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DEVELOPMENT OF AUTOMATED ANALYTICAL
CAPABILITY FOR THE EARLY DETECTION OF
DIABETES MELLITUS

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TABLE OF CONTENTS

	Page
SUMMARY	
1. ASPECTS OF DIABETES MELLITUS	
1.1 Pathophysiological abnormalities	
1.2 Acute decompensations	
1.3 Vascular complications	
1.4 Stages of diabetes mellitus	
1.5 Classification of overt diabetes mellitus	
1.6 Diagnosis of diabetes mellitus	
2. STATEMENT OF THE PROBLEM	
3. ANALYTICAL TECHNIQUES	
3.1 Sample preparation	
3.2 Gas chromatographic separation	
3.3 Mass spectrometric identification	
3.4 Mass fragmentography	
4. RESULTS AND DISCUSSION	
4.1 Profiles of volatile metabolites in normal urine.	
4.2 Profiles of volatile metabolites in urine of patients with diabetes mellitus	
5. REFERENCES	

1. ASPECTS OF DIABETES MELLITUS

1.1 Pathophysiological abnormalities

Diabetes mellitus is a metabolic disorder characterized by a deficiency of effective insulin. The deficiency may be caused by a reduced secretion of insulin by the beta cells of the pancreas, by tissue resistance to insulin or by other factors causing insufficient action of insulin.

As a consequence the blood glucose level is increased (hyperglycemia caused by reduced metabolism of glucose and increased gluconeogenesis) and glucose is excreted in urine (glucosuria in conjunction with polyuria, because glucose is not sufficiently reabsorbed by the renal tubules).

Furthermore, insufficient insulin activity causes increased lipolysis resulting in high concentration of free fatty acids in blood. The triglyceride synthesis is reduced. The excess of free fatty acids and of acetyl - Co A leads to the formation of the ketone bodies β -hydroxybutyrate, acetoacetate and acetone. Their concentration is increased in blood and they are excreted in urine.

A third consequence is an insufficient protein synthesis and an increased metabolism of proteins.

1.2 Acute decompensations

Three forms of acute decompensation require immediate intervention : ketoacidotic, hyperosmolar and hypoglycemic situations.

Under severe conditions the patient falls into coma.

Hyperglycemia can lead to diabetic ketoacidosis. As a result of the excess of free fatty acids and because of reduced utilization of acetyl-Co A the synthesis of acetoacetic acid and β -hydroxybutyric acid is accelerated causing an increase of hydrogen ions. During ketoacidosis the pH may drop to below 7.0 . Acetone is high in serum, in urine and in breath. The blood glucose levels are between 400 and 700 mg/100 ml.

Whereas ketoacidotic coma occurs mostly in patients with insulin deficiency diabetes (juvenile diabetes) another form of hyperglycemic coma, the hyperosmolar coma, occurs in patients with adult diabetes. With blood glucose levels of sometimes more than 1000 mg/100 ml and without acidosis heavy glucosuria occurs. Osmotic diuresis causes the loss of body water.

The decompensation of the blood glucose level in form of hypoglycemia may be caused by therapy: changed insulin requirement or error in insulin dose.

1.3 Vascular complications

Vascular diseases occur as chronic diabetic complications. They affect kidneys, eyes, heart, central and peripheral nervous system and appear as microangiopathy, nephropathy, retinopathy, atherosclerosis, neuropathy or polyneuropathy. Pathogenesis and cause of these diabetic complications are still controversial.

1.4 Stages of diabetes mellitus

(a) Prediabetes: It is the period between conception and first manifestation of diabetes and is usually determined retrospectively.

During this period fasting blood glucose level and glucose tolerance test are normal and no symptoms are observed.

Normally the identical twin of a diabetic patient and children of two diabetic parents are classified as prediabetic.

(b) Latent diabetes: The patients have normal fasting blood glucose and under standard conditions normal glucose tolerance. Under stress conditions (infection, pregnancy, periods of overweight) they develop pathological glucose tolerance.

