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Determination of Furosine as a Measure for Irreversibly Bound Glucose in Human Fibrinogen

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Summary. An analytical procedure is presented which enables for the determination of irreversibly, nonenzymatic glycation of fibrinogen isolated from human blood. The method is based on determination of furosine, which is formed during hydrolysis of Amadori-products. For validation of the worked out furosine assay, synthesis of N_α-formyl-N_ε-(desoxy-1-*D*-fructosyl-1)-*L*-lysine as a model substance and furosine as a calibration standard were necessary. Several hydrolysis experiments showed the extent of furosine formation from fructosyllysine and from human fibrinogen. The increase of fibrinogen glycation by incubation with *D*-glucose could also be confirmed according to the literature. Using well-known techniques for sampling, work up and in performing reduction and hydrolysis steps, quantitative determination of furosine by high-performance liquid chromatography is possible. By application of the analytical assay, the extent of glycation of human fibrinogen can be analyzed with good precision. Calibration is performed by means of a prepared furosine standard. The practical handling of the method is shown.

Keywords. Human fibrinogen; Furosine; Glycation; HPLC; N_α-Formyl-N_ε-(desoxy-1-*D*-fructosyl-1)-*L*-lysine.

Bestimmung von Furosin als Maß für irreversibel gebundene Glucose in Human-Fibrinogen

Zusammenfassung. In der vorliegenden Arbeit wird ein Analysenverfahren vorgestellt, das eine Bestimmung der irreversiblen, nichtenzymatischen Glucosylierung von Fibrinogen aus menschlichem Blut ermöglicht. Die Methode beruht auf der Bestimmung von Furosin, welches während der Hydrolyse von Amadori-Produkten gebildet wird. Für die Validierung der erarbeiteten Furosin-Methode waren die Synthese von N_α-Formyl-N_ε-(desoxy-1-*D*-fructosyl-1)-*L*-lysin als Modell und von Furosin als Eichstandard erforderlich. In Hydrolyseversuchen wurde das Ausmaß der Furosinbildung aus Fructoselysin und Fibrinogen untersucht. In Übereinstimmung mit der Literatur wurde gezeigt, daß es möglich ist, den Glucosylierungsgrad von Fibrinogen durch In-vitro-Inkubation mit *D*-Glucose zu erhöhen. Die Fibrinogengewinnung aus Blut erfolgte mit bekannten Techniken der Probenahme und Aufarbeitung. Durch Reduktions- (Reaktionsblindwert) und Hydrolyseschritte ist es möglich, Furosin flüssigkeitschromatographisch (UV-Detektion) quantitativ zu erfassen. Durch Anwendung dieses Analysenverfahrens kann das Ausmaß der Glucosylierung von menschlichem Fibrinogen mit guter Reproduzierbarkeit vergleichend erfaßt werden. Die Kalibrierung erfolgt gegen den synthetisierten Furosinstandard. Die praktische Durchführbarkeit wurde gezeigt.

Introduction

D-Glucose can be reversibly bound in a protein (ε-aminogroup of *L*-lysine, N-terminal sites neglectable) as an 1-imino-desoxyglucose (Schiff-base, aldimine). This

reversible bonding can be transferred to an irreversible (1-amino-1-desoxyfructose) by Amadori-rearrangement. This reaction is known as irreversible, nonenzymatic glycation.

Hydrolysis of glycated proteins by HCl is resulting, beside of amino acids, in characteristic products for fructosyllysine. They are furosine and pyridosine [1–4]. Yields depend on HCl-concentration, temperature and time. Pyridosine is less important, however, furosine can be quantified and is specific for at the ε-amino group of *L*-lysine irreversibly bound glucose [5].

Erbersdobler and Zucker [2] reported ionchromatographic investigations on acidic hydrolized milk powder and found a substance, which increased after extensive heat treatment of milk powder. Heyns et al. [6] and Finot et al. [3] identified this substance as "furosine". Hofmann et al. (first stage N_α-formyl-*L*-lysine) [7] and Finot et al. [3, 8] published a synthesis. This synthesis leads to very complex reaction mixtures with widely unknown composition. Characterization of furosine standards is performed by chromatographic purity. GC-determination with previous derivatization is known [9–11]. More important is the LC-technique. The use of ion exchange resins [1, 2, 12–14] or RP-packings is reported [15–18]. Further characterization by IR, respectively ^{1}H-NMR [6, 3, 19], MS [3], melting point and elemental analysis [19] is reported. A furosine standard is not available. We synthesized a furosine standard according to Finot with improved purification steps to obtain furosine as a calibration standard.

