Glucosamine Acetoacetate Condensate
II. Isolation and Identification from Human Diabetic Urines[1] (34201)

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Previous work in our laboratories indicated that the condensation product of D-glucosamine and ethyl acetoacetate, ethyl 2-methyl-5-(D-arabino-tetrahydroxybutyl)-3-pyrrole carboxylate (pyrrole condensate) (1), acts as an antialloxan-diabetes factor (2). Also chromatographic evidence for the presence of the pyrrole condensate in the human diabetic urine was found. Its isolation and identification became the next goal in our research studies.

Materials and Methods At least 75 ml of urine is refrigerated for 24 hr to cause precipitation of inorganic salts and after filtration, it is concentrated by blowing cold air through a semipermeable membrane filled with the specimen. The yield of dry material is approximately 1–2 g. The dry material is dissolved in 50% alcohol and applied to thin-layer chromatography (Eastman silica gel), both one dimensional (butanol–acetic acid–water, 4:1:5) and two dimensional (chloroform–acetic acid–water, 2:1:1 and 20% KCl). The isolated pyrrole condensate was eluted with 95% alcohol and used in the identification experiments after drying. The yield of isolated pyrrole condensate is approximately 1.5–2.0 mg. This material was used for the instrumental analyses.

The KBr pellets (1 mg of sample to 10 mg of KBr) were prepared from the dried isolated pyrrole condensate for infrared studies. Solutions of the isolated pyrrole condensate in absolute ethanol were used for the ultraviolet absorption studies.

For the gas chromatography analysis, the dried isolated pyrrole condensate and the known pyrrole condensate were treated with Tri-Sil (trimethylcholorosilane from Pierce Chemical Company) in order to make the more volatile trimethysilyl ether derivations to be separated on a 5% SE 52 on 60/70 Anakrom ABS column under the following conditions: detector temperature, 250°; injector temperature, 150°; flow rate, 60 ml/min helium; current, 125 mA; polarity, positive; attenuator, 2; and sample size, 40 μl. Column temperature was programmed to increase from 125° at the rate of 4.5°/min.

Results. The presence of pyrrole condensate in 13 out of 14 diabetic urines was established using ultraviolet, infrared spectrophotometry, and gas chromatography. Of the 14 urines from patients with diabetes mellitus[2] isolated pyrrole condensate from 6 urines show characteristic absorption maxima of 230 and 265 mμ which agrees with the data obtained for the known condensate, and six additional urines show characteristic absorption maxima of 225 and 275 mμ which agrees with that obtained for the salt form of the condensate. On the other hand, the condensate was not found in any of 14 normal urines. (Table I, Column 1). A comparison of a typical curve obtained from the UV absorption characteristic of the known pyrrole condensate as compared with the isolated one from a diabetic urine is shown in Fig. 1A and B; and the curves for the known and isolated salts are compared in Fig. 1C and D. Both the shape of the curve and the absorption maxima correspond not only with each other but with the data published by Gomez Sanchez *et al.* (3) for the pyrrole condensate prepared by them. These results seem to indicate that the total electronic configuration of the isolated compound

[1] Supported by a grant from the Research Corporation.

[2] Diagnosed and treated by local physician.