(c) Asymptomatic (subclinical or chemical) diabetes: These patients have normal or slightly elevated fasting blood glucose but abnormal glucose tolerance under standard conditions. Except reactive hypoglycemia only occasional symptoms are observed.

(d) Overt diabetes: Fasting blood glucose and glucose tolerance test are abnormal. Glucosuria, polyuria, ketonemia and ketonuria are common.

1.5 Classification of overt diabetes mellitus

(a) Infantile diabetes: first manifestation and diagnosis between 0 and 14 years of age. In most cases the patients develop an abrupt onset, severe symptoms and depend on insulin.

(b) Juvenile diabetes: first manifestation between 15 and 24 years of age. Usually acute symptoms occur and the patients depend on insulin.

(c) Adult diabetes: first manifestation after 25 years of age. Often the patients do not depend on insulin but can be treated by diet or by oral antidiabetic medication.

1.6 Diagnosis of diabetes mellitus

Overt diabetes mellitus is usually recognized by elevated blood glucose levels and by glucosuria. Fasting blood glucose levels of ≥ 140 mg/100 ml in capillary blood or ≥ 130 mg/100 ml in venous blood indicate diabetes mellitus. The values depend somewhat on the method applied for determination.

Hexokinase-method, glucose oxidase-method and glucose dehydrogenase-method are most frequently used.

Less severe cases of diabetes, borderline cases and latent diabetes are diagnosed by tolerance tests. Commonly, the oral glucose tolerance test and the intravenous glucose tolerance test are applied.

Oral glucose tolerance test: after drawing blood for determination of the fasting blood glucose concentration the patient is given 100 g of glucose in 400 ml of water or tea. Blood is taken in definite intervals, e.g. after 30, 60, 90 and 120 minutes.

Normal values: maximum glucose level < 160 mg/100 ml; 2-h glucose level < 120 mg/100 ml. Clearly pathological values: maximum glucose level > 180 mg/100 ml, 2-h glucose level > 140 mg/100 ml.

Intravenous glucose tolerance test: the patient is administered a solution of 25 g of glucose or 0.5 - 0.8 g of glucose per kg body-weight. The blood glucose is determined under fasting conditions and then every 10 minutes over a period of 80 minutes.

The mean glucose elimination in percent per minute (K-rate) is

≥ 1.2 for normal persons. $K \leq 1.0$ indicates diabetes.

2. STATEMENT OF THE PROBLEM

By ketogenesis the ketone bodies β -hydroxybutyrate, acetoacetate and acetone are formed from acetyl-CoA. Acetone has been long known to occur in urine of patients with diabetes mellitus. Its qualitative and semiquantitative determination belongs to the routine controls of diabetics, and very simple and rapid tests are available. Acetone is a normal metabolite and is excreted in urine of every individual. In urine of diabetics its concentration may be increasing by the factor of 100 or even more.

The goal of this investigation was to study the total profile of volatile metabolites in urine of patients with diabetes mellitus. Because of the drastic abnormalities in the metabolism of carbohydrates, lipids and proteins connected with diabetes we expected that apart from acetone further characteristic abnormalities occur in the profiles of volatile urinary metabolites in case of diabetes mellitus. We found quantitative and qualitative changes in these urines as compared to the urines of normal subjects.

3. ANALYTICAL TECHNIQUES

3.1 Sample preparation

For profile analyses the constituents have to be enriched. Our first approach was a continuous extraction of 450 ml of a 24-h urine with 80 ml of ether followed by distillation of the extract at low temperature and under reduced pressure (1). The disadvantage of this procedure is the possibility of introducing contaminants

sampling time: 1 h
extracting gas: helium, 20 ml/min
water condenser between sampling flask
and adsorbent tubes: 12°C
adsorbing material: Tenax GC, .35 - 60 mesh, 2 cm³
adsorbent tubes: 2 tubes in parallel, 11 cm x 10 mm O.D.

The optimization of sampling temperature, sampling time and water bath temperature is described in (11).

