Middle Molecules in Uraemia

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Introduction

According to the ‘middle-molecule’ hypothesis introduced by Babb et al (1972) uraemic solutes in the molecular range of 350 to 2000 daltons are assumed to be toxic at least as far as neuropathy is concerned. However, this hypothesis for uraemic toxicity is still controversial since it is largely dependent upon indirect measures for accessing its validity. Recently evidence has been brought forward that uraemic patients accumulate middle-molecular solutes in their body fluids (Dall’Aglio et al, 1972; Gajdos and Dzúrik, 1973; Man et al, 1973). However, further separation and quantisation of these potential toxic substances have not been achieved with the methods used.

We have recently developed methods by which uraemic middle molecules in the molecular-weight range of 1000–2000 daltons can be detected and quantified. In the present communication the methodology will be briefly surveyed and some preliminary results will be shown and commented upon.

MATERIAL AND METHOD

Plasma, urine and dialysis fluid from uraemic patients and plasma and urine from non-uraemic controls were analysed.

Plasma samples were obtained in the fasting state of two hours postprandially. Control studies revealed no difference between specimens obtained at these times.

Urine specimens were either an aliquot of a 24-hr specimen, the first-morning urine or in certain cases the first urine obtained after hospitalisation.

Peritoneal dialysate specimens were obtained after an abdominal dwell time of two hours on the first exchange and after 24 and 36 hr of dialysis using LKB PD 700 automatic peritoneal dialysis equipment. Collected dialysis fluid was carefully mixed and an aliquot sample was used for analysis.
Analytical Technique

Samples Serum and occasionally peritoneal dialysate were ultrafiltered using an Amino Centrifle® membrane (224-CF-50) which has a molecular cut-off at approximately 50 000 dalton.

Molecular filtration (HSGF) Molecular filtration of the different biological fluids was carried out on a microcolumn 75 × 0.3 cm using Sephadex G-15 (Pharmacia, Uppsala, Sweden). After equilibration of the column with 0.01 M Tris HCl buffer, pH 8.6, the sample was loaded on top of the column. The outflow rate of the eluate was maintained at 4.6 ml/hr.

The column was coupled to a dual-channel ultra-violet absorptiometer (LKB Uvicord III, Sweden). The absorption was measured at 206 nm and 254 nm simultaneously using a two-channel recorder (W + W Electronic Recorder 1200) and was collected in a fraction collector (LKB, Ultrorac 7000) for further analysis, e.g., ion-exchange chromatography. The HSGF technique has been described in a previous communication (Gordon et al., 1974; Fürst et al., 1974).

Gradient elution chromatography (GEC) Ion-exchange chromatography on different fractions from the separation on Sephadex G-15 (mostly peak 7) was carried out on a microcolumn 30 × 0.4 cm using DEAE Sephadex A-25 (Pharmacia, Uppsala, Sweden).

A sample column and a reference column were packed and equilibrated with 0.01 M Tris HCl buffer, pH 8.6. The columns were coupled to a dual-channel ultra-violet absorptiometer (LKB, Uvicord III) equipped with two 3 mm optical pathway cells, volume 0.1 ml (reference and measuring cells).

Using the system with specially designed sample and reference columns, with reference and measuring cells used simultaneously, changes in absorption caused by changes in ionic strength of the buffer are eliminated. Using a reference column and cell is of special importance when detecting absorption at 206 nm.

After loading the sample on top of one column, both columns were eluted with 0.01 M Tris HCl buffer, pH 8.6, with a linear salt gradient to 1 M NaCl using two pumps with identical flow, one for the sample column and one for the reference column and then passed through a gradient mixer (LKB ULTROGRAD 11300). The flow rate was 8 ml/hr and the time for the separation about 3 hr. The absorption was measured at 206 nm and 254 nm simultaneously using a two-channel recorder (W + W Electronic Recorder). Automatic scale expansion ensures that the recorder will not go off-scale even if the output signal from the Uvicord varies greatly. The recorder was operated at a constant speed of 10 cm/hr. The GEC technique has earlier been described in detail (Fürst et al., 1974).

