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GLYCOSYLATED HAEMOGLOBIN: HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF 5-(HYDROXYMETHYL)-2-FURFURALDEHYDE AFTER HAEMOGLOBIN HYDROLYSIS

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SUMMARY

A specific and accurate method for the quantitation of the azomethine linkage present in non-enzymatically glycosylated haemoglobin is described. This protein is hydrolysed for 5 h in 1 *M* oxalic acid at 100°C to yield 5-(hydroxymethyl)-2-furfuraldehyde (5-HMF), known as a specific degradation product of hexoses linked to the protein. 5-HMF is then purified through a Sep-Pak C_{18} cartridge and measured by its absorption at 280 nm after separation on a C_{18} reversed-phase silica column. Quantitation is made accurate by using 1-methylxanthine as internal standard throughout the whole procedure. The identity and the purity of the 5-HMF chromatographic peak was ascertained by UV spectroscopy, gas chromatography on a glass capillary column and mass spectrometry. The method has been successfully used for 5-HMF determinations in monitoring diabetes mellitus patients. The mean values, expressed as nmol of 5-HMF per mg of haemoglobin were 0.64 ± 0.13 (S.D.) for 27 controls and 1.32 ± 0.39 for 78 diabetic patients. Unlike the usually employed thiobarbituric acid assay, the present procedure is truly specific for the 5-HMF determination.

INTRODUCTION

With the discovery of the formation of a glucose adduct of haemoglobin[1] and serum proteins[2], the interest in such reactions has increased. The level of glycosylated haemoglobin (G-Hb) is widely recognized as a reliable indicator in the diagnosis and management of diabetes mellitus[3]. Therefore, there is an increasing requirement for the determination of G-Hb in diabetic patients, and for suitable methods for assaying G-Hb.

An extensive review of relevant techniques and their clinical utility was recently compiled by Mayer and Freedman[4]. Among them, direct chromatographic methods have been widely adapted for isolating[5,6] and assaying G-Hb or Hb A_1c. The latter designation refers to the fact that this Hb fraction is eluted in open column chromatography as the last peak among the "fast" haemoglobins. However, these methods require strict control of several analytical variables[7,8].

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Several indirect assays of G-Hb have also been reported[9–14]. All of them are based upon the same experimental technique: they require heating of a sample of haemolyzed blood with a weak acid, such as oxalic acid, to hydrolyse the haemoglobin hexose and convert it into 5-(hydroxymethyl)-2-furfuraldehyde* (5-HMF). This compound is then converted into a coloured complex with thiobarbituric acid and thus can be measured by spectrophotometry. However, careful standardization of the assay is essential for obtaining reliable results. The main disadvantage of such procedures still resides in the lack of specificity due to interfering coloured compounds[15].

This paper is concerned with an improved assay for 5-HMF in biological samples by reversed-phase high-performance liquid chromatography (HPLC). This method has the advantages of specificity, accuracy and reproducibility.

MATERIALS AND METHODS

Reagents

(a) Hydrolysis reagent: 60 μM 1-methylxanthine (1-MX) (Interchim, Montluçon, France) in 1 M oxalic acid (Merck, Darmstadt, F.R.G.).

(b) Standard stock solution: 2.3 mM 5-hydroxymethylfurfural (Merck) containing 0.5 g/l sodium azide, kept at −18°C, and diluted before assay in distilled water to obtain the required concentration range (23–2.3 μM).

(c) Sep-Pak® C_{18} cartridges (Waters Associates, Milford, MA, U.S.A.): washed before use successively with 5 ml of dichloromethane, 2 ml of tetrahydrofuran, 5 ml of methanol and 3 × 5 ml of distilled water.

HPLC conditions

The Spectra Physics (Packard-France, Rungis, France) HPLC system consisted of a continuous constant-flow delivery module, SP 8700, equipped with a Rheodyne Model 7125 syringe-loading sample injector valve (20-μl loop), mounted on the SP-8750 organizer module and a Pye Unicam LC-UV detector operated at 280 nm. The HPLC column (20 cm × 4 mm I.D.) was a LiChrosorb RP-18, 7 μm (Merck), plate height H = 37 μm, reduced plate height h = 5, with a guard column (6 cm × 4 mm I.D.) of LiChrosorb RP-18, 10 μm. The chromatographic system was operated at room temperature. The mobile phase was methanol–tetrahydrofuran–acetic acid–water (6.2:0.3:1:93.5).