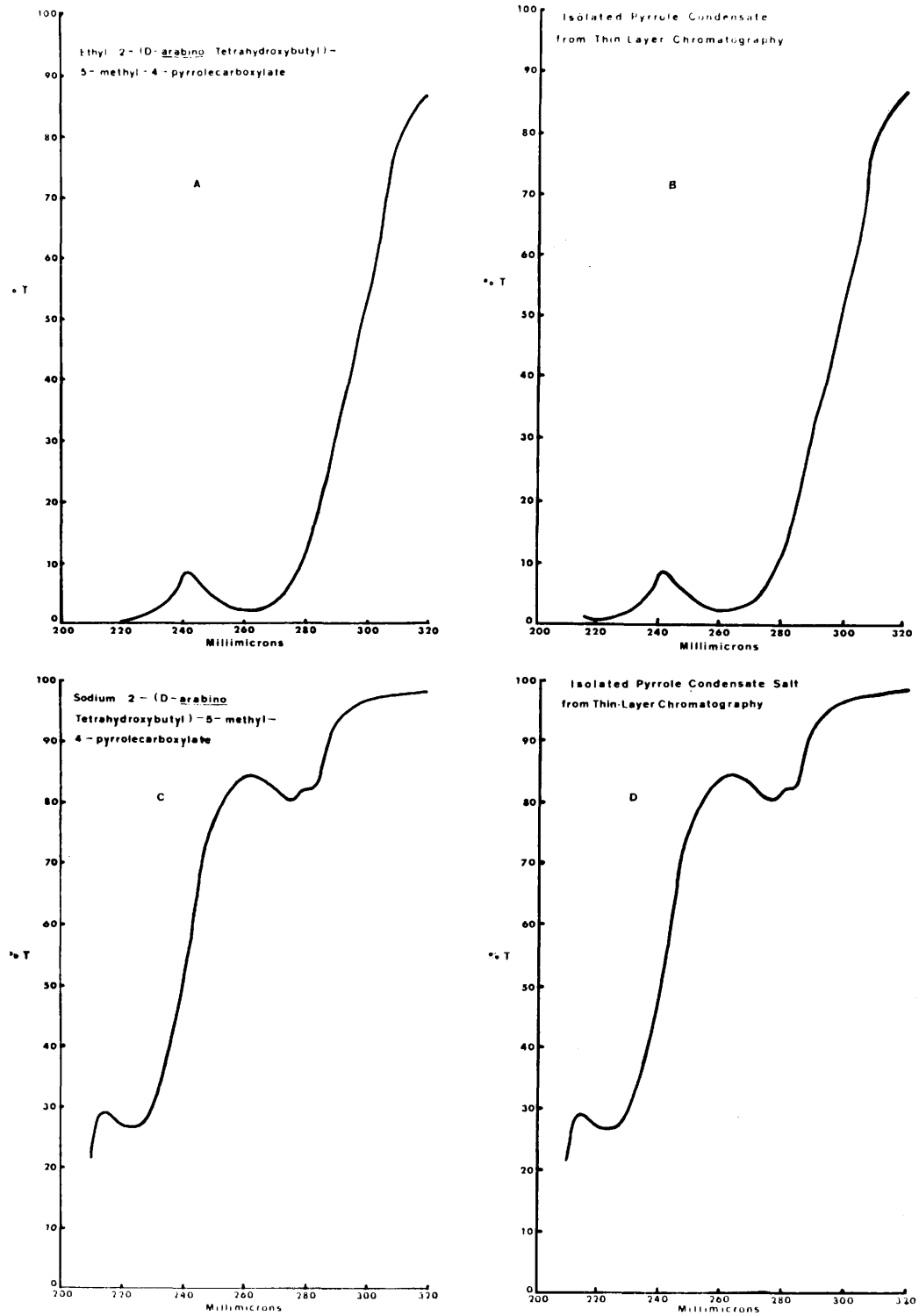

FIG. 1. Ultraviolet absorption spectra: (A) known pyrrole condensate; (B) isolated pyrrole condensate; (C) sodium salt of known pyrrole condensate; (D) sodium salt of isolated pyrrole condensate; cell length: 1 cm; concentration of samples: 1 mg/10 ml.

TABLE I. Spectrophotometric and Gas Chromatographic Data.

Sample	1 UV maxima (mμ)	2 IR maxima (μ)	3 Gas chromatography retention time (min)
Diabetic, 1	230, 265 (Ester)	3.00, 5.85 (Ester)	17.5
2	225, 275 (Salt)	3.10, 6.40 (Salt)	17.3
3	225, 275 (Salt)	3.00, 6.35 (Salt)	17.9
4	230, 265 (Ester)	3.05, 5.85 (Ester)	17.1
5	230, 265 (Ester)	3.00, 5.95 (Ester)	18.0
6	230, 265 (Ester)	3.00, 5.85 (Ester)	17.7
7	230, 265 (Ester)	3.01, 5.85 (Ester)	17.9
8	230, 265 (Ester)	3.02, 5.95 (Ester)	17.7
9	225, 275 (Salt)	3.05, 6.35 (Salt)	17.9
10	225, 265 (Salt)	3.03, 6.32 (Salt)	17.6
11	—	—	—
12	225, 275 (Salt)	3.05, 6.35 (Salt)	17.2
13	225, 275 (Salt)	3.05, 6.35 (Salt)	17.5
14	—	—	—
Pyrrole condensate	230, 265	3.05, 5.85	17.5
Pyrrole salt	225, 275	3.05, 6.35	

matches closely that of the known condensate.

