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DETERMINATION OF MYOGLOBIN IN HUMAN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH CHEMILUMINESCENCE DETECTION

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SUMMARY

A highly sensitive method for the determination of myoglobin in serum is described, based on high-performance size-exclusion chromatography with chemiluminescence detection. Serum proteins are separated according to their molecular masses on columns packed with TSK-SW gel and those containing haem are detected selectively by post-column chemiluminescence reaction with luminol using a conventional fluorimetric detector. The method is rapid (30 min) and sufficiently sensitive for the diagnosis of myocardial infarction. The minimum detectable myoglobin concentration is 10 ng/ml.

INTRODUCTION

One of the most important problems in clinical biochemistry is the determination of tissue-specific proteins in serum. Injuries to the tissues of various organs may be reliably diagnosed when a protein that is not characteristic of normal blood but is specific for a certain organ is excreted into the blood channel. For instance, in order to diagnose myocardial infarction, apart from analysis of lactate dehydrogenase (LD) and creatine kinase (CK) isoenzymes, the determination of myoglobin in blood serum is also used. This is usually carried out by means of radioimmunoassay (RIA) [1]. According to RIA data, the myoglobin content in normal serum is 6–85 ng/ml and after myocardial infarction it increases to approximately 400 ng/ml, or even to 500–600 ng/ml in a case complicated by circulatory insufficiency [2]. The myoglobin concentration in blood attains its maximum 7.5 h after an anginal attack and reduces to the normal level in 28–36 h [2]. Hence the determination of myoglobin offers considerable advantages over other biochemical indices, such as activity levels of LD1, LD2 and CK-MB isoenzymes, as it ensures the earliest diagnosis of myocardial infarction.

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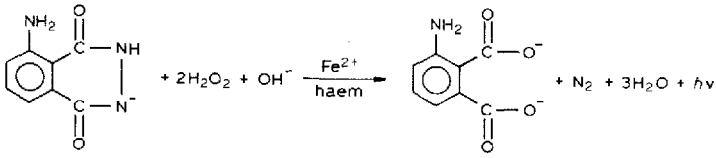
In the RIA method, highly specific and pure monoclonal antibodies should be used; only then reliable results can be obtained. Hence, it seemed desirable to develop an alternative method for the determination of myoglobin in serum that is as specific and sensitive as RIA but more readily available to biochemical laboratories.

EXPERIMENTAL

Method

The proposed method is based on high-performance liquid chromatography (HPLC), which has been used successfully for the assay of LD [3,4] and CK [5, 5] isoenzymes. The required specificity of chromatographic isoenzyme analysis was attained by means of activity-based detection with the use of post-column reactors [3].

Myoglobin and other haemoproteins can be detected selectively using the chemiluminescent luminol reaction:



(1)

Luminol (aminophthalic hydrazide) reacts with hydrogen peroxide in an alkaline medium to give a carboxylate ion. This reaction is accompanied by a pronounced flash of chemiluminescence [7]. Reaction 1 is catalysed by Fe^{2+} and, in particular, by haem containing this ion [8]. When hydrogen peroxide is present in excess, the chemiluminescence intensity is proportional to the Fe^{2+} concentration. The maximum intensity of chemiluminescence is observed in the blue region of the spectrum ($\lambda_{max} = 430$ nm). Attempts had been made to use reaction 1 for the direct chemiluminescence assay of the whole pool of haemoproteins in serum without a preliminary separation of individual components [8]. However, it does not allow individual components, and in particular myoglobin, to be determined. Application of reaction 1 to continuous effluent monitoring in HPLC seems very promising, as the high resolving power of HPLC may be combined with the excellent selectivity and unsurpassed sensitivity of chemiluminescent detection.

Apparatus

Luminometers [9] with a coiled capillary flow cell located in the photomultiplier plane are generally used for recording the chemiluminescent flash. We used for this purpose a Kratos FS-970 standard fluorimetric detector (Kratos Analytical Instruments, Ramsey, NY, U.S.A.) in which the deuterium lamp was closed with a shutter, thus converting it into a flow-through luminometer. The apparatus used for the analysis of haem-containing proteins is shown schematically in Fig. 1.