The solvents were filtered through a 0.45-μm membrane filter (Millipore, Bedford, MA, U.S.A.). The flow-rate was adjusted to 1.5 ml/min, generating a pressure of 1200–1300 p.s.i. All the solvents from Merck were of chromatographic grade.

Sample preparation

Hydrolysis. Venous blood samples were collected in EDTA Vacutainer® tubes. Red cells were separated by centrifugation (1400 g for 10 min) and washed three times with saline solution. The haemolyzates were prepared by the addition of one volume of distilled water and 0.4 volume of carbon tetrachloride. The upper layer (obtained by centrifugation at 2000 g for 10 min) was dialysed against 0.15 M sodium

* Common name 5-hydroxymethylfurfural.

chloride for 18 h at 4°C and the haemoglobin concentration was estimated by a Coulter Counter (Model 5; Coultronics, Andilly, France). The incubation mixture, containing 0.2 ml haemolyzate (*ca.* 100 g/l), 1.8 ml distilled water and 2.0 ml hydrolysis reagent containing the internal standard, was incubated for 5 h on a water-bath at 100°C.

Purification of haemolyzate. An 80-μl volume of 40% (20 μl/ml) trichloroacetic acid was added to the haemolyzate and the samples were centrifuged (2000 *g* for 10 min). A 2-ml volume of supernatant was transferred to a Sep-Pak C_{18} cartridge (prepared as previously described) and washed with 1 ml of distilled water. The 5-HMF and 1-MX were eluted with 2 ml of methanol–water (45:55).

HPLC quantitative analysis. The factor response of pure 5-HMF relative to the 1-MX internal standard was determined by analysing 5-HMF standard solutions according to the same procedure as for biological samples, *i.e.*, to 1 ml of diluted stock standard solution was added 1 ml of hydrolysis reagent containing the internal standard and then it was processed on a Sep-Pak C_{18} cartridge like the biological samples. A 20-μl volume of standard or biological sample was injected for HPLC, *i.e.*, about 0.5% of the haemolyzate sample. The sensitivity of the UV detector was set at 0.01 A.U.F.S. Chromatograms were recorded with a potentiometric recorder. Peak height measurements were used for quantification. Under the conditions described, 5-HMF and 1-MX had retention times of 3.40 and 5.20 min respectively.

The results are expressed in nmol of 5-HMF per mg haemoglobin. For convenience, the extent of glycosylation of Hb can be expressed as the "HMF index", where "HMF index" = 5-HMF (nmol)/Hb (mg).

Specificity of the analytical method

Gas–liquid chromatography (GLC). The compound eluted by HPLC with the retention time of 5-HMF was subjected to GLC after freeze-drying of the eluate. This analysis was carried out on a glass capillary column, 30 m × 0.3 mm, coated with OV-73, film thickness 0.15 μm. Injection was carried out with an on-column injector. The oven temperature, being at 40°C during injection, was programmed at a rate of 6°C/min. The carrier gas was hydrogen at 0.5 bar. The gas chromatograph was a Model 2150 (Carlo Erba, Milan, Italy) equipped with a flame ionization detector.

Mass spectrometry. On-line liquid chromatography–mass spectrometry (LC–MS) was carried out with a R 10-10 apparatus (Nermag, Rueil-Malmaison, France) by using the LC solvent as reactant for chemical ionization (CI). The other analytical parameters were: positive ion detection, electron energy, 90 eV; temperature of the coupling device oven, 250°C; splitting ratio, 1/1000; scan speed, 20 msec a.m.u. from 100 to 240 a.m.u.

UV spectrophotometry. UV spectra of 5-HMF and 1-MX standards were recorded on a Pye-Unicam SP 8-100 spectrophotometer. Peaks eluted from the HPLC column with the retention times of 5-HMF and 1-MX, respectively, were recorded after freeze-drying in order to increase concentration.