3.2 Gas chromatographic separation -

After adsorption of the volatile compounds one adsorbent tube was used for gas chromatographic separation. It was inserted into the injector block of the gas chromatograph whose dimensions were designed to accept the trap. In the injector block the substances were desorbed and re-condensed in a pre-column cooled with liquid air. After recondensation pre-column and separation column were connected and the gas chromatographic separation was performed.

gas chromatograph: Model 900, Perkin-Elmer
integrator: Model 480, Varian Aerograph
column: 100 m x 0.5 mm I.D., coated with
Emulphor ON-870 (polyoxyethylated fatty
alcohol), Supelco, Bellefonte, Pa.
pre-column: 1 m x 0.75 mm I.D., coated with Emulphor ON-870.
column temperature: 60°C for 16 min, then programmed to
175°C at 2°C/min

carrier gas: N₂, at 5 ml/min
attenuation: 256
detector: FID
desorption: 300°C, 15 ml N₂/min for 10 min

Comparisons of several desorption temperatures are described in (11).

3.3 Mass spectrometric identification

The second adsorbent trap was used for mass spectrometric identification and for mass fragmentography. The mass spectrometer was coupled with the gas chromatograph (GC-MS combination) over a platinum column of 30 cm in length. The total effluent from the GC column entered the ion source of the mass spectrometer. No separator was applied.

The mass spectrometer was connected on-line with a computer for data acquisition and data processing. Mass spectra were recorded exponentially at a scan rate of 2.5 sec/decade applying automatic repetitive scanning. One spectrum was recorded approximately every 7 sec. All spectra were recorded on magnetic tape and were processed after the analysis was completed. Calibration, background subtraction, GC-peak subtraction, calculation of mean values of intensities from several mass spectra and various other operations were performed to facilitate the identification of the substances. The total ion current of a second ion source (total pressure monitoring source) was the signal for the gas chromatogram. The following mass spectrometric conditions were chosen :

electron energy of ion source	70 eV
electron energy of total pressure	
monitoring source	20 eV
emission current	100 uA
accelerating voltage	3 kV
multiplier voltage	2 kV
ion source temperature	220°C
interface temperature	220°C
resolution	750
operation pressure	4.10^{-5} torr

3.4 Mass fragmentography

From the mass spectrometric data on magnetic tape computer reproductions of the full chromatogram were obtained by plotting the sum of the intensities of all ions. By selecting a specific ion selective chromatograms of groups of compounds or of a single compound were plotted. This selective detection of compounds by computer-produced mass fragmentograms was described for primary alcohols, ketones and sulfur compounds (10, 12).

4. RESULTS AND DISCUSSION

4.1 Profiles of volatile metabolites in normal urine

Combining the adsorption technique for concentration of the compounds, thermal desorption and gas chromatography for their separation, mass spectrometry for their identification and computerized mass fragmentography for their selective detection a powerful

analytical system is available to study the profiles of low molecular weight volatile substances and to analyze pathological variations in these profiles.

The analysis of several hundred normal urines showed that a large number of volatile substances (approximately 250 were counted in the chromatograms) are found in urine. Their molecular weights range from 40 to approximately 160 and their concentrations vary widely. They are ketones, alcohols, aldehydes, sulfides, allyl isothiocyanates, pyrroles, furan derivatives and pyrazines. A list of the substances identified by mass spectrometry is given in Table I.

As key components in the profiles we found ketones with three to nine carbon atoms. The concentrations of the constituents in the profile are subject to physiological variations which are very pronounced for some compounds, e.g. allyl isothiocyanate and carvone. These compounds appear as large peaks in some profiles, whereas in others they are completely absent. The dependence on the diet is not yet clearly established. Despite these variations we can characterize a normal profile. Figure 1 is an example of a normal urinary profile (28-year old male person), which was run at high attenuation. Only substances of high concentration are detected resulting in a simple and interpretable profile. The major components are :

1 = acetone, 2 = 2-butanone, 3 = ethanol, 5 = 2-pentanone, 7 = dimethyl disulfide, 9 = N-methylpyrrole, 10 = 3-penten-2-one, 12 = 4-heptanone, 14 = 2-heptanone, 17 = pyrrole. In this profile no allyl isothiocyanate and carvone are present. The person participating in the

screening study were healthy individuals age 20 to 70 years, male and female. They were allowed to follow their normal eating habits except to avoid alcoholic beverages. Exogenous ethanol is partially excreted in urine and appears as a high peak. It was observed that intraindividual variations in the profiles were smaller than the interindividual variations.

