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PROFILING OF UREMIC SERUM BY HIGH-RESOLUTION GAS CHROMATOGRAPHY—ELECTRON-IMPACT, CHEMICAL IONIZATION MASS SPECTROMETRY

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SUMMARY

A fast and reliable procedure for gas chromatographic profiling of components in ultrafiltrated uremic serum has been developed, using glass capillary columns. Sample pretreatment consists of ultrafiltration, evaporation and silylation. Some twenty components are identified by electron-impact and chemical ionization mass spectrometry. A comparison is made between profiles of sera from a series of uremic patients, before and after hemodialysis, and from non-uremic sera. Significant differences are found between these profiles. A "dialysis ratio" is introduced as a parameter for the removal of retained components by hemodialysis treatment.

INTRODUCTION

Patients with endstage renal failure have to be submitted to regular treatment with an artificial "kidney". These patients show a complex of clinical symptoms, usually called "the uremic syndrome", or "uremia". Many of these symptoms are related to a disturbance in the homeostatic or regenerative function of the kidney, which results in retention of metabolic products and in disorders of hormonal and metabolic function.

There are indications that retained components can act as cell toxins or as inhibitors of enzyme action. The identity of these components is still subject to discussion. In recent years several authors have mentioned the importance of compounds of medium molecular weight [1—3].

Others re-emphasized the role of components of lower molecular weight, such as methylguanidine, guanidino acids, amines, phenolic acids, polyols, inositol and other compounds [4—9]. Although experiments were carried out to test the various hypotheses, no definitive conclusions could be drawn [10].

Therefore it is difficult to improve "artificial kidney" strategies and equipment in a planned and efficient way. For this purpose, it is necessary to develop analytical techniques which give data on the effectiveness of the treatment. Moreover, analytical information can lead to clinical tests on the toxicological behaviour of certain compounds, and could contribute to a better understanding of biochemical and physiological processes in uremia. Several profiling techniques have been applied in the analysis of body fluids of uremic patients. These profiling techniques give information for a whole range of compounds. Dzúrik et al. [11], Chang [12], Gordon et al. [3], Cueille [13] and others tried to characterize uremic plasma by gel permeation chromatography, especially with reference to "middle molecules". Senftleber et al. [14] and Veening [15] analysed serum and hemodialysis fluid with reversed-phase liquid chromatography. Mikkers et al. [16] reported on an isotachophoretic profiling technique which gives information on ionic compounds with low as well as high molecular weight. Masimore et al. [17] examined volatile components in hemodialysis fluid by gas chromatography—mass spectrometry (GC—MS) using packed columns. Bultitude and Newham [18] applied the same technique in the analysis of uremic serum, also using packed columns. In the latter, a laborious and time-consuming sample pretreatment procedure of several days, including fractionation by gel permeation chromatography and freeze-drying, was used. In this report a reliable GC-profiling technique, using glass capillary columns, and a fast pretreatment procedure is described.

EXPERIMENTAL

Samples, reagents and materials

Blood samples of ten uremic patients, before and after hemodialysis on polyacrylonitrile RP6 (Rhone-Poulenc, Paris, France) and cuprophane GM (Gambro-Major, Lund, Sweden) membranes, were obtained from the Nephrological Division of the University Hospital of Ghent (Belgium). After centrifugation, serum samples were stored at —18° until used. A pool of serum from non-uremic persons was prepared. Removal of high molecular weight substances was carried out by ultrafiltration on Amicon (Lexington, Mass., U.S.A.) XM 50 membranes. Chemical derivatization was performed in borosilicate reaction vessels (Hewlett-Packard, Avondale, Pa., U.S.A.) with bistrimethylsilyltrifluoroacetamide (BSTFA) from Pierce (Rockford, Ill., U.S.A.). Straight-chain C13- and C22-hydrocarbons [(from Phillips Petroleum (Bartlesville, Okla., U.S.A.) and Applied Science Labs. (State College, Pa., U.S.A.)] were used as internal standards. A standard solution was prepared by dissolving 21 mg of C13 and 4.9 mg of C22 in 50 ml *n*-hexane. Reagent gas for chemical ionization (CI) was isobutane (CH 35) from l'Air Liquide (Paris, France).