Rapid "time-of-flight" scannings of the absorption spectra of the 5-HMF and 1-MX chromatographic peaks were recorded by means of a Model 165 UV detector (Beckman, Berkeley, CA, U.S.A.). The purity of the chromatographic peaks was checked by determining the absorbance ratio at two wavelengths, 280 and 265 nm, with the same detector without stopping the liquid flow.

RESULTS

Chromatographic analysis

The chromatograms of five biological samples are shown in Fig. 1. The chromatographic profiles illustrate the complete peak resolution without interference. LiChrosorb RP-18 was found to be a suitable reversed-phase material with regard to selectivity and stability. The quality of this packing material did not noticeably decline with injections of purified samples. Since the injected samples showed a pH of about 1.7, the analytical column should be protected by a guard column in order to minimize column damage.

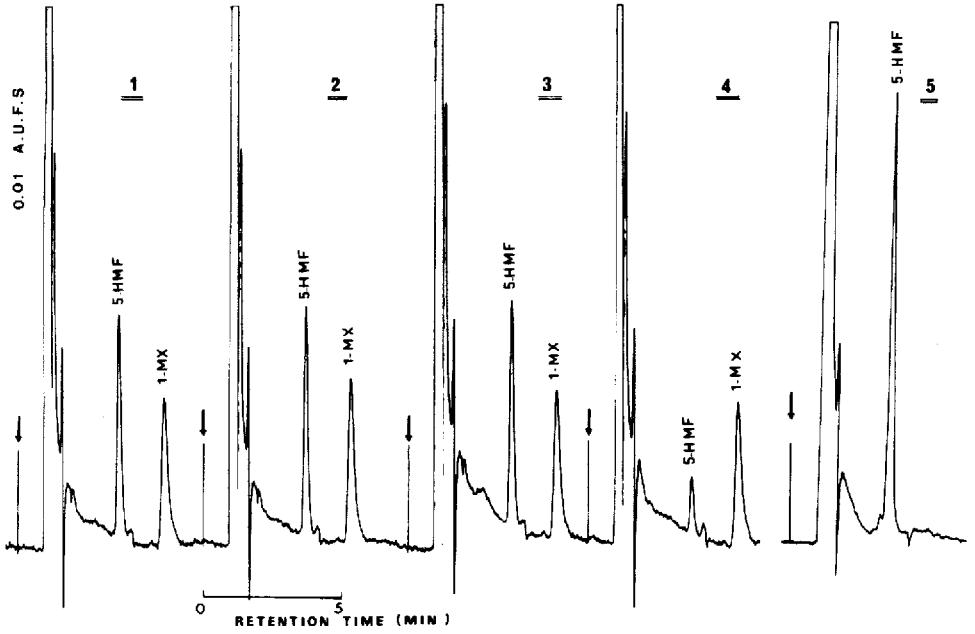

Fig. 1. Chromatogram of five biological samples after Sep-Pak C_{18} cartridge purification. Samples 1–4 contain 1-methylxanthine as internal standard; sample 5 does not contain 1-methylxanthine. The arrows indicate the injection times.

The chromatographic behaviour was studied on different reversed phases (Table I) in order to obtain an adequate retention (*i.e.*, $2 < k' < 5$) of a hydrophilic compound such as 5-HMF[16]. The eluent was selected so that the capacity factor of 5-HMF was about 2 and the efficiency was optimized. This involved the use of two organic modifiers, methanol and tetrahydrofuran. The addition of acetic acid at 1% decreased the tailing of the internal standard peak while the pH of the mobile phase was *ca.* 3.0.

