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LIQUID COLUMN CHROMATOGRAPHY OF OXO COMPOUNDS AS THEIR 2,4-DINITROPHENYLHYDRAZONES WITH SPECIAL REFERENCE TO GLUCOSE–GLYCINE MAILLARD REACTION PRODUCTS

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SUMMARY

Oxo compounds (2,4-dinitrophenylhydrazine-reactive) formed during the Maillard reaction between glucose and glycine were converted into 2,4-dinitrophenylhydrazones and were separated into five main fractions by reversed-phase chromatography on Separon SGX C_{18} using a methanol–water gradient. Four of these fractions were homogeneous, as shown by thin-layer chromatography. One of the fractions still consisted of two zones, which, however, were easy to separate by preparative thin-layer chromatography on silica gel with tetrachloromethane–methanol–*n*-butanol (90:5:5, v/v/v) with dual development. The nature of the compounds separated is discussed.

INTRODUCTION

Analysis of the Maillard reaction products, as well as analysis of the products of non-enzymatic glycation of proteins, is a difficult task because of the complexity of the resulting mixture. In the present study we have attempted to separate aldehydic components present among the Maillard reaction products as they represent the most likely candidates involved in cross-linking reactions which are followed by a decrease in the metabolic turnover of proteins and finally extracellular chromophore formation with age[1–4].

Separation of oxo compounds represents a traditional analytical problem and all types of chromatography have been used for this purpose in the past[5,6]. Routinely, before analysis oxo compounds are converted into 2,4-dinitrophenylhydrazones[5]. The resulting derivatives can be separated by high-performance liquid chromatography (HPLC) in the reversed-phase mode using methanol–water[7,8] or acetonitrile–water[9–12] gradients or isocratic elution, but other HPLC methods can be used[13–15] as well. Unmodified silica gel is the most popular sorbent used for this purpose in planar chromatography[16–19] and a large number of mobile phases have been reported. Also, gas chromatography can be used for this separation[12,20]. Successful separations of aliphatic C_1–C_{12} aldehydes[7,8,10–12,14,16–20], C_3–C_{11} ketones[7,8,14,16–20], aromatic

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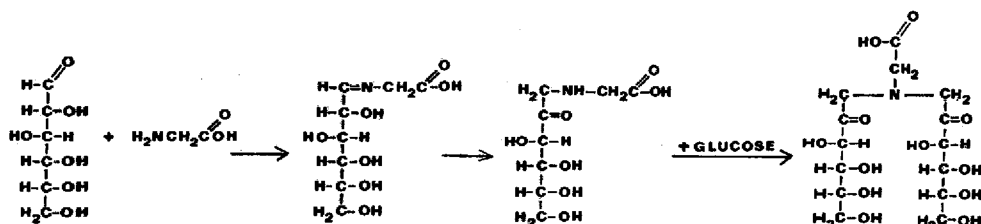

Fig. 1. Formation of di-D-fructose–glycine from D-glucose and D-glycine.

compounds[12,14,18,20,21] and keto acids[9,13,15,16], containing one or two oxo groups per molecule, have been described.

An exhaustive review on oxo compounds arising during the Maillard reaction was published by Paulsen and Pflughaupt[22]. Based on a previous finding by Anet[23], the following set of reactions leading to di-oxo compounds can be visualized. In the reaction of D-glucose and D-glycine, D-fructose–glycine was found, which by way of the Amadori rearrangement resulted in the formation of di-D-fructose–glycine (Fig. 1). Di-D-fructose–glycine is at pH 5.5 and 100°C readily split into 3-deoxy-D-erythro-hexosulose and unsaturated side products. It was proposed that the extremely reactive dicarbonyl intermediates can easily react further with free amino groups, leading to the formation of brown and/or polymeric products. Generally, the Maillard reaction products represent extremely complex mixtures. Separation and isolation of individual components of such mixtures is unusually difficult and this is probably the reason why only single components but not a category of compounds constituting Maillard products have been succesfully separated[24–30].

The above reaction products[22,23] were obtained at elevated temperature (100°C) in acid media (pH 5.5). In preliminary experiments carried out *in vitro* at 37°C and pH 7.4 we have observed a considerably slower formation of the Maillard reaction products. Profiling of the mixture resulting from the reaction of different amino acids and sugars indicated the importance of the nature of the sugar involved. Equilibria were reached only after several weeks of incubation. In experiments with proteins (elastin) rapid insolubilisation was observed, indicating profound cross-linking of the protein. Similar incubations with serum albumin lead to the formation of 2-formyl-5-(hydroxymethyl)pyrrole-1-norleucine after alkaline hydrolysis[24]. Reversed-phase chromatography in 0.1 *M* triethylammonium formate–methanol (9:1, v/v) enabled the determination of the above compounds after alkaline hydrolysis. However, the presence of other reaction products in the resulting mixture was observed as well[24].