Based on work by Wieland [20] and Mirshahi et al. [21], an analytical method had to be improved for the determination of irreversibly bound glucose in human fibrinogen, calibrated with furosine [49]. Practicability in clinical laboratories is aimed for further investigations of relations between fibrinogen glycation and vascular damage. Glycation of fibrinogen may interfere with fibrin-monomer polymerization causing changes in clot structure. A possible influence on crosslinking by factor XIII is discussed. It may enhance atherosclerosis and late complications of diabetes mellitus and reduce susceptibility of fibrin degradation by plasmin [51].

For calculation of the degree of glycation, some quantitative information about hydrolysis of glycated fibrinogen has to be known. Therefore experiments using a model substance are necessary. A possible model is N_ε-(desoxy-1-*D*-fructosyl-1)-*L*-lysine (short N_ε), used by given authors. We chose N_α-formyl-N_ε-(desoxy-1-*D*-fructosyl-1)-*L*-lysine (short N_α) as our model. Compared with N_ε, this model promises a shorter way of synthesis and better stability [6, 8, 22]. The formyl group in the N_α-position is cleaved under acidic conditions so that N_ε is produced during hydrolysis.

We used our model for the validation of furosine yield during hydrolysis and also for preparation of furosine to avoid the disadvantages of N_ε for calibration during routine work in a clinical laboratory. For this purpose, the following steps are described: a) synthesis of the model substance and furosine, b) selection of a separation system, c) investigation of the behaviour of the model substance, furosine and lysine under conditions of hydrolysis, d) influence of free, respectively reversibly bound glucose on the hydrolysis and e) hydrolysis of human fibrinogen including reaction blank test.

The results of these experiments should lead to an analytical method which contains the steps blood sampling, separation of fibrinogen, hydrolysis and analysis

of furosine by external calibration using a furosine standard. This method should be useful for determination of normal and pathologic glycation of human fibrinogen.

Experimental

Liquid chromatography system, Perkin-Elmer Series 3 B/LC-100/LC-85/56; sample valve Rheodyne 7125; HPLC-columns Lichrosorb® RP-18 5 µm, ID = 4.6 mm, L = 25 (resp. 12.5) cm – Lichrospher® 100 RP-18 5 µm, ID = 4.6 mm, L = 25 cm – Knauer Lichrosorb® RP-8 7 µm, ID = 16 mm, L = 25 cm; gas chromatograph Perkin-Elmer F 22/FID; GC-column packed with 10% polyethyleneglycol on silica, OD = 0.25″, ID = 2 mm, L = 4 m, glass; LAS Hewlett-Packard HP 3353 A (1732)-REV 1847/HP 1000; FTIR-spectrometer Digilab FTS 20 B/D; NMR-spectrometer Varian FT-80 A, 80 MHz; KF-titrator Metrohm E 551/E 415; DC-applicator AIS TLC multi spotter; DC-scanner Zeiss KM 3/MQ 3/PMQ 3; ultrasonic bath Bandelin Sonorex RK 255; peristaltic pump Ismatec mp 25 GJ-4; microbalance Mettler ME 30.

Chemicals, as far as available, in analytical grade. Water was deionized and double distilled, methanol was rectified.

N_α-Formyl-L-lysine

The synthesis was performed analogous to Hofmann et al. [7]. *L*-lysine was used instead of the hydrochloride, so separation of Cl^- was not necessary. 7.3 g (0.05 mol) *L*-lysine and 105 ml (2.73 mol) formic acid were stirred and ice-cooled. Then 47.5 ml (0.50 mol) acetic anhydride were added during 20 min. The temperature rised from 5°C to 15°C. Stirring was continued for 60 min. Now 100 g ice and 100 ml water were added. After this the solvents were removed on a water bath at 40°C under reduced pressure by distillation. The residual oil was crystallized from ethanol.

The product was white and melted at 187–189°C. The ^{1}H-NMR-spectrum was equivalent to literature [23]. A recorded IR-spectrum was compatible with supposed structure.

N_α-Formyl-N_ε-(desoxy-1-D-fructosyl-1)-L-lysine

Synthesis according to Finot and Mauron [8]. The product was chromatographed (glass column, Dowex® 50 WX4, 200–400 mesh, L = 130 cm (packing), ID = 4 cm; mobile phase 0.2 M pyridine, HCOOH/*pH* = 3.25; flow = 330–340 ml/h, peristaltic pump, Δp = 0.4 bar). N_α eluated at 3 400–6 700 ml. Due to the aim of the presented work, this problematic chromatographic step was not optimized. The eluate was rotated on a water bath (40°C, reduced pressure). The oil was then dissolved in methanol (absolute) and precipitated from 300 ml methylethylketone (*MEK*).