In order to separate proteins according to molecular mass, commercial 60×0.8

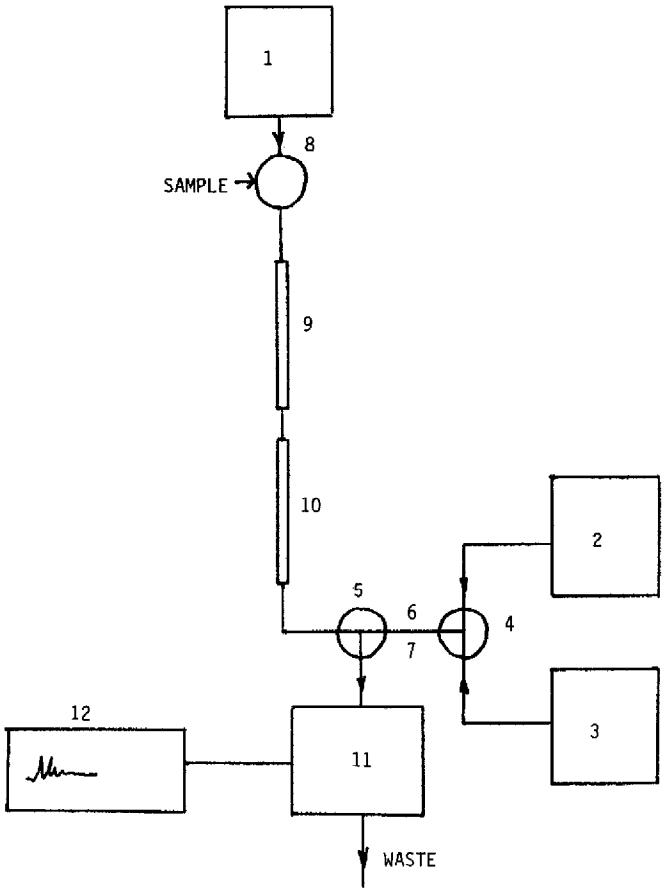


Fig. 1. Schematic diagram of the HPLC system for the determination of myoglobin in serum. 1, 2, 3 = Pumps; 4, 5 = mixing tees; 6, 7 = connecting capillaries; 8 = sample injector; 9, 10 = columns with Spherogel TSK 2000 SW and TSK 3000 SW; 11 = chemiluminometer; 12 = recorder.

cm I.D. columns, packed with Spherogel TSK 2000 SW and TSK 3000 SW (Beckman, Berkeley, CA, U.S.A.) were used. The sample was injected by means of an Altex 210 injector (Beckman) with a 150-μl loop. The eluent was delivered using an Altex 110 A pump (Beckman Instruments) (1 in Fig. 1) at a flow-rate of 1.3 ml/min.

A system of two T-shaped mixers was used for mixing the effluent with hydrogen peroxide solution and with alkaline luminol reagent. Both reagents were delivered by LKB 1200 peristaltic pumps (LKB, Bromma, Sweden) at a flow-rate of 0.3 ml/min (2 and 3 in Fig. 1). The dimensions of the connecting capillaries (6 and 7 in Fig. 1) between mixing tees (4 and 5 in Fig. 1) are very important for attaining the highest sensitivity of detection, because the chemiluminescence intensity of the luminol reaction reaches its maximum 1–2 s after the oxidant is added and then decreases (for approximately 1.5 min) exponentially. A short capillary (7 in Fig. 1) (0.2 mm I.D.) with a volume of 40 μl

ensured the maximum yield of the chemiluminescent reaction, whereas additional band broadening caused by this capillary was negligible compared with the peak width. On the other hand, a relatively long capillary (600×0.5 mm I.D.) was used on the tees 4 and 5 line (7 in Fig. 1). The resulting lag time was sufficient to provide the decay of the background chemiluminescence, appearing after hydrogen peroxide was added to luminol.

Reagents

Hydrogen peroxide (30%), potassium chloride and potassium dihydrogen phosphate were of analytical-reagent grade (Reakhim, U.S.S.R.). Luminol of analytical-reagent grade was obtained from Fluka (Buchs, Switzerland). Horse myoglobin from a Serva MS-II Kit (Serva, Heidelberg, F.R.G.) was used for calibration.