In the present study we report the results of our attempts to separate the carbonyl (2,4-dinitrophenylhydrazine-reactive) compounds arising during the Maillard reaction between glucose and glycine; this model system was selected to gain fundamental information on the possibilities of their separation.

EXPERIMENTAL

Chemicals

Glycine (Reanal, Budapest, Hungary), glucose monohydrate (Lachema, Brno,

Czechoslovakia) and 2,4-dinitrophenylhydrazine (Lachema) were all of analytical grade. Solvents for the preparation of the Maillard products were used without further purification. The following aldehyde standards were used: formaldehyde, formamide, acetone (Lachema); acetaldehyde, salicylaldehyde (Fluka, Buchs, Switzerland); butyraldehyde (Prolabo, Rhone-Poulenc, Paris, France); glutaraldehyde (Merck, Darmstadt, F.R.G.). All, except formaldehyde, were purified by distillation. Methanol for chromatography (Merck) and double-distilled water were used for gradient preparation.

Preparation of Maillard products

Glucose–glycine Maillard products were prepared according to the following procedure[24]: 18 mmol of glycine (1.35 g) were reacted with 4.5 mmol of glucose monohydrate (0.9 g) in 60 ml of ethanol with 20 drops of glacial acetic acid under reflux. After 24 h of boiling, the reaction mixture was filtered, the filtrate was dried and reconstituted in double-distilled water (*ca.* 10 mg/ml).

Preparation of 2,4-dinitrophenylhydrazones

The reagent was prepared as a saturated solution of 2,4-dinitrophenylhydrazine in 2 *M* hydrochloric acid[5]. Reaction was performed by addition of 6 ml of reagent solution to 1 ml of the solution of the Maillard products. After 1 h, the sediment was filtered off and washed with distilled water on the filter. The resulting 2,4-dinitrophenylhydrazines were dissolved in ethyl acetate (*ca.* 5 mg/ml). The same procedure was used for the preparation of the standard solution of oxo compounds. The final concentration of this standard was 1.5 mg/ml.

Reversed-phase chromatography

Separations were carried out with an SP 8100 liquid chromatograph (Spectra-Physics, San Jose, Ca, U.S.A.) connected to an SP 4100 computing integrator (Spectra-Physics). The eluent was monitored at 360 nm using a Waters 490E programmable multi-wavelength detector (Waters, Milford, MA, U.S.A.). A glass column (150 × 3.3 mm I.D.) packed with Separon SGX C_{18} (7 μm; Tessek, Prague, Czechoslovakia) was mounted into the instrument.

The column was conditioned with methanol–distilled water (30:70, v/v) for 15 min before each analysis. The flow-rate was 1.0 ml/min. The sample was applied to the column in ethyl acetate and elution was started with a linear methanol–water gradient from (30:70, v/v) to (65:35, v/v) in 15 min; isocratic elution with methanol–water (65:35, v/v) followed for another 15 min. Finally, the column was washed with 100% methanol for 10 min. The column temperature was held at 30°C. Preparative separations were achieved with the same system.

Thin-layer chromatography

HPTLC silica gel 60 F_{254} plates (10 × 10 cm) for nano-TLC (Merck) were used for thin-layer chromatography (TLC). The plates were developed twice with tetrachloromethane–ethanol–*n*-butanol (90:5:5, v/v/v). The compounds were detected as quenching spots under ultraviolet light at 254 nm (Min UVIS; Desaga, Heidelberg, F.R.G.). For preparative purposes the same solvent system (dual development) with PLC silica gel 60 F_{254} plates (20 × 20 cm) from Merck was used.

RESULTS

In preliminary experiments the system was optimized by using a standard mixture of simple oxo compounds. Fig. 2 shows the elution profile of this standard mixture derivatized with 2,4-dinitrophenylhydrazine. As expected, the retention times of the aliphatic aldehydes increased with increasing number of carbon atoms. Glutaraldehyde yielded mono- and bis-disubstituted derivatives, which were eluted as the last of our test mixture. Salicylaldehyde was only incompletely resolved from butyraldehyde. The most readily eluted compound was formamide.

From these preliminary experiments, it was concluded that the chromatographic system used allowed the separation of simple aldehydes and ketones and, therefore, its applicability to the separation of oxo compounds (2,4-dinitrophenylhydrazine-reactive) formed during the Maillard reaction was studied. It should be emphasized that, even in a simple test mixture, attempts to separate the Maillard reaction products without pre-column derivatization by reversed-phase chromatography were entirely unsuccessful. On the other hand, chromatography of products containing an oxo group, derivatized with 2,4-dinitrophenylhydrazine, yielded satisfactory results (Fig. 3).