The resulting product was a white amorphous powder. ^{1}H-NMR and IR-spectra were in agreement to the supposed structure. Observed melting point (microscope): 103–105°C. Water (Karl Fischer): 1.8%. *MEK* residue (GC, FID, oven/injector = 60°C, detector = 270°C, flow (He) = 20 ml/min): 0.13%. Element analysis; found: 45.0% C, 7.5 H, 7.6 N, 39.8 O; theory including 1.8% H_2O and 0.13% *MEK*: 45.6 C, 7.3 H, 8.2 N, 38.9 O. Chromatographic purity control: Following Finot and Mauron it is possible to use a two dimensional DC on cellulose as the stationary phase [8]. Found after ninhydrin reaction [24]: R_{f1}/R_{f2} = 0.66/0.69.

We were able to confirm the R_f-values given in the literature, due to the quality of the ninhydrin spots we could not identify other spots properly. According to Schleicher and Wieland [17] we performed a DC-separation on silica 60. As reagents nihydrin (prim. and sec. amines) and Dragendorff-reagent (*tert.* amines, [24]) were used. With ninhydrin we found three spots (1 main – R_f = 0.23, 2 small – R_{f1} = 0.18/R_{f2} = 0.34), while Dragendorff-reagent gave no reaction. A remission measurement at λ = 570 nm showed a 93%-area for the main spot. Quantification of the impurities was done by HPLC [25] (Lichrospher® RP-18/OPA, gradient 2 (T = 0 min/0% B, linear increase to T = 10 min/

5% B, linear increase to T = 12 min/12% B, constant to T = 19 min, linear increase to T = 51 min/100% B, constant to T = 54 min, linear decrease to T = 56 min/0% B; A% = 100 − B%). This showed if the impurities were primary or secondary amines. N_α should not react with OPA. Only two substances were found ($k'_1 = 8.8$, $k'_2 = 16.9$). *L*-Lysine could be excluded because of its capacity ratio ($k' = 24.4$). Considering these facts, the response factor of *L*-lysine can be used for quantification of the impurities. Measurement at $\lambda = 340$ nm showed 10%-area for the impurities.

Concerning element analysis and chromatographic investigations, the purity of N_α can be assumed to be 90–92%.

Furosine

The synthesis was following Finot et al. [3], work up had to be modified. 3.5 g (0.01 mol) N_α were refluxed in 500 ml 6 *N* HCl for 4 h [3, 4, 19, 47, 48]. The resulting oil was dissolved in 5 ml 2 *N* HCl and chromatographed (glass column, DOWEX® 50 WX4, 200–400 mesh, L = 120 cm (packing), ID = 2 cm; mobile phase 2 *N* HCl; flow = 100 ml/h, peristaltic pump). The composition of the eluate was checked by HPLC (Lichrosorb® RP-18, 10 µm, L = 25 cm, ID = 4.6 mm; mobile phase = [1 000 ml H_2O + 2 ml $(C_2H_5)_2NH$ + H_3PO_4 ad *pH* = 2, 1 ml/min; detection UV λ = 210 nm] for furosine and pyridosine. After 2 250 ml preparative chromatography, a furosine peak occurred in the HPLC chromatogram (R_t = 4.3 min, identification see later) accompanied by a second peak (R_t = 8.8 min, spectral identification of pyridosine in later experiments [unpublished data]. The used DOWEX®-column was not sufficient for separation of furosine and pyridosine. We were not able to reproduce the chromatographic data given by Finot et al. [3, 4]. Due to these reasons we used the big fraction 1 800–9 000 ml for keeping all furosine but also pyridosine for the necessary additional separation step. The solvent was removed by distillation (water bath, 40°C, reduced pressure). The residue was dissolved in water and separated by preparative HPLC (Lichrosorb® RP-8, prep. column; mobile phase 0.1% trifluoroacetic acid; flow = 10 ml/min; UV λ = 280 nm) [26]. Furosine was eluted at $k'_{\text{furosine}} = 2.1$, pyridosine at $k'_{\text{pyridosine}} = 5.5$. The use of trifluoroacetic acid was leading to relatively high retention times but offers the possibility of total solvent evaporation.

Fractions were rotated on a water bath at 40°C under reduced pressure and dissolved in a few ml dry methanol. After addition of 1 droplet HCl conc., the product was precipitated from 300 ml *MEK* (24 h stirring, see N_α). Work up of pyridosine can be performed similar to furosine.