Apparatus

Pressure-ultrafiltration was carried out in a microcell [19]. For GC separations a Perkin-Elmer F-30 instrument was used. The standard sample introduction system was replaced by a moving-needle injector [20]. Average carrier gas velocity (helium) was 28 cm/sec. An oven temperature programme was used starting with an isothermal period of 2 min at 110°, an increase of 5°/min to 200° and remaining isothermic at 200° for 35 min. Injection and detection temperatures were maintained at 250°. Glass capillary columns (47 m), deactivated [21] with Carbowax 20M and coated with SE-30 (layer thickness 0.2 μm) were prepared by the static coating procedure of Rutten and Rijks [22]. The flame ionization detector signal was recorded on two traces because of large concentration differences for different compounds (2 mV and 50 mV full scale corresponding to $4 \cdot 10^{-12}$ A and $2 \cdot 10^{-10}$ A respectively). Mass spectra were obtained with a 4000 GC—MS system from Finnigan (Sunnyvale, Calif., U.S.A.) coupled to a D 116E minicomputer (Digital Computer Controls). A platinum—iridium capillary was used as a GC—MS interface. In MS analysis the electron energy was 70 eV in both the electron-impact (EI) and the CI mode. Source temperature was 250° under EI and 220° under CI, sensitivity 10^{-8} and 10^{-9} A/V, multiplier voltage 1675 V, and reagent gas pressure 13 Pa (0.1 Torr.) in chemical ionization.

Procedure

Serum samples are pressure-ultrafiltrated under nitrogen to remove high molecular weight components such as proteins (cut-off at 50,000). Aliquots of 250 μl of the ultrafiltrated material are evaporated to dryness under a nitrogen stream in a sandbath at 70°. The dried samples are derivatized to enable GC separation and are allowed to react with 250 μl of BSTFA reagent at 80° for 2 h. After dilution with 250 μl *n*-hexane, aliquots of 50 μl of the standard solution (C13 and C22 in hexane) are added.

Samples of 0.5 μl are applied to the tip of the moving needle. After 90 sec, during which solvent, volatile reaction products and unreacted BSTFA are allowed to evaporate, the sample is injected.

RESULTS AND DISCUSSION

Reliability of the method

As a retention parameter the relative retention is used. In our experiments this parameter appeared to be more reliable than the retention index. The reproducibility of the temperature programme was tested by injecting the same sample six times within a short period. Five peaks (peak numbers 35, 49, 67, 75, 78 in Fig. 1) throughout the whole temperature range show coefficients of variation for the relative retention (with respect to C22) which are less than 0.4%. This demonstrates that the reproducibility of the temperature programme is good.

The same peaks were tested in chromatograms that were recorded in the course of two months. Coefficients of variation between 0.2 and 2% for different peaks represent long-term changes in column performance and carrier gas flow. This result is satisfactory, however, the maximum value of 2% can

cause difficulties in distinguishing one peak from another for certain components (e.g. peaks 34 and 35 in Fig. 1).

Peak height is used as a quantitative measure. For near-baseline peaks or unresolved peaks this gives better results than peak area measurement. Reproducibility of the normalized peak heights, from four injections, is measured for 15 major peaks in the chromatograms. The coefficients of variation of different peaks were between 2 and 9%. The influence of the sample pretreatment on peak height variation was studied by derivatizing an ultrafiltrated serum sample four times. Each sample was then injected four times. A Student's t-test (on the mean) and a F-test (on variance ratio) were applied to peak heights "within and between" samples. This led to the conclusion that variance due to sample pretreatment does not differ from variance from the analysis step (95% probability level).