4.2 Profiles of volatile metabolites in urine of patients with diabetes mellitus

As described in section "Aspects of Diabetes Mellitus" , a variety of abnormalities occurs in the metabolism of carbohydrates, lipids and proteins in patients with diabetes. The biochemistry of many of these abnormalities is well known. Studying the profiles of low molecular weight volatile compounds in urine several so far unknown abnormalities were found.

The most characteristic abnormality is the increase in the concentrations of five primary aliphatic alcohols : ethanol, n-propanol, isobutanol, n-butanol and isopentanol. In 80 urine samples from 31 patients, we found the following results on elevated alcohol concentrations: ethanol in 90%, n-propanol in 82%, isobutanol in 67%, n-butanol in 58% and isopentanol in 34% of the cases.

An example of the abnormalities in the profiles of patients with diabetes mellitus is shown in Figure 2. It is the urine of a 75 year old patient with diabetic retinopathy. The concentrations of all five alcohols are increased, especially of ethanol, n-propanol and isopentanol (peak 3 = ethanol, 6 = n-propanol, 8 = isobutanol, 11 = n-butanol, 13 = isopentanol). Using computerized mass fragmen-

tography it is possible to plot selective profiles for the alcohols. The fragment ion m/e 31, $H_2C = OH$, is suitable for profiling the entire group of primary alcohols (Figure 3). From the spectra of the repetitive scan the computer plots the intensity of this fragment over the total chromatogram. In Figure 3 the abscissa indicates the spectrum number of the GC-MS scan. Interference with detection of primary alcohols occurs only when aldehydes and ketones are present in very high concentration. Even more specificity is achieved when additional characteristic ions are used. n-Propanol is detectable by the molecular ion m/e 60.

With 90% and 82% respectively of the cases studied, ethanol and n-propanol are most often elevated in urine of patients with diabetes mellitus and appear suitable for screening purposes. Isobutanol and isopentanol are less frequently elevated. According to our findings their concentrations are increased in cases with complications as described in section 1 (13).

The second characteristic abnormality occurs in the group of ketones. In the example of Figure 2 the concentration of 4-heptanone (peak 12) is decreased, of cyclohexanone (peak 15) increased. Selective profiling for the C_7 -ketones is possible with the molecular ion m/e 114 (Figure 4) and for cyclohexanone with the molecular ion m/e 90 or the fragment ions m/e 83 and m/e 80 (12).

A dependence of the concentration of cyclohexanone and 4-heptanone on severenss and control of diabetes was observed (11,13). However, the abnormalities in the group of the ketones are much more

differentiated than in the group of the alcohols. The number of cases investigated so far does not allow the classification of the findings and the correlations.

Currently we are increasing the number of cases within classified groups of diabetics: infantile, juvenile or adult diabetes mellitus; insulin deficiency diabetes or insulin resistance diabetes; presence or absence of complications.

For screening patients in order to detect diabetes the alcohols may be most useful. Before introducing their detection as a routine procedure besides blood and urine glucose determinations and glucose tolerance tests a broad study will be required in order to prove their significance in diagnosing diabetes. Furthermore it must be shown that other diseases, e.g. of the pancreas, the liver, or of the kidneys do not result in similar abnormalities. If this can be shown profiling the alcohols in urine would be a noninvasive test which could easily be automated.