The resulting furosine was a slightly yellow colored powder. ^{1}H-NMR- and IR spectra according to literature [3, 19]. Melting point (microscope): 108–110°C. Water (Karl Fischer): 1.5%. *MEK* residue (GC, FID, oven/injector = 60°C, detector = 270°C, flow (He) = 20 ml/min): 0.9%. Element analysis; found: 42.7% C, 6.2 H, 8.2 N, 20.3 O, 21.2 Cl; theory including 1.5% H_2O and 0.9% *MEK*: 43.6 C, 6.3 H, 8.3 N, 20.6 O, 21.2 Cl. Chromatographic purity control: DC (silica/ninhydrin, (mobile phase = (1-butanol: CH_3COOH: H_2O = 80:20:20) [17, 24]): chromatographic pure (R_f = 0.1). HPLC (Lichrosorb®/RP-18, 10 µm, 250 × 4.6 mm; mobile phase 1 000 ml H_2O + 2 ml $(C_2H_5)_2NH$ + H_3PO_4 (85%) to pH = 2; flow = 1 ml/min; UV λ = 280 nm): chromatographic pure.

Furosine occurs as dihydrochloride. Concerning elemental analysis and chromatographic investigations, the purity of furosine can be assumed to be sufficient for an analytical standard. The substance is slightly hygroscopic. Storage of all synthesized substances in a desiccator under reduced pressure, ambient temperature and in the dark.

Furosine Standard Solutions

Stability of an aqueous solution (stock solution, 5.2 mg furosine · 2 HCl/10 ml) was tested on three days over a period of one month. For analysis, 50 µl stock solution were brought to 10 ml. For 41 injections a coefficient of variation of 3.23% was found. Between days of analysis, the stock solution was stored at −20°C. For injections on one day a coefficient of variation of 0.70% was observed.

For calibration in sample analysis, solutions of 0.2–3.1 nmol/50 µl were checked to give linear response.

Remarks on Syntheses

Alternative ways of syntheses were not found in the literature. Furosine could be formed from 2-bromoacetylfurane (furane → 2-acetylfurane → 2-bromoacetylfurane) and *L*-lysine (protection for α-amino group). This synthesis is presently subject of investigation. In case of pyridosine a possible way should be the reaction of *allo*-maltol (Kojic acid → chloro-Kojic acid → *allo*-maltol) with *L*-lysine (protection for α-amino group) [27–33]. As a protection group the copper chelate of *L*-lysine seems to be one possibility. It is stable under alkaline conditions and can be removed by precipitation of CuS.

Blood Samples

1 ml of 2% aqueous NaF was added into a 10 ml centrifugal tube. 9 ml blood were sampled into the tube and centrifugated (3 500 rpm, 2 × 15 min). From 5 ml plasma, fibrinogen was precipitated by means of 2.5 ml $(NH_4)_2SO_4$ (sat., ambient temperature) and separated by centrifugation (4 000 rpm, 20 min). Simultaneously, using another sample of blood, a determination of fibrinogen in blood was carried out (citrate plasma, 45 mg $Na_3C_6H_5O_7 \cdot 2\,H_2O$ in 1 ml H_2O/9 ml blood, e.g. Clauss [50]) [34–37].

The storage of plasma at −20°C was checked over a period of three weeks. Eight identical plasma samples were worked up. Including one outlier (Dixon-test) a coefficient of variation of 6.32% was found. After rejection of the outlier, the value decreased to 1.98%.

Hydrolysis

About 20 mg fibrinogen (known weight) were dissolved in 10 ml 7.8 *N* HCl and melted into a glass ampoule. Hydrolysis was carried out at 115°C for 18 h [1]. After cooling to room temperature the ampoule was opened and the brown colored liquid was transferred to a distillation flask by water. This solution was then distilled to dryness (40°C, reduced pressure). The residue was dissolved (ultrasonic bath, 1 min) in 5 ml water (final volume) and filtered.

Reduction by Sodiumborohydride

This step was necessary for determination of a reaction blank (formation of furosine). The protein (≈ 20 mg, known weight), 20 mg $NaBH_4$ and 3.5 ml of a solution of 0.4 g NaCl and 1.2 g $Na_3C_6H_5O_7$ (sodiumcitrate) in 100 ml water were kept at room temperature for 18 h. Excess of $NaBH_4$ was eliminated by addition of 1 drop 37% HCl. After this the mixture was degassed by slightly warming. The final mixture for hydrolysis was prepared by adding 6.5 ml 37% HCl [17, 38].