The efficiency tests of columns packed with different bonded phases are reported in Table I. The reduced plate height and the peak asymmetry factor were measured on the 1-methylxanthine peak. Many reversed-phase packings gave inadequate column specifications, especially for the xanthine peak, suggesting a mixed

TABLE I

CHROMATOGRAPHIC PARAMETERS OF 5-HMF AND 1-MX ON DIFFERENT OCTADECYL REVERSED PHASES

See Materials and Methods for mobile phase conditions. k' = Capacity factor; α = relative retention factor; h = reduced plate height = H/d_p; H = plate height = L/N; d_p = particle diameter; As = peak asymmetry factor = 0.548 $W_{10\%}/W_{1/2}$; $W_{10\%}$ and $W_{1/2}$ = peak widths at 10 or 50% peak height.

Column packing	*k' (5-HMF)*	*k' (1-MX)*	*α*	*h (1-MX)*	*As (1-MX)*
Partisil ODS-3, 5 μm	2.00	2.88	1.44	14.8	1.07
Partisil ODS-2, 5 μm	2.65	4.63	1.75	22.0	1.49
LiChrosorb RP-18, 7 μm	2.09	3.02	1.44	5.0	1.04
Hypersil ODS, 5 μm	1.35	1.90	1.41	12.2	1.28
Radial Pak A, 10 μm	2.00	4.07	2.04	19.0	1.01

retention mechanism. Only LiChrosorb RP-18 gave satisfactory column specifications, in agreement with the previously published study[17].

Internal standard

The concentration of 5-HMF in biological samples is calculated from its response relative to that of the internal standard added in a known amount. Such a procedure enables a noticeable enhancement in precision.

The internal standard was selected after many assays. 1-Methylxanthine met the requirements of stability during the hydrolysis step, a response factor similar to 5-HMF, a satisfactory retention time near that of 5-HMF and absence from the biological samples (see sample 5 in Fig. 1). Among the investigations on methylxanthine metabolism, very few reports concern the plasma metabolites[18]. However, although 1-MX was a major urinary metabolite of caffeine, it could not be identified and measured in plasma[19]. Moreover, as the haemolyzate was dialysed before hydrolysis and addition of the internal standard, this compound would be eliminated from biological samples.

Sample preparation

The Sep-Pak C_{18} purification technique is designed for rapid sample preparation. When used for preliminary sample clean-up, Sep-Pak cartridges effectively eliminate interfering contaminants which can severely impair accurate quantitation, as shown in Fig. 2. Without purification the 5-HMF peak is contaminated by interfering compounds, which produce tailing. Throughout the purification procedure, the recovery of 5-HMF and 1-MX was found to be between 50 and 80%, but for every 5-HMF concentration the same 5-HMF/1-MX ratio was observed, as shown by the linearity of the standard curve (Fig. 3).

Evaluation of the method

Linearity. For standards (1.15–11.5 nmol/ml) with or without Sep-Pak C_{18} purification, the mathematical expressions of the calibration curves are, respectively, $y = 7.74\,x - 0.57$ and $y = 7.78\,x - 0.16$. The correlation coefficients are, respectively, 0.9998 and 0.9995, indicating good linearity between the concentration of 5-HMF and the detector response.