Application to a series of uremic patients

Predialysis and postdialysis serum samples were submitted to the described procedure. Typical gas chromatograms are shown in Fig. 1. Differences in
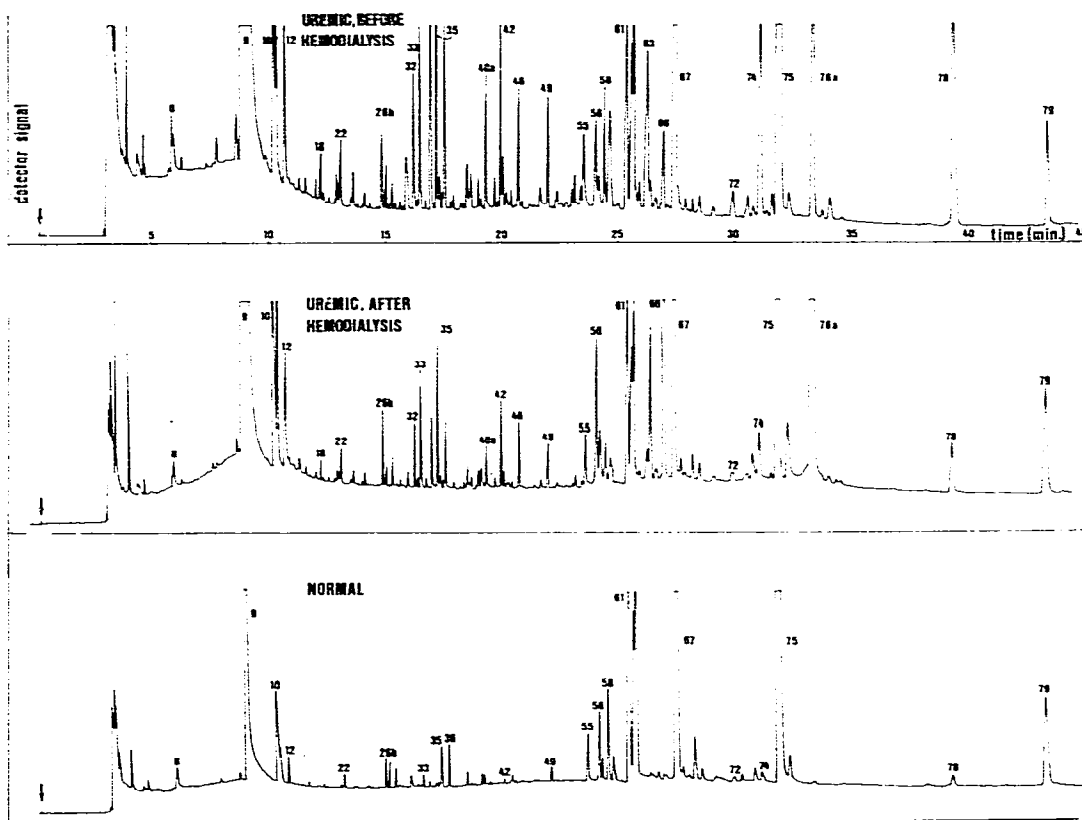

Fig. 1. Gas chromatographic profiles of ultrafiltrated serum from an uremic patient, before and after hemodialysis treatment, and from a pool of non-uremic sera. Glass capillary column coated with SE-30. The 2-mV trace corresponding to a signal of $4 \cdot 10^{-12}$ A f.s.d is shown.

TABLE I

CLASSIFICATION ACCORDING TO DIALYSIS RATIO

Group	$\overline{D}_x$	Number of peaks	Major GC peaks
1	$0.6 \leqslant D < 1.4$	8	22, 27, 28, 67, 75
2	$D \geqslant 1.4$	36	18, 24, 26, 26b, 30, 32, 33, 34, 35, 36, 40a, 42, 43, 46, 49, 53, 54, 54a, 58, 62a, 72, 73, 74, 78
3	$D < 0.6$	1	56
4	variating	13	23, 44, 55, 61, 62, 63, 66, 76a

concentrations of various components are very obvious. All ten patients showed similar profiles, however, individual deviations, both qualitative and quantitative, did occur. For quantification of the effect of hemodialysis treatment a "dialysis ratio" (D) is defined in the following way:

$$D_x = \frac{H'_{x, \text{ before}}}{H'_{x, \text{ after}}}$$

where H'_x is the normalized peak height for component x.