OPA-Derivatization

50 mg phthaldialdehyde (*OPA*) were dissolved in 1 ml methanol. After addition of 40 µl mercapto-ethanol the solution was brought to a final volume of 10 ml by 0.2 *M* $Na_2B_4O_7$ ($pH = 9.5$). 100 µl amino acid test solution and 100 µl reagent were mixed and kept at 30°C for 1 min [25].

Incubation with D-Glucose

A known amount of fibrinogen (≈ 20 mg) was filled in a glass ampoule together with a known amount of *D*-glucose and 3.5 ml of a solution of 0.4 g NaCl and 1.2 g $Na_3C_6H_5O_7$ (sodiumcitrate) in 100 ml water. Incubation was performed at 37°C (water bath, time as fixed). The final mixture for hydrolysis was prepared by adding 6.5 ml 37% HCl. For long incubation times 0.02% NaN_3 can be added to prevent growth of microorganisms [21].

Results and Discussion

Hydrolysis of L-Lysine, Furosine and N_α

To transform the hydrolysis data of N_ε given by Erbersdobler et al. [1] to the model N_α used in this paper, the stability of *L*-lysine and furosine under conditions of hydrolysis as well as the extent of formation of these substances from the model had to be investigated.

Hydrolysis was carried out as given in the experimental section. Sample size and volumes were adjusted. 3 mg (20 µmol) *L*-lysine (final volume 20 ml), 5 mg (15 µmol) N_α (final volume 25 ml) and 2 mg (6 µmol) furosine (final volume 20 ml) were hydrolized in separate experiments.

The chromatographic separation of the hydrolysis products (*OPA* derivatives) was carried out by HPLC according to Bober [25]. To reduce the analysis time, we modified the gradient (gradient 1: T = 0 min/5% B, linear increase to T = 30 min/100% B, linear decrease to T = 32 min/5% B; A% = 100 − B%). Component A was 0.05 *M* sodiumacetate buffer (*pH* = 6.8): methanol : tretrahydrofurane = 80 : 19 : 1, component B was 0.05 *M* sodiumacetate buffer (*pH* = 6.8): methanol = 20 : 80. As the stationary phase we used Lichrosorb® RP-18, 5 µm, L = 12.5 cm, ID = 4.6 mm (flow 1 ml/min, sample size 20 µl). The preparation of *OPA* derivatives is described in the experimental section. In the present case UV detection at λ = 340 nm was sufficient. External calibration was used, linearity was checked. For derivatisation we adjusted the amounts as given: *L*-lysine, 40 µl (40 nmol) sample solution + 300 µl (11 µmol) *OPA* reagent (1 : 280); N_α, 40 µl (25 nmol) sample solution + 500 µl (18 µmol) *OPA* reagent (1 : 750); furosine, 300 µl (90 nmol) sample solution + 300 µl (11 µmol) *OPA* reagent (1 : 125).

For each substance ten experiments were performed. Results are given in Table 1. We found 95.4% yield for *L*-lysine. This value was taken as the recovery of this particular procedure.

For furosine, the experiment was repeated. Now the final determination of furosine was done similar to the final method (HPLC, RP-18, H_2O/H_3PO_4, UV λ = 280 nm). The yield of furosine was 89.0% with a coefficient of variation of

Table 1. Hydrolysis of *L*-lysine, furosine and N_α [M ± T%-mol (s, P, n)], M = mean (arith.), T = range of scattering = s · t, s = standard deviation, t = Student-factor for confidence level P = 95%, n = number of experiments

	L-Lysine	
95.4 ± 3.0%	*L*-Lysine	(± 1.3; 95; 10)
	Furosine	
92.2 ± 0.9%	Furosine	(± 0.4; 95; 10)
2.2 ± 0.7%	*L*-Lysine	(± 0.3; 95; 10)
N_α-Formyl-N_ε-(desoxy-1-*D*-fructosyl-1)-*L*-lysine		
45.6 ± 1.2%	Furosine	(± 0.5; 95; 10)
35.0 ± 5.2%	*L*-Lysine	(± 2.3; 95; 10)

4.31.%. This is considered to be equivalent to results found after *OPA*-derivatization.