The analytical system (HSGF + DEAE Sephadex gradient elution) proved to give stable and reproducible values. The retention-time reproducibility is better than 1.3%, allowing the nine different sub-peaks to be identified and measured. Recovery from the combined HSGF-GEC technique was 92%. The coefficient of variation in one single determination of the relative integral of a single peptide
peak, determined from duplicate determinations (n = 20), was 11.2% at 206 nm and 11.3% at 254 nm. The errors in duplicate determinations were 7.9% and 8.0% respectively.

Although the absolute magnitude of each peak in relation to the other peaks cannot yet be determined, since we lack data of the specific absorptions, it should still be possible to compare different patients with each other with regard to the size of individual peaks and also to calculate clearance ratios, mass transfer rates, etc, since in these formulas the relative absorption is the factor of importance. The quantification of the peaks is performed by integrating the area of the individual peaks.

RESULTS

High-speed gel filtration (HSGF) Utilising HSGF technique we have made comparative analyses of 612 samples on 109 uraemic patients, 4 hypercatabolic non-uraemic patients, four severe rheumatic patients, and on normal controls.

Various body fluids have been separated into 10–11 component ultra-violet absorbing peaks, based primarily upon difference in molecular weight and configuration. The significance of the different peaks has been discussed previously (Fürst, 1973; Gordon et al, 1974).

<table>
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<tr>
<th>TABLE I. The Estimated Magnitude of Peak 7 in Non-uraemic Controls and in Uraemic Patients from Different Biological Fluids</th>
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<tr>
<td>Non-Uraemic Controls</td>
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<td>Healthy subjects</td>
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<td>Rheumatological patients</td>
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<td>Hypercatabolic patients</td>
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<td>Progressive neuropathy</td>
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<td>Peritoneal-dialysis treated</td>
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<td>Haemodialysis treated</td>
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<td>Transplanted</td>
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<td>Peritoneal dialysate</td>
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<td>Haemodialysate</td>
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NB The estimated magnitudes after respective treatments (eg peritoneal dialysis, hemodialysis and transplantation) are given in parentheses.
Peak 7 was of special interest because it contains middle molecular solutes in the molecular-weight range of 1000–2000 daltons. This method, however, permits only semi-quantitative discrimination between different categories of patients. The magnitude of peak 7 in different biological fluids from non-uraemic controls and uraemic patients is presented in Table I. The table demonstrates that peak 7 is absent in plasma from non-uraemic subjects but present in plasma from uraemic patients of all categories. The magnitude of peak 7 appeared to be related to the severity of uraemia. An especially prominent peak was found in patients with pericarditis and progressive neuropathy. Peak 7 was present in urine from non-uraemic as well as uraemic subjects.

Haemodialysis and peritoneal dialysis brought about a decrease in peak 7 in serum. The peak appeared in peritoneal dialysate and in haemodialysate, thus proving that the solutes of peak 7 are dialysable.

**Gradient elution chromatography of peak 7** Peak 7 has further been separated into 7–9 different sub-peaks using the technique of gradient elution on DEAE Sephadex microcolumns (GEC). With this technique 108 samples from 52 uraemic patients and from normal subjects have been analysed. The peaks which were labelled 7a, b, c, d, e, f and g are consistently found in severe uraemia. Only two of the sub-peaks, f and g, were detected in normal serum although in much lower quantities than in uraemia (Figure 1). On the other hand normal urine contained all the peaks found in uraemic serum and urine, although most of them in lower concentrations than in uraemic urine. Especially, one of the sub-peaks, 7c, was prominent in uraemia. There is evidence that this peak consists of more than one peptide since it sometimes separates into two peaks (c₁ and c₂).