This dialysis ratio (D) was determined for some 70 peaks in the chromatograms of the ten patients. From these data an average D ($\overline{D}$) was calculated for each compound. Then the components were classified into four groups, according to their $\overline{D}$. The maximal error in D can be calculated from that in peak height. A maximal variation coefficient of 9% in peak height was found. This leads to a maximum value of approximately 20% for the variation coefficient (V.C.) of D. On this basis all components with D-values between 1—0.4 and 1+0.4 were considered to be unaffected by hemodialysis treatment ($D \pm 2$ V.C.). These components are classified in group 1 (see Table I). Group 2 represents components that show a decreased concentration as a result of dialysis treatment ($D \geqslant 1.4$). Only very few components showed higher concentrations after hemodialysis, and are placed in group 3 ($D < 0.6$). Group 4 represents components that show a great variation in D in the samples of different patients. The table demonstrates that group 2 components are very well removed during dialysis, and that concentrations of group 3 components appear to be raised as a result of the treatment. From peak heights and D-values for samples of different patients it is concluded that the higher the concentrations, the higher the dialysis ratio. The ten patients show substantial differences in "overall" concentrations. Some of them have postdialysis profiles that approach those for non-uremic sera.

The underlined peak numbers in Table I refer to compounds that were identified by GC—MS and from GC retention data. Table II lists these compounds.

TABLE II

IDENTIFIED COMPONENTS IN UREMIC SERUM

Peak number	Relative retention	Compound*	Dialysis ratio ($\overline{D}$)	Group
9	0.153	urea	1.40	2
10	0.178	phosphoric acid	2.40	2
11	0.183	glycerol	—	—
18	0.228	tartronic acid (tent.)	2.71	2
22	0.251	threonine	1.05	1
26b	0.296	homoserine (tent.)	1.47	2
30	0.322	Δ-pyrrolidone-5-carboxylic acid	2.70	2
32	0.327	threitol (tent.)	2.30	2
33	0.336	erythritol	2.10	2
35	.0.352	erythronic acid	3.27	2
40a	0.403	tartaric acid	3.42	2
42	0.417	2-deoxy-erythropentonic acid	1.96	2
49	0.469	arabinitol	2.93	2
54/54a	0.498/0.504	hydroxy or dicarboxylic acids	2.98/3.24	2/2
55	0.507	arabinonic acid	—	4
56	0.520	citric acid	0.57	3
58	0.530	fructose	1.61	2
61	0.553	galactose	—	4
63	0.574	3-deoxy-arabinohexonic acid (tent.)	—	4
66	0.591	glucono-1,4-lactone	—	4
67	0.605	α-D-glucose	1.25	1
72/73	0.665/0.682	mannitol and/or glucitol	6.09/3.72	2/2
74	0.695	isomer of myo-inositol (tent.)	3.52	2
75	0.716	β-D-glucose	1.28	1
76a	0.751	mannonic or gluconic acid	—	4
78	0.900	myo-inositol	7.00	2

*Tent. = tentatively identified.

Identification by mass spectrometry

Many carbohydrate-related trimethylsilyl derivatives have EI mass spectra that look very similar. Although different classes of these compounds (e.g. aldoses, aldonic acids and polyols) demonstrate some characteristic fragment or rearrangement ions, no molecular ions are found [23—26]. Because of this similarity, reference spectra from different origin were used [24, 25, 27]. Moreover extra information on molecular weight was obtained for some components by recording CI mass spectra. Although polyols and aldonic acids showed molecular ions in these spectra, aldoses did not. Differences between EI and CI spectra for several classes of compounds will be discussed in a separate publication. Table III shows the highest mass ions in EI and CI (isobutane) spectra of some components.