As a result it is pointed out that furosine is not totally stable under these conditions. Therefore it is necessary to keep the hydrolysis conditions constant. The furosine yield for hydrolysis of N_α is approximately equivalent to the results for N_ε reported in [1]. Our yield is about 5% higher. Some furosine was transferred to *L*-lysine. Lysine formation from N_α is significantly lower than the results of Erbersdobler et al. found for their model, but is equivalent to a report of Steinig et al. [39] who also used the model N_ε. All results depend highly on the special conditions (HCl concentration, time and temperature). According to the literature we found that the given conditions are resulting in a sufficient yield for a furosine assay.

Influence of Free-D-Glucose on Hydrolysis of L-Lysine, Furosine and N_α

In the previous hydrolysis experiments the influence of irreversibly bound *D*-glucose (an Amadori product) on the formation of furosine was investigated. Now it is necessary to show how reversibly bound (Schiff base) or free *D*-glucose influences the yield of furosine. The concentration of *D*-glucose in these experiments was 10 mg/ml (= 1 000 mg%). This is significantly higher than normal concentrations of *D*-glucose in blood of 45–95 mg% (GOD/POD method, equivalent 70–120 mg% by Hagedorn-Jensen) [34]. We repeated the previous experiments with addition of 20 mg *D*-glucose. To proof that no Amadori rearrangement takes place, incubation of *D*-glucose on the substrate must be prevented. This can be done by immediate hydrolysis. Because of the high concentrations of *D*-glucose the hydrolysates become darker than before. This browning can be explained by caramelization reactions at higher temperatures in the presence of mineral acid. *OPA* derivatisation and chromatography were similar to above.

Again ten hydrolysis experiments were performed for each substance. Results are shown in Table 2. The yield of *L*-lysine was 95.1%. This value was considered to be the recovery of this procedure.

Table 2. Influence of free *D*-glucose on hydrolysis of *L*-lysine, furosine and N_α [M ± T%-mol (s, P, n)], M = mean (arith.), T = range of scattering = s · t, s = standard deviation, t = Student-factor for confidence level P = 95%, n = number of experiments

	L-Lysine	
95.4 ± 14.1%	*L*-Lysine	(± 6.2; 95; 10)
	Furosine	
91.5 ± 6.4%	Furosine	(± 2.8; 95; 10)
2.6 ± 0.7%	*L*-Lysine	(± 0.3; 95; 10)
	N_α-Formyl-N_ε-(desoxy-1-*D*-fructosyl-1)-*L*-lysine	
39.7 ± 3.2%	Furosine	(± 1.4; 95; 10)
37.1 ± 9.7%	*L*-Lysine	(± 4.3; 95; 10)

Increased scattering can be explained by the increase of matrix effects. For *L*-lysine the variation coefficient 6.52% is slightly higher than a value given in literature for a model mixture of amino acids (6.14%) [25]. The influence of free, respectively reversibly bound glucose on the formation of furosine is not significant.

Hydrolysis of Fibrinogen

Separation system for determination of furosine in hydrolysate of fibrinogen. As discussed, there are different methods for HPLC of furosine. In latest publications RP-18 and H_2O/H_3PO_4 were used as stationary and mobile phases. Detection at a sufficient sensitivity level was carried out by UV absorption at $\lambda = 280$ nm [17, 18, 21]. The concentration of H_3PO_4 (*pH* adjustment) depends on the condition (e.g. age, past) of the stationary phase. First attempts of separation of *OPA* derivatives were not successful because of lacking separation power. A separation according to Chiang [15] (RP-18/acetate buffer, $pH = 4.3$) was also not realized due to the matrix. Finally separation was sufficient with Lichrospher® 100 RP-18, 5 µm (L = 25 cm, ID = 4.6 mm) and the mobile phase 0.03 *M* H_3PO_4 at a flow of 1 ml/min, sample size of 50 µl and detection at UV $\lambda = 280$ nm.

During this experiment we used a fibrinogen preparation available in Austria (Human Fibrinogen KABI 1 g). This is produced from batches of plasma (each batch from 5000 Scandinavian donators) [40]. The white, lyophilizated material contains 1 g fibrinogen human, 0.4 g NaCl and 1.2 g tri-sodiumcitrate (dry). After addition of 100 ml water a colloidal solution with $pH = 6.8$ is formed.

For hydrolysis we used the procedure given in the experimental section with a final volume of 10 ml. External calibration was performed using synthesized furosine (synthesis and purity check above), linearity was checked.

From ten experiments we found (M ± T [unit] (s; P%; n)): 0.15 ± 0.06 mol furosine/mol fibrinogen (± 0.03; 95; 10).

