.0	94	92
3.0	53	42
10.0	0	4

which was obtained by dilution (twenty-fold) of Polybuffer 96. Fig. 2 illustrates the partial separation of ferricyano HbA from its IAA alkylated form. The chromatofocusing conditions were as described in Experimental. The sample had been alkylated with 3 m*M* IAA.

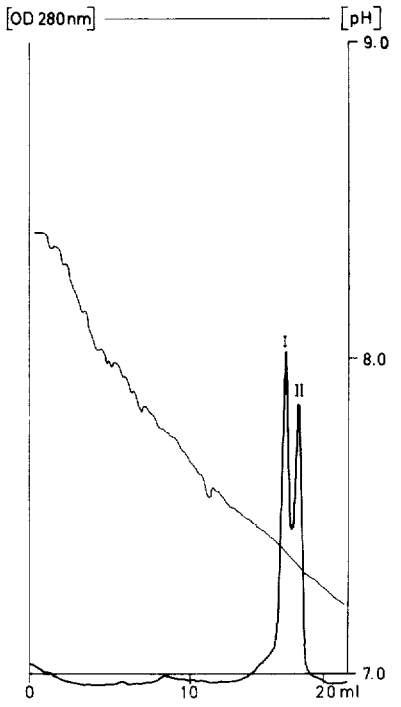

Fig. 2. Separation of ferricyano HbA (I) from ferricyano HbA alkylated with IAA (II) by chromatofocusing on a Mono P column.

DISCUSSION

It was possible to separate HbA alkylated with IAA from its native form with fast protein ion-exchange chromatography on a Mono Q column. Partial separation was obtained by chromatofocusing on a Mono P column. Of the two methods developed, the ion-exchange procedure is the more promising. It is faster (including a regeneration time of less than 40 min versus ca. 2.5 h for each analysis) and in its current state better separations are obtained. Furthermore, it offers many possiblities for optimization. For instance, the oxidation and ligand-binding state of the haemoglobins largely influences their chromatographic behaviour, and control of these features can be used for further optimization.

The fast preparative purification of haemoglobins alkylated at the β93 cysteine, which can be performed with these novel techniques, will be of great importance for the development of methods that determine the amount of haemoglobin alkylated for the purpose of monitoring primary effects due to biologically alkylating agents. In its present form FPLC already offers a tremendous purification step in procedures aimed at determination of cysteine adducts after hydrolysis of the protein. Procedures based on gas chromatography–mass spectromety (GC–MS) of animo acids purified in several lengthy ion-exchange steps have been described for S-methylcysteine [10] and S-(2-carboxyethyl)cysteine [11]. The use of an FPLC step prior to protein hydrolysis in such procedures would

have the advantage that the alkylated cysteines are purified with respect to all other amino acids in equal proportions. Thus a large part of the amino acids that are difficult to remove from the alkylated cysteine by amino acid chromatography, such as methionine, will be removed prior to hydrolysis.

Since the FPLC methods can be used preparatively they also offer the possibility to develop methods for determination of the low levels of adducts that are found in practice by combination with other protein purification steps, such as IEF and other FPLC methods.

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