A 1.3 m*M* solution of luminol in 0.2 *M* sodium hydroxide solution and a 0.1% aqueous solution of hydrogen peroxide were used as reagents.

The quality of the water used for the preparation of the solutions was found to be of utmost importance. In order to avoid background chemiluminescence, which would decrease the sensitivity of detection, distilled water was freed from Fe^{2+} by passage through a laboratory water purification system similar to the Super-Q system (Millipore, Bedford, MA, U.S.A.). The resistance of the purified water was greater than 1 MΩ cm.

It was also found that the background chemiluminescence may result from slow corrosion of the stainless-steel mixers and the pump head, which are in contact with the eluent or reagents. Hence, a complexing agent that binds Fe^{2+} ions, Trilon B (a sodium salt of ethylenediaminetetraacetic acid), was introduced into the eluent [0.01 *M* potassium phosphate buffer (pH 6.8)–0.2 *M* potassium chloride solution] at the level of 1 g/l.

The use of purified water with the addition of Trilon B reduces the background chemiluminescence by approximately two orders of magnitude and makes it possible to detect minor components of haem-containing proteins in serum.

Serum preparation

Serum samples were prepared by centrifugation for 5 min at 10 000 *g* with subsequent microfiltration through a GS-022 membrane (Millipore). Prior to the injection, serum was diluted 1:10 with 0.1 *M* Tris–HCl buffer (pH 7.3).

RESULTS AND DISCUSSION

The molecular mass of myoglobin (17 800) is relatively small compared with most serum proteins, which is why the size-exclusion mode of HPLC was chosen for the separation of myoglobin from other proteins. Fig. 2 shows the chromatograms of (A) serum and (B) a reference sample of myoglobin with non-selective photometric detection at 254 nm.

It has been shown [8] that the presence of other macromolecular components of serum (albumins, globulins) supresses the chemiluminescence of luminol and leads to a non-linear concentration dependence of the signal generated by a hae-

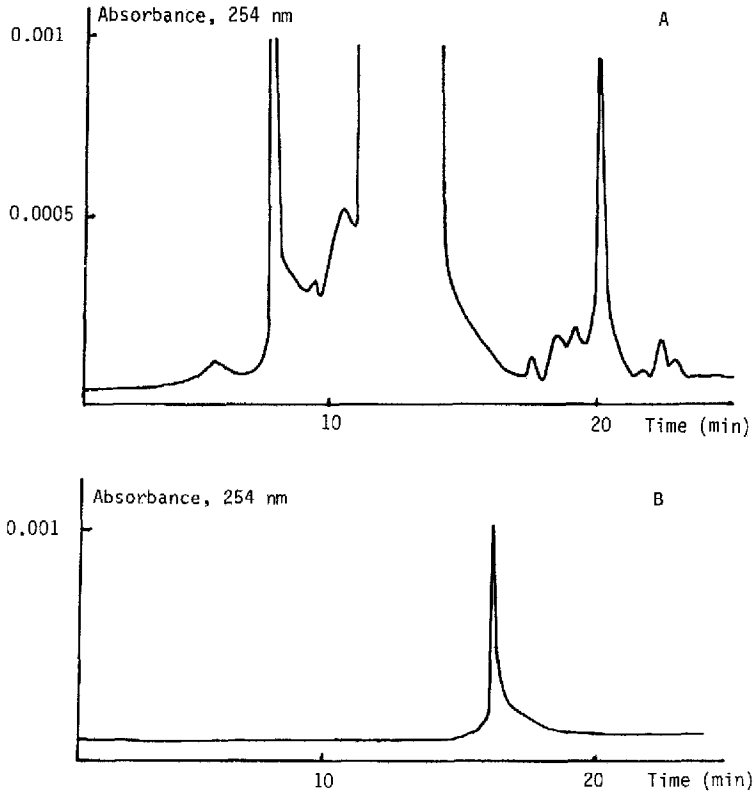


Fig. 2. Chromatograms of (A) serum and (B) myoglobin on a Spherogel TSK 3000 SW column. Flow-rate, 1.3 ml/min; sample volume, 20 μl; dilution of serum, 1:10.