TABLE I - COMPOUNDS IN NORMAL URINE

Acetone

2 - Butanone

Ethanol

Propionaldehyde

3 - Methyl - 2 - butanone

2,3 - Butanedione

2,3 - Dimethylfuran

2 - Pentanone

2,4 - Dimethylfuran

Chloroform

4 - Methyl - 2 - pentanone

3 - Methyl - 2 - pentanone

Methyl - ethylfuran

Toluene

Dimethyl disulfide

3 - Hexanone

2, 3, 5 - Trimethylfuran

5 - Methyl - 3 - hexanone

3 - Penten - 2 - one

N - Methylpyrrole

1 - Buranol

4 - Methyl - 3 - penten - 2 - one

4 - Heptanone

2 - Heptanone

Table 1 (cont)

3 - Methylcyclopentanone

6 - Methyl - 3 - heptanone

3 - Octanone

Methylpyrazine

2 - n - Pentylfuran

2,6 - or 2,5 - Dimethylpyrazine

Allyl isothiocyanate

2,3 - Dimethylpyrazine

Furfural

Vinylpyrazine

2, 3, 5 - Trimethylpyrazine

2 - Methyl - 6 - ethylpyrazine

Pyrrole

2 - Nonanone

Acetylfuran

Benzaldehyde

- Pinene

2 - Methylpyrrole

Dimethylpyrrole

1 - Butylpyrrole

Carvone

Piperitone

p - Cresol

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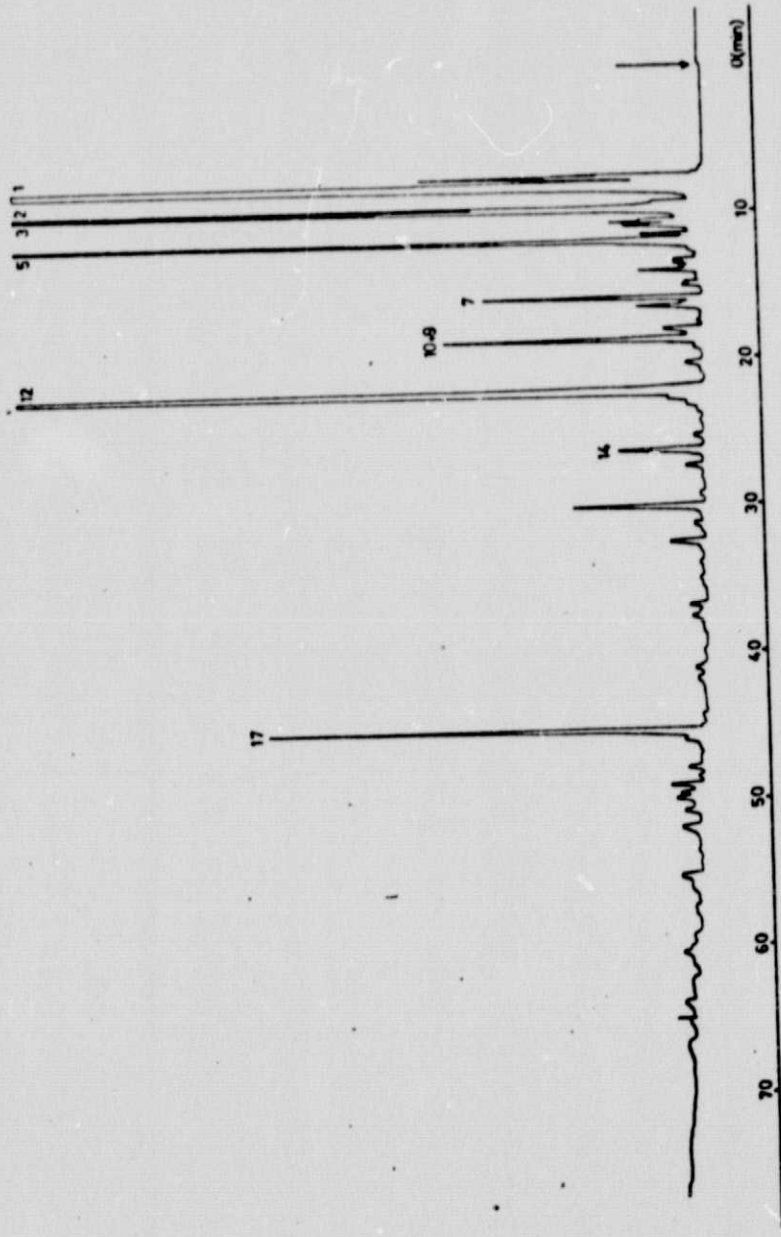