Isolated pyrrole condensate showed characteristic absorption maxima for NH(OH) and C–O groups coinciding with those of the known condensate and salt (Table I, Column 2). Similarly a comparison of typical infrared absorption spectrum for the known condensate with that of the isolated condensate (Fig. 2) shows significant correspondence both in the functional group region (2.5–10 μ) and in the fingerprint region

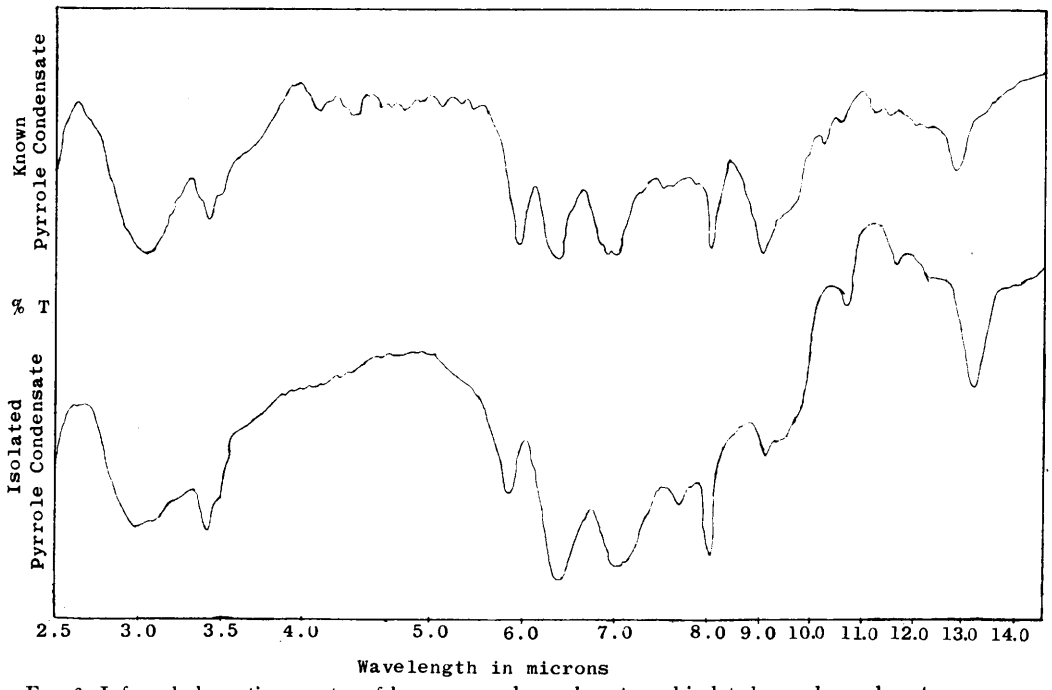

FIG. 2. Infrared absorption spectra of known pyrrole condensate and isolated pyrrole condensate.