Peak 7 consists of a mixture of peptides and the different peptides vary independently concerning specific ultra-violet absorption. On the other hand, the sub-peaks of peak 7, which are readily separated from each other, would lend themselves better to quantitative evaluation. Comparison between various categories of patients revealed important differences in the absolute and relative absorbance of the different sub-peaks of peak 7. We present here some typical findings in different groups of patients — the preliminary nature of our results should be emphasized.

Patients with untreated symptomatic uraemia had very high peak-7 sub-peaks, especially those with uraemic pericarditis. An example is presented in Figure 1. In this patient and in three other patients with pericarditis peak 7c was especially high whereas in two other patients with pericarditis peaks a, b and f were more prominent than peak c.

The effect of treatment with protein-poor diet and essential amino acids orally is shown in Figure 2. In this patient one week’s treatment with protein-poor diet resulted in a decrease in peak 7c. A further decrease occurred after another week during which essential amino acids were supplied together with the protein-poor diet. The change in peak 7c occurred in parallel with a decrease in serum urea nitrogen, the serum creatinine concentration remaining unchanged. On the other hand
Figure 1. Sub-peaks of peak 7, fractionated by GEC from uraemic and normal (b) serum. Uraemic serum was obtained from a patient with uraemic pericarditis. Serum-creatinine 9.0 mg/100 ml, urea-nitrogen 133 mg/100 ml.

The other sub-peaks were not affected by the dietary treatment. Low peak 7 sub-peaks were also observed in four other patients treated with protein-poor diet and essential amino acids.

The effect of peritoneal dialysis is illustrated in Figure 3. This preliminary investigation was made in a patient with severe uraemic symptoms (nausea, vomiting and mental confusion) who was undergoing his first dialysis treatment. The figure shows that all the sub-peaks present in serum appeared in the dialysis-fluid, proving that the middle molecules are dialysable. Peaks 7a, b and c decreased markedly after 24-hr peritoneal dialysis, whereas 7f and g remained essentially unchanged.
Figure 2. The effect of dietary treatment on uraemic middle molecules. Serum was obtained before treatment, after seven days on protein-poor (PPD) 2.7 g N/day and after another seven days on the diet with addition of essential amino acids (EAA) 2.65 g N/day. Peak c decreased during treatment, whereas the other peaks were not consistently changed. EAA was given in the form of tablets of Aminess (registered trade name, Astra Läkemedel, Sweden).

Despite apparently being dialysed to the same extent as the other peaks. This suggests that peak f and g material was produced continuously during dialysis in sufficient amounts to substitute for the removal by dialysis.

In pre-dialysis serum from four patients on intermittent haemo-dialysis (Gambro Lundia 17 μm, 8 hr, 3 times a week) who were free of uraemic symptoms, low peak 7 sub-peaks were found. An example is given in Figure 4. Peak 7 sub-peaks, measured at wavelength 254 nm, decreased after dialysis. However, at 206 nm (the wavelength at which peptide bonds are detected) peak 7f was unchanged and peak 7g even increased after dialysis. These findings among others demonstrate the complex nature of peaks 7f and g. In three uraemic patients who were transplanted with kidneys from living donors, with immediate transplant function, peak 7 and its sub-peaks disappeared from the serum within 24 hr after transplantation, i.e. before serum creatinine and urea had normalized. An example is given in Figure 5. These results indicate that the presence of a normally-functioning kidney brings about a rapid elimination of the uraemic middle molecules.
Figure 3. The effect of peritoneal dialysis on uraemic middle molecules in a patient undergoing his first treatment. Serum was obtained before dialysis and after 24 hr of treatment. The dialysate was collected during the first 2 hr of dialysis.

DISCUSSION

The results presented here indicate that middle-molecular solutes accumulate in the body-fluids of uraemic patients.