moprotein. Hence, it was necessary to establish whether other components that might suppress chemiluminescence were eluted from the column with myoglobin. The results of an experiment carried out for this purpose are shown in Fig. 3. In order to amplify the effect of chemiluminescence quenching, Fe^{2+}-containing (unpurified) water was specially used. Fig. 3 shows that the chromatogam of blood serum exhibits two peaks of proteins suppressing luminol chemiluminescence (negative peaks in Fig. 3). However, in the region of myoglobin elution, no chemiluminescence quenching is observed. This favourable circumstance ensures the linearity of the myoglobin signal with respect to its concentration in serum, which is an essential condition for quantitative analysis. The calibration graph was obtained using additions of horse myoglobin to normal serum, resulting in myoglobin concentrations of 1000, 500, 250 and 100 ng/ml. For this purpose, serum from a healthy subject with a myoglobin content not exceeding 10 ng/ml was used. The peak area computed by the CDS-111 integrator (Varian, Palo Alto, CA, U.S.A.) was linear, with a correlation coefficient of 0.98.

Typical chromatograms of haemoproteins in serum from patients at the Leningrad Cardiologic Centre are shown in Fig. 4. The chromatogram of the serum samples exhibits five Fe^{2+}-containing peaks. Peak 5 corresponds to myoglobin and peak 1 to haemoglobin. The other peaks were not identified. However, the

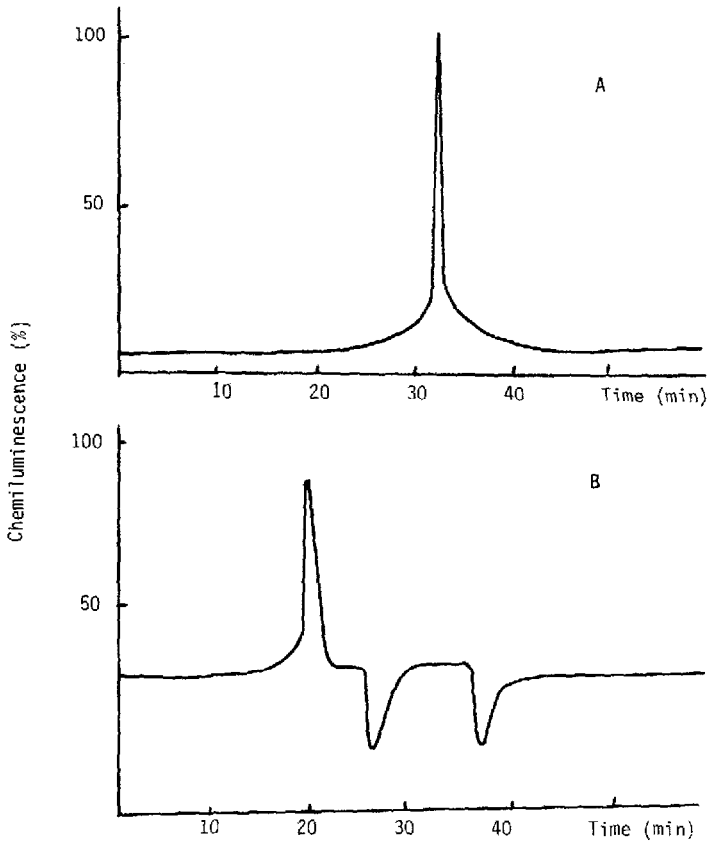


Fig. 3. Chromatograms of (A) horse myoglobin (0.001 mg/ml) and (B) serum (dilution 1:1000).

corresponding molecular masses were determined by calibrating the column set with reference proteins: peak 2, 57 000; peak 3, 33 000; and peak 4, 22 000. Peak 2, which was poorly separated from haemoglobin, was revealed only on high dilution of the serum (Fig. 4). Peak 4 was absent in some samples. In all serum samples peak 3 was characterized by severe tailing, but the system of two TSK gel SW columns allowed a satisfactory resolution to be achieved.