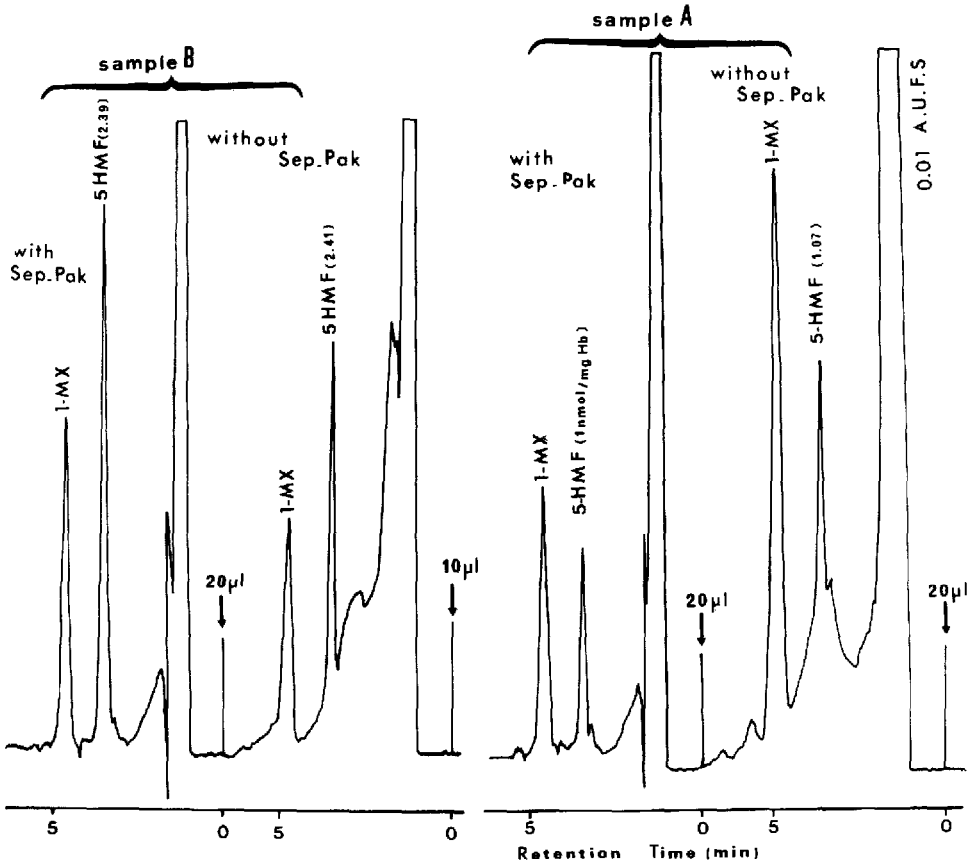

Fig. 2. Chromatograms of two haemolyzates, A and B, before and after Sep-Pak C_{18} cartridge purification. The concentrations of 5-HMF are indicated by brackets.

Precision. The precision of the assay was determined for a haemolyzate within-assay (n = 10, $\bar{x}$ = 0.96 nmol 5-HMF per mg Hb, coefficient of variation (C.V.) = 1.9%) and between-assay (n = 12, $\bar{x}$ = 0.80 nmol 5-HMF per mg Hb, C.V. = 4.5%).

Accuracy. 5-HMF and 1-MX were added to an haemolyzate to give six samples in the concentration range 2–17 nmol/ml corresponding to 0.4–3.4 nmol per mg Hb. The results are presented in Table II and confirm the accuracy of this method.

Limit of detection. According to Curie's[20] definition, a quantitative detection limit, Lq, can be defined as the level at which the precision of the measurement will be less than 10%. This term Lq is calculated as Lq = $10\sigma_0$, where σ_0 is the standard deviation of measurement at very low levels. Under these conditions, Lq = 0.5 nmol/ml, *i.e.*, 0.1 nmol per mg Hb in the case where the Hb concentration was adjusted to 100 g/l.

Specificity. Definitive proof that 5-HMF resulted from the hydrolysis reaction of azomethine-linked hexoses was achieved by ascertaining the identity of HPLC peak 1. Therefore, our method is based upon the reaction described by Flückiger and

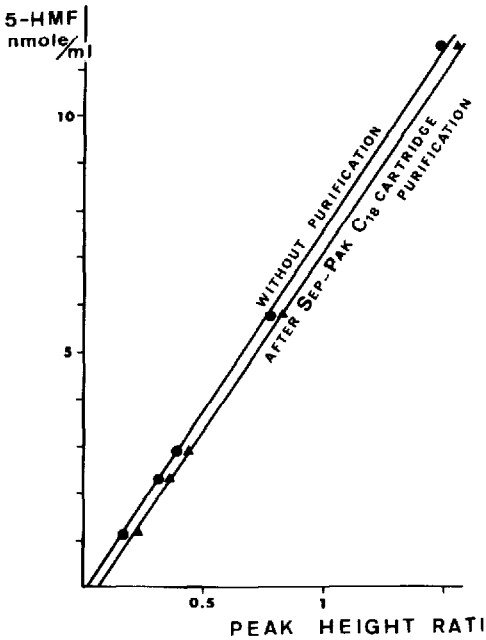

Fig. 3. Standard curves for 5-HMF, 1-MX solutions with and without Sep-Pak C_{18} cartridge purification.

