Accumulation and Excretion of Middle Molecules

H ASABA, J BERGSTROM, P FURST, R OULES, L ZIMMERMAN
St Erik's Hospital, Stockholm, Sweden

Summary

By using gel filtration combined with gradient elution chromatography, we have evaluated quantitatively the concentration of middle molecule fractions (7a, b, c, and d) from the integrated peak areas of the chromatograms. Plasma and urine from patients with varying degrees of renal failure were analysed.

Among 66 patients studied, measurable plasma peaks were only found when the plasma creatinine concentration was above 400 - 500 μmol/l or creatinine clearance below 12 ml/min. No correlation was found between the plasma urea or creatinine concentration, respectively, and the concentration of middle molecules. There were significant correlations between creatinine clearance and the renal clearance of the middle molecule fractions. The clearance of 7a far exceeded the creatinine clearance indicating either tubular excretion or production by the kidney.

The relative size of the middle molecule peaks varied greatly from one patient to the other. The rates of excretion of individual middle molecules in the urine varied considerably between different patients; peaks found to be high in plasma were also found to be high in the urine. Taking the excretion rate to be equal to the generation rate, the results indicate that great differences in production (by a factor of 5 or more) exist between different patients. This also explains the lack of correlation between accumulation of middle molecules and urea or creatinine in plasma.

In consequence, mathematical models for calculating the level of middle molecules based on the assumption of constant production rates are bound to give erroneous results.
Introduction

The middle molecule hypothesis was originally derived from clinical observations in connection with different modes of dialysis treatment (Babb et al, 1972b). More recently direct evidence has been brought forward that uraemic patients accumulate middle molecular solutes in their body fluids (Dall’Aglio et al, 1972; Gajdos and Dzurik, 1973; Man et al, 1973; Fürst et al, 1974; Migone et al, 1975).

By using high speed gel filtration followed by gradient elution chromatography it was possible to separate eight to ten UV absorbing fractions in the middle molecular range, which were present in plasma of severely uraemic patients but not in normal subjects or in patients with other severe diseases (Fürst et al, 1976). These fractions could be quantitated by integrating the peaks’ areas on the chromatograms. Using this technique we studied the accumulation and urinary excretion of middle molecules in uraemic patients.

MATERIAL AND METHODS

The patient material comprised 66 uraemic patients. Thirty-five patients were either untreated or under conservative treatment with protein-poor diet and essential amino acids; 24 patients were treated with intermittent haemodialysis and 7 patients with intermittent peritoneal dialysis.

In 20 non-dialysed patients, blood and 24 hr urine samples were collected for determination of endogenous creatinine and middle molecule clearances.

Heparinised blood samples from the dialysis patients were obtained immediately before dialysis.

Middle molecules were determined in plasma and urine by high speed gel filtration (HSGF) followed by gradient elution chromatography (GEC) (Fürst et al, 1976). The solutes were detected at 254 and 206 nm. The molecular weight range of the middle molecule fraction (peak 7) isolated by HSGF was assessed against standards of known molecular weight, and estimated to be between 1000 and 2000. The middle molecule peaks obtained by the GEC technique were quantitated by integrating the peak areas of the chromatograms obtained at 254 nm. The coefficient of variation in one single determination was 4.6–11%, depending on the peak size.

Urea was determined enzymatically according to Chaney and Marbach (1962). Creatinine was determined either by using a Technicon autoanalyser or by using slope photometry applying 2-point kinetics for the creatinine reaction according to a recently developed technique (to be published).

The integrated areas of peaks 7a, b, c, and d were presented in the graphs, each patient being represented by one spot. Peak 73 only occurred sporadically and peaks 7f and g were unsuitable for integration, since they probably consisted of a mixture of several peaks, which were poorly separated.
Figure 1. Relationships between the area of peaks 7a and 7b, respectively, and the concentration of creatinine and urea in plasma.
Figure 2. Relationships between the area of peaks 7c and 7d, respectively, and the concentration of creatinine and urea in plasma.
RESULTS

The relationships between the size of peaks 7a, b, c and d, respectively, and the plasma creatinine and urea concentrations are shown in Figures 1 and 2. The results show that high middle molecule peaks were observed only when the creatinine concentration was higher than 400 – 500 μmol/l.

Above this creatinine level, the values were widely scattered, with no apparent correlation between the area of any of the peaks and the plasma creatinine and urea concentrations, respectively. It should be pointed out that many dialysis patients had very low middle molecule peaks in spite of high plasma creatinine concentrations.

The relationship between the area of peaks 7a, b, c, and d, respectively, and the endogenous creatinine clearance is shown in Figure 3. Accumulation of middle molecule material occurred only when the creatinine clearance was lower than 12 ml/min.

Figure 4 shows the relationship between the endogenous creatinine clearance and the endogenous clearance of middle molecule fractions 7a, b, c, and d. There was a significant linear correlation between the clearance of each of the middle molecule peaks and the endogenous creatinine clearance.

The slope of the regression line indicates that the clearance of 7a was on an average about 3 times higher than the creatinine clearance. The clearance of 7b and c was also on an average slightly higher than the creatinine clearance, whereas the clearance of 7d was about equal to the creatinine clearance.

A significant correlation was found between the areas of peaks 7a and 7b in plasma in the non-dialysed patients. The regression equation is: 7a = -0.83 + 0.74 · 7b, r = 0.81 (p < 0.01). A significant correlation was found between 7b and 7d in the dialysed patients.

In single patients, the plasma and urine middle molecule patterns were similar to each other. Peaks found to be high in plasma were also found to be excreted in increased amounts in the urine. Figure 5 shows chromatograms from plasma and urine obtained in 4 uraemic patients. Quantitative data from these patients are given in Table I.

DISCUSSION

One prerequisite for the appearance of uraemic middle molecules in plasma, as measured by us, was that the glomerular filtration rate should be substantially reduced. A correlation between the size of middle molecule peaks and urea and creatinine, respectively, would have been expected if the middle molecules mainly accumulate passively as a result of decreased renal function. However, the areas of the middle molecule peaks varied considerably between different patients, apparently independent of the accumulation of creatinine and urea in plasma – in other words, a uraemic middle molecule does not behave like a
'big creatinine or urea.

The renal clearance of the various middle molecule fractions were linearly correlated with the endogenous creatinine clearance. Especially for 7a the clearance appeared to be higher than the creatinine clearance, suggesting either local production in the kidney or the urinary tract or active tubular excretion. The finding that the plasma and urinary middle molecule patterns are very similar in the individual patient (Figure 5) indicates that the middle molecule fractions found in urine are mainly derived from plasma.

When comparing the excretion rates (U x V values) for each peak, significant differences were found between different patients (cf. Table I). Assuming excretion rate to be equal to production rate these differences (by a factor of
five or more) are too great to be accounted for only by differences in protein intake and degradation (urea production rate). The explanation has to be sought among other extrarenal factors as well. We have consistently found that 'sick' uraemic patients, e.g. with pericarditis, neuropathy, malnutrition, and severe fluid
Figure 5. Chromatograms of plasma and urine, obtained from 4 uraemic patients.
retention, tend to accumulate (produce) middle molecule material, whereas patients free from symptoms, e.g. ‘adequately’ dialysed patients maintained on successful conservative treatment have low middle molecule peaks (Fürst et al, 1974; Bergström et al, 1975).

No significant correlations were found between the relative size of the different peaks in plasma, except between the areas of 7a and 7b (in the non-dialysed patients) and between the areas of 7b and 7d in the dialysed patients. This indicates that the accumulation of one middle molecule fraction may occur independent of another one; as a consequence the peak pattern may vary markedly from one patient to another (Figure 5). The correlations found may, however, suggest that certain middle molecules are related with regard to production and elimination.

TABLE I. Renal excretion rates (relative values) of middle molecule fractions in the patients illustrated in Figure 5.

<table>
<thead>
<tr>
<th>EXCRETION (GENERATION?) RATE OF PEAK 7 SUBPEAKS (U-V, cm²/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient</td>
</tr>
<tr>
<td>---------</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
</tbody>
</table>

Most haemodialysers in use today have a much lower clearance for middle molecules (vitamin B₁₂) than for urea and creatinine (von Hartitzsch et al, 1973). Haemodialysis patients generally have lower residual renal function than non-dialysed patients. Thus, one would expect that the plasma concentration of middle molecules in relation to the concentration of creatinine would be higher in haemodialysis patients than non-dialysed uraemics, provided that the production rates were approximately equal in both groups of patients. On the contrary, we generally found very low peaks in patients doing well on dialysis, high peaks only being found in ‘sick’ patients with infections or other complications independent of the dialysis schedule. This suggests that the production rate is probably lower in most dialysis patients than in non-dialysed uraemics, except during periods of complications.

Various mathematical models have been worked out to predict the accumulation and the transfer of middle molecules (Babb et al, 1972a; Kjellstrand et al, 1972 and 1973; Milutinovic et al, 1974; Popovich and Moncrief, 1974; Sargent and
Gotch, 1974; Babb et al, 1975; Popovich et al, 1975). However, these mathematical models for calculation of endogenous middle molecule levels (from dialyser characteristics and residual renal function) do not take into consideration the great individual variation in production rate of different middle molecule fractions. For this reason these models are not valid for prediction of the in vivo concentration of endogenous middle molecules.

The fact that patients with severe symptoms of uraemia (pericarditis, neuropathy) produced more middle molecule material than patients free from symptoms indicates that middle molecules may play a role as uraemic toxins (Fürst et al, 1974; Bergström et al, 1975).

The direct evidence for toxicity of middle molecules is so far limited to a few in vitro studies which show an inhibiting effect on glucose utilisation (Dzurik et al, 1973), fibroblast proliferation (Man et al, 1974), phagocytic activity of leucocytes (Odeberg et al, 1973), and proliferation of lymphocytes (Bergström et al, 1975; Touraine et al, 1975). Although corresponding symptoms and signs have been found in uraemic patients (glucose intolerance, susceptibility to infection, partial inhibition of immune response) it is still an open question whether these are caused by the middle molecules. There remains the possibility that these molecules are non-toxic but accumulate as a consequence of uraemic toxicity.

Acknowledgments

This work has been supported by grants from the NIAMDD (contract no NO1-AM-2-2215), Bethesda, Maryland, U.S.A.

References

Babb, AL, Farrell, PC, Uveli, DA and Scribner, BH (1972b) Transactions. American Society for Artificial Internal Organs, 18, 98
Chaney, AL and Marbach, EP (1962) Clinical Chemistry, 8, 130
Wells. Page 417
Gajdos, M and Dzúrik, R (1973) International Urology and Nephrology, 5, 331
von Hartitzsch, B, Hoenich, NA, Peterson, RJ, Buselmeier, TJ, Kerr, DNS and
Kjellstrand, CM (1973) Proceedings of the European Dialysis and Transplant
Association, 10, Pitman Medical, London. Page 522
Kjellstrand, CM, Evans, RL, Petersen, RJ, Rust, LW, Shideman, J, Buselmeier, TJ
and Rozelle, LT (1972) Proceedings of the Clinical Dialysis and Transplant
Forum, 1, 127
Kjellstrand, CM, Petersen, RJ, Evans, RL, Shideman, JR, von Hartitzsch, B and
Buselmeier, TJ (1973) Transactions of the American Society for Artificial
Internal Organs, 19, 325
Man, NK, Cueuille, G, Zingraff, J, Drueke, T, Junger, P, Sausse, A, Brillon, JP
and Funck-Brentano, JL (1974) Proceedings of the European Dialysis and
Transplant Association, 11, Pitman Medical, Tunbridge Wells. Page 214
(1973) Transactions of the American Society for Artificial Internal Organs,
19, 320
Migone, L, Dall ’Aglio, P and Buzio, C (1975) Clinical Nephrology, 3, 82
Milutinovic, J, Strand, M, Casaretto, A, Follette, W, Babb, AL and Scribner, BH
(1974) Transactions of the American Society for Artificial Internal Organs,
20, 410
Odeberg, H, Olsson, I and Thysell, H (1973) Transactions of the American
Society for Artificial Internal Organs, 19, 484
Popovich, RP, Hlavinka, DJ, Bomar, JB, Moncrief, JW and Decherd, JF (1975)
Transactions of the American Society for Artificial Internal Organs, 21, 108
Popovich, RP and Moncrief, JW (1974) Transactions of the American Society
for Artificial Internal Organs, 20, 377
Sargent, JA and Gotch, FA (1974) Transactions of the American Society for
Artificial Internal Organs, 20, 395
Touraine, JL, Navarro, J, Corre, C and Traeger, J (1975) Biomedicine, 23, 180