The method, designated high-speed gel filtration (HSGF), permits a greater degree of resolution to be achieved than is possible with standard techniques of gel filtration. A high degree of sensitivity is also achieved which has permitted the development and use of ‘micro’ columns of Sephadex and ‘micro’ fluid sample volumes. With regard to peptides and proteins the sensitivity is considerably higher
at 206 nm than at the conventionally-used wave lengths 254 or 280 nm.

As shown in Table I the method of HSGF proved to be at best semiquantitative, thus only permitting us to make rough discriminations between different categories of patients. The reason for this is evidently that peak 7 consists of a compound mixture of various peptides. These different peptides vary independently from each other. Since each one may be expected to have a specific absorption of ultraviolet light, which is unique for that special peptide, strictly quantitative data could possibly not be obtained.

With the following gradient elution technique it was possible to obtain at least eight different ultra-violet-absorbing fractions in the molecular-weight range of 1000–2000 in uraemic serum most of which were not detectable in normal serum. There are reasons to suspect that some of these fractions consist of mixtures of different molecular species. Especially peaks 7f and g (and extra peaks in their neighbourhood), which contain uncharged or basic solutes, appear to be complex, the present techniques of gradient elution not being optimal for their separation.

The fact that a number of middle-molecular peaks were detected at wavelength 206 nm, indicates that they represent peptide material. This was also confirmed by amino-acid analysis of whole peak 7 before and after hydrolysis (unpublished observations).
Figure 5. The effect of transplantation on uraemic middle molecules in a patient treated with protein-poor diet and essential amino acids (see caption to Figure 3). Serum was obtained before and one day after transplantation with a graft from a living donor.

Although still preliminary, our results suggest that the degree of accumulation of middle molecules as determined by our methods, correlates better with uraemic symptomatology than commonly measured parameters such as serum urea and creatinine. This is apparent from Figures 1 to 4 which present results in patients with approximately similar serum concentrations of urea nitrogen and creatinine.

Patients with marked uraemic symptoms such as pericarditis (Figure 1) mental confusion and gastro-intestinal symptoms (Figure 3) exhibited far higher middle-molecular peaks than patients who were treated either by dialysis (Figure 4) or by diet (Figure 2) and who were virtually free of symptoms.

Our results demonstrate that we are dealing with a complex mixture of middle-molecular solutes which accumulate in uraemia apparently independently of each other, and behave differently from each other in connection with dialysis treatment. This implies that it is probably not possible to define their role in uraemia by a single unifying hypothesis. The results raise a number of new questions regarding their nature, origin, biological significance and mode of elimination. It may be suggested that some of these substances are products of protein catabolism which accumulate because of impaired urinary excretion, whereas other substances are peptide hormones which accumulate as a consequence of the disturbed homostasis in uraemia. Whether some of these substances are uraemic toxins or are formed in larger amounts than in normal subjects as a consequence of uraemic toxicity is also an open question.

Acknowledgement

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References

Fürst, P (1973) Opuscula Medica, 10, 61
Gajdos, M and Dzúrik, R (1973) International Urology and Nephrology, 5, 331

Open Discussion

F P BRUNNER (Basle). Does your peak 7 have natriuretic properties?
FÜRST I don’t know, but the solutes have the properties of peptides.
C GOLD (South Africa) On your first slide, there was something in peak 8 and 9 which was reduced in uraemic serum and present in normal serum. Perhaps the removal of substances could explain some of the complications of uraemia?
FÜRST Peak 9 corresponds to high-molecular-weight solutes such as proteins, below mw 50,000. Peak 8 correspond to peptide material between mw 2,000 and mw 5,000. However, the method for separation is not optimal. To discuss this range of compounds, we would first have to optimise the separation for this range.
A DRUKKER (Israel) Have you had the opportunity to measure your peaks in recently transplanted patients before urinary excretory function came back?
BERGSTROM Perhaps I could reply to that question. The two patients we studied before and after transplantation had transplants from living donors with immediate function. We have not up to now had the opportunity to study any patient after cadaveric transplantation with a delayed onset of renal function. I think the question is interesting and very important for us.