Six prominent peaks were visible in the chromatogram of the Maillard reaction products, along with a number of small peaks. The first of the dominant peaks, with a

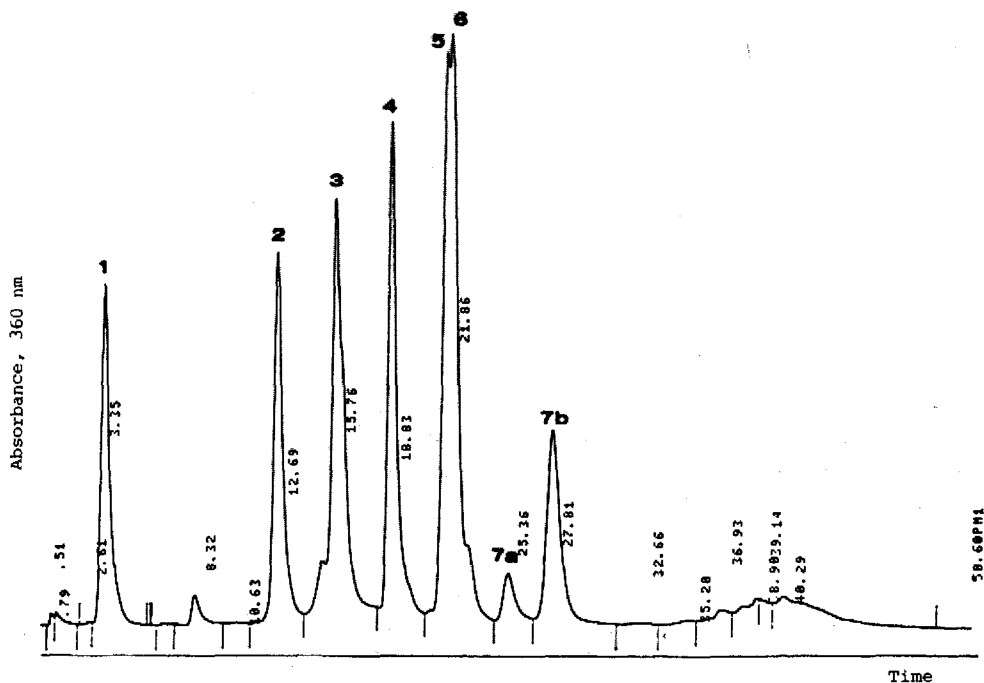

Fig. 2. Separation of the 2,4-dinitrophenylhydrazones of standard oxo compounds. Peaks: 1 = formamide, 2 = formaldehyde, 3 = acetaldehyde, 4 = acetone, 5 = salicylaldehyde (2-hydroxybenzaldehyde), 6 = butyraldehyde, 7a and 7b = glutaraldehyde. Time in min.

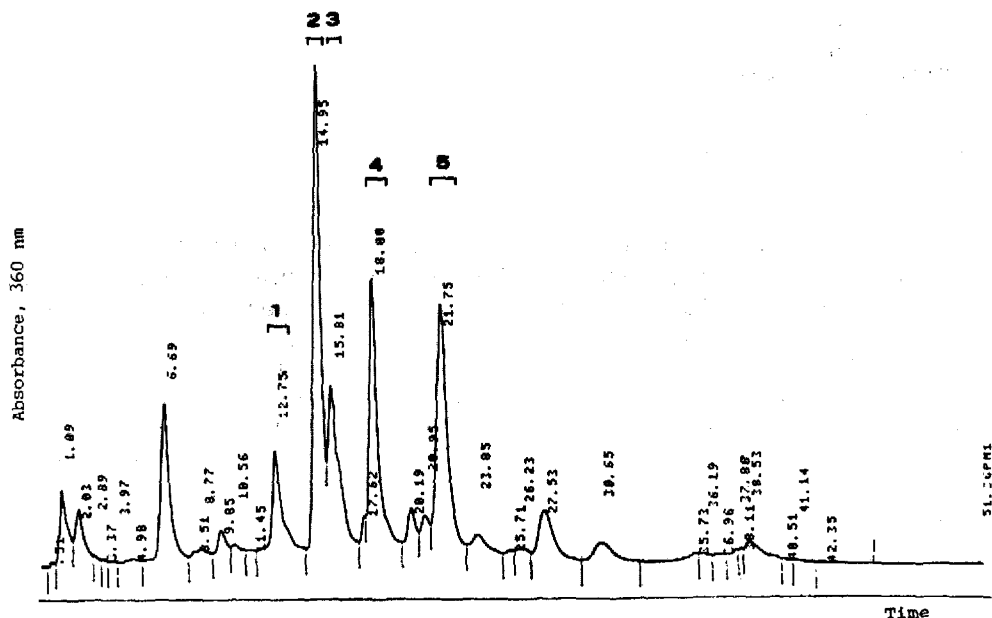

Fig. 3. Separation of the Maillard products reacting with 2,4-dinitrophenylhydrazine. Nos. 1–5 indicate the fractions that were collected. Horizontal bars indicate the extent of fraction collection. Time in min.