Figure 1 Urinary Profile of Normal Individual

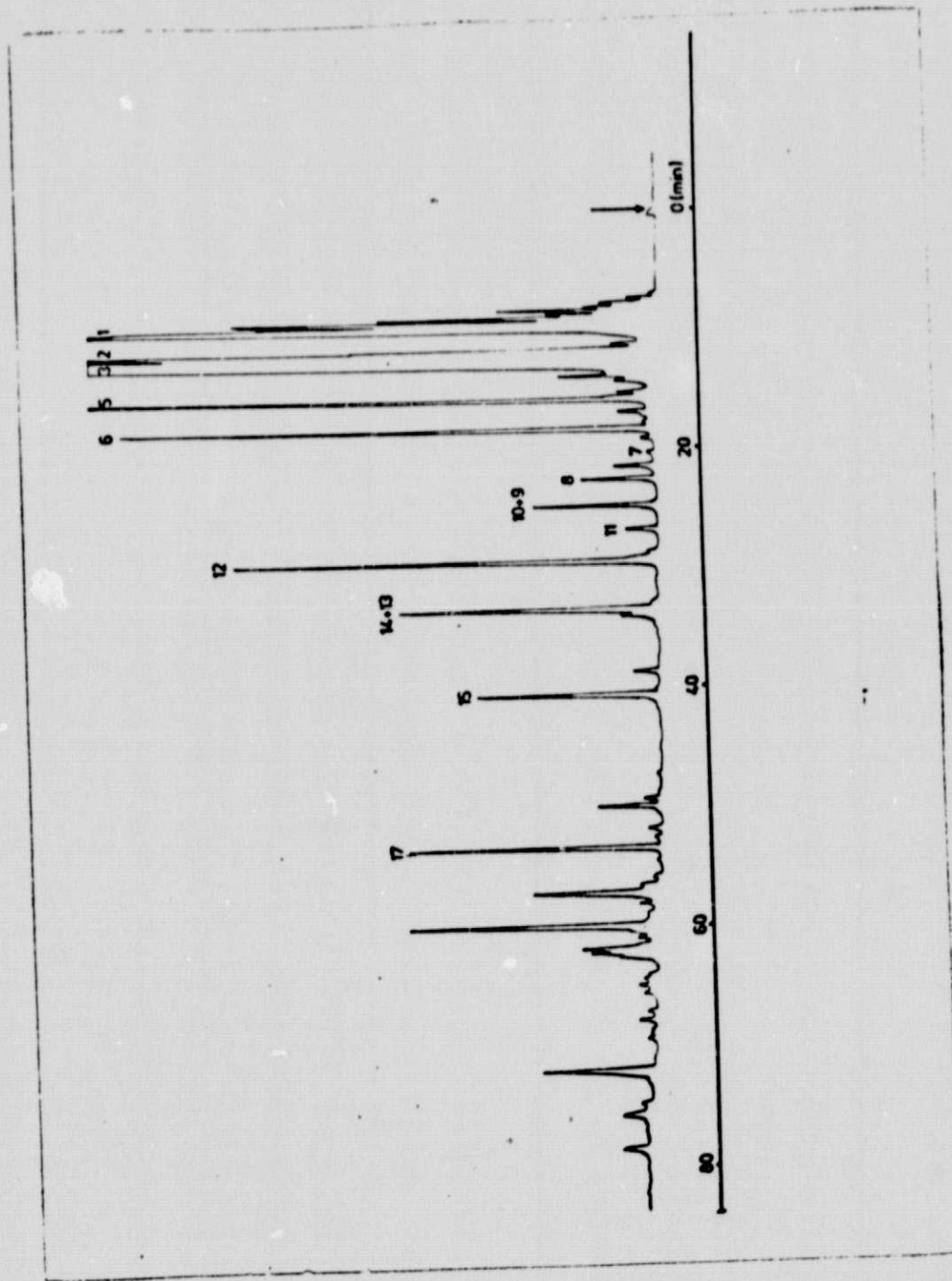


Figure 2 Urinary Profile of Patient with Diabetic Retinopathy