Winterhalter[9]. In this reaction, azomethine-linked hexoses were converted into 5-HMF by limited hydrolysis under weakly acidic conditions.

The UV spectra of peaks 1 and 2 of Figs. 1 and 2, eluted with retention times of 5-HMF and 1-MX, respectively, were compared with those of pure compounds (Fig. 4). In order to detect compounds possibly interfering with the 5-HMF peak, the ratio of the peak heights, A 280 nm/A 265 nm, was recorded and found to be constant at a value of 3.8. Such a value is identical with that of pure 5-HMF. Moreover, the rapid "time-of-flight" UV scanning of peak 1 confirms the identity and the homogeneity of this chromatographic peak.

As 5-HMF can be determined by gas chromatography, the HPLC eluate after freeze-drying was submitted to GC on a capillary column (Fig. 5). A retention index of 1269 was measured for both biological and pure 5-HMF. However, the detection of a second GC peak in the chromatogram for the biological sample could suggest

TABLE II

ACCURACY OF 5-HMF DETERMINATION FOLLOWING HAEMOGLOBIN HYDROLYSIS

5-HMF was added to a biological sample with a 5-HMF content of 1.32 nmol/ml.

5-HMF added (nmol/ml)	*5-HMF found (nmol/ml)*	*Recovery (%)*
0.77	0.81	105
1.53	1.64	107
2.55	2.62	103
3.83	3.47	91
7.70	8.04	104
15.4	15.7	102
	Overall mean recovery	102 ± 6%

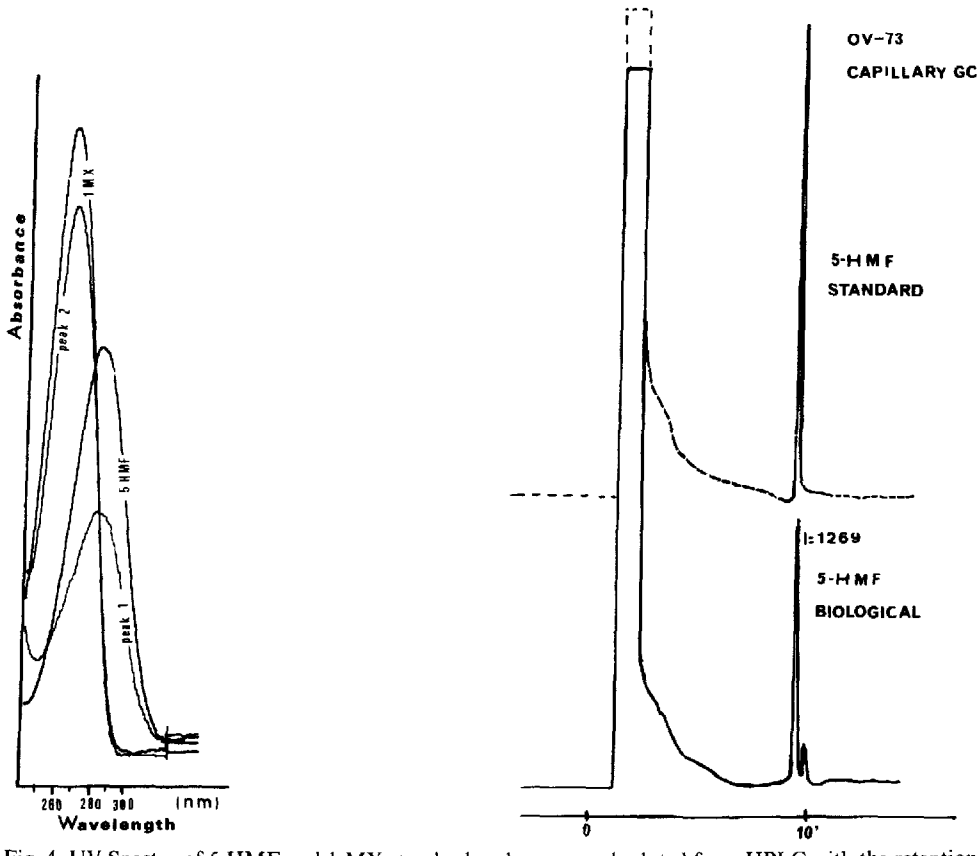

Fig. 4. UV Spectra of 5-HMF and 1-MX standard and compounds eluted from HPLC with the retention times of 5-HMF (peak 1) and 1-MX (peak 2) for a biological sample.