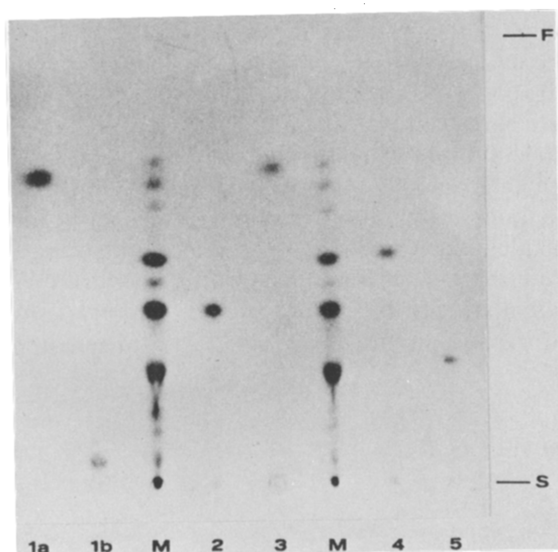

Fig. 4. TLC of isolated peaks from HPLC. Nos. 1–5 indicate individual peaks from HPLC; M = mixture of Maillard reaction products; 1a and 1b represent the result of preparative TLC separation of compounds present in peak 1 of the HPLC step; 2 μl of a solution containing 1 mg/ml were spotted on the start. S and F indicate start and front, respectively.

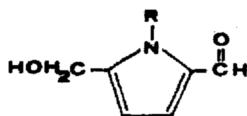

Fig. 5. Structure of N-substituted 2-formyl-5-(hydroxymethyl)pyrrole.

retention time of 6.69 min, corresponded to free 2,4-dinitrophenylhydrazine. The remaining five peaks were collected for further studies (as indicated). For this purpose the same chromatographic system and equipment was used and individual peaks were collected from 30 consequent runs. Next, the homogeneity of individual fractions was shown by TLC using silica gel plates developed twice with tetrachloromethane–ethanol–*n*-butanol (90:5:5, v/v/v). Fig. 4 shows that all fractions, except fraction 1, were sufficiently pure. Fraction 1, which yielded two zones on TLC, could, however, be easily separated into two subfractions (1a and 1b) by preparative TLC. By comparison with appropriate standards, fractions 1a and 3 were tentatively identified as formaldehyde and acetaldehyde, respectively (they exhibit identical retention and R_F with standard under the specified conditions).

DISCUSSION

Derivatization with 2,4-dinitrophenylhydrazine of the oxo compounds formed during the Maillard reaction between glycine and glucose offered the possibility of a single-step separation by reversed-phase chromatography. Preparative separation of the Maillard products resulted in pure fractions, as shown by TLC (except for fraction 1 which was further separated by preparative TLC into fractions 1a and 1b). Abundant literature data on the structure of the products formed during the Maillard reaction of glycine, lysine or aliphatic amines with glucose have indicated the presence of various pyrrole or furane derivatives[24–28]. In addition, it was shown that two and three carbon atom fragments, *e.g.* glycolaldehyde and methylglyoxal, may arise[29]. In other reports from the same authors it was demonstrated that unstable imines derived from glyoxal and methylglyoxal may be formed as well[30]. Under the conditions specified, vicinal dioxo compounds may be subjected to Streckner degradation[31], yielding, for example, formaldehyde, which may further modify the products formed. At least four different groups[24–27] have found N-substituted 2-formyl-5-(hydroxymethyl)pyrrole in the reaction mixture (Fig. 5). Regarding the furanyl derivatives there is currently a controversy whether or not 2-(2-furoyl)-4(5)-

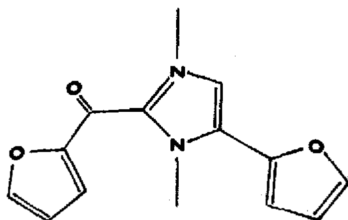

Fig. 6. Structure of 2-(2-furoyl)-4(5)-(2-furanyl)-1H-imidazole.

(2-furanyl)-1H-imidazole[28] (Fig. 6), the presence of which was once generally accepted, is, indeed, present in these reaction products[32,33].

On the contrary, in our model system no evidence was obtained for the presence of either a pyrrole or a furan ring. This, however, does not exclude the possibility that under slightly modified conditions such derivatives could arise.

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