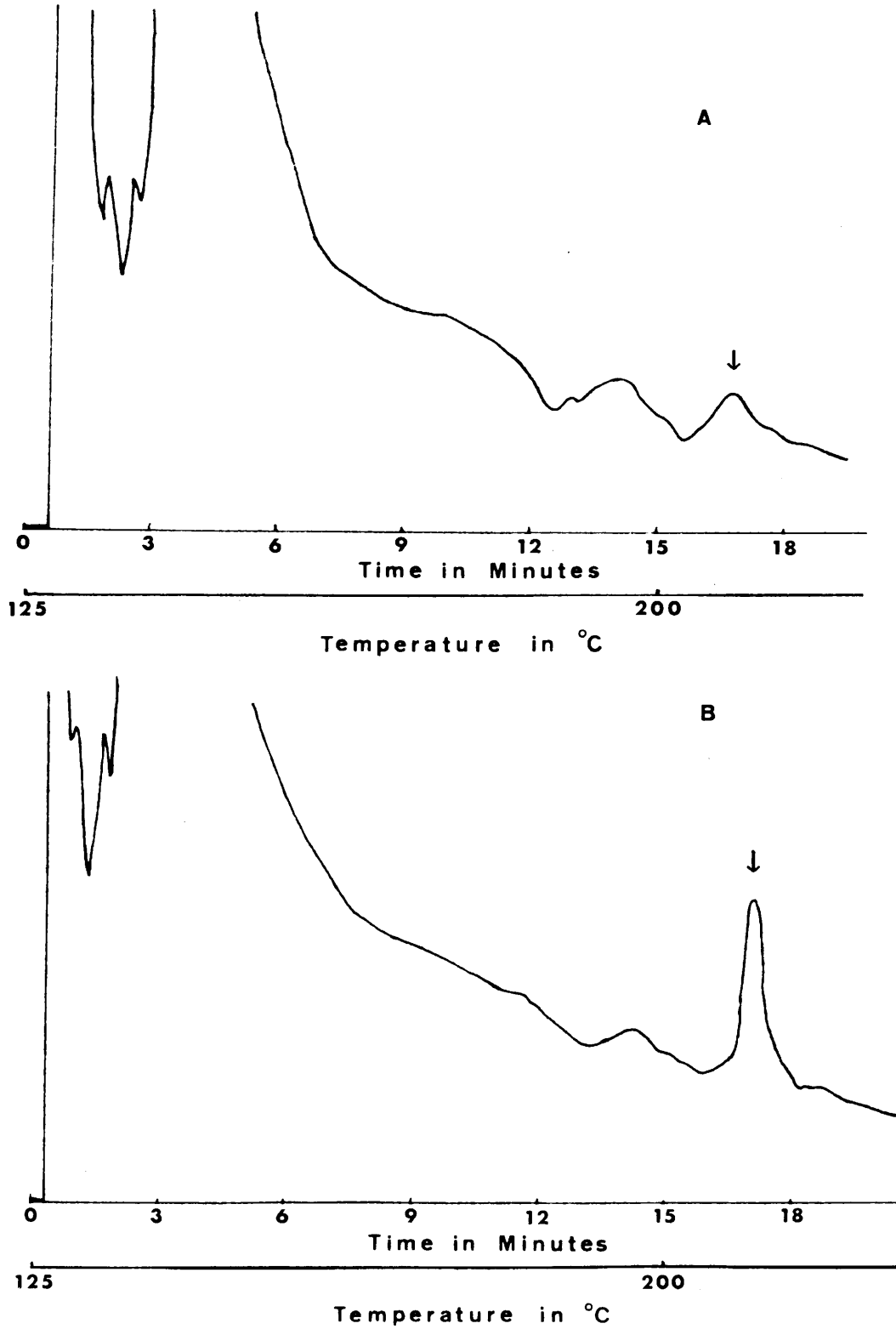

FIG. 3. Gas chromatogram of: (A) diabetic urine with peak under arrow of 17.5-min retention time corresponding to known pyrrole condensate; (B) diabetic urine with added known pyrrole condensate with peak under arrow of 17.5-min retention time.

TABLE II. Elemental Analysis.[a]

Analytical report	(Ratios, C:H:N) C	H	N
Pyrrole condensate			
Calculated	52.74	7.01	5.13
Known	52.63	7.03	5.09
Isolated	52.56	6.57	4.83

[a] Combustion analysis done by Spang Microanalytical Laboratories, Ann Arbor, Michigan.

(10–16.5 μ). These results indicate that the total atomic structure of the isolated pyrrole condensate and the known pyrrole condensate are nearly in complete agreement.

A typical gas chromatogram is shown in Fig. 3A where a peak with retention time of 17.5 $\pm$ 0.5 min and 205° corresponds to that for the known pyrrole condensate. In Fig. 3B, is shown the chromatogram obtained when a small amount of the known derivative was added to the same sample and injected. In each case the known increased only the peak suspected to be the isolated pyrrole condensate, giving strong evidence of the presence of the pyrrole condensate in diabetic urine. Table I, Column 3 is a summary of retention times for the known condensate and five of the isolated condensates from diabetic urine, showing complete agreement of these retention times. No correlation was found between the qualitative presence of pyrrole condensate in the urine of diabetic patients and the levels of ketonuria, glycosuria, and blood sugar.

Conclusion. Glucosamine acetoacetate condensate or its salt form was isolated and identified by ultraviolet and infrared spectra and by gas chromatography from 12 out of 14 human diabetic urines; the condensate was not found in any of the 14 normal[3] urines. The isolated condensate preparations were also subjected to elemental analysis and found to have ratios of C:H:N corresponding with the standard condensate. These results indicate that the pyrrole condensate of glucosamine and acetoacetate occurs as a metabolite in patients with diabetes.

[3] College students without any clinical history of diabetes mellitus or other metabolic disease.

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2. Padron, J. L., Sulman, M., and Lewis, N. R., Nature **213**, 1254 (1967).

3. Gomez Sanchez, A., Rey Romero, L., Garcia Gonzalez, F., Anales Real Soc. Espan. Fis. Quim. **60**, 505 (1964).

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