The observed extent of scattering can be explained from sampling out of a lyophilizated sample pool. The given result is equivalent to 0.32 mol fructosyllysine/mol fibrinogen. To get information about the extent of glycation of *L*-lysine in fibrinogen it is necessary to know the content of *L*-lysine in fibrinogen. This value (8.1%, 210 mol *L*-lysine/mol fibrinogen) can be calculated from known sequence [41–45]. Assuming this, a degree of glycation of 0.15% can be calculated for the used Human Fibrinogen KABI (Lot.-No. 84337).

In the present case it is necessary that the measured furosine peak is within ample room of the chromatogram. Reducing the keto group, necessary for furosine formation, by means of $NaBH_4$, offers the opportunity for a reaction blank test as given in the experimental section.

The experiment showed that no peak occurred within the retention window in question.

Incubation of fibrinogen with D-glucose. Mester [43] refers that fibrinogen always contains "free" sugar molecules (e.g. bovine fibrinogen 1.64–3.20% hexoses, human ≈ 1% [36]). To see if additional free glucose is able to increase the number of keto groups an in vitro experiment was performed.

The following experimental conditions were used: ≈ 69 mg (26 mg respectively 80 nmol, known weight) Human Fibrinogen KABI, ≈ 15 mg (80 µmol, known

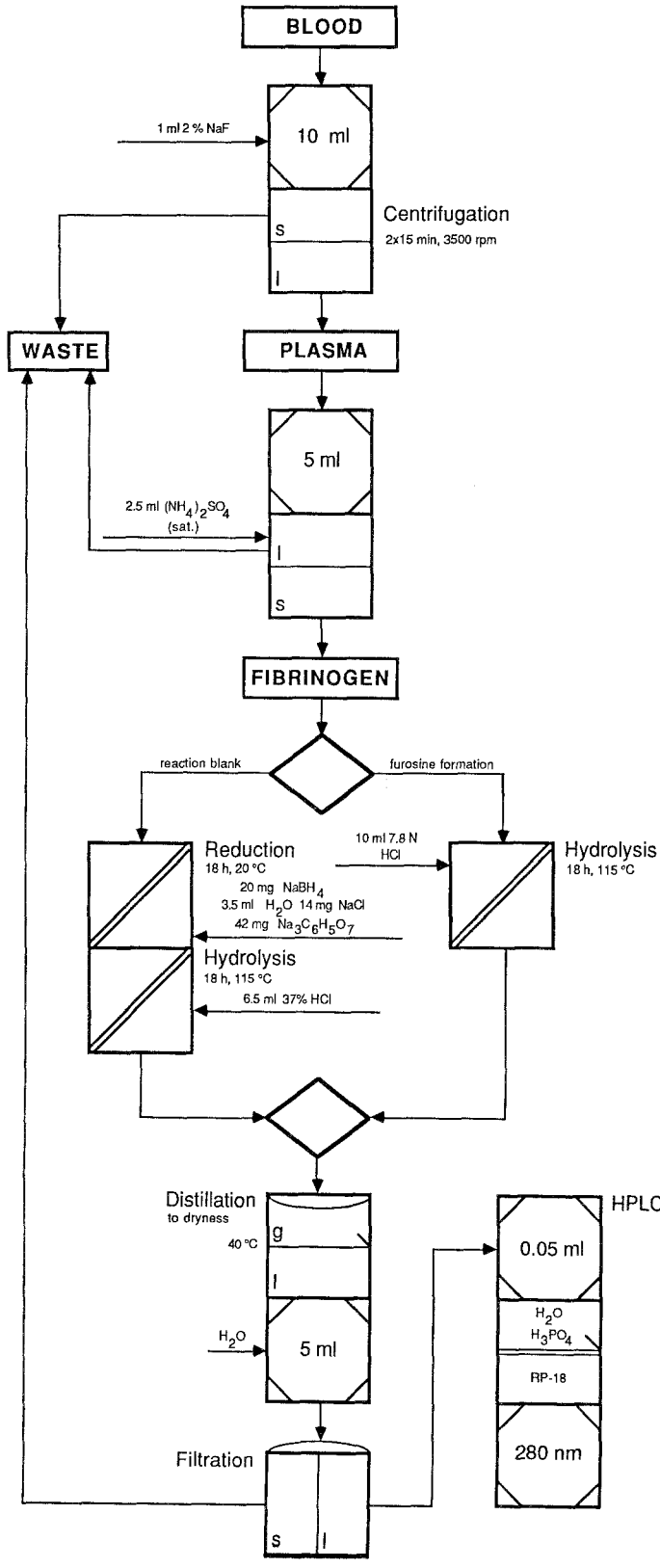

Fig. 1. Diagram showing the procedure for determination of furosin in fibrinogen hydrolysate

weight) *D*-glucose (1 : 1 000 mol), 3.5 ml H_2O. Incubation time was 137 h at 37°C, the final volume was 10 ml. Chromatography was performed as above.