Fig. 5. Gas chromatogram of HPLC peak 1 on a glass capillary column of OV-73. See Materials and methods for analytical conditions. I = retention index.

that the HPLC peak of 5-HMF may contain about 20% impurity. This lack of homogeneity could certainly not have been of importance with regard to quantitation because this interfering compound was not detected by UV, as shown by the rapid UV scanning of the HPLC peak.

The mass spectrum of the 5-HMF standard was compared with that of the compound from the biological source, eluted at the same retention time by LC (Fig. 6). The base peak at m/z 127 was obtained by CI with methanol–water as reactant. This peak corresponds to the quasi-molecular ion (Q M^+) at $(M + 1)^+$. A peak at m/z 157, corresponding to $(M + 31)^+$, was also found for both samples. Thus, the mass spectra were quite similar and confirmed the identity of 5-HMF in the biological sample. If the spectrum B had a much more complex fragmentation pattern than the spectrum A, this was probably due to the presence of interfering compounds, which were not detected by UV. Finally, the specificity of the method was principally based upon the UV detection.

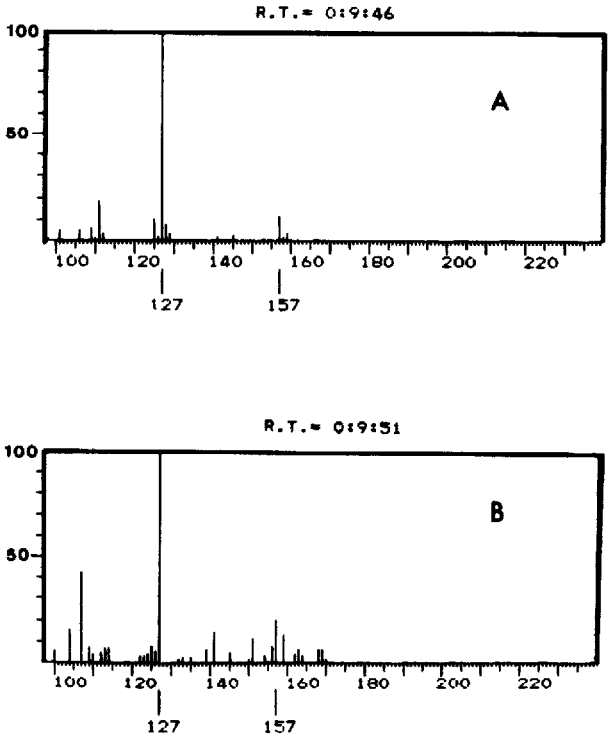

Fig. 6. CI mass spectra of 5-HMF standard (A) and biological compound (B). The LC eluent, methanol water (7:93), was used as reactant for CI. Retention times (R.T.) are indicated.

Application of the method. This method has been extensively applied to monitoring diabetes mellitus patients. The mean values, expressed as nmol of 5-HMF per mg of haemoglobin, were 0.64 ± 0.13 (mean ± S.D.) for 27 normal controls and 1.32 ± 0.39 for 78 diabetic subjects. The amount of carbohydrate bound to Hb in diabetic subjects was significantly higher ($p < 0.001$) than that bound in controls. The comparison of the values for haemoglobin hexose in normal *versus* diabetic subjects indicated that 5 of 27 normal subjects overlap 7 of 78 diabetics, as shown in Fig. 7. This overlap corresponded to diabetics that were well equilibrated or gestational diabetics.