The sensitivity of this method for the determination of myoglobin is sufficiently high for a reliable diagnosis of myocardial infarction. The minimum detectable concentration is 10 ng/ml, which is near the lowest level of myoglobin concentration in normal blood.

Using this method over 40 blood samples from cardiology patients were investigated at different times after the attack. In 100% of the cases, myocardial infarction determined according to the myoglobin test was confirmed later by other biochemical indices (MB-CK and LD1, LD2 isoenzymes).

The relative standard deviation of the method calculated on the basis of peak area (ten runs) was 3.2%.

The only chromatographic procedure for the determination of myoglobin in biological fluids that has been proposed previously [10] lacks sensitivity, as it

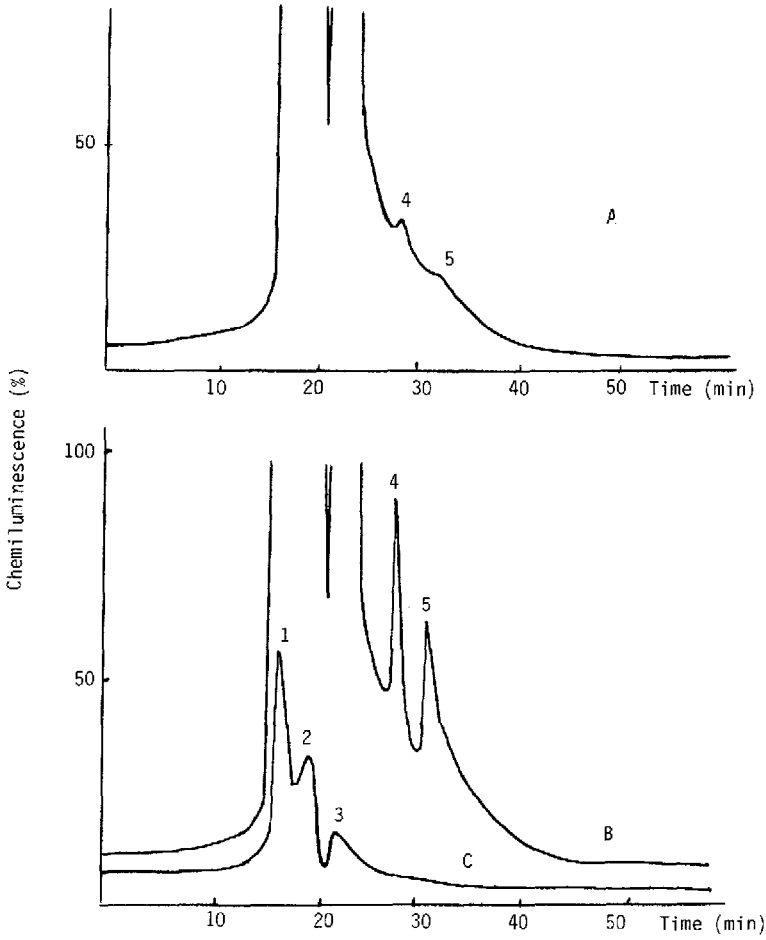


Fig. 4. Chromatograms of (A) normal serum and (B and C) serum after myocardial infarction. Peaks: 1 = haemoglobin; 2, 3, 4 = unidentified; 5 = myglobin. Dilution of serum: (A, B) 1:10 and (C) 1:1000. Sample B contained 2.6 μg/ml myoglobin. Columns connected in series, Spherogel TSK 2000 SW and TSK 3000 SW. Sample volume, 150 μl; flow-rate, 1.3 ml/min.

employs the absorbance detection at 405 nm. This technique is based on ion-exchange HPLC and provides a detection limit of 2 μg/ml, which is sufficient for the diagnosis of myoglobinuria but extremely far from the desired level of concentration sensitivity corresponding to the myoglobin content in normal serum.

The method described here ensures precise quantitative results and is very sensitive. Moreover, using the same equipment it is possible to determine in serum all three proteins that are diagnostically important in cardiology: LD isoenzymes by NADH fluorescence, generated in the post-column reactor; myoglobin by chemiluminescence post-column reaction with luminol; and CK isoenzymes by bioluminescence post-column reaction of ATP with lucipherase [11].

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