Quantitative evaluation resulted in 0.40 mol furosine/mol fibrinogen, equivalent to 0.88 mol fructosyllysine/mol fibrinogen. This result shows that the degree of glycation increased from 0.15% to 0.42%.

Final Method for Determination of Furosine in Fibrinogen Hydrolysate

The description of the method follows the system suggested by Rohleder and Gorbach [46] (Fig. 1). Sampling of blood was done according to the experimental section. Then, fibrinogen was hydrolized by means of 10 ml 7.8 *N* HCl for 18 h at 115°C. After cooling to room temperature the acid was removed by distillation on a water bath at 40°C under reduced pressure. The brown colored residue was dissolved in 5 ml water (ultrasonic bath, 1 min). The sample solution was filtered and 50 µl of it were chromatographed on Lichrospher® 100 RP-18 (5 µm, L = 25 cm, ID = 4.6 mm) using H_3PO_4 (e. g. 0.1 *M*, flow = 1 ml/min) as the mobile phase (UV detection, λ = 280 nm). External calibration by means of aqueous furosine solution was performed as given in the experimental section.

If it is necessary to show the identity of a furosine peak in a chromatogram, a reaction blank test is indicated. Disturbing peaks rarely occur during analysis of actual blood samples. Figure 2 shows an example for this case.

For determination of the reaction blank (no formation of furosine during hydrolysis) the fibrinogen of a second sample of blood was separated in the same manner and suspended in 3.5 ml of an aqueous solution of 14 mg NaCl and 42 mg tri-sodiumcitrate in 100 ml water. After addition of 20 mg $NaBH_4$ reduction took place during 18 h at ambient temperature. The mixture for hydrolysis was obtained by addition of 6.5 ml 37% HCl. Hydrolysis and further steps see above.

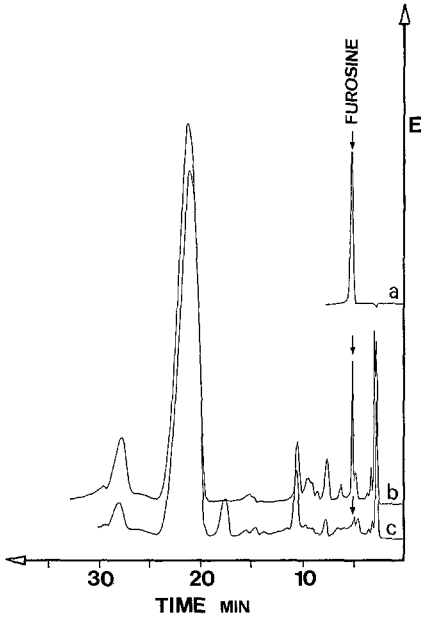

Fig. 2. Hydrolysis of fibrinogen obtained from blood sample; stationary phase: Lichrospher® 100 RP-18, 5 µm, L = 25 cm, ID = 4.6 mm; mobile phase: H_3PO_4/H_2O, 1 ml/min; detection: UV λ = 280 nm; *a* external calibration using furosine standard; *b* sample; *c* reaction blank

Analysis of Blood Samples

The given method was tested on different blood samples. Sampling was performed under medical supervision. Fibrinogen assays (Clauss) were done by a medical laboratory.

Sample analysis. Ten samples of five nondiabetic patients (two of each) were taken. Result: 248–285 mg% fibrinogen; 2.67 ± 1.14 mol fructosyllysine/mol fibrinogen (0.35; 99%; 10); 1.27 ± 0.54% glycation; no outliers (Dixon-test).

Precision. Ten portions of blood (one donator) were worked up at once (eight for furosine determination, two for reaction blank) (Fig. 2). Result: 280 mg% fibrinogen; 2.56 ± 0.29 mol fructosyllysine/mol fibrinogen (0.08; 99%; 8); 1.22 ± 0.14% glycation; no outliers (Dixon-test). The coefficient of variation was found to be 3.27%. This value is equivalent to the value given by Schleicher et al. [17] for serumalbumin (2.94%, n = 12).

As shown, the presented method can be used for determination of irreversibly glycation of fibrinogen for routine work in clinical laboratories. The results of this paper are subject of further investigations.

For determination of the true glycation, the used procedure for fibrinogen preparation [34, 37] must be revised with respect to necessary protein purity. This aspect is also a subject of further work.

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