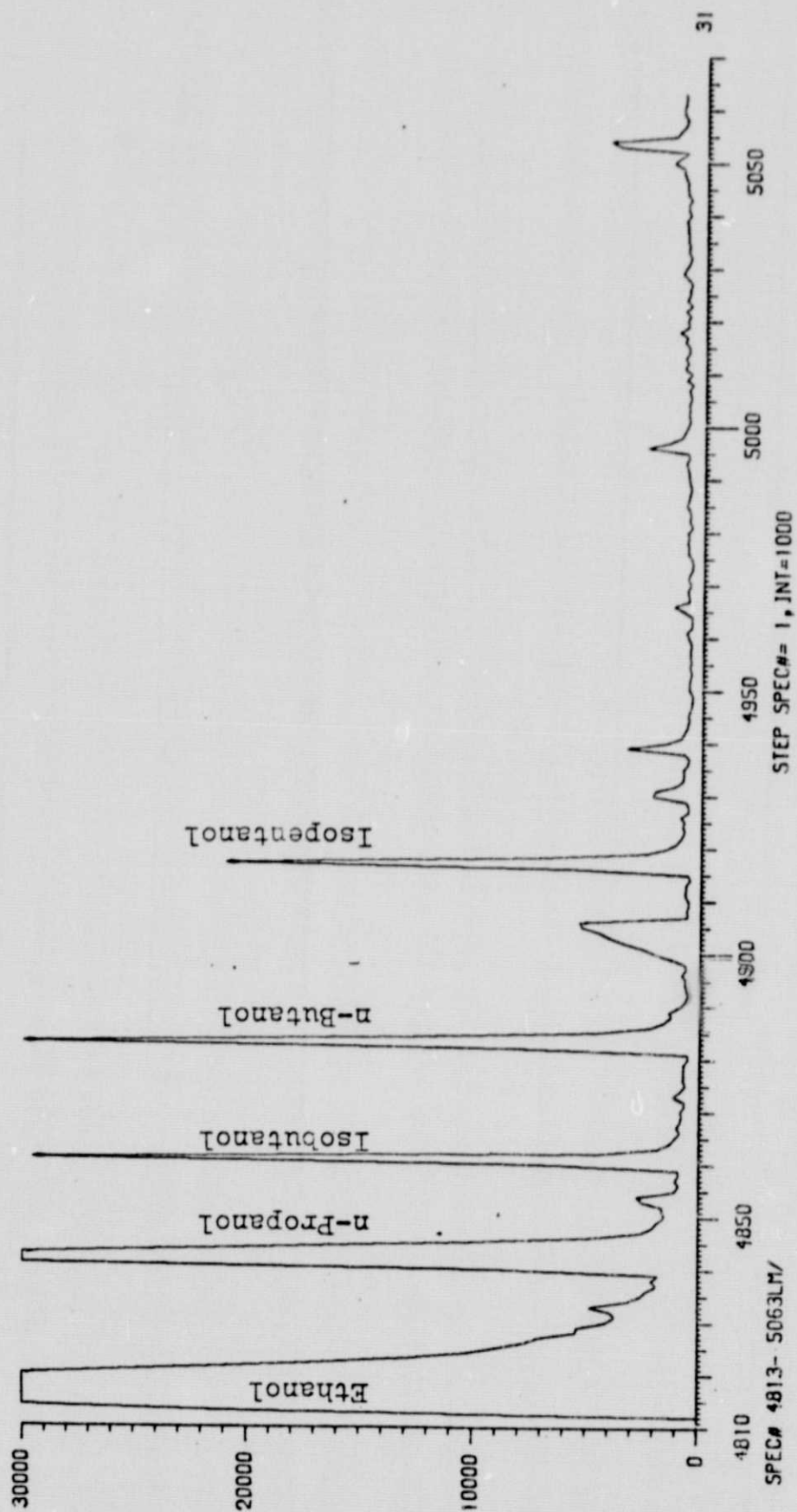


Figure 2 Mass Fragmentogram of Primary Alcohols, M/E 31 (diabetic urine) - patient shown in Figure 1.