Peak 18 (Fig. 1, Table II) must be a hydroxy acid or a dicarboxylic acid with molecular weight of 336. Its spectrum (EI) shows an abundant peak at m/z 292, which probably results from a McLafferty-type rearrangement of a trimethylsilyl group [28]. The m/z 292 ion is the highest mass ion in the EI spectrum. The CI spectrum shows a peak at m/z 337, which is probably the $(M+1)^+$ molecular ion. Peak 18 is therefore tentatively identified as tartronic

TABLE III

COMPARISON OF EI AND CI SPECTRA

Peak number	Compound name	Mol. weight	Highest mass ions in	
			EI (70 eV)	CI (70 eV, 13 Pa)
33	erythritol-4TMS	410	320, 307, 293, 277	411, 321, 305, 293
35	erythronic acid-4TMS	424	409, 379, 319, 292	425, 409, 335, 307
40a	tartaric acid-4TMS	438	423, 333, 305, 292	439, 423, 321, 292
42	2-deoxyerythropentonic acid-4TMS	438	348, 335, 333, 321	439, 423, 349, 333
49	arabinitol-5TMS	512	320, 319, 317, 307	513, 333, 307, 303
56	citric acid-4TMS	480	465, 375, 363, 347	481, 465, 363, 319
78	inositol-6TMS	612	507, 432, 393, 367	613, 433, 393, 367

acid (hydroxymalonic acid). The spectra of peaks 54 and 54a also show ions at m/z 292, but no molecular weight information is available. Hippuric acid, which can not be derivatized in a reproducible way, eluted in a few chromatograms simultaneously with arabinonic acid (peak 55). Peak 74 shows an EI spectrum that is similar to the spectrum of myo-inositol (peak 78). Moreover peak heights of peaks 74 and 78 seem to be related to each other, so it is to be concluded that it is an isomer of myo-inositol.

The components at peak numbers 22, 26b, and 30 were included in Table II at the last moment. Obviously they are related to amino acid metabolism. Peak 30 was identified as Δ-pyrrolidone-5-carboxylic acid. This is a product of an intramolecular peptide bonding (cyclisation) in glutamic acid. It is not known whether this compound is really present in uremic serum in this quantity or is formed from glutamic acid in the derivatization step [29].

CONCLUSIONS

A reproducible and reliable GC method for profiling of uremic serum has been described. Profiles from pre- and postdialysis serum show that hemodialysis treatment results in a significant decrease of the concentration of many components. However, it is observed that different components are not removed to the same extent. Therefore a component-specific parameter, the dialysis ratio, is introduced. It could be seen that different patients showed substantial differences in "overall" concentration. Some patients showed postdialysis profiles that seemed "worse" than predialysis profiles of other patients. Some postdialysis profiles had "overall" concentrations comparable to those for non-uremic serum. Components that are detected by this method are related to carbohydrate metabolism, such as aldoses, aldonic acids and polyols. Also other organic acids and some nitrogen containing compounds are detected. The toxicological behaviour of these components is not yet well understood [4].

In order to include other classes of compounds it is necessary to apply several techniques simultaneously. The isotachophoteric profiling technique developed in this laboratory by Mikkers et al. [16], is very suitable for profiling of ionic substances in serum.

In the described GC procedure sample pretreatment consisted of ultra-

filtration, evaporation and silylation. Total analysis time is only 6 h including sample pretreatment. Bultitude and Newham [18] reported on a laborious pretreatment procedure of several days including fractionation by gel permeation chromatography. Despite the fact that no such fraction technique was applied in our GC method, the same range of compounds (and some more) are detected.

The use of glass capillary columns gives more detailed information than earlier investigations using packed columns.

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