DISCUSSION

Further research on non-enzymatic glycosylation of proteins depends to a great extent on the development of analytical procedures for their specific and sensitive quantitation. The method reported in this paper is based upon the properties of the azomethine linkage, as described by Flückiger and Winterhalter[9]. These authors reported the release of bound hexose from glycosylated haemoglobin by mild acid hydrolysis and their conversion into 5-HMF. Until today the formation of such a compound was indicated only by the typical spectrum obtained with thiobarbituric

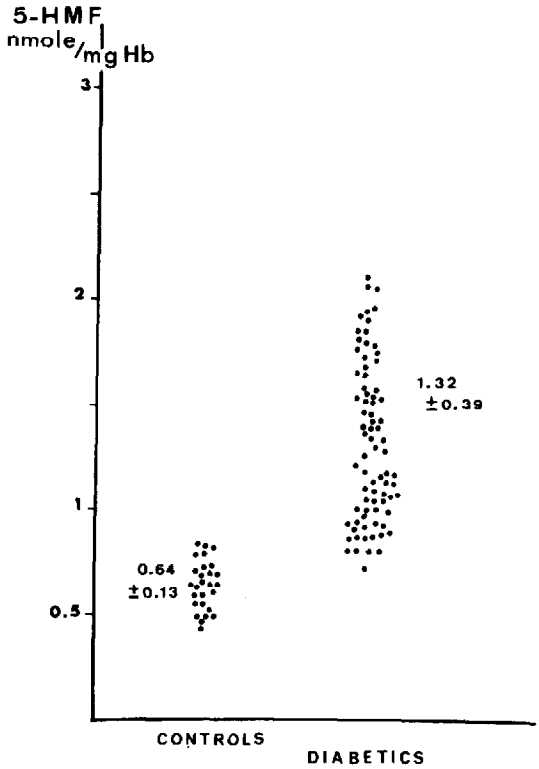

Fig. 7. Amount of 5-HMF liberated from haemoglobin in control and diabetic subjects, as determined by HPLC (mean ± S.D.).

acid, as previously described[21]. In our paper other criteria are reported to confirm such a conversion of bound hexose into 5-HMF. The hydrolysis conditions have been extensively discussed by several authors[10,11]. However, because the yield of 5-HMF depends on the hydrolysis conditions, many parameters, such as temperature, acid concentration and incubation time, must be strictly standardized.

Some authors have criticized the colorimetric 5-HMF method for a lack of reproducibility, especially the manual techniques[22,23]. Therefore, automated procedures have been developed[13,24,25]. Meanwhile, the accuracy of the thiobarbituric acid test is limited by a variable degree of background absorbance from non-glycosylated haemoglobin[15]. Important differences between HPLC values (0.64 ± 0.13 for controls and 1.32 ± 0.39 nmol per mg Hb, mean ± S.D., for diabetics) and thiobarbituric acid values (1.62 ± 0.14 for controls and 2.24 ± 0.38 nmol per mg Hb for diabetics)[25] were observed when the same hydrolysis procedure was used. The increase in 5-HMF values was about 1 nmol per mg Hb for the thiobarbituric acid method, compared with the HPLC procedure. This difference is due to background absorbance, which depends on the haemoglobin concentration[26]. Accordingly the HPLC method undoubtedly enhances the accuracy of 5-HMF measurements.

It is noteworthy that the ratio of mean values for normal and diabetic subjects is about 2 for the HPLC method while it is only 1.4 for the thiobarbituric acid

procedure. Thus, the HPLC method seems to be more useful in the management of diabetes mellitus.

Although ion-exchange chromatography or isoelectric focusing is useful for routine work, especially since they are commercially available as test kits, these methods cannot be considered sufficiently specific. Indeed, the HPLC measurement of 5-HMF appears to be more suitable, because it allows (a) reliable assays of stored, frozen blood samples and (b) estimation of glycosylation in samples that contain significant amounts of haemoglobins other than Hb A_1 such as Hb F and Hb C, (c) there is no interference with additional minor Hb components that are eluted with Hb A_1c in ion-exchange chromatography and found in uremic[27], alcoholic[28] and lead-poisoned individuals and (d) obviously, such a method would be adaptable to the measurement of other glycosylated proteins in blood, such as glycosylated albumin[29].

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