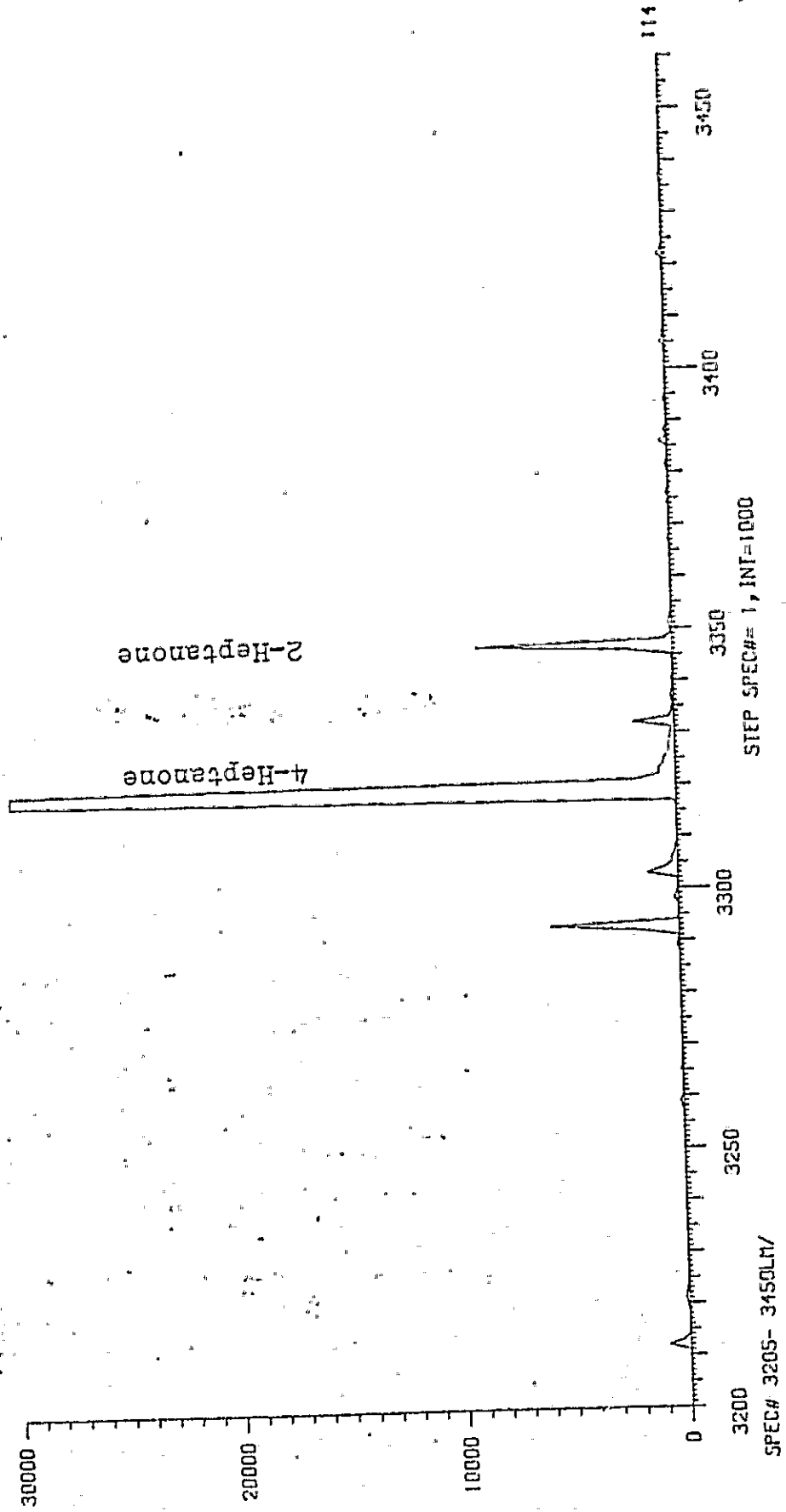


Figure 4 Mass Fragmentogram of C₇ Ketones, M/E 114 (diabetic urine) - patient